DNA stable-isotope probing delineates carbon flows from rice residues into soil microbial communities depending on fertilization

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Abstract

Decomposition of crop residues in soil is mediated by microorganisms whose activities varied with fertilization. The complexity of active microorganisms and their interactions by utilizing residues is impossible to disentangle without isotope applications. Thus, $^{13}$C-labeled rice residues were employed and DNA stable-isotope probing (DNA-SIP) combined with high-throughput sequencing was applied to identify microbes active in assimilating residue carbon (C). Manure addition strongly modified microbial community compositions involved in the C flow from rice-residues. Relative abundances of bacterial genus *Lysobacter* and fungal genus *Syncephalis* were increased, but that of bacterial genus *Streptomyces* and fungal genus *Trichoderma* were decreased in soils receiving mineral fertilizers plus manure (NPKM) compared to soils receiving only mineral fertilizers (NPK). Microbes involved in the flow of residue-C formed more complex network in NPKM than that in NPK soils, because of necessity to decompose more diverse organic compounds. The fungal species (*Jugulospora rotula* and *Emericellopsis terricola* in NPK and NPKM soils, respectively) were identified as keystone species in network and may significantly contribute to residue-C decomposition. Most of fungal genera in NPKM soils, especially *Chaetomium*, *Staphylotrichum*, *Penicillium* and *Aspergillus*, responded faster to residue addition than those in NPK soils. This is connected with the changes in the composition of the rice residue during the degradation and with fungal adaptation (abundance and activity) to continuous manure input. Our findings provide fundamental information about the roles of key microbial groups in residue decomposition and offer important cue on manipulating the soil microbiome for residue utilization and C sequestration in soil.

**Keywords:** Fertilization; $^{13}$C-labeled rice residue; DNA stable-isotope probing; High-throughput sequencing; Active microbes
Importance

Identifying and understanding the active microbial communities and interactions involved in plant-residue utilization is a key question to elucidate the transformation of soil organic matter (SOM) in agricultural ecosystems. Microbial community composition responds strongly to management, but little is known about specific microbial groups involved in plant-residue utilization and consequently microbial functions under distinct fertilization. We combined DNA stable-isotope (\(^{13}\)C) probing and high-throughput sequencing to identify active fungal and bacterial groups degrading residues in soils after 3-year mineral fertilization with and without manure. Manuring changed the active microbial composition and complexified microbial interactions involved in residue-C flow. Most fungal genera, especially Chaetomium, Staphylotrichum, Penicillium and Aspergillus, responded to residue addition faster in soils historically received manure. We generated a valuable “library of microorganisms” involved in plant residue utilization for future targeted research to exploit specific functions of microbial groups in organic matter utilization and C sequestration.
Introduction

The importance of microbial residues for soil organic matter (SOM) formation has been recognized for over two decades (1, 2), indicating the crucial roles of microbes in the sustainability and soil fertility. Therefore, dominant microbes involved in the decomposition play important role for biologically driven carbon (C) in C flows. The development of molecular-biology techniques has greatly advanced our understanding of microorganisms that mediate the decomposition of plant residue in soil (3-6). To sequester more C in soil during plant residue decomposition, we need to consider the C pathways from the residue into the soil organic matter through microbial processing (7). Therefore, precise identification of the microbial groups involved in C utilization and sequestration is the key to explore the functional roles microbes improving soil fertility.

Approaches using stable-isotope probing (SIP) provide novel insights into C flows in soil microbial communities. The identity of microorganisms was linked with their activities and functions using SIP, for example, in tracing the fate of low molecular weight organic substrates, such as glucose (8-12), methanol (13, 14), and propionate (15). A few studies considering more complex organic compounds focused on root exudates (16-20) or high molecular weight organic substrates, such as cellulose (21, 22). A number of studies used SIP to characterize specific microbial groups utilizing plant residues differing in qualities or types, but all of these studies concentrated on only a single soil conditions (23-29). Limited information is available to address whether the active microbiome involved in residue decomposition will be affected by specific fertilization practices, although the total soil microbial community have been widely reported to be changed (30, 31).

In agricultural soils, changes in the C availability due to fertilization, such as mineral or organic fertilization, always alter microbial community structure (32, 33), which further leads to alterations in the enzyme activities related to C and N cycling (34-38). The application of organic fertilizers (i.e. cattle manure and compost) consistently resulted in higher levels of cultivable microorganisms in soil and enzyme
activities, which can be used as an environmentally friendly and rapid measure for
restoring degraded cropland (39). The organic amendments also significantly impact
the decomposition rate of crop residues (40). So far, no attempt has been made to
evaluate the effects of mineral and organic fertilizers on microbial groups
decomposing plant residues. Considering the degradation of plant residues is a
microbe-mediated process and fertilization selects for specifically adapted microbial
communities, it is reasonable to propose that distinct active microbial communities
responding to residue decomposition can exist in soils experiencing contrasting
fertilization regimes. Mineral vs. organic inputs alter the general balance of soil
stoichiometry, thereby resulting in adaptative resident selection as copiotrophic (low
C use efficiency, fast-growing) versus more oligotrophic taxa (high C use efficiency,
slow-growing). Wang et al. (41) reported that the application of organic fertilizers
increased the abundance of generally copiotrophic bacterial groups, while application
of mineral fertilizers increased the abundance of oligotrophic groups. Thus, we
hypothesized that there will be a shift in dominance and response strategies of specific
microbial groups involved in residue assimilation in soils after manuring compared to
only mineral fertilization. To clarify this, soils were collected from a field experiment
with 3-year different fertilization regimes including fertilization with solely mineral
fertilizers (NPK) and fertilization with mineral fertilizers plus manure (NPKM), and a
microcosm experiment with $^{13}$C-labeled rice residues was conducted coupled with
DNA-SIP technology.

