



1 **Title: Straw return with diverse nitrogen fertilizer application rates modulate ecosystem services**

2 **and microbial traits in a meadow soil**

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21 **Abstract:**

22 Nitrogen (N) fertilization has received worldwide attention due to its benefits to soil fertility and  
23 productivity, but excess N application also causes an array of ecosystem dis-services, such as  
24 greenhouse gas emissions. Generally, soil microorganisms are considered to be involved in upholding a  
25 variety of ecosystem services and dis-services. However, the linkages between soil ecosystem services  
26 and microbial traits under different N fertilizer application rates remain uncertain. To address this, a  
27 4-year in situ field experiment was conducted in a meadow soil on the Northeast China Plain after  
28 straw return with the following treatments combined with regular phosphorus (P) and potassium (K)  
29 fertilization: (i) regular N fertilizer (N+PK); (ii) 25% N fertilizer reduction (0.75N+PK); (iii) 50% N  
30 fertilizer reduction (0.5N+PK); and (IV) no N fertilizer (PK). Ecosystem services, dis-services and  
31 microbial traits responded distinctly to the different N fertilizer rates. Treatment 0.75N+PK had overall  
32 positive effects on soil fertility, productivity, straw decomposition, and microbial abundance and  
33 function and alleviated greenhouse effects due to N deficiency. Meanwhile, 0.75N+PK upregulated  
34 aboveground biomass and soil C:N and thus increased the abundance of genes encoding  
35 cellulose-degrading enzymes, which may imply the potential ability of C and N turnover. In addition,  
36 most observed changes in ecosystem services and dis-services were strongly associated with microbial  
37 modules and keystone taxa. Specifically, the *Lasio-sphaeriaceae*-driven module 1 community promoted  
38 straw degradation and C and N release, while the *Terrimonas*-driven module 3 community contributed  
39 to production improvement, which was conducive to soil multifunctionality. Therefore, our results  
40 suggest that straw return with 25% chemical N fertilizer reduction is optimal for achieving ecosystem  
41 services. This study highlights the importance of abiotic and biotic factors in soil health and supports  
42 green agricultural development by optimizing N fertilizer rates in meadow soil after straw return.



43

44 **Keywords:** Ecosystem services; Straw return; Nitrogen fertilization; Microbial community; Crop yield

45

## 46 **1. Introduction**

47 Multiple soil ecosystem services are indicators of soil health (Kihara et al., 2020; Lehmann et al.,  
48 2021). Soil ecosystem services refer to the ability of soil to function as a vital living system to  
49 sustainably increase crop productivity, improve environmental quality, tackle climate change and  
50 promote plant and animal health (de Bello et al., 2010; Tang et al., 2019). In recent decades,  
51 anthropogenic activity, such as intensive agriculture, has posed a wide range of threats to  
52 agroecosystem services (Robertson et al., 2014; Allen et al., 2015). Irrational or excessive application  
53 of chemical fertilizers, especially nitrogen (N), is ubiquitous to achieve high crop yields in response to  
54 population surges globally (Shi et al., 2019). In fact, N is considered the essential macronutrient for all  
55 biota, while excessive N fertilizer inputs not only reduce soil fertility and productivity but also lead to  
56 environmental burdens (Trost et al., 2016). For example, previous studies emphasized that N fertilizer  
57 abuse may accelerate greenhouse gas emissions (Huang et al., 2006; Wu et al., 2015) and degrade  
58 groundwater quality (Rhymes et al., 2016). Therefore, how to achieve agroecosystem services by  
59 regulating N fertilizer application rates is a critical issue that needs to be fully assessed.

60 Straw return has also been widely applied as a major measure to moderate soil ecosystem services  
61 (Xu et al., 2021). Plant residues, as natural organic bioenergy resources, contain abundant N that  
62 further affects soil fertility and productivity (Pan et al., 2009; Liu et al., 2014). Thus, the straw-derived  
63 N released during degradation is an important source that may serve as a partial substitute for chemical  
64 N fertilizer application (Wang et al., 2017; Latifmanesh et al., 2020). However, crop fields suffering



65 from abundant organic materials usually have low reutilization efficiency (Hou et al., 2020). Generally,  
66 the majority of N in straw is released into the atmosphere as oxynitride, such as nitrous oxide (N<sub>2</sub>O),  
67 resulting in lower soil organic matter (SOM) formation efficiency (Wang et al., 2019; Sun et al., 2021).  
68 Subsequent literature highlighted that straw return significantly elevates greenhouse gas emissions so  
69 that less than 15% of straw-derived N can be transformed into soil and become SOM (Yin et al., 2018;  
70 Wu et al., 2019). However, the potential for the partial substitution of straw for chemical N fertilizer  
71 application is still unclear. Revealing the mechanisms of efficient straw utilization under diverse N  
72 fertilizer input rates is essential to achieving ecosystem multifunctionality.

73 Compared with plants and animals, soil contains more microorganisms living in an opaque  
74 environment, making the evaluation of soil ecosystem services more complex (Handa et al., 2014;  
75 Wagg et al., 2014). Agronomic management for such “multifunctionality” has prompted research into  
76 the role that microbes play in providing desired rates of multiple ecosystem processes (Gong et al.,  
77 2020). To our knowledge, fertilization-induced changes in microbial communities and functions are  
78 fundamental to the regulation of a variety of ecosystem multifunctionalities, including SOM formation,  
79 greenhouse gas emissions, litter decomposition, and crop production (Dominati et al., 2014). To date,  
80 we still lack empirical evidence of the linkages among N fertilizers, specific microbial communities or  
81 functions and multiple ecosystem services, and the diverse cropland services driven by complex  
82 microbial traits under different N fertilizer rates are seldom clarified.

83 Microorganisms contribute to ecosystem services by modulating microbial function, community  
84 composition and succession, which makes understanding the consequences of the changes in microbial  
85 traits crucial for determining different N fertilizer input levels (Bradford et al., 2014). The role of  
86 microorganisms in ecosystem functioning is unequivocal, and these organisms can be recognized as the



87 key drivers of ecosystem services (Chen et al., 2019a). Generally, bacteria and fungi are the main  
88 drivers of straw labile and recalcitrant component decomposition, respectively (Frey et al., 2013; Ge et  
89 al., 2017). Therefore, the ratio of fungi to bacteria is always considered an indicator during straw  
90 degradation periods (Hogberg et al., 2007). Specifically, the expression levels of the *cbhI* and *GH48*  
91 genes were identified as biomarkers of cellulolytic fungi and bacteria, respectively (Zhang et al., 2017).  
92 Previous studies revealed that the N input level dominated the associations between microbial  
93 composition and cellulolytic gene abundance with SOM physical fractions (Duan et al., 2021). In  
94 addition, microbial module communities and keystone taxa have been used to provide satisfactory  
95 explanations for ecosystem services. Chen et al. (2019b) found that particular microbial modules  
96 participated in N and phosphorus (P) accumulation and CO<sub>2</sub> emissions in a Cambisol. Moreover,  
97 specific taxa are involved in agrosystem services. For example, Actinobacteria have been extensively  
98 studied and can be considered the main degraders of straw by secreting cellulase (Bao et al., 2021).  
99 *Mortierella* has been proven to increase soil fertility and crop yield due in part to its strong C  
100 sequestration capacity (Ning et al., 2020). Notably, it is also well known that microbial traits are  
101 mediated by nutrient availability and stoichiometry (Chen et al., 2014). C, N and P stoichiometry has  
102 profound impacts on microbial in vivo metabolism and ex vivo modification processes (Chen et al.,  
103 2016). Multiple studies have indicated that soil C:N and N:P ratios are the key factors mediating  
104 microbial functions and soil health (Ning et al., 2020; Duan et al., 2021). Nevertheless, the knowledge  
105 of the microbial mechanisms that modulate ecosystem services in response to N fertilizer input levels  
106 are still rudimentary.

107 As an important grain-producing region, the Northeast China Plain contributes to more than 20%  
108 of the total grain yield in China (Li et al., 2017). However, excessive chemical N fertilizer inputs have



109 caused ecosystem dis-services over the past decades (Zhao et al., 2018). Therefore, a field experiment  
110 was conducted to reveal the influences of N input levels on soil ecosystem multifunctionality and  
111 associated microbial traits and to try to establish the linkages between them. In the present study, two  
112 hypotheses were tested: (i) soil ecosystem services and dis-services would show distinct responses to N  
113 fertilizer input levels, and (ii) the changes in cropland ecosystem services and dis-services would be  
114 linked to specific microbial traits.

