Title: Moderate N fertilizer reduction with straw return modulates cropland functions and microbial traits in a meadow soil

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Abstract:

Nitrogen (N) fertilization has received worldwide attention due to its benefits to soil fertility and productivity, but excess N application also causes an array of ecosystem degeneration, such as greenhouse gas emissions. Generally, soil microorganisms are considered to be involved in upholding a variety of soil functions. However, the linkages between soil cropland properties and microbial traits under different N fertilizer application rates remain uncertain. To address this, a 4-year in situ field experiment was conducted in a meadow soil on the Northeast China Plain after straw return with the following treatments combined with regular phosphorus (P) and potassium (K) fertilization: (i) regular N fertilizer (N+PK); (ii) 25% N fertilizer reduction (0.75N+PK); (iii) 50% N fertilizer reduction (0.5N+PK); and (IV) no N fertilizer (PK). Cropland properties and microbial traits responded distinctly to the different N fertilizer rates. Treatment 0.75N+PK had overall positive effects on soil fertility, productivity, straw decomposition, and microbial abundance and function and alleviated greenhouse effects. Specifically, no significant difference was observed in SOC, total N, P content, straw C, N release amounts, microbial biomass C, N content, as well as celluase and N-acetyl-D-glucosamindase activities, which were all significantly higher than 0.5N+PK and PK. Greenhouse gas emissions was reduced with the decresing of N input levels. Moreover, the highest straw biomass and yield were measured in 0.75N+PK, which were significantly higher than 0.5N+PK and PK. Meanwhile, 0.75N+PK upregulated aboveground biomass and soil C:N and thus increased the abundance of genes encoding cellulose-degrading enzymes, which may imply the potential ability of C and N turnover. In addition, most observed changes in cropland properties were strongly associated with microbial modules and keystone taxa. The Lasiosphaeriaceae within module 1 community showed significant positive correlations with straw degradation rate, C and N release, while the Terrimonas within module

3 community showed a significant positive correlation with production, which were conducive to soil multifunctionality. Therefore, our results suggest that straw return with 25% chemical N fertilizer reduction is optimal for achieving soil functions. This study highlights the importance of abiotic and biotic factors in soil health and supports green agricultural development by optimizing N fertilizer rates in meadow soil after straw return.

Keywords: Soil functions; Straw return; Nitrogen fertilization; Microbial community; Crop yield

1. Introduction

Multiple soil functions are indicators of soil health (Kihara et al., 2020; Lehmann et al., 2021), which refer to the ability of soil as a vital living system to sustainably increase crop productivity, improve environmental quality, tackle climate change and promote plant and animal health (de Bello et al., 2010; Tang et al., 2019). Intensive agriculture has posed a wide range of threats to agroecosystem functions (Robertson et al., 2014; Allen et al., 2015). Irrational application of chemical fertilizers, especially nitrogen (N), is ubiquitous to achieve high crop yields in response to population surges globally (Shi et al., 2019). In fact, N is considered the essential macronutrient for all biota, while excessive N fertilizer inputs not only reduce soil fertility and productivity but also lead to environmental burdens (Trost et al., 2016). Recent researched indicated that appropriate reduction of N fertilizer input can not only maintain crop yield by increasing N fertilizer use efficiency, but promote soil health by regulating soil C: N ratio (Chen et al., 2014). However, excessive reduction of N fertilizer input can lead to a "N-mining" effect, resulting in the loss of soil organic matter, which reduces crop yields (Chen et al., 2014). Therefore, how to achieve agroecosystem functions by regulating N fertilizer application rates needs to be fully assessed.