Results

0.05 g (rice residue) / g (dry weight soil) was employed to conduct microcosms
experiment. Similar amount about 0.04 - 0.05 g residue/g soil (dry weight basis) was
also employed in previous SIP studies investigating microbial communities involved
in residue assimilation (26-28, 42). The choice of 60 days’ incubation duration was
based on a previous study showing that the cumulative CO$_2$ efflux at first 60 days was
nearly 70% of the total 160 days’ cumulative CO$_2$ efflux after the addition of rice
residues under 25°C (43). Our data (Fig. S1) also showed that the CO$_2$ efflux rates
were relatively stable during 30-60 days post the residue amendment. Besides, microbial communities are mainly affected by the addition of residue or carbon substrates (such as, glucose, cellulose) during the first 7-31 days of incubation (26, 28, 44, 45). In this study, due to the disturbance of soil during the rice residue amendment, soils were allowed to equilibrate for 7 days prior to the first sampling. Thus, 7, 15, 30 and 60 days after rice residue addition were selected as sampling points.

**Microbial communities utilizing residue-derived C**

The CO₂ efflux rates and both the abundances of soil total bacteria and fungi were significantly increased after the rice residue addition (Fig. S1). The dynamics of microbial abundances were similar in soils with and without manure addition (NPK+residue and NPKM+residue) (Fig. S1). According to the relative abundance distribution of the fungal (ITS) and bacterial (16S) genes in the gradient density (Fig. S2), the fungal and bacterial DNA from microorganisms utilizing $^{13}$C residues were clearly separated in the density gradient from 1.720-1.730 and 1.730-1.740 g mL$^{-1}$, respectively. The $^{13}$C-labeled-DNA from the NPK+$^{13}$C-residue and NPKM+$^{13}$C-residue soil samples and the corresponding fractions from the NPK+$^{12}$C-residue and NPKM+$^{12}$C-residue soils were isolated for use in sequencing of the fungal ITS1 and bacterial 16S rRNA genes (V4-V5 region).

The OTUs that were identified as being significantly labeled based on the log₂ fold change analysis. A total of 219 and 212 bacterial OTUs were $^{13}$C labeled in the NPK and NPKM soils, respectively (Fig. 1a), whereas, 157 OTUs of them were shared in soils with NPK and NPKM fertilization. Proteobacteria accounted for 46.5% and 39.6% of the total $^{13}$C labeled bacterial communities in the NPK and NPKM soils, respectively, while Actinobacteria accounted for 48.6% and 54% (Fig. 1b). This indicates that Proteobacteria and Actinobacteria dominated the rice residue decomposition. The relative abundance of Proteobacteria was higher in NPKM soil than in NPK soil, whereas the relative abundance of Actinobacteria had the opposite trend. Besides these phyla, 5 other phyla, namely, Chloroflexi, Gemmatimonadetes, Acidobacteria, Planctomycetes, and Candidate division WPS-1, also participated in
The number of $^{13}$C enriched fungal OTUs was less than that of bacterial OTUs, with 45 fungal OTUs $^{13}$C labeled in NPK soil and 64 $^{13}$C labeled in NPKM soil (Fig. 2a). In NPK soil, residue-derived C was assimilated mainly by Ascomycota and Basidiomycota (Fig. 2b). The relative abundance of $^{13}$C labeled Ascomycota gradually increased with time, from 64.4% on day 7 to 97% on day 60. In NPKM soil, beside the members of Ascomycota and Basidiomycota, Zoopagomycotina was also responsible for the residue-C decomposition and utilization. The relative abundance of Zoopagomycotina gradually decreased from 29.6% on day 7 to 1.92% on day 15, and it was absent on day 30. In contrast, the relative abundance of Ascomycota gradually increased from 53.8% on day 7 to 95.6% on day 60 (Fig. 2b).

NMDS ordination of the $^{13}$C-labeled bacterial (stress = 0.049) and fungal (stress = 0.12) communities varied between the NPK and NPKM treatments on the horizontal axis and also clustered according to the different times on the vertical axis (Fig. 3). In addition, adonis analysis also showed that the $^{13}$C-labeled bacterial and fungal communities were significantly changed by fertilization and sampling time (Table S1). Therefore, in the following analyses, we mainly focused on the microbes that played dominant roles in the C flow depending on fertilization and their dynamics based on the analysis of response strategies.

**Dominant microbes involved in the flow of residue-derived C**

OTUs with relative abundances higher than 1% were identified as the dominant microbes. The dominant bacterial OTUs were mainly Proteobacteria and Actinobacteria, and the annotated OTUs mainly belonged to *Lysobacter, Devosia, Actinomadura, Glycomyces, Nonomuraea, Rhodanobacter, Luteibacter, Kribbella* and *Streptomyces* (Fig. 4). The relative abundances of *Lysobacter, Devosia, Actinomadura, Glycomyces* and *Nonomuraea* were higher in NPKM soil compared to NPK soil, whereas the relative abundances of *Rhodanobacter, Luteibacter, Kribbella* and *Streptomyces* showed the opposite trend.