115

## 116 2. Materials and methods

### 117 2.1 Site description and sampling

118 A field experiment under contrasting inorganic N fertilizer input levels was established in 2018 in  
119 Wenchun town (44°59'61" N, 129°59'18" E), Mudanjiang city, Heilongjiang Province, Northeast China  
120 Plain, which is an important grain-producing area. This region has a typical temperate continental  
121 monsoon climate with an average annual temperature of 4.3 °C and a mean annual precipitation of  
122 579.7 mm. The soil is classified as a meadow soil according to US Soil Taxonomy (USST). The  
123 cropping system was continuous maize (*Zea mays* L.) monoculture. Four treatments received different  
124 N fertilizer input levels after straw return to the field for 4 years as follows: (1) regular chemical  
125 fertilization, N+PK (300 kg urea (N 46%) ha<sup>-1</sup> yr<sup>-1</sup>, 250 kg diammonium phosphate (P<sub>2</sub>O<sub>5</sub> 48%) ha<sup>-1</sup> yr<sup>-1</sup>,  
126 150 kg potassium chloride (K<sub>2</sub>O 50%) ha<sup>-1</sup> yr<sup>-1</sup>); 25% reduction of N fertilizer, 0.75N+PK (225 kg urea  
127 ha<sup>-1</sup> yr<sup>-1</sup>, 250 kg diammonium phosphate ha<sup>-1</sup> yr<sup>-1</sup>, 150 kg potassium chloride ha<sup>-1</sup> yr<sup>-1</sup>); 50% reduction  
128 of N fertilizer, 0.50N+PK (150 kg urea ha<sup>-1</sup> yr<sup>-1</sup>, 250 kg diammonium phosphate ha<sup>-1</sup> yr<sup>-1</sup>, 150 kg  
129 potassium chloride ha<sup>-1</sup> yr<sup>-1</sup>); and no N fertilizer, PK (250 kg diammonium phosphate ha<sup>-1</sup> yr<sup>-1</sup>, 150 kg  
130 potassium chloride ha<sup>-1</sup> yr<sup>-1</sup>). All straw and chemical fertilizers were applied with shallow tillage to 20



131 cm. Straw was cut into pieces less than 5 cm and input after the harvest in October, while the chemical  
132 fertilizers were applied during ploughing in May of the next year. All other normal management  
133 practices were consistent among treatments during the experiment. Before the experiment, the initial  
134 soil contained 18.74 g kg<sup>-1</sup> SOC, 1.03 g kg<sup>-1</sup> total N and 0.54 g kg<sup>-1</sup> total P with a pH of 7.37 (H<sub>2</sub>O).  
135 The yield and some of the soil chemical properties under different bulk soil treatments during the  
136 experimental process are shown in Supplemental material [Table S1](#).

137 Soils were sampled after the maize harvest in October 2021. A randomized complete block design  
138 consisting of 5 treatments with 3 replications was adopted in this study. Each field plot was 4.5 m × 15  
139 m. We took nine soil cores (5 cm diameter) from the top 20 cm of bulk soil in each plot. Each soil  
140 sample consisted of a mixture of subsamples randomly collected at nine different positions in the same  
141 plot. In total, 12 soil samples were collected from 4 treatments with 3 replicates. Soils were sieved  
142 through a 2 mm mesh, the mineral particles and plant roots were carefully removed, and then the soils  
143 were homogenized and stored in an incubator at 4 °C in a 40% moisture environment. One part of the  
144 soil sample was air-dried to measure basal soil properties, and the other part was used for microbial  
145 molecular analysis.

## 146 **2.2 The field straw decomposition and carbon and nitrogen release experiments**

147 The ditch-buried straw decomposition experiment was conducted using litter nylon bags. Maize  
148 straw materials were collected after maize harvesting in 2021 and air-dried. Ten grams of maize straw  
149 was cut to 2 cm in length and put into nylon litter bags, which were then sealed via heat sealing. The  
150 nylon bags were 6 cm × 10 cm in size and were made of 200 mesh nylon fabric, which permitted the  
151 free transfer of microorganisms between the nylon bags and soil. Before maize cultivation in 2021,  
152 litter bags containing straw were buried at 10 cm depth in a spatially random design to prevent bags



153 associated with a given decomposition stage being placed together in space. The litter bags were  
154 collected after the harvest in October 2021.

155 The straw decomposition ratio was calculated based on dry weight loss as (dry initial mass - dry  
156 final mass)/dry initial mass. The straw-C concentration was measured by titrimetry after oxidation with  
157 a mixture of H<sub>2</sub>SO<sub>4</sub> and K<sub>2</sub>Cr<sub>2</sub>O<sub>7</sub>. Total N, P and K were determined using the Kjeldahl, molybdenum  
158 blue colorimetry, and flame photometry methods, respectively. All methods have been described by Lu  
159 (2000). The initial and sampled maize straw material properties are shown in Supplemental material  
160 Table S2. The amounts of total straw C and N released were calculated by the following equation:

161 The amounts of total straw C and N released = (initial C (or N) content × dry initial mass - final C  
162 (or N) content × dry final mass) × aboveground biomass

### 163 2.3 Measurement of soil properties and assessment of ecosystem services

164 Soil pH was measured at a soil:water ratio of 1:2.5 (weight/weight). Air-dried soil and 25 ml of  
165 deionized water were shaken together for 1 min and left to settle for 30 min, and the soil pH was  
166 determined using an electrode. Soil organic carbon (SOC) was measured by titrimetry after oxidation  
167 with a mixture of H<sub>2</sub>SO<sub>4</sub> and K<sub>2</sub>Cr<sub>2</sub>O<sub>7</sub>. Total N and P were determined using the Kjeldahl and  
168 molybdenum blue colorimetric methods, respectively. All of these methods have been described by Lu  
169 (2000).

170 Microbial biomass C (MBC) and microbial biomass N (MBN) were analysed using the  
171 fumigation-extraction method. Ten grams of fresh soil was fumigated with chloroform in the dark for  
172 24 h, and then the fumigated and nonfumigated soils were extracted with 0.5 M K<sub>2</sub>SO<sub>4</sub> and shaken at  
173 200 rpm for 0.5 h. Soil extracts were filtered through a 0.45-µm Millipore filter, and the C and N in the  
174 extracts were determined using a multi C/N 3100 analyser (Analytik Jena AG). The C and N contents





175 in extracts of the nonfumigated soil were subtracted from C and N extracted from the fumigated soil to  
176 give the C and N extracted from the soil microbial biomass. Values of 0.45 and 0.54 were used to  
177 calibrate the contents of MBC and MBN, respectively (Vance et al., 1987; Wu et al., 1990).

178 The activities of cellulose and N-acetyl- $\beta$ -glucosaminidase (NAG) were measured using  
179 *p*-nitrophenyl- $\beta$ -D-cellobioside and *p*-nitrophenyl-N-acetyl- $\beta$ -D-glucosaminide as substrates,  
180 respectively. Fresh soil (1.0 g) was mixed with 2.5 mL of 0.2 M acetate buffer (pH 5.0) and 2.5 mL of  
181 0.02 M substrates and then shaken at 200 rpm and 37 °C for 1 h. The reaction was stopped by adding 1  
182 mL of 0.5 M CaCl<sub>2</sub> and 4 mL of 0.1 M Tris buffer (pH 12.0). The mixture was suspended with a vortex,  
183 the supernatant was filtered, and the concentration of *p*-nitrophenol (PNP) was measured by  
184 colorimetry at 400 nm. The same procedure was followed for the controls, with the exception that the  
185 substrate was added after the incubation, and CaCl<sub>2</sub> and Tris buffer were added (Dick, 2011; Geisseler  
186 and Horwath, 2009).

187 To estimate the greenhouse gas emission potential, we conducted a 60-day incubation experiment.  
188 Briefly, 20 g of fresh soil was placed in a 250-mL flask and then sealed with a gas-tight lid that had a  
189 rubber stopper in the middle. Gas samples (10 mL) were taken from the headspace of each flask at 1, 3,  
190 7, 15, 30, and 60 days after sealing using a plastic syringe. The gas sample was immediately injected  
191 into a preevacuated 10-mL glass vial. Concentrations of methane (CH<sub>4</sub>), N<sub>2</sub>O and carbon dioxide (CO<sub>2</sub>)  
192 were determined using a gas chromatograph (Agilent 7890) equipped with a flame ionization detector  
193 for CO<sub>2</sub> and CH<sub>4</sub> and a <sup>63</sup>Ni electron capture detector for N<sub>2</sub>O. The gas standards were provided by the  
194 National Research Center for Certified Reference Materials, Beijing, China. The precision for  
195 greenhouse gas emission concentrations was  $\pm 0.5\%$  based on repeated measurements of gas standards.  
196 When the maize plants matured, all plants and grains were harvested from each plot, oven-dried at



197 60 °C for 48 h and weighed. Aboveground biomass and crop yield were converted into weight per  
198 hectare.