Straw return has also been widely applied as a major measure to moderate cropland traits (Xu et

al., 2021). Plant residues contain abundant N that further affects soil fertility and productivity (Pan et al., 2009; Liu et al., 2014). Thus, the straw-derived N released during degradation is an important source that may serve as a partial substitute for chemical N fertilizer application (Wang et al., 2017; Latifmanesh et al., 2020). However, crop fields suffering from superabundant exogenous materials may result in ecosystem negative effects. For example, excess organic materials usually have low reutilization efficiency (Hou et al., 2020); the majority of N in straw is released into the atmosphere as oxynitride, such as nitrous oxide (N2O) (Wang et al., 2019; Sun et al., 2021); Subsequent literature highlighted that straw return significantly elevates greenhouse gas emissions so that less than 15% of straw-derived N can be transformed into soil and become SOM (Yin et al., 2018; Wu et al., 2019). (Yin et al., 2018; Wu et al., 2019). However, the potential for the partial substitution of straw for chemical N fertilizer application is still unclear.

Soil microorganisms were the drivers of soil functions (Handa et al., 2014; Wagg et al., 2014). Agronomic management for such "multifunctionality" has prompted research into the role that microbes play in providing desired rates of multiple ecosystem processes (Gong et al., 2020). Fertilization-induced changes in microbial communities and functions are fundamental to the regulation of a variety of ecosystem multifunctionalities, including SOM formation, greenhouse gas emissions, litter decomposition, and crop production (Dominati et al., 2014). For example, Ning et al. (2020) found that long-term manure increased the abundance of specific fungi, which was involved in yield improvment. Duan et al. (2021) indicated that adequate N input improved cellulose-degrading ability of bacteria. To date, we still lack empirical evidence of the linkages among N fertilizers, specific microbial communities or functions and multiple cropland traits, and the diverse soil functions driven by complex microbial traits under different N fertilizer rates are seldom clarified.

Microorganisms contribute to soil functions by modulating microbial traits (e.g., function, community composition and succession), which are influenced by different N fertilizer input levels (Bradford et al., 2014; Chen et al., 2019a). Generally, bacteria and fungi are the main drivers of straw labile and recalcitrant component decomposition, respectively (Frey et al., 2013; Ge et al., 2017; Hogberg et al., 2007). In addition, microbial module communities and keystone taxa have been used to provide satisfactory explanations for soil functions. Chen et al. (2019b) found that particular microbial modules participated in N and phosphorus (P) turnover in a Cambisol. Actinobacteria have been extensively studied and can be considered the main degraders of straw by secreting cellulase (Bao et al., 2021). C, N and P stoichiometry has profound impacts on microbial in vivo metabolism and ex vivo modification processes (Chen et al., 2016). Nevertheless, the knowledge of the microbial mechanisms that modulate cropland traits in response to N fertilizer input levels are still rudimentary.

As an important grain-producing region, the Northeast China Plain contributes to more than 20% of the total grain yield in China (Li et al., 2017; Zhao et al., 2018). Here, a field experiment was conducted to reveal the influences of N input levels on soil ecosystem multifunctionality and associated microbial traits. In the present study, two hypotheses were tested: (i) soil cropland traits would show distinct responses to N fertilizer input levels, and (ii) the changes in cropland traits would be linked to specific microbial traits. The purpose of this study is to optimize the N fertilizer application rate to achieve soil ecosystem multifunctionality and explore the potential microbial mechanism in Mollisol.

2. Materials and methods

2.1 Site description and sampling

A field experiment under contrasting inorganic N fertilizer input levels was established in 2018 in Wenchun town (44°59′61″ N, 129°59′18″ E), Mudanjiang city, Heilongjiang Province, Northeast China

Plain, which is an important grain-producing area. This region has a typical temperate continental

monsoon climate with an average annual temperature of 4.3 °C and a mean annual precipitation of

579.7 mm. The soil is classified as a meadow soil according to US Soil Taxonomy (USST). The

cropping system was continuous maize (*Zea mays* L.) monoculture. Four treatments received different

N fertilizer input levels after straw return to the field for 4 years as follows: (1) regular chemical

fertilization, N+PK (300 kg urea (N 46%) ha^{-1} yr^{-1}, 250 kg calcium triple superphosphate (P$_2$O$_5$ 46%)

ha^{-1} yr^{-1}, 150 kg potassium chloride (K$_2$O 50%) ha^{-1} yr^{-1}); (2) 25% reduction of N fertilizer, 0.75N+PK