The dominant OTUs of fungi in the NPK and NPKM soils were Ascomycota and...
Zoopagomycotina, and most were classified into the genera *Aspergillus*, *Thielavia*, *Trichocladium*, *Chaetomium*, *Emericellopsis*, *Cladorrhinum*, *Cercophora*, *Purpureocillium*, *Trichoderma*, *Fusarium* and *Myrmecridium* (Fig. 5). Other identified OTUs were classified into the genera *Penicillium*, *Aspergillus*, *Talaromyces*, and *Staphylotrichum* of the Eurotiomycetes. OTU27, identified on day 7 as accounting for approximately 29.6% of the total labeled fungal sequences in NPKM soil, was identified as a member of *Syncephalis*; this OTU was entirely absent from NPK soils. Compared to NPK, NPKM decreased the relative abundances of *Trichoderma*, *Purpureocillium*, *Talaromyces*, *Cercophora*, *Cladorrhinum*, *Chaetomium*, *Staphylotrichum* and *Thielavia*, and increased the relative abundances of *Fusarium*, *Aspergillus*, *Paecilomyces*, *Emericellopsis*, *Myrmecridium*, *Trichocladium*, and *Syncephalis*.

**Microbial networks involved in the C flow from rice residues**

The microbes that played key roles in the flow of residue-C were explored using microbial molecular ecology networks (Fig. 6). The bacterial and fungal OTUs with average relative abundances higher than 0.1% were selected for network construction by Random Matrix Theory (RMT). There were 106 nodes in the NPK soil network, including 80 bacterial nodes and 26 fungal nodes. There were 128 nodes in the NPKM soil network, including 92 bacterial nodes and 36 fungal nodes. The average degree of the NPK network was 4.40, whereas that of the NPKM network was 11.4 (Table S2), indicating that the 3-year manure application increased the complexity of the microbial network related to the utilization of residue-derived C.

Two key network nodes were found in the NPK soil (Fig. 6 and Table S3): the fungal OTU30, affiliated to Ascomycota, acted as a module hub, and the Uncultured bacteria, OTU140, acted as an important connector between modules. Four key network nodes were present in the NPKM soil, including a module hub and three connectors. Specifically, the fungal OTU25 (Ascomycota), the unclassified fungi (OTU13) and the bacterial OTU1393 affiliated to Gemmatimonadetes acted as
connectors in the network, and a module hub was found to be the unclassified fungi, OTU14. The average relative abundances of the two fungal OTUs that were served as module hubs in NPK and NPKM soils were 2.3% and 5.81%, respectively (Table S3).

However, the average relative abundances of bacterial module connectors in both NPK and NPKM soils were less than 1%. The fungal OTU30 acted as the module hub in NPK soil was identified as *Jugulospora rotula*, and the fungal OTU25 that acted as a connector in the NPKM soil was classified as *Emericellopsis terricola*.

**Response strategies of dominant microbes to residue amendment**

In order to assess the temporal dynamics of the microorganisms that use the residue-derived C, the abundant genera (Fig. 3 and Fig. 4) having significant changes between any two sampling points were selected. We assumed that the relative abundance changes between sampling points reflect microbial activities. Response strategies were assigned according to the time point, at which the relative abundance of the genus was maximal. Genera that displayed highest relative abundance at the outset (7 d after residue addition) were referred to as rapid responders; those that displayed highest relative abundance at intermediate sample time points (between 15 d and 30 d) were referred to as intermediate responders; those that displayed highest relative abundance at 60 d after residue addition were referred to as delayed responders. In NPK and NPKM fertilized soils, the response strategies of the genera *Devosia*, *Glycomyces* and *Kribbella* were the most rapid, whereas those of *Rhodanobacter* and *Actinomadura* responded much slower, as well as *Streptomyces* in NPK soil and *Nonomuraea* in NPKM soil. In contrast to the similar response strategies of bacteria between NPK and NPKM soils, fungi behaved quite diverse (Fig. 7). Only one genus, *Fusarium*, had the same response rate in the NPK and NPKM soils. In NPK soil, the genus *Cercophora* was the most rapid to respond to the addition of residue; *Thielavia*, *Cladorrhinum*, and *Paecilomyces* had slower response rates, and *Staphylotrichum*, *Chaetomium*, *Penicillium*, and *Aspergillus*, the delayed responders, were the slowest. In NPKM soil, *Syncephalis* responded rapidly, while *Myrmecridium*, *Chaetomium*, *Staphylotrichum*, *Talaromyces*, *Purpureocillium*,
Penicillium and Aspergillus responded at intermediate levels, and Paecilomyces and Cladorrhinum exhibited delayed responses.

Discussion

Fertilization changed the composition of microorganisms utilizing rice residues

Fertilization regimes significantly changed the microbial community composition involved in rice residue assimilation (Fig. 3, Fig. 4 and Fig. 5). The changes in the composition of the residue-assimilating microbial community are affected by the differences in nutrient content between NPK and NPKM soils (28, 46) as well as by organic matter availability. The modification of the microbial community by fertilization can be direct (addition of microorganisms living in manure) (47) and indirect consisting on: i) pH buffering capacity improved by manure application (48, 49), ii) by addition of diverse available organic compounds or nutrients with manure, and iii) by modification of C:N:P ratios and so, the change of stoichiometric ratio of food resources for microorganisms (50, 51), iv) by increasing and stabilizing soil aggregates and so, modification of microbial habitats (52, 53).