199 We selected 15 soil properties to estimate cropland ecosystem services, i.e., the soil fertility index  
200 (SOC, total N, total P, MBC and MBN), greenhouse gas emission amount (mainly CO<sub>2</sub>, N<sub>2</sub>O and CH<sub>4</sub>),  
201 straw decomposition and C and N released, soil extracellular enzymes (cellulase and  
202 N-acetyl-D-glucosaminidase), and maize biomass (aboveground biomass and crop yield). Generally,  
203 SOC, total N and total P are the major soil fertility factors and indicate the present nutrient status in  
204 croplands, which can be used to explain soil fertility conditions. Microbial biomass reflects ecosystem  
205 productivity. Greenhouse gas emissions are related to climate change, which can be regulated by  
206 fertilization regimes and soil microbial activities. Soil extracellular enzymes catalyse the  
207 decomposition of a range of organic polymers, resulting in C and N turnover. Maize biomass (such as  
208 aboveground biomass and crop yield) reflects soil productivity. As a whole, all of these variables  
209 together contributed to the cropland function. To evaluate the function of the cropland ecosystem under  
210 different fertilization conditions, we calculated an integrative soil ecosystem multifunctionality index  
211 for further analysis. Due to the lack of a specific definition of multifunctionality, we first calculated the  
212 Z scores of the 15 measured variables and obtained a multifunctionality value for each plot by  
213 averaging the Z scores of the 15 variables.

#### 214 **2.4 DNA extraction and quantification of general fungal ITS, bacterial 16S rRNA and genes** 215 **encoding cellulose-degrading enzymes**

216 Total DNA was extracted from 0.5 g freeze-dried soil by using a Fast DNA Spin Kit for Soil  
217 (MPbio, USA) according to the manufacturer's instructions and then dissolved in 50 µl of Tris-EDTA  
218 buffer. The success of the DNA extraction was characterized by electrophoresis on 1% (wt/vol) agarose



219 gels. The quantity and quality of DNA were checked using a Nanodrop spectrophotometer (Nanodrop,  
220 PeqLab, Germany). The extracted DNA samples were stored at  $-80^{\circ}\text{C}$  before molecular analysis.

221 Bacterial and fungal abundances were determined to reveal the changes in microbial community  
222 compositions. The abundances of bacteria and bacteria fungi were measured according to modified  
223 procedures (Fierer and Jackson., 2005). We selected the primers *338F/518R* (*338F*:  
224 *CCTACGGGAGGCAGCAG*; *518R*: *ATTACCGCGGCTGCTGG*) and *NSII/58A2R* (*NSII*:  
225 *GTAGTCATATGCTTGCT*; *58A2R*: *CATTCCCGTTACCCGTT*) for the qPCR assay. The thermal  
226 qPCR profiles for the bacteria and fungi were as follows:  $95^{\circ}\text{C}$  2 min, 35 cycles ( $95^{\circ}\text{C}$  30 s,  $60^{\circ}\text{C}$  30  
227 s,  $72^{\circ}\text{C}$  30 s,  $80^{\circ}\text{C}$  15 s), and data collection at  $81^{\circ}\text{C}$  for 10 s;  $95^{\circ}\text{C}$  10 min, 40 cycles ( $95^{\circ}\text{C}$  15 s,  
228  $52^{\circ}\text{C}$  30 s,  $72^{\circ}\text{C}$  30 s,  $79^{\circ}\text{C}$  30 s), and data collection at  $81^{\circ}\text{C}$  for 10 s, respectively. The initial  
229 concentrations of the two plasmids used as the standards for the bacterial and fungal abundance  
230 analyses were  $1.22 \times 10^{10}$  and  $9.05 \times 10^9$ , respectively.

231 The fungal *cbhI* gene and bacterial *GH48* gene were selected as functional biomarkers of  
232 cellulolytic fungi and bacteria, respectively. The primers *GH48\_F8/GH48\_R5* (*GH48\_F8*: 5 -  
233 *GCCADGHTBGGCG ACTACCT* - 3; *GH48\_R5*: 5 - *CGCCCCABGMSWWGTACCA* - 3) and *cbhI*  
234 *F/cbhI\_R* (*cbhI\_F*: *ACCAAYTGCTAYACIRGYAA*; *cbhI\_R*: *GCYTCCCAIATRCCATC*) were used for  
235 the qPCR assay. The abundance of bacterial *GH48* and fungal *cbhI* genes was quantified according to  
236 modified procedures (Zhang et al., 2017). The thermal profiles of qPCR for the target genes of *GH48*  
237 and *cbhI* were as follows:  $95^{\circ}\text{C}$  for 5 min,  $40 \times$  ( $94^{\circ}\text{C}$  for 30 s,  $60^{\circ}\text{C}$  for 45 s, and  $72^{\circ}\text{C}$  for 90 s),  
238 and data collection at  $84^{\circ}\text{C}$  for 10 s; and  $94^{\circ}\text{C}$  for 4 min,  $40 \times$  ( $94^{\circ}\text{C}$  for 45 s,  $50^{\circ}\text{C}$  for 30 s, and  
239  $72^{\circ}\text{C}$  for 60 s), and data collection at  $81^{\circ}\text{C}$  for 10 s, respectively. The initial concentrations of the two  
240 plasmids as the standards for bacterial *GH48* and fungal *cbhI* gene abundance analysis corresponded to



241  $1.85 \times 10^{11}$  and  $2.65 \times 10^{10}$  copies  $\text{g}^{-1}$  dry soil, respectively. qPCR was performed in triplicate, and  
242 amplification efficiencies higher than 95% were obtained with  $r^2$  values  $> 0.99$ .

### 243 **2.5 Bacterial 16S rRNA genes and fungal ITS amplification and sequencing**

244 High-throughput sequencing was performed with the Illumina MiSeq sequencing platform  
245 (Illumina Inc.). Both the forward and reverse primers were tagged with an adapter and linker sequence,  
246 and 8-bp barcode oligonucleotides were added to distinguish the amplicons from different soil samples.

247 The primers *515F* ( $5'$ -GTGCCAGCMGCCGCGTAA-3') and *907R*  
248 ( $5'$ -CCGTCAATTCMTTTRAGTT-3') were chosen to amplify the 16S rRNA genes in the V4–V5  
249 hypervariable region. PCR was conducted in a 50- $\mu\text{L}$  reaction mixture containing 27  $\mu\text{L}$  of ddH<sub>2</sub>O, 2  
250  $\mu\text{L}$  (5  $\mu\text{M}$ ) of each forward/reverse primer, 2.5  $\mu\text{L}$  (10 ng) of template DNA, 5  $\mu\text{L}$  (2.5 mM) of  
251 deoxynucleoside triphosphates, 10  $\mu\text{L}$  of 5 $\times$  Fastpfu buffer, 0.5  $\mu\text{L}$  of bovine serum albumin, and 1  $\mu\text{L}$   
252 of TransStart Fastpfu polymerase (TransGen, Beijing, China). The PCR conditions were 94 °C for 5  
253 min; 30 cycles of 94 °C for 30 s, 52 °C for 30 s and 72 °C for 30 s of extension; followed by 72 °C for  
254 10 min (Caporaso et al., 2010).

255 The fungal ITS1 region was amplified using the primer pair *ITS1F*  
256 ( $5'$ -CTGGTCATTAGAGGAAGTAA-3')/*ITS2* ( $5'$ -GCTGCGTCTTCATCGATGC-3') (Ghannoum et al., 2010).

257 The 50- $\mu\text{L}$  reaction mixture of each reaction mix consisted of 1  $\mu\text{L}$  (30 ng) of DNA, 4  $\mu\text{L}$  (1  $\mu\text{M}$ ) of each  
258 forward/reverse primer, 25  $\mu\text{L}$  of PCR Master Mix, and 16  $\mu\text{L}$  of ddH<sub>2</sub>O. PCR amplification was  
259 conducted at 98 °C for 3 min, followed by 30 cycles (98 °C for 45 s, 55 °C for 45 s, and 72 °C for 45 s),  
260 with a final extension at 72 °C for 7 min (Ghannoum et al., 2010). All amplicons were cleaned and  
261 pooled in equimolar concentrations in a single tube, after which they were subjected to library  
262 preparation, cluster generation, and 250-bp paired-end sequencing on an Illumina MiSeq platform



263 (Illumina Inc., San Diego, CA, USA).

264 The raw sequence data were processed using the Qualitative Insights into Microbial Ecology  
265 (QIIME) pipeline (Caporaso et al., 2010). Sequences that fully matched the barcodes were selected and  
266 distributed into separate files for the bacterial 16S rRNA and fungal ITS genes. Poor-quality sequences  
267 with lengths less than 200 bp (for fungal ITS) and 500 bp (for bacterial 16S) and quality scores less  
268 than 20 were discarded, and the chimaeras were removed using the UCHIME algorithm (Edgar et al.,  
269 2010). The remaining sequences were assigned to operational taxonomic units (OTUs) with a 97%  
270 similarity threshold using UCLUST (Edgar, 2010). Alpha diversity and Bray–Curtis distances for  
271 principal coordinate analysis of the soil microbial community were calculated after rarefying all  
272 samples to the same sequencing depth.