(225 kg urea ha^{-1} yr^{-1}, 250 kg calcium triple superphosphate ha^{-1} yr^{-1}, 150 kg potassium chloride ha^{-1}

yr^{-1}); (3) 50% reduction of N fertilizer, 0.50N+PK (150 kg urea ha^{-1} yr^{-1}, 250 kg calcium triple

superphosphate ha^{-1} yr^{-1}, 150 kg potassium chloride ha^{-1} yr^{-1}); and (4) no N fertilizer, PK (250 kg

calcium triple superphosphate ha^{-1} yr^{-1}, 150 kg potassium chloride ha^{-1} yr^{-1}). Urea is the only nitrogen

source in the soil. All straw and chemical fertilizers were applied with shallow tillage to 20 cm. Straw

was cut into pieces less than 5 cm and input after the harvest in October, while the chemical fertilizers

were applied during ploughing in May of the next year. All other normal management practices were

consistent among treatments during the experiment. Before the experiment, the initial soil contained

18.74 g kg^{-1} SOC, 1.03 g kg^{-1} total N and 0.54 g kg^{-1} total P with a pH of 7.37 (H$_2$O). The yield and

some of the soil chemical properties under different bulk soil treatments during the experimental

process are shown in Supplemental material Table S1.

Soils were sampled after the maize harvest in October 2021. A randomized complete block design

consisting of 4 treatments with 3 replications was adopted in this study. Each field plot was 4.5 m × 15

m. We took nine soil cores (5 cm diameter) from the top 20 cm of bulk soil in each plot. Each soil

sample consisted of a mixture of subsamples randomly collected at nine different positions in the same

131   plot. In total, 12 soil samples were collected from 4 treatments. Each treatment contained 3 replicates.

132   Soils were sieved through a 2 mm mesh, the mineral particles and plant roots were carefully removed,

133   and then the soils were homogenized and stored in an incubator at 4 °C in a 40% moisture environment.

134   One part of the bulk soil sample was air-dried to measure basal soil properties, and the other part was

135   used for microbial molecular analysis.

136   **2.2 The field straw decomposition and carbon and nitrogen release experiments**

137   The ditch-buried straw decomposition experiment was conducted using litter nylon bags. Maize

138   straw materials were collected after maize harvesting in 2020 and air-dried. Ten grams of maize straw

139   was cut to 2 cm in length and put into nylon litter bags, which were then sealed via heat sealing. The

140   nylon bags were 6 cm × 10 cm in size and were made of 200 mesh nylon fabric, which permitted the

141   free transfer of microorganisms between the nylon bags and soil. At 2ed, May 2021, litter bags

142   containing straw were buried at 10 cm depth in a spatially random design to prevent bags associated

143   with a given decomposition stage being placed together in space. The litter bags were collected after

144   the harvest at 1st, October 2021.

145   The straw decomposition ratio was calculated based on dry weight loss as (dry initial mass - dry

146   final mass)/dry initial mass. The straw-C concentration was measured by titrimetry after oxidation with

147   a mixture of H_2SO_4 and $K_2Cr_2O_7$. Total N, P and K were determined using the Kjeldahl, molybdenum

148   blue colorimetry, and flame photometry methods, respectively. All methods have been described by Lu

149   (2000). The initial and sampled maize straw material properties are shown in Supplemental material

150   Table S2. The amounts of total straw C and N released were calculated by the following equation:

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

152   (or N) content × dry final mass) × aboveground biomass

2.3 Measurement of soil properties and assessment of cropland traits

Soil pH was measured at a soil:water ratio of 1:2.5 (weight/weight). Air-dried soil and 25 ml of deionized water were shaken together for 1 min and left to settle for 30 min, and the soil pH was determined using an electrode. Soil organic carbon (SOC) was measured by titrimetry after oxidation with a mixture of H_2SO_4 and $K_2Cr_2O_7$. Total N and P were determined using the Kjeldahl and molybdenum blue colorimetric methods, respectively. All of these methods have been described by Lu (2000).