In both NPK and NPKM soils, the rice residue facilitated the growth of specific microbial groups, such as members of Proteobacteria, Actinobacteria, and Ascomycota (Fig. 1 and Fig. 2). Bacterial genera Lysobacter and Streptomyces, which have been described to be involved in the breakdown of potato tissue, wheat straw, rice straw and corn cellulose (26, 29, 54, 55), are also the dominant bacteria utilizing the rice-residue (Fig. 4). Streptomyces is commonly used in industrial applications to produce laccase and xylanase, using rice or wheat straw as substrates (56, 57). Microbial laccase production is usually related to the ability of the microbe to degrade lignin (58), and lignin is also the most difficult decomposable straw component. The higher abundance of Streptomyces in NPK compared to that in NPKM soil may be related to the higher content of recalcitrant components in NPK soil resulting from the 3-year application of mineral-only fertilizers (59).

Most of the fungal groups were associated with the breakdown of cellulose (Fig.
5), as they can produce a variety of cellulolytic enzymes. Fungal genera Trichocladium, Thielavia, Chaetomium and Aspergillus can produce cellulase or xylanase (60-64). Chaetomium and Fusarium have been identified in previous SIP studies as fungal genera that can utilize crop straw or cellulose (21, 22, 65). The application of manure increased the relative abundance of Syncephalis, whereas the relative abundance of Trichoderma was reduced. Trichoderma possess a well-developed cellulase system containing many endoglucanases and two exoglucanases (66, 67). Various organic substances, such as monosaccharides, polysaccharides and chitin has been shown to induce enzyme secretion (68), which may indicate that Trichoderma is more competitive for rice residue than other genera. Trichoderma has superior capacities to mobilize and take up nutrients compared to many other soil microbes, especially in oligotrophic conditions, making it more efficient and competitive (69). Thus, the manure application in NPKM soil during the three years reduced Trichoderma development.

Fertilization changed the co-occurrence patterns of microbes driving C flow from rice-residues

Network analyses provide a tool for identifying co-occurrence patterns and reflecting community organization (70-72) of microbes involved in the $^{13}$C-labeled residue C flow. Most previous studies have explored microbial molecular ecological networks in individual kingdom, such as only fungi or only bacteria, but few showed the co-occurrence patterns between these groups. As microbial groups are not existing in isolation but potentially share niches in the given environment, thus, network analyses on both bacteria and fungi were conducted (Fig. 6a). Linkages in microbial molecular networks can represent covariation between microbes or niche sharing (73). As our analysis might not have enough data points to construct a reliable network at each sample time, the network analyses were based on the combination of the samples across all sampling times for each fertilization regime. Pooled four sampling time points from each fertilization treatment in the network analysis not only can increase sensitivity for co-occurrence events, but also can be able to show the co-occurrence patterns of microbes driving C flow from rice-residues across the time points in each
treated soil. Despite the microbial network will be also changed because of cross-feeding, the cross-feeding (microbes feeding on labeled microbial residues or microbial metabolites) also represents a type of C flow from rice residue through microbial community. It is likely that the $^{13}$C-label was turned over as the experiment progressed and that a second generation of microbial cells became labeled not only from $^{13}$C-labeled rice residue but also from $^{13}$C-labeled microbial metabolites, residues and necromass of $r$ strategists that were formed earlier. Hence, it follows that our DNA-SIP data enabled the probing on C flow from rice residue into microbial communities in soils. Consequently, species that tended to negative correlations in the networks (Fig. 6a) indicate potential reuse of the microbial residue by some other microbes or potential competition in using rice residue. Reversely, any two species with positive correlation reflect the potential cooperative or syntrophic in residue-C utilization (Fig. 6a).

Compared with the network in NPK soil, the network in NPKM soil was much more complex, with higher number of nodes, edges, average clustering coefficient and average degree of the co-occurrences (Table S2, Fig. 6a). The complexity of network is related to the changes of environmental factors, such as soil pH and C availability (31). An increased C input increases the functional complexity of soil microbial networks (31, 74). 3-year manure application fosters greater inter-microbial correlation and/or establish more diverse shared niches, which represents a fundamental difference between NPKM and NPK soil. Keystone taxa acting as hubs or connectors in the network play critical roles in maintaining network structure relative to the other taxa (75). Fungal species, *Jugulospora rotula* in the NPK soil and *Emericellopsis terricola* in NPKM soil, were identified as keystone species (Fig. 6c, TableS3). *Emericellopsis terricola* was proved to have strong cellulase, protease and laminarinase activities (76), which might be an essential factor for organizing microorganisms to cooperate in residue C utilization and led to a positive effect on the residue decomposition in NPKM soil. These keystone species, which are recognized as initiating components in networks, also can selectively alter microbial composition (77). Identifying these keystone groups is critical for predictive understanding of
subsequent potential for microbial-mediated C sequestration when rice residue is returned to soil. To better understand the role of these organisms in co-occurrence networks, uncultured keystone species should be focused in future work.

Bacteria and fungi connected to each other in the network, and the organic amendment changed the ratio of the edges between bacteria and fungi to the total edges (Fig. 6b). In general, recalcitrant organic compounds in the residues (such as lignin and cellulose) are mainly degraded by fungi, and the released water-soluble substances can be utilized also by bacteria (78). Thus, fungi provide bacteria with resources that they cannot directly make from residues themselves, such as intermediate decomposition products. The 3-year input of manure increased C sources and their availability in the soil, including some soluble organics, which reduces the dependence of bacteria on fungi (79). In the NPK soil, which did not receive manure during the three years, bacteria will strengthen their connection with fungi to get more soluble organics from residue decomposition. Thus, compared to NPK soil, NPKM fertilization decreased the ratio of edges between fungi and bacteria to the total edges (Fig. 6b). Therefore, the results provide some evidences for that the co-occurrence patterns between the microbes are impacted by 3-year manure amendment. However, network co-occurrence patterns are ultimately based on statistical correlations, and they must be interpreted with caution, as correlation does not directly demonstrate real microbial interactions. The more solid experimental evidence including more reasonable inferring methods or promising culturing approaches is still needed to explore complex ecological relationships in natural conditions and assess the effect of keystone species.