## 273 **2.6 Statistical analysis**

274 The soil ecosystem multifunctionality index, crop yields, microbial traits and other relevant soil  
275 variables among treatments were subjected to a chi-square test for independence of variance.  
276 Significant differences were determined by one-way analysis of variance (ANOVA) based on the post  
277 hoc Tukey test at the 5% level. Prior to ANOVA, the normality and homogeneity of variances were  
278 tested by the Kolmogorov–Smirnov test and Levene’s test, respectively. If normality was not met, log  
279 or square-root transformation was implemented. One-way ANOVA was performed using SPSS 21.0  
280 (SPSS Inc., Chicago, IL, USA).

281 Nonmetric multidimensional scaling (NMDS) analysis was used to describe and evaluate the  
282 microbial community composition. Redundancy analysis (RDA) was performed to visualize the  
283 associations between the microbial community composition and selected soil properties. The NMDS  
284 and RDA were performed in the “Vegan” package of R (4.0.2). To describe the complex co-occurrence



285 patterns in various organisms, we constructed co-occurrence networks. We focused on the abundant  
286 microbial phylotypes (with average relative abundance  $> 0.01\%$  for bacteria and fungi) for network  
287 construction. Nodes with Pearson correlations greater than 0.70 and  $p < 0.05$  were retained. Network  
288 visualization between microbial taxa was conducted by Gephi software. To obtain the keystone species  
289 of each network, a  $Zi-Pi$  plot series was constructed to determine the role of each OTU. According to  
290 Deng et al. (2012), the plot includes (a) peripheral nodes ( $Z \leq 0.25, P \leq 0.62$ ). (b) module hubs ( $Z >$   
291  $0.25, P \leq 0.62$ ), (c) connectors ( $Z \leq 0.25, P > 0.62$ ) and (d) network hubs ( $Z > 0.25, P > 0.62$ ). From an  
292 ecological perspective, OTUs in module hubs, connectors and network hubs may be regarded as the  
293 microbial keystone taxa of the network systems.

294 A heatmap was constructed to reveal the associations between microbial traits and fertilizers, soil  
295 properties, greenhouse emissions and ecosystem multifunctionality. The random forest algorithm was  
296 performed in the R package “RandomForest” to estimate the importance predictors of soil properties  
297 and microbial traits on ecosystem multifunctionality.

298

### 299 **3. Results**

#### 300 **3.1 Cropland ecosystem services**

301 Data collection after a continuous 4-year in situ field experiment under different N input levels  
302 revealed changes in cropland ecosystem services (Fig. 1). In terms of soil fertility, compared with the  
303 N-limitation treatments (PK and 0.5N+PK), the SOC and total P contents were increased significantly  
304 by the N+PK and 0.75N+PK treatments (Fig. 1a, c) ( $P < 0.05$ ), while there were no significant changes  
305 in the total N content (Fig. 1b). After straw decomposition (Fig. 1d), the amounts of straw C (Fig. 1e)  
306 and N (Fig. 1f) released showed different responses to varying N fertilizer input levels. Generally,



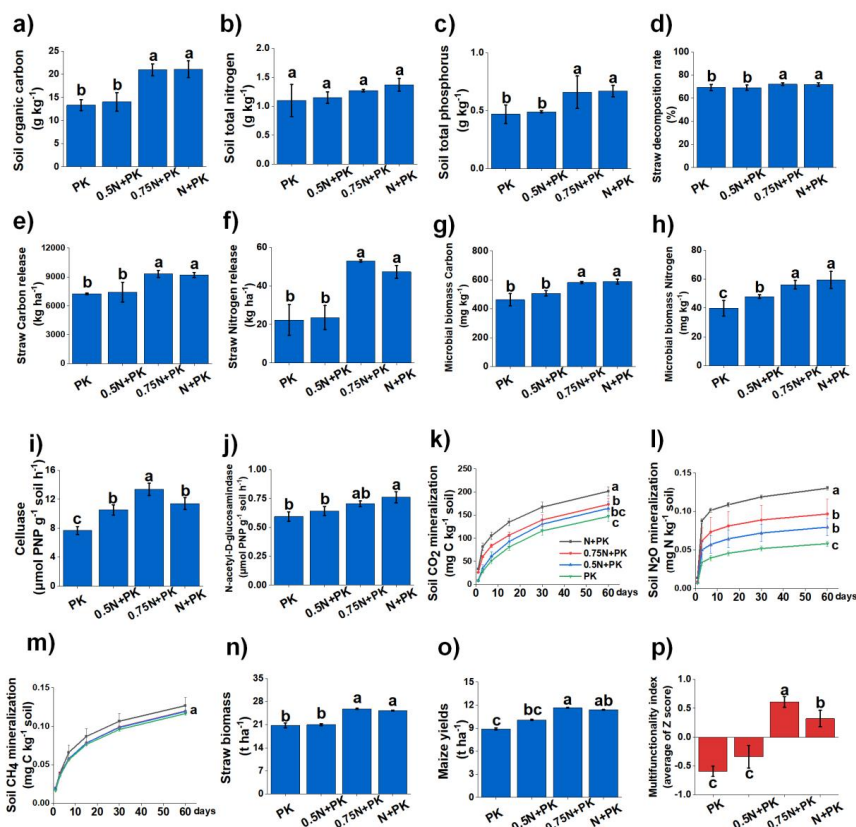
307 N-rich treatments (N+PK and 0.75N+PK) significantly increased the straw decomposition rate and  
308 achieved higher amounts of straw C and N release than the N-limitation treatments ( $P < 0.05$ ).  
309 However, there was no significant difference between N+PK and 0.75N+PK. Microbial biomass and  
310 function were also sensitive to N fertilizer application (Fig. 1g, h, i and j). The MBC (Fig. 1g) and  
311 MBN (Fig. 1h) contents were significantly higher in the N-rich treatments than in the other treatments.  
312 However, the highest cellulase activity was observed in the 0.75N+PK treatment, which was  
313 significantly higher than that in the other treatments (Fig. 1i) ( $P < 0.05$ ), and the  
314 N-acetyl-D-glucosaminidase activity decreased with the reduction in N application (Fig. 1j).

315 For the ecosystem dis-services (greenhouse gas emissions), with the increase in N fertilizer  
316 application levels, CO<sub>2</sub> and N<sub>2</sub>O emissions gradually increased (Fig. 1k, m). No significant difference  
317 was observed in CH<sub>4</sub> emissions under the different fertilization treatments (Fig. 1l). In addition, the N  
318 fertilizer levels also had a strong influence on maize yields and aboveground biomass (Fig. 1n, o). Our  
319 results indicated that the N+PK and 0.75N+PK treatments resulted in higher maize yields and  
320 aboveground biomass than the other treatments ( $P < 0.05$ ), suggesting that a 25% N fertilizer reduction  
321 could be satisfactory for maize growth. As expected, the 0.75N+PK treatment achieved the highest  
322 multifunctionality index (0.61), followed by N+PK (0.32), 0.5N+PK (-0.34) and PK (-0.59) (Fig. 1p).

323 However, although the 0.75N+PK treatment increased the straw N release amount and may meet  
324 the requirements for plant growth, the total N input was still dominated by inorganic N input (Fig. S1).  
325 Therefore, the N released from the straw cannot offset the deficiency of N fertilizer. Additionally,  
326 contrasting N fertilizer input levels significantly changed the stoichiometry of C, N and P (Fig. S2).  
327 Notably, the 0.75N+PK treatment significantly increased the C:N ratio compared with the 0.5N+PK  
328 and PK treatments ( $P < 0.05$ ). The lowest C:N ratio was shown for the 0.5N+PK treatment (Fig. S2a).



329 The N:P and C:P ratios showed no significant difference regardless of nutrient excess or limitation (Fig.  
 330 S2b and c).



331  
 332 **Fig. 1** The 15 cropland variables and multifunctionality index under different N input levels after straw  
 333 return. Abbreviations: N+PK, straw return plus regular inorganic N-P-K fertilizers; 0.75N+PK, straw  
 334 return plus regular inorganic P-K with 25% N fertilizer reduction; 0.5N+PK, straw return plus regular  
 335 inorganic P-K with 50% N fertilizer reduction; PK, straw return plus regular inorganic P-K without N  
 336 fertilizer.

### 337 3.2 Abundances of bacteria, fungi and genes encoding cellulose-degrading enzymes

338 N fertilizer input levels had marked impacts on the abundances of fungi and bacteria (Table S3).  
 339 The highest fungal abundance was observed in the 0.75N+PK treatment, which was significantly





340 higher than that in the other treatments ( $P < 0.05$ ). The N+PK treatment significantly increased  
341 bacterial abundance compared with the PK treatment ( $P < 0.05$ ), while there were no obvious  
342 differences among the N+PK, 0.75N+PK and PK treatments. The ratios of fungi to bacteria also  
343 showed contrasting responses to N fertilization (Table. S3). The 0.75N+PK treatment significantly  
344 increased the ratio of fungi to bacteria compared with the other treatments ( $P < 0.05$ ), and the lowest  
345 ratio of fungi to bacteria was found in the PK treatment.