Microbial biomass C (MBC) and microbial biomass N (MBN) were analysed using the fumigation-extraction method. Ten grams of fresh soil was fumigated with chloroform in the dark for 24 h, and then the fumigated and nonfumigated soils were extracted with 0.5 M K_2SO_4 and shaken at 200 rpm for 0.5 h. Soil extracts were filtered through a 0.45-μm Millipore filter, and the C and N in the extracts were determined using a multi C/N 3100 analyser (Analytik Jena AG). The C and N contents in extracts of the nonfumigated soil were subtracted from C and N extracted from the fumigated soil to give the C and N extracted from the soil microbial biomass. Values of 0.45 and 0.54 were used to calibrate the contents of MBC and MBN, respectively (Vance et al., 1987; Wu et al., 1990).

The activities of cellulose and N-acetyl-β-glucosaminidase (NAG) were measured using *p*-nitrophenyl-β-D-cellobioside and *p*-nitrophenyl-N-acetyl-β-D-glucosaminide as substrates, 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 0.02 M substrates and then shaken at 200 rpm and 37 °C for 1 h. The reaction was stopped by adding 1 mL of 0.5 M $CaCl_2$ and 4 mL of 0.1 M Tris buffer (pH 12.0). The mixture was suspended with a vortex, the supernatant was filtered, and the concentration of *p*-nitrophenol (PNP) was measured by colorimetry at 400 nm. The same procedure was followed for the controls, with the exception that the

175 substrate was added after the incubation, and $CaCl_2$ and Tris buffer were added (Dick, 2011; Geisseler

176 and Horwath, 2009).

177   To estimate the greenhouse gas emission potential, we conducted a 60-day incubation experiment.

178 Briefly, 20 g of fresh soil was placed in a 250-mL flask and then sealed with a gas-tight lid that had a

179 rubber stopper in the middle. Gas samples (10 mL) were taken from the headspace of each flask at 1, 3,

180 7, 15, 30, and 60 days after sealing using a plastic syringe. The gas sample was immediately injected

181 into a preevacuated 10-mL glass vial. Concentrations of methane (CH_4), N_2O and carbon dioxide (CO_2)

182 were determined using a gas chromatograph (Agilent 7890) equipped with a flame ionization detector

183 for CO_2 and CH_4 and a ^{63}Ni electron capture detector for N_2O. The gas standards were provided by the

184 National Research Center for Certified Reference Materials, Beijing, China. The precision for

185 greenhouse gas emission concentrations was $\pm0.5\%$ based on repeated measurements of gas standards

186 (Qiu et al., 2019). When the maize plants matured, all plants and grains were harvested from each plot,

187 oven-dried at 60 °C for 48 h and weighed. Straw aboveground biomass and crop yield were converted

188 into weight per hectare.

189   We selected 15 soil properties to estimate cropland traits, i.e., the soil fertility index (SOC, total N,

190 total P, MBC and MBN), greenhouse gas emission amount (mainly CO_2, N_2O and CH_4), straw

191 decomposition and C and N released, soil extracellular enzymes (cellulase and

192 N-acetyl-D-glucosaminidase), and maize biomass (aboveground biomass and crop yield). Generally,

193 SOC, total N and total P are the major soil fertility factors and indicate the present nutrient status in

194 croplands, which can be used to explain soil fertility conditions. Microbial biomass reflects ecosystem

195 productivity. Greenhouse gas emissions are related to climate change, which can be regulated by

196 fertilization regimes and soil microbial activities. Soil extracellular enzymes catalyse the

197 decomposition of a range of organic polymers, resulting in C and N turnover. Maize biomass (such as

198 aboveground biomass and crop yield) reflects soil productivity. As a whole, all of these variables

199 together contributed to the cropland function. To evaluate the function of the cropland ecosystem under

200 different fertilization conditions, we calculated an integrative soil ecosystem multifunctionality index

201 for further analysis. Notably, the opposite value was chosen for greenhouse gas emissions. Due to the

202 lack of a specific definition of multifunctionality, we first calculated the Z scores of the 15 measured

203 variables and obtained a multifunctionality value for each plot by averaging the Z scores of the 15

204 variables (Chen et al., 2019).