Response strategies of microbes by utilization of rice-residues depending on fertilization

Plant residues contain complex organic compounds, such as water-soluble substances (low molecular weight organic substrates), polymeric carbohydrates (hemicellulose and cellulose), lignin and phenolics (80-82). Each component can be decomposed or utilized mainly by specific taxonomic groups of microorganisms. Thus, rice-residue
degradation could be linked to a continuous change of microbial beta diversity over time (Fig. 3). The dynamics of microbial community structure was often related to the chemical composition of straw (83, 84). Decomposition of easily degradable components can stimulate the growth of early $r$-strategists (85), and as the availability of resources decreases, the relative abundance of microorganisms within this group will gradually decrease, while the late $K$-strategist groups will gradually come to dominate (86). Thus, decomposition of labile components during the first week leads to the rapid growth of certain groups of fungi and bacteria, such as the bacterial genera *Devosia*, *Glycomyces* and *Kribbella*, and then the relative abundance of these groups decreases as the liable organic components are gradually consumed (Fig. 7). The relative abundances of *Rhodanobacter*, *Actinomadura*, *Streptomyces* and *Nonomuraea* increased gradually, as these bacteria could decompose recalcitrant components in the later succession (Fig. 7). However, since the classification the response strategies was at the genus level, thus little can be known whether these members were directly involved in rice-residue degradation or only indirectly by cross-feeding on the intermediate or metabolic products and necromass of the $r$ strategists.

Bacterial groups had similar response strategies in the two fertilized soils by assimilation of rice-residue-derived C. The pure culture methods have shown that bacteria could not grow on cellulose or crop residues unless they were cultured together with fungi (87). Thus, bacteria rely on fungi and their oxidative enzymes when using substrates that are difficult to decompose. Consequently, the oxidative exoenzymes secreted by fungi (e.g. (per)oxidases) degrade the recalcitrant components into low molecular weight organic substrates that can be further utilized by bacteria (Fig. 7). Thus, no significant differences in the response strategies of the bacteria were found between the two fertilization regimes. However, the response strategies of the fungal genera were quite different between NPK and NPKM soils, which may be due to changes in the composition of the rice residue during the degradation (87).
Conclusions

The DNA-SIP approach enabled to identify the key bacterial and fungal taxa microbes involved in the rice residue decomposition and utilization in soils under NPK and NPKM fertilization. The C from the rice residues was predominantly incorporated into bacteria phyla of Proteobacteria and Actinobacteria and fungal phyla of Ascomycota. Fertilization regimes had strong effects on microbial community composition, microbial response strategies and microbial interactions involved in the C flow from rice-residues. Since complete residue degradation involves multiple enzymatic steps that are conducted by many community members, our community-based analyses detected the microbial groups using the residues itself and the secondary products (metabolites) of initial residue cleavage. Bacterial response strategies for assimilating residue were independent on fertilization regimes. However, most of fungal genera in soil receiving manure responded faster to residue addition than those in soil receiving solely mineral fertilizers. This is connected with the changes in the composition of the rice residue during the degradation and with fungal adaptation (abundance and activity) to continuous manure input. This is confirmed by more complex microbial network involved in the flow of rice-residue-derived C in NPKM than that in NPK soils. Presumably, fungi release enzymes decomposing recalcitrant organic compounds in the residues (e.g. cellulose and lignin) and bacteria subsequently use the available soluble compounds produced by fungal exoenzymes. Future studies should be designed to discriminate the cross-feeding C flow between the species, to understand mechanistic interactions of soil microbes that are directly and indirectly involved in residue-derived C utilization at any time point.

Materials and methods

Rice residue labeling

Rice seeds (Oryza sativa cv. Zhendao11) were sterilized by exposure to 30% hydrogen peroxide for 30 minutes. Following washing, the rice seeds were cultured in the dark until germination. The seedlings were transferred into a transparent air-tight
chamber and grown under hydroponic conditions with Hogland nutrient solution. The chamber was continually injected with $^{13}$CO$_2$ (99% atom, Cambridge Isotope Laboratories, Inc.) at a concentration of 400 µL L$^{-1}$. Additionally, before $^{13}$CO$_2$ injection, high-purity air, which contained N$_2$ (70% v/v) and O$_2$ (30% v/v), was used to flush the chamber to remove the $^{12}$CO$_2$. The environmental parameters of the illumination incubator were set as 12 h photoperiod, temperature of 30 °C day/20 °C night, and relative humidity of 70% day/80% night. Following 30 days of labeling, the above parts of the rice seedlings (including leaves and stems) were harvested and had a $^{13}$C atom percentage of 46-48%. The rice residue was oven dried to constant weight at 65 °C for further use.