346 **Table 1 The abundances of genes encoding cellulose-degrading enzymes**  
347 **across different N fertilizer level treatments after straw return**

Treatment	<i>cbhI</i> gene abundance ( $\times 10^6$ copies $g^{-1}$ soil)	<i>GH48</i> gene abundance ( $\times 10^7$ copies $g^{-1}$ soil)	<i>cbhI</i> : <i>GH48</i> ratio
N+PK	4.75 $\pm$ 0.16 a	1.68 $\pm$ 0.01 a	0.28 $\pm$ 0.01 a
0.75N+PK	4.95 $\pm$ 0.19 a	1.60 $\pm$ 0.04 a	0.31 $\pm$ 0.02 a
0.5N+PK	4.01 $\pm$ 0.12 b	1.54 $\pm$ 0.08 a	0.26 $\pm$ 0.03 b
PK	3.76 $\pm$ 0.13 b	1.40 $\pm$ 0.06 b	0.27 $\pm$ 0.02 b

348 The results show means  $\pm$  standard deviations (n = 3). Different lowercase letters after values  
349 indicate significant differences between each treatment,  $P < 0.05$ . N+PK, straw return plus  
350 regular inorganic N-P-K fertilizers; 0.75N+PK, straw return plus regular inorganic P-K with  
351 25% N fertilizer reduction ; 0.5N+PK, straw return plus regular inorganic P-K with 50% N  
352 fertilizer reduction; PK, straw return plus regular inorganic P-K without N fertilizer.

353

354 N fertilizer input levels led to changes in the expression levels of genes encoding  
355 cellulose-degrading enzymes (Table 1). The N-rich treatments achieved higher fungal *cbhI* and  
356 bacterial *GH48* gene abundance than the N-limitation treatments. In contrast, the highest *cbhI* gene



357 abundance was shown in the 0.75N+PK treatment, while the highest *GH48* gene abundance was shown  
358 in the N+PK treatment. Compared with the PK treatment, the ratio of the fungal *cbhl* gene to the  
359 bacterial *GH48* gene increased significantly under the 0.75N+PK treatment ( $P < 0.05$ ).

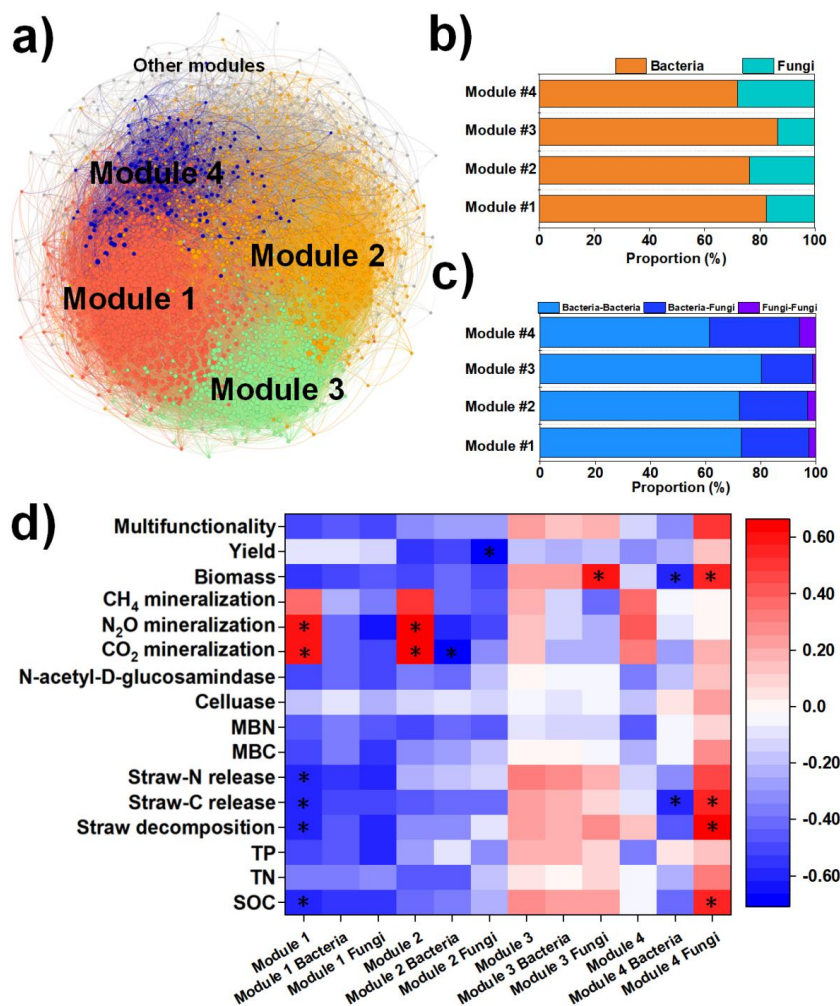
360

### 361 3.3 Co-occurrence network analysis of the microbial community

362 Regarding fungal alpha diversities, there were no significant differences in the Chao1 index across  
363 treatments. The N+PK treatment significantly increased fungal richness compared with the PK  
364 treatment ( $P < 0.05$ ) (Table S4). In addition, the PK treatment resulted in lower bacterial richness than  
365 the other treatments ( $P < 0.05$ ). No significant difference was observed in the bacterial Chao1 index  
366 across treatments (Table S4). NMDS plots showed that diverse N input levels significantly changed the  
367 fungal (Fig. S3a) and bacterial communities (Fig. S3b) ( $P < 0.05$ ).

368 We further conducted network analysis to identify co-occurrence patterns between specific  
369 microbial taxa (Fig. 2). The cooccurrence network was aggregated into smaller coherent modules that  
370 were examined to determine important module-trait relationships. The present network comprised 1963  
371 nodes (composed of 1520 bacterial taxa and 443 fungal taxa) and 62206 edges with 52.49% positive  
372 associations (Fig. 2a). The results showed four dominant ecological modules (1-4) that strongly  
373 co-occurred within the multitrophic network, which contributed 86.10% of the whole network. Among  
374 the four modules, bacteria accounted for the highest proportion in each module, contributing more than  
375 70% of the total (Fig. 2b). The percentage of edges linking bacteria to bacteria (B-B) was higher than  
376 that linking fungi to fungi (F-F) and bacteria to fungi (B-F). The highest proportion of B-B (80.32%)  
377 was found in Module 3, while the highest proportion of B-F (32.66%) and F-F (6.00%) was found in  
378 Module 4 (Fig. 2c).

379



380

381 **Fig. 2** The relationships of microbial module communities with soil ecosystem services and

382 dis-services. Multitrophic network including multiple ecological modules. The colours of the

383 nodes represent different ecological modules (a). OTU number proportions of bacteria and fungi

384 (b). The proportions of the edges linking bacteria to bacteria (B-B), bacteria to fungi (B-F) and

385 fungi to fungi (F-F) in the major ecological modules (c). Links between the specific module

386 communities with soil ecosystem services and dis-services (d). \* indicates significance at  $P < 0.05$ .

387 Abbreviations: SOC, soil organic carbon; C: N, the ratio of the SOC content to the total N content;



388 N: P, the ratio of the total N content to the total P content.

389 Individual nodes represented different roles in the microbial network based on the intramodule

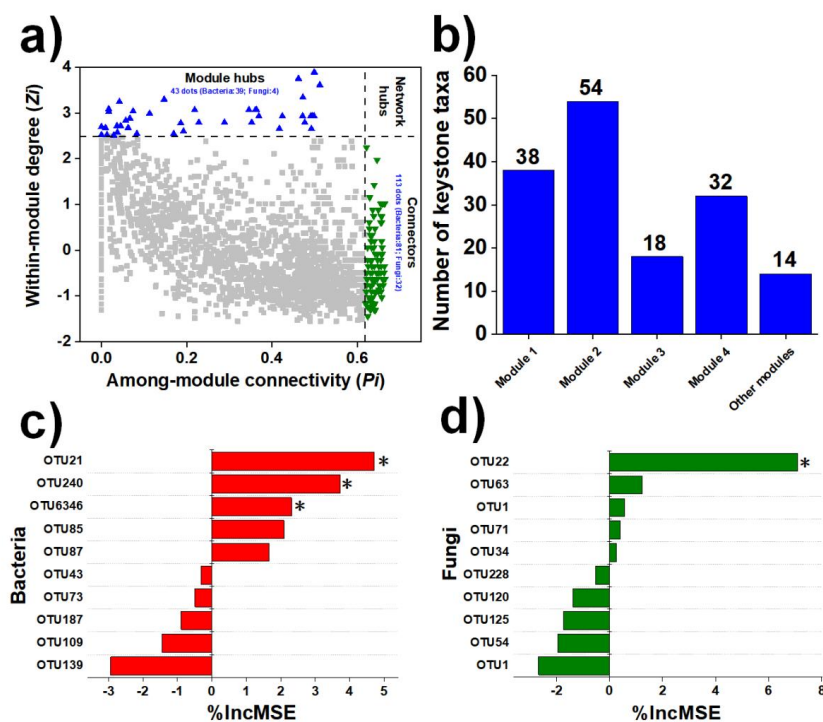
390 connectivity  $Z_i$  and the intermodule connectivity  $P_i$ .  $ZP$  plots were constructed to identify the

391 topological roles of each node in the network (Fig. 3a). As shown in Fig. 3b, 113 microbial taxa (81

392 bacterial species and 32 fungal species) were regarded as connectors, and 43 microbial taxa (39

393 bacterial species and 4 fungal species) were regarded as module hubs. Specifically, module 2 (54

394 contained the most keystone taxa, followed by module 1 (38) and module 3 (32).