205 **2.4 DNA extraction and quantification of general fungal ITS, bacterial 16S rRNA and genes**

206 **encoding cellulose-degrading enzymes**

207   Total DNA was extracted from 0.5 g freeze-dried soil by using a Fast DNA Spin Kit for Soil

208 (MPbio, USA) according to the manufacturer's instructions and then dissolved in 50 μl of Tris-EDTA

209 buffer. The quality of the DNA extraction was characterized by electrophoresis on 1% (wt/vol) agarose

210 gels. The quantity and quality of DNA were checked using a Nanodrop spectrophotometer (Nanodrop,

211 PeqLab, Germany). The extracted DNA samples were stored at −80 °C before molecular analysis.

212   Bacterial and fungal abundances were determined to reveal the changes in microbial community

213 compositions. The abundances of bacteria and bacteria fungi were measured according to modified

214 procedures (Fierer and Jackson., 2005). We selected the primers *338F/518R (338F:*

215 *CCTACGGGAGGCAGCAG; 518R: ATTACCGCGGCTGCTGG)* and *NSI1/58A2R (NSI1:*

216 *GTAGTCATATGCTTGTCT; 58A2R: CATTCCCCGTTACCCGTT)* for the qPCR assay. The thermal

217 qPCR profiles for the bacteria and fungi were as follows: 95 °C 2 min for DNA denaturation, 35 cycles

218 (95 °C 30 s, 60 °C 30 s, 72 °C 30 s, 80 °C 15 s) for DNA annealing, and 81 °C, 10 s for DNA extension;

219 95 °C 10 min for DNA denaturation, 40 cycles (95 °C 15 s, 52 °C 30 s, 72 °C 30 s, 79 °C 30 s) for

220 DNA annealing, and 81 °C 10 s for DNA extension, respectively. The initial concentrations of the two

221 plasmids used as the standards for the bacterial and fungal abundance analyses were 1.22×10^{10} and

222 9.05×10^9, respectively.

223 The fungal *cbhI* gene and bacterial *GH48* gene were selected as functional biomarkers of

224 cellulolytic fungi and bacteria, respectively. The primers *GH48 F8/GH48 R5* (*GH48_F8*: 5 -

225 GCCADGHTBGGCG ACTACCT - 3; *GH48_R5*: 5 - CGCCCCABGMSWWGTACCA - 3) and *cbhI*

226 *F/cbhI R* (*cbhI F*: ACCAAYTGCTAYACIRGYAA; *cbhI R*: GCYTCCCAIATRTCCATC) were used for

227 the qPCR assay. The abundance of bacterial *GH48* and fungal *cbhI* genes was quantified according to

228 modified procedures (Zhang et al., 2017). The thermal profiles of qPCR for the target genes of *GH48*

229 and *cbhI* were as follows: 95 °C, 5 min for DNA denaturation, 40 × (94 °C for 30 s, 60 °C for 45 s, and

230 72 °C for 90 s) for DNA annealing, and 84 °C, 10 s for DNA extension; and 94 °C, 4 min for DNA

231 denaturation, 40 × (94 °C for 45 s, 50 °C for 30 s, and 72 °C for 60 s) for DNA annealing, and 81 °C,

232 10 s for DNA extension, respectively. The initial concentrations of the two plasmids as the standards

233 for bacterial *GH48* and fungal *cbhI* gene abundance analysis corresponded to 1.85×10^{11} and 2.65×10

234 ^{10} copies g $^{-1}$ dry soil, respectively. qPCR was performed in triplicate, and amplification efficiencies

235 higher than 95% were obtained with r ^{2} values > 0.99.