Soil microcosm set-up

Soils used for this study were collected from an experimental farm in Rugao, Jiangsu, China (120°51'E, 32°00'N). Rice (Oryza sativa cv. Zhendao11) was rotated annually with winter wheat (Triticum aestivum cv. Yangmai16). Soil samples were collected from a short-term (3-year) experimental site that included treatment with solely mineral fertilizers (NPK) and treatment with mineral fertilizers plus manure (NPKM). The NPK-treated soil annually received 440 kg N ha$^{-1}$ applied as urea (240 kg N ha$^{-1}$ for rice season and 200 kg N ha$^{-1}$ for wheat season), whereas the NPKM-treated soil similarly received a total of 440 kg N ha$^{-1}$ in both season, but of which 80% was from urea and 20% was from 7.41 tons ha$^{-1}$ compost of pig manure. Both the NPK and NPKM treatments were applied annually with 128 kg P ha$^{-1}$ and 116 kg K ha$^{-1}$ as calcium superphosphate and potassium chloride, respectively. Each treatment was applied to three plots, which served as three replicates. Soils were collected from the top 0–20 cm after rice harvest in October, 2015. Three soil samples for each fertilization treatment were obtained from the corresponding plots. Each soil sample consisted of 10 soil cores that were then sieved through 2 mm sieves. The NPK-treated soil had pH of 6.34, 20.34 g SOM kg$^{-1}$, 1.22 g total N kg$^{-1}$, 12.97 mg Olsen-P kg$^{-1}$ and 92.00 mg NH$_4$OAc-K kg$^{-1}$; the NPKM-treated soil had pH of 6.68,
Each soil sample was subjected to three microcosm treatments including negative control (soil without rice residue), positive control (soil with $^{12}$C-labeled rice residue) and $^{13}$C-treatment (soil with $^{13}$C-labeled rice residue), and each microcosm treatment group contained six replicates. Each replicate comprised a microcosm of 10 g (oven-dried basis) of soil in a 125-ml hermetically sealed serum bottle. In total, 36 microcosms [(2 fertilization treatments × 3 replicates) × (3 microcosm treatments × 2 replicates)] were employed in the incubation experiment. All microcosms were subjected to a 14-day preincubation with soil water content adjusted to 60% of the soil maximum water holding capacity and with incubation temperature at 25°C before beginning the experiments. This pre-incubation adjusted all the treatments to the environmental conditions used in the incubation stage to make the rice residue addition the unique variable in the microcosms incubations. After preincubation, 0.05 g of $^{12}$C-labeled rice residue or $^{13}$C-labeled rice residue (5 mg g$^{-1}$ d.w.s.) was thoroughly amended into the positive control or $^{13}$C-treatment soils, respectively. For each microcosm treatment, one was used for the measurement of microbial respiration and the remaining one was used for sampling for DNA isolation. Sterile water was supplied to each microcosm to adjust the soil moisture content to 60% of the soil maximum water holding capacity. All soil microcosms were incubated at 25°C in the dark for 60 days, with daily adjustment of the soil water content. Soil samples used for DNA extraction were collected after 7, 15, 30, and 60 days of incubation.

**Measurements of soil respiration**

The measurement of microbial respiration was conducted at day 1, 7, 15, 30, 60 after the addition of rice residue. The vials were closed with an airtight butyl rubber stopper and incubated for 12 h at 25 °C. Afterwards, the headspace CO$_2$ concentration was analysed by a gas chromatograph with a thermal conductivity detector operating at 60 °C (Agilent 7890, Santa Clara, CA, USA). Separation was performed using a 177/149 mm (80/100 mesh) Chromosorb 102 column (Advanced Minerals, Santa
Barbara, CA, USA) at 40 °C. The temperature of the injecting port was 100 °C. The carrier gas (H₂) flow rate was 80 ml min⁻¹.

DNA extraction and gradient centrifugation

Soil DNA was extracted by the FastDNA Spin Kit for Soil (MP Biomedicals, Cleveland, OH, USA) according to the manufacturer’s protocol. A Nanodrop ND-2000 UV–vis Spectrophotometer (Nano Drop, ND2000, Thermo Scientific, Wilmington, DE) was used to measure the concentration and quality of extracted soil DNA. DNA density gradient centrifugation and fractionation was performed according to the methods of Kong (88). Briefly, 3 µg DNA was added into 1.85 g mL⁻¹ of CsCl gradient buffer (0.1 M Tris-HCl, 0.1 M KCl, 1 mM EDTA, pH = 8.0) with an initial CsCl buoyant density of 1.725 g mL⁻¹, which was prepared by adjusting the refractive index to 1.4025 with an AR200 digital hand-held refractometer (Reichert Inc., Buffalo, NY, USA). Density gradient centrifugation was performed in a 5.1-mL Quick-Seal polyclamor ultracentrifugation tube (Beckman Coulter, Palo Alto, CA, USA) in a VTi 65.2 rotor (Beckman Coulter), which was subjected to centrifugation at 177000 g (45000 r.p.m.) for 44 h at 20 °C (Beckman Coulter, Optima-XPN-100, USA). Centrifuged gradients were fractionated into 14 equal volumes (~340 µL) by displacing the gradient medium with sterile water at the top of the tube using a syringe pump (Longer Pump. LSP01-1A. China). Then, 50 µL aliquots were used to measure the refractive index to determine the buoyant density of each collected fraction. Fractionated DNA was precipitated from CsCl by adding 500 µL precipitating agent (30% PEG 6000 and 1.6 M NaCl), incubating for 1 h at 37 °C and then washing two times with 70% ethanol. The samples were then dissolved in 30 µL of Tris-EDTA buffer.