395

396 **Fig. 3** The topological roles of microbial taxa and their effect on the soil multifunctionality index.

397 The topological role of each OTU was determined according to the scatter plot of within-module

398 connectivity ( $Z$ ) and among-module connectivity ( $P$ ) (a). The distribution of keystone taxa in each

399 ecological module (b). Contribution of bacterial (c) and fungal OTUs (d) to the soil



400 multifunctionality index. \*, \*\* and \*\*\* indicate significance at  $P < 0.05$ , 0.01 and 0.001,  
401 respectively.

### 402 **3.4 Linkage between microbial traits and soil ecosystem multifunctionality**

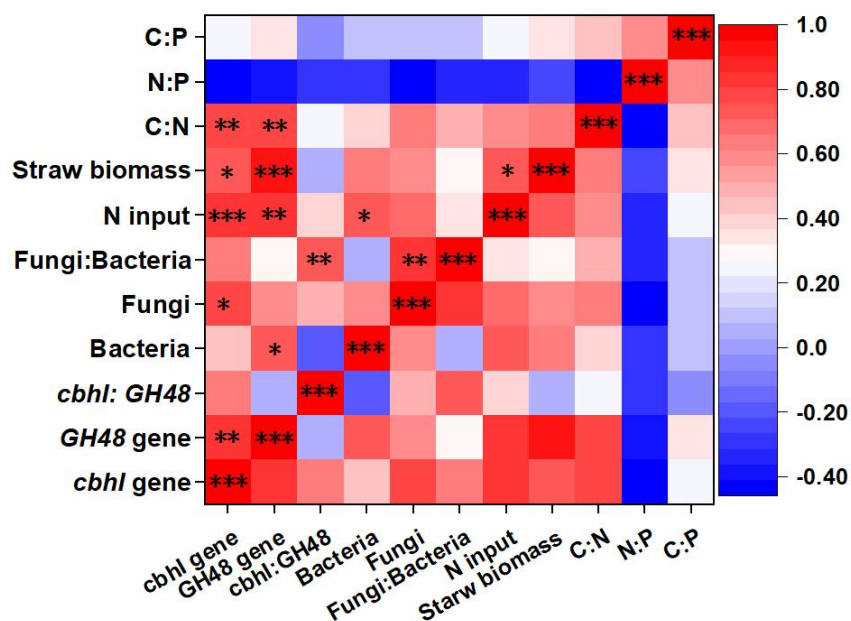
403 The heatmap assumed close correlations between fertilizers (N input and straw), as well as soil  
404 stoichiometry, and microbial traits (Fig. 4). Overall, the N input level, straw biomass and C:N ratio  
405 upregulated the abundance of genes encoding cellulose-degrading enzymes. In addition, N input was  
406 positively correlated with bacterial abundance, while a significant correlation was observed between  
407 straw biomass and the N input level. The random forest model was also used to identify abiotic and  
408 biotic attributes correlated with soil ecosystem multifunctionality (Fig. 5). The model explained  
409 83.89% of the variance in ecosystem multifunctionality. The results indicated that the N input level,  
410 straw biomass and soil C:N ratio were the most prominent abiotic factors affecting the ecosystem  
411 multifunctionality index, while some biotic factors, such as the abundance of genes encoding  
412 cellulose-degrading enzymes, significantly affected the ecosystem multifunctionality index.

413

414 Moreover, to clarify the potential main specific drivers of soil ecosystem services, correlations  
415 between the microbial physiological traits and soil properties were determined to illuminate the role of  
416 the microbial community in soil ecosystem multifunctionality (Fig. 3d). The results indicated that the  
417 particular microbial module community was significantly correlated with soil ecosystem services. The  
418 communities of modules 1 and 2 and the fungal community in module 4 showed potential in soil  
419 ecosystem services (Fig. 3d). Specifically, significant correlations were observed between the SOC  
420 content, straw decomposition, straw C/N release,  $\text{CO}_2/\text{N}_2\text{O}$  mineralization and the module 1  
421 community; the module 2 community was positively correlated with greenhouse gas emissions (except



422 for CH<sub>4</sub>); and the fungal community in module 4 was positively correlated with the SOC content, straw  
 423 decomposition, straw C/N release and straw biomass. Furthermore, the bacterial and fungal  
 424 communities belonging to module 2 and the fungal community belonging to module 3 were  
 425 significantly correlated with CO<sub>2</sub> emission, maize yield and straw biomass.



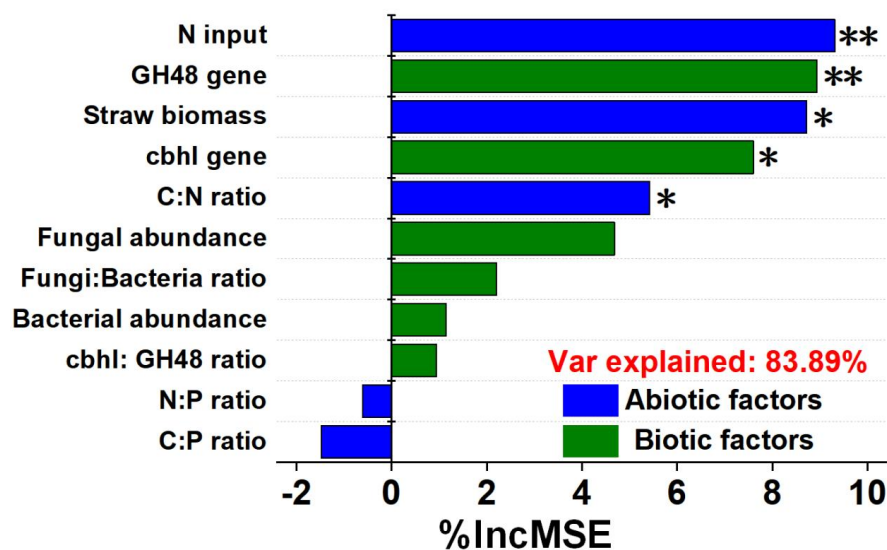
426  
 427 **Fig. 4** Heatmap revealing the correlation coefficients between microbial traits with fertilization  
 428 and soil stoichiometry. \*, \*\* and \*\*\* indicate significance at  $P < 0.05$ ,  $0.01$  and  $0.001$ ,  
 429 respectively. Abbreviations: C: N, the ratio of the SOC content to the total N content; N: P, the  
 430 ratio of the total N content to the total P content.

431

432 At the scale of microbial species, we selected the 20 keystone taxa (10 bacterial and 10 fungal  
 433 taxa) with the highest relative abundance for further analysis. The random forest models indicated that  
 434 the specific keystone taxa strongly influenced soil ecosystem multifunctionality (Fig. 4c and d).



435 Bacterial OTU21 (in module 1), OTU240 (in module 2) and OTU6346 (in module 3) were highlighted  
 436 as essential predictors of soil ecosystem multifunctionality, and fungal OTU22 (module 3) was also  
 437 found to be an important variable for predicting its changes. Subsequently, the relative abundances of  
 438 selected keystone taxa were different across different N fertilizer level treatments after straw return  
 439 (Table S5). The relative abundances of fungal OTU22 and bacterial OTU21 were higher in the N-rich  
 440 treatments than in the N-limitation treatments. Moreover, compared with the N+PK treatment, the  
 441 0.75N+PK treatment increased the relative abundances of fungal OTU22 by 38.20% and bacterial  
 442 OTU21 by 40.63%.



443  
 444 **Fig. 5** Contribution of abiotic and biotic variables to the soil multifunctionality index. \*, \*\* and  
 445 \*\*\* indicate significance at  $P < 0.05$ , 0.01 and 0.001, respectively. Abbreviations: C: N, the ratio  
 446 of the SOC content to the total N content; N: P, the ratio of the total N content to the total P  
 447 content.  
 448



449 **4. Discussion**

450 **4.1 Effect of N fertilizer reduction on cropland ecosystem services after straw return**

451 Soil fertility, straw decomposition, C and N release amounts, and crop productivity showed an  
452 overall positive effect with the increase in the N fertilizer input level and together contributed to  
453 ecosystem services. Moreover, high N applications may also cause ecosystem dis-services due to the  
454 surge in greenhouse gas emissions (Fig. 1). Our results indicated that the soil fertility index (SOC, total  
455 N and P contents) increased under N-rich treatments as a result of high net primary production, in  
456 accordance with previous reports (Liu et al., 2010; Williams et al., 2013). Higher microbial biomass C  
457 and N, as well as relevant enzyme activities, were also observed under N-rich treatments, indicating the  
458 strong positive impact of abundant N fertilizer application (Fig. 1g, h, i, j). It was reported that straw  
459 return with N fertilizer application can stimulate microbial activity and promote biomass accumulation  
460 (Treseder, 2008). The substantially increased straw decomposition and straw C and N release under  
461 N-rich treatments may be primarily attributed to the activation of microbial activity (Fig. 1d, e, f),  
462 which is consistent with previous research (Ramirez et al., 2012). Recent studies have also proven that  
463 rational N input can stimulate microbial ex vivo production of extracellular enzymes to accelerate  
464 straw decomposition and nutrient transformation (Chen et al., 2016). Moreover, it is well known that  
465 fungi have high nutrient utilization efficiency; thus, more straw-derived C and N would be stored in  
466 soil under N-rich treatments than under N-limited treatments (Hou et al., 2020). Higher N availability  
467 is also the premise of straw decomposition and SOM formation due to the microbial “stoichiometry  
468 decomposition” theory, while the “N-mining” theory in N-limitation treatments reveals that  
469 oligotrophic species (such as K-strategists) degrade native SOM because of the lack of N fertilizer  
470 inputs (Chen et al., 2014). Finally, the increases in SOC, total N, and P contents and straw C and N





471 release, as well as microbial biomass and function, are commonly attributed to high aboveground  
472 biomass and maize yields (Fig. 1n, o), which are favourable from the viewpoint of ecosystem services.

473 However, the overuse of N inputs also causes ecosystem dis-services, such as unintended  
474 environmental consequences (Tang et al., 2019). In the present study, greenhouse gas emissions were  
475 quantified to evaluate the ecosystem dis-services under different N fertilizer input levels (Fig. 1k, l, m).  
476 Straw return with N fertilizer addition might be the crucial driver of CO<sub>2</sub> and N<sub>2</sub>O emissions from  
477 agroecosystems and has been widely studied in previous literature (Gregorich et al., 2005). CO<sub>2</sub> and  
478 N<sub>2</sub>O emissions increased significantly compared with those under the PK treatment, likely by  
479 stimulating the activity of copiotrophs when sufficient C and N substrates were provided. For example,  
480 Dieleman et al. (2010) implied that N fertilizer addition significantly increased CO<sub>2</sub> and N<sub>2</sub>O by  
481 increasing bacterial abundance through meta-analysis and field experiments, respectively. Qiu et al.  
482 (2019) indicated that the emission of CO<sub>2</sub> enhanced root and mycorrhizal N uptake and increased N<sub>2</sub>O  
483 emissions, which was related to the changes in the soil denitrifier community composition in favour of  
484 N<sub>2</sub>O-producing taxa (nirK- or nirS-type). In addition, there was no difference in CH<sub>4</sub> emissions among  
485 treatments, although contradictory results have been widely reported in previous literature (Tang et al.,  
486 2019). Mapanda et al. (2011) and Liu et al. (2012) indicated that the emission of CH<sub>4</sub> depended highly  
487 on the soil water content in maize crops, which is in line with our results.

488 In summary, compared with the N+PK treatment, the 0.75N+PK treatment supported multiple  
489 ecosystem services, including promoting soil fertility, straw nutrient release and microbial activity and  
490 alleviating greenhouse gas emissions (Fig. 1p). Therefore, a reduction of 25% in chemical N fertilizer  
491 input with straw return may be the appropriate regime to promote ecosystem services in meadow soils  
492 on the Northeast China Plain.



493 **4.2 Responses of the microbial composition and function to straw return with N fertilizer**  
494 **reduction**

495 Fungal and bacterial abundances, as well as the ratio of fungi to bacteria, were sensitive to the  
496 changes in the N fertilizer input levels (Table S3 and Fig. 2). Straw addition with N fertilizer input  
497 supplied enough C and N for microbial metabolism, thus promoting microbial proliferation (Chen et al.,  
498 2016). Generally, bacterial abundance decreased with reduced N fertilizer input. This is mainly because  
499 bacteria are more sensitive to N availability than fungi, which is in line with a previous study (Ramirez  
500 et al., 2020). Interestingly, it is worth noting that a 25% reduction of N fertilizer significantly increased  
501 fungal abundance compared with regular N inputs. This result might be attributed to the negative effect  
502 of excess N fertilizer (Wan et al., 2015). Moreover, Ning et al. (2020) demonstrated that the C:N ratio  
503 was the pivotal factor in fungal community compositions after performing 7 long-term field  
504 experiments under different fertilization conditions across China and reported a significant positive  
505 correlation between them. Therefore, the 0.75N+PK treatment with a higher C:N ratio may facilitate  
506 the proliferation of microorganisms and promote an increase in microbial abundance.

507 Subsequently, our results showed that N-rich treatments resulted in higher microbial  
508 cellulose-degrading gene abundances than the PK treatment (Table 1), which demonstrated the  
509 irreplaceable role of N inputs in straw degradation (Zhang et al., 2017). Additionally, compared with  
510 bacterial *GH48* gene abundance, the increase in fungal *cbhI* gene abundance required adequate N  
511 fertilizer inputs and was regulated by the soil C:N ratio, which suggests that rational N fertilizer inputs  
512 could promote fungal function for further degradation of recalcitrant straw components (Hou et al.,  
513 2020). Therefore, the ratio of *cbhI* gene abundance to *GH48* gene abundance was higher under  
514 0.75N+PK than under the N-limitation treatments since the increased expression of a fungal



515 cellulose-degrading gene implies more straw C and N release.

516 Our results indicated that adequate N fertilizer upregulated fungal and *cbhI* gene abundances,  
517 which may lead to multiple ecosystem services. It is therefore necessary to further explore the potential  
518 associations between microbial traits and ecosystem services under diverse N fertilizer input levels.

#### 519 **4.3 Linkages of cropland ecosystem services with microbial traits**

520 To clarify the effect of abiotic and biotic factors on soil ecosystem services, we then quantified the  
521 contributions of abiotic and biotic attributes to the ecosystem multifunctionality index across N input  
522 treatments (Fig. 4 and 5). Biotic factors, such as *cbhI* and *GH48* gene abundances, as well as abiotic  
523 factors, including the C:N ratio, straw biomass and N input level, are also pivotal regulators of  
524 ecosystem multifunctionality (Fig. 5). In general, promoting the rapid degradation of straw is an  
525 important way to convert straw-C into SOM, thus improving soil fertility, aboveground biomass and  
526 crop yield. In addition, fungi have a higher C utilization efficiency than bacteria; thus, a high fungal  
527 *cbhI* gene abundance may achieve better soil multifunctionality (Hou et al., 2020). For abiotic factors,  
528 the soil C:N ratio, straw biomass and N fertilizer input are always regarded as the main indicators of  
529 soil fertility and health, likely due to providing various nutrient accessibilities and influencing the  
530 microbial community composition (Ning et al., 2020).

531 Numerous studies have shown that core microbiota play a vital role in maintaining the stability of  
532 soil microbial function and the complexity of microbial networks and then promoting soil nutrient  
533 cycling ecosystem services (Ghannoum et al., 2015), and keystone species may show great explanatory  
534 power in terms of specific network (or module) structure and function (Chen et al., 2019b). Heatmaps  
535 and random forest models were used to illuminate the relationships of module communities with  
536 ecosystem services (Fig. 2d and Fig 3c, d). In the present study, *Terrimonas* (bacterial species in