Real-time PCR quantification

Quantitative real-time PCR was performed on an ABI 7500 real-time PCR system (Applied Biosystems, America) to determine the abundances of bacterial 16S rRNA
genes and fungal ITS1 using primer pairs 338F/518R (338F: 5’-ACTCCTACGGGAGGCAGCAG-3’/ 518R: 5’-ATTACCCGCGCTGCTGG-3’) (89) and ITS1/ 5.8s (ITS1: 5’-TCCGTAGGTAACCTCGG-3’/ 5.8s: 5’-CGCTGCGTTCCTTCATCG-3’) (90, 91), respectively, in the total soil DNA and fractionated DNA. Each reaction was performed in 25 µL total volume containing 12.5 µL SYBR Premix Ex Taq (TaKaRa Biotechnology, Otsu, Shiga, Japan), 0.5 µL of each primer (10 µM), 0.5 µL of ROX Reference Dye II (50×), 1 µL of DNA template (2-20 ng) and 10.5 µL of sterile water. Amplification conditions were as follows: 95 °C for 5 min, 40 cycles of 15 s at 95 °C, 34 s at 64 °C and a final temperature increase to 95 °C for 15 s. Data were collected after each annealing step. One PCR product for each bacterial 16S rRNA gene and fungal ITS1 was gel purified using an Axygen PCR purification kit (Axygen Bio, USA), and the fragments were cloned separately into the pMD19-T vector (TaKaRa Cloning ® Kit). Then, ligated plasmid was transformed into competent DH5α Escherichia coli cells (TaKaRa). The re-amplification and sequencing of white-positive clones using the vector-specific primers M13f/M13r were used to identify the correct inserted DNA fragments. Plasmids with correct inserts were extracted using the Axygen Plasmid Extraction Kit (Axygen Bio, USA) for use as plasmid standards. A 10-fold dilution series of the plasmid standards was used to generate the standard curves. The PCR efficiency and correlation coefficients for the standard curves were 95.68% and R² = 0.9977, respectively, for 16S rRNA genes and 96.11% and R² = 0.9978, respectively, for ITS1. The specificity of the amplification products was checked using melt curve analysis at the end of each PCR run and was then confirmed by standard agarose gel electrophoresis.

High-throughput sequencing analysis

Based on the relative abundance distribution patterns of the bacterial 16S rRNA genes and fungal ITS1 in 14 CsCl gradients, heavy fractions of the 13C-treatment samples and the corresponding fractions from the positive controls were chosen for sequencing.
In total, 24 DNA samples (2 soils × 3 replicates × 4 sampling time points) were collected from the 13C-treatment heavy fractions and 24 DNA samples (2 soils × 3 replicates × 4 sampling time points) were collected from the corresponding positive control fractions. Sequencing of the V4-V5 regions from bacterial 16S rRNA genes (92) and of the fungal ITS1 (93) was conducted using an Illumina MiSeq System (Roche, Switzerland) at the Nanjing Genepioneer company (Nanjing, China). The raw data were processed using the Quantitative Insights Into Microbial Ecology (QIIME) toolkit (94) and UPARSE standard pipeline (95). Reads with a quality score less than 20 and length less than 220 bp were discarded. Paired reads were merged using the “fastq_mergepairs” module of USEARCH (95, 96), pairs with mismatches in the overlap were eliminated, and only sequences >220 bp in length were included in subsequent analyses. Then singletons were discarded using UPARSE. In addition, SILVA reference database was used to identify and remove the putative chimeras.

Taxonomic assignment was accomplished by clustering the sequences into operational taxonomic units (OTUs) at a 97% similarity level. Each sample was rarefied to the same number of reads (18,000 16S rRNA reads and 20,000 ITS1 reads) for downstream analyses using Mothur software. One representative sequence from each OTU was selected using the UPARSE pipeline, and in total 5029 bacterial OTUs and 1864 fungal OTUs were obtained in the OTU table (Table S4). Each representative sequence was assigned to an RDP classifier for identification using a threshold of 0.8. The sequences obtained in this study have been submitted to NCBI SRA under the accession numbers PRJNA520803 and PRJNA518924.

Statistical analysis

The OTUs involved in the decomposition and assimilation of 13C-labeled residue were filtered by comparing their relative abundances in the heavy fraction of 13C-treatment samples to those in the positive control samples using R software with the DESeq 2 package (97, 98). Unfortunately, the 16S rRNA sequencing data for one replicate in the NPKM positive control at 30 d was observed to be of much lower
quality; thus, the mean of the NPKM positive control at 30 d was calculated based on
the remaining 2 replicates for this analysis. Based on the result that the atomic
percentage of $^{13}$C in the rice residue in the present study was lower than 50%, both the
padj value (FDR adjusted $P$ value) and the log2 fold change value were strictly
controlled in subsequent analyses. The $^{13}$C-labeled OTUs were defined as the OTUs
with padj values lower than 0.01 and log2 fold change values higher than 1.
Nonmetric multidimensional scaling (NMDS) based on Bray-Curtis distances of all
labeled OTUs and the PERMANOVA test were performed using R (2.15.0) with the
Vegan package. $^{13}$C-labeled OTUs with relative abundances higher than 1% were
defined as the dominant OTUs involved in $^{13}$C-labeled residue decomposition and
assimilation. Only genera that passed our filters for significant changes over time by
ANOVA were included the assessment of response strategies for rice residue
assimilation. Labeled OTUs observed in four sampling time points from each
fertilization treatment were pooled to assess the roles of the microbes in driving the
flow of rice-residue-derived carbon using molecular ecological networks. The labeled
OTUs with average relative abundances greater than 0.1% were selected to construct
molecular ecological networks using the Random Matrix Theory (RMT) (99), and the
networks were visualized by Gephi (0.9.2). The threshold values of $Z_i$ (within-module
connectivity) and $P_i$ (among-module connectivity) for categorizing OTUs in networks
are 2.5 and 0.62, respectively (75, 100). Furthermore, three categories that are
organised as module hubs ($Z_i > 2.5$), connectors ($P_i > 0.62$) and network hubs ($Z_i >
2.5$ and $P_i > 0.62$) in the networks are defined as keystone microbes in the
decomposition and assimilation of $^{13}$C-labeled rice residue. Origin 8.0 was used to
draw the histogram, variance analysis was conducted using SPSS software (SPSS
16.0 for Windows, IBM Corp., Armonk, NY, USA), and $P < 0.05$ was defined as
statistically significant.