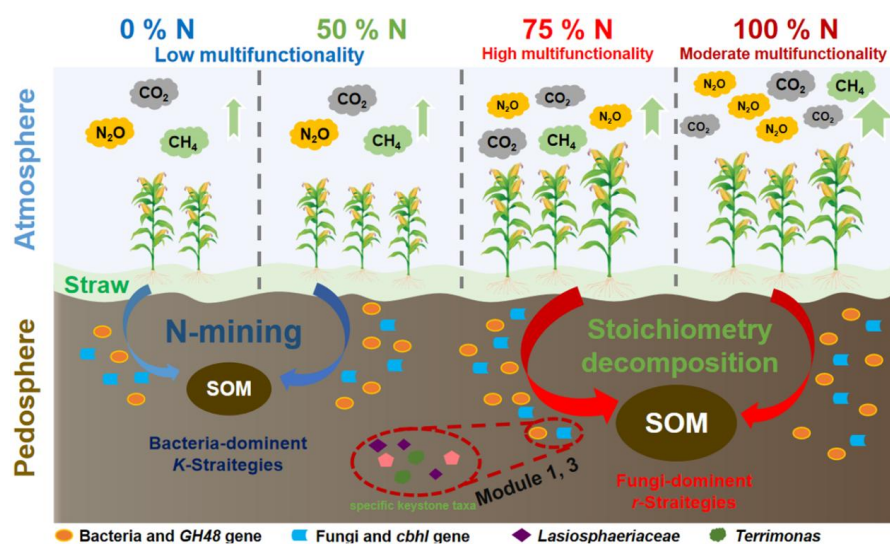


537 module 1) and *Lasiosphaeriaceae* (fungal species in module 3) were detected as the keystone taxa in  
538 influencing soil multifunctionality of the cooccurrence network (Table S5). A previous study  
539 demonstrated that straw addition significantly increased the relative abundance of *Lasiosphaeriaceae*,  
540 which implied straw decomposition ability (Song et al., 2020). Afterwards, *Lasiosphaeriaceae* was  
541 proven to promote straw-derived C and N accumulation by secreting multiple extracellular enzymes  
542 (Guo et al., 2022). Meanwhile, Sun et al. (2023) revealed that *Lasiosphaeriaceae* abundance was  
543 regulated by the soil C:N ratio, especially changes in mineral N. Therefore, *Lasiosphaeriaceae* can  
544 effectively promote straw degradation and straw C and N release while driving the function and  
545 community of module 1, which is consistent with our results (Fig. 2d). However, relatively few studies  
546 have focused on the function of *Terrimonas*, so this study focused on *Chitinophagaceae*. As reported in  
547 the previous literature, straw return was the main method to increase *Chitinophagaceae* abundance (Li  
548 et al., 2021). Furthermore, *Chitinophagaceae* was indicated to have a strong ability to accumulate soil  
549 C and N and degrade cellulose (Zhong et al., 2022), facilitating production improvement by regulating  
550 the module 3 community and function, which is in line with our results (Fig. 2d).

551 Overall, straw return with sufficient N fertilizer application can increase the C:N ratio and  
552 stimulate microbial traits, which ultimately achieve soil ecosystem multifunctionality (Fig. 6). Straw  
553 return without enough N supply cannot support ecosystem services due to the decomposition of native  
554 SOM and the out-of-balance microbial community composition, according to the “N-mining” theory  
555 (Chen et al., 2014); straw return with sufficient N application (N+PK and 0.75N+PK) can promote soil  
556 fertility, straw release, microbial activity and crop productivity, which can be explained by the  
557 “stoichiometry decomposition” theory (Chen et al., 2014). Meanwhile, N+PK also caused more serious  
558 ecosystem dis-services, such as greenhouse gas emissions, than the 0.75N+PK treatment. Moreover,



559 compared with the N+PK treatment, the 0.75N+PK treatment increased the soil C:N ratio and  
 560 stimulated microbial module 1 and 3 communities function, *cbhl* gene abundance, and keystone taxa  
 561 abundances, which were significantly positively correlated with soil ecosystem multifunctionality. Our  
 562 study provides evidence that a 25% reduction of chemical N fertilizer after straw return was the  
 563 optimal agronomic measure for ecosystem services in meadow soil on the Northeast China Plain.



564  
 565 **Fig. 6** A graphical sketch of the changes in ecosystem services and potential microbial mechanisms in  
 566 response to different chemical N fertilizer application rates after straw return. N, nitrogen; SOM, soil  
 567 organic matter

568

569 **5. Conclusion**

570 Straw return combined with different chemical N fertilizer application rates significantly changed  
 571 ecosystem services and dis-services. Collectively, our work indicates that compared with the N+PK  
 572 treatment, straw return with a 25% reduction in chemical N fertilizer has the potential to improve  
 573 ecosystem services by maintaining soil fertility, productivity, microbial biomass and function,



574 promoting straw decomposition and C and N release and alleviating greenhouse gas emissions. The  
575 0.75N+PK treatment achieved higher soil ecosystem multifunctionality than all other treatments. In  
576 addition, the N input level, straw biomass and soil C:N ratio can upregulate the abundances of the *cbhl*  
577 and *GH48* genes, which may together achieve soil ecosystem multifunctionality.

578 Furthermore, the changes in multiple soil ecosystem services were strongly associated with  
579 microbial module communities and keystone taxa. The relationships between ecosystem services and  
580 microbial traits were examined here to confirm that the *Lasiosphaeriaceae* driving the function and  
581 structure of the module 1 community leads to the promotion of straw degradation and straw C and N  
582 release, while *Terrimonas* driving the function and structure of the module 3 community probably  
583 contributes to production improvement under 0.75N+PK treatment. Therefore, a 25% reduction in  
584 chemical N fertilizer with straw return might be a win–win strategy that not only produces considerable  
585 ecological benefits for the pedosphere and atmosphere but also reduces fertilizer expenditures in  
586 meadow soil on the Northeast China Plain.



### **Declaration of competing interests**

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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### Figure captions

**Fig. 1** The 15 cropland variables and multifunctionality index under different N input levels after straw return. Abbreviations: N+PK, straw return plus regular inorganic N-P-K fertilizers; 0.75N+PK, straw return plus regular inorganic P-K with 25% N fertilizer reduction; 0.5N+PK, straw return plus regular inorganic P-K with 50% N fertilizer reduction; PK, straw return plus regular inorganic P-K without N fertilizer.

**Fig. 2** The relationships of microbial module communities with soil ecosystem services and dis-services. Multitrophic network including multiple ecological modules. The colours of the nodes represent different ecological modules (a). OTU number proportions of bacteria and fungi (b). The proportions of the edges linking bacteria to bacteria (B-B), bacteria to fungi (B-F) and fungi to fungi (F-F) in the major ecological modules (c). Links between the specific module communities with soil ecosystem services and dis-services (d). \* indicates significance at  $P < 0.05$ . Abbreviations: SOC, soil organic carbon; C: N, the ratio of the SOC content to the total N content; N: P, the ratio of the total N content to the total P content.

**Fig. 3** The topological roles of microbial taxa and their effect on the soil multifunctionality index. The topological role of each OTU was determined according to the scatter plot of within-module connectivity ( $Z$ ) and among-module connectivity ( $P$ ) (a). The distribution of keystone taxa in each ecological module (b). Contribution of bacterial (c) and fungal OTUs (d) to the soil multifunctionality index. \*, \*\* and \*\*\* indicate significance at  $P < 0.05$ , 0.01 and 0.001, respectively.

**Fig. 4** Heatmap revealing the correlation coefficients between microbial traits with fertilization and soil stoichiometry. \*, \*\* and \*\*\* indicate significance at  $P < 0.05$ , 0.01 and 0.001,



respectively. Abbreviations: C: N, the ratio of the SOC content to the total N content; N: P, the ratio of the total N content to the total P content.

**Fig. 5** Contribution of abiotic and biotic variables to the soil multifunctionality index. \*, \*\* and \*\*\* indicate significance at  $P < 0.05$ , 0.01 and 0.001, respectively. Abbreviations: C: N, the ratio of the SOC content to the total N content; N: P, the ratio of the total N content to the total P content.

**Fig. 6** A graphical sketch of the changes in ecosystem services and potential microbial mechanisms in response to different chemical N fertilizer application rates after straw return. N, nitrogen; SOM, soil organic matter



**Table 1 The abundances of genes encoding cellulose-degrading enzymes across different N fertilizer level treatments after straw return**

Treatment	<i>cbhI</i> gene abundance ( $\times 10^6$ copies $g^{-1}$ soil)	<i>GH48</i> gene abundance ( $\times 10^7$ copies $g^{-1}$ soil)	<i>cbhI</i> : <i>GH48</i> ratio
N+PK	4.75 $\pm$ 0.16 a	1.68 $\pm$ 0.01 a	0.28 $\pm$ 0.01 a
0.75N+PK	4.95 $\pm$ 0.19 a	1.60 $\pm$ 0.04 a	0.31 $\pm$ 0.02 a
0.5N+PK	4.01 $\pm$ 0.12 b	1.54 $\pm$ 0.08 a	0.26 $\pm$ 0.03 b
PK	3.76 $\pm$ 0.13 b	1.40 $\pm$ 0.06 b	0.27 $\pm$ 0.02 b

The results show means  $\pm$  standard deviations (n = 3). Different lowercase letters after values indicate significant differences between each treatment,  $P < 0.05$ . N+PK, straw return plus regular inorganic N-P-K fertilizers; 0.75N+PK, straw return plus regular inorganic P-K with 25% N fertilizer reduction ; 0.5N+PK, straw return plus regular inorganic P-K with 50% N fertilizer reduction; PK, straw return plus regular inorganic P-K without N fertilizer.