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Figure captions:

Fig. 1 Effects of fertilization on the number (a) and relative abundance at the phylum level (b) of the $^{13}$C-labeled bacterial OTUs. The values 7D, 15D, 30D and 60D represent the sample time (day) after the addition of the rice residues. NPK indicates soils with mineral-only fertilizers and NPKM indicates soil with mineral fertilizers combined with manure.

Fig. 2 Effects of fertilization regimes on the number (a) and relative abundance at the phylum level (b) of the $^{13}$C-labeled fungal OTUs. The values 7D, 15D, 30D and 60D represent the sample time (day) after the addition of the rice residues. NPK indicates soil with mineral-only fertilizers and NPKM indicates soil with mineral fertilizers combined with manure.

Fig. 3 NMDS analyses of the $^{13}$C-labeled bacterial communities (a) and fungal communities (b) involved in the utilization of rice residues. The values 7D, 15D, 30D and 60D represent the sample time (day) after the addition of the rice residues. NPK indicates soil with mineral-only fertilizers and NPKM indicates soil with mineral fertilizers combined with manure.

Fig. 4 Dominant OTUs (relative abundance higher than 1%) in the bacterial communities involved in the C flows from rice residues. The size of the circle represents the relative abundance of the OTU. The color of the circle represents the fertilization treatment, with green for NPK and purple for NPKM. The values 7D, 15D, 30D and 60D represent the sample time (day) after the addition of the rice residues. The yellow asterisk (*) at right of the circle represents instances wherein the relative abundance was higher in the indicated fertilization treatment than in the other. The green line represents bacterial phylum Actinobacteria, and the orange line represents Proteobacteria. The histogram on the right represents the average relative abundance of the dominant genera at the four sampling time points.

Fig. 5 Dominant OTUs (relative abundance higher than 1%) in the fungal
communities involved in the C flow from rice-residues. The size of the circle represents the relative abundance of the OTU. The color of the circle represents the fertilization treatment, with green for NPK and purple for NPKM. The values 7D, 15D, 30D and 60D represent the sample time (day) after the addition of the rice residue. The yellow asterisk (*) at right of the circle represents instances wherein the relative abundance was higher in the indicated fertilization treatment than in the other. The green line represents Zoopagomycotina and the orange line represents Ascomycota. The purple line represents uncultured fungus. The histogram on the right represents the average relative abundance of the dominant genera at the four sampling time points.

Fig. 6 Molecular ecology network analyses of microbes involved in the C flow from rice residues in soils depending on fertilization regimes. Labeled OTUs observed in four sampling time points from each fertilization treatment were pooled to construct networks that represent random matrix theory co-occurrence models. (a) Networks in soils treated with NPK or NPKM. The circles represent nodes. Circles with red edge represent bacterial OTUs, whereas circles with black edge represent fungal OTUs. Lines connecting two nodes represent the significant correlations between OTUs. Red lines represent significant positive correlation and blue lines represent significant negative correlations. (b) Proportion of inter- (edges between bacterial OTU and bacterial OTU, or between fungal OTU and fungal OTU) and intra-kingdom edges (edges between bacterial OTU and fungal OTU) in the networks of NPK- and NPKM-fertilized soils. (c) The distribution of $Z_i$ and $P_i$ values for the OTUs established from their module-based topological roles. Symbols represent OTUs in NPK (circle) or NPKM (triangle) network. The threshold values of $Z_i$ and $P_i$ for categorizing OTUs were 2.5 and 0.62, respectively (75, 100).

Fig. 7 Conceptual diagram of microbial groups responsible for utilization and decomposition of rice residues in soils depending on fertilization: NPK- mineral fertilizers, NPKM- mineral fertilizers combined with manure. The line colors
correspond to the colors of the bacterial and fungal groups response strategies in rice residue utilization. Red dashed line represents rapid responders (genera that displayed highest relative abundance at the outset (7 d after residue addition)); Green dashed line represents intermediate responders (genera that displayed highest relative abundance at intermediate sample time points (between 15 d and 30 d)); Purple dashed line represents delayed responders (genera that displayed highest relative abundance at 60 d after residue). The bottom parts reflect the C availability and predominance of microbial groups. The vertical arrows going from fungi to bacteria in the lower part of the figure represent the interactions between them in residue utilization, such as some carbon resources provided by fungi to bacteria (see detail in below). As time goes on, the proportion of labile C in rice residue decreased, while the proportion of recalcitrant C increased gradually. And most bacteria had weak capacity to decompose recalcitrant components in the later succession. Intermediate or delayed fungal genera responders can provide bacteria with resources that they cannot directly make from residues themselves, such as intermediate decomposition products (see detail in text).
a

NPK: 12

NPKM: 33

b

Relative abundance (%)

- Ascomycota
- Basidiomycota
- Zoopagomycotina
- Unclassified

7D 15D 30D 60D

NPK NPKM