236 **2.5 Bacterial 16S rRNA genes and fungal ITS amplification and sequencing**

237 High-throughput sequencing was performed with the Illumina MiSeq sequencing platform

238 (Illumina Inc.). Both the forward and reverse primers were tagged with an adapter and linker sequence,

239 and 8-bp barcode oligonucleotides were added to distinguish the amplicons from different soil samples.

240 The primers *515F* (5'-GTGCCAGCMGCCGCGGTAA-3') and *907R*

241 *(5'-CCGTCAATTCMTTTRAGTTT-3')* were chosen to amplify the 16S rRNA genes in the V4–V5

242 hypervariable region. PCR was conducted in a 50-µL reaction mixture containing 27 µL of ddH₂O, 2

243 µL (5 µM) of each forward/reverse primer, 2.5 µL (10 ng) of template DNA, 5 µL (2.5 mM) of

244 deoxynucleoside triphosphates, 10 µL of 5× Fastpfu buffer, 0.5 µL of bovine serum albumin, and 1 µL

245 of TransStart Fastpfu polymerase (TransGen, Beijing, China). The PCR conditions were 94 °C for 5

246 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

247 10 min (Caporaso et al., 2010).

248 The fungal ITS1 region was amplified using the primer pair *ITS1F*

249 *(CTTGGTCATTTAGAGGAAGTAA)/ITS2 (GCTGCGTTCTTCATCGATGC)* (Ghannoum et al., 2010).

250 The 50-µL reaction mixture of each reaction mix consisted of 1 µl (30 ng) of DNA, 4 µl (1 µM) of each

251 forward/reverse primer, 25 µl of PCR Master Mix, and 16 µl of ddH₂O. PCR amplification was

252 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),

253 with a final extension at 72 °C for 7 min (Ghannoum et al., 2010). All amplicons were cleaned and

254 pooled in equimolar concentrations in a single tube, after which they were subjected to library

255 preparation, cluster generation, and 250-bp paired-end sequencing on an Illumina MiSeq platform

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

257 The raw sequence data were processed using the Qualitative Insights into Microbial Ecology

258 (QIIME) pipeline (Caporaso et al., 2010). Sequences that fully matched the barcodes were selected and

259 distributed into separate files for the bacterial 16S rRNA and fungal ITS genes. Poor-quality sequences

260 with lengths less than 200 bp (for fungal ITS) and 500 bp (for bacterial 16S) and quality scores less

261 than 20 were discarded, and the chimaeras were removed using the UCHIME algorithm (Edgar et al.,

262 2010). The remaining sequences were assigned to operational taxonomic units (OTUs) with a 97%

263 similar threshold using UCLUST (Edgar, 2010). Alpha diversity and Bray–Curtis distances for

264 principal coordinate analysis of the soil microbial community were calculated after rarefying all

265 samples to the same sequencing depth.

266 **2.6 Statistical analysis**

267 The soil ecosystem multifunctionality index, crop yields, microbial traits and other relevant soil

268 variables among treatments were subjected to a chi-square test for independence of variance.

269 Significant differences were determined by one-way analysis of variance (ANOVA) based on the post

270 hoc Tukey test at the 5% level. Prior to ANOVA, the normality and homogeneity of variances were

271 tested by the Kolmogorov–Smirnov test and Levene's test, respectively. If normality was not met, log

272 or square-root transformation was implemented. One-way ANOVA was performed using SPSS 21.0

273 (SPSS Inc., Chicago, IL, USA).

274 Nonmetric multidimensional scaling (NMDS) analysis was used to describe and evaluate the

275 microbial community composition. The NMDS was performed in the "Vegan" package of R (4.0.2).

276 Analysis of similarities (ANOSIM) was used to examine the significant differences in microbial

277 community structure under different fertilization.To describe the complex co-occurrence patterns in

278 various organisms, we constructed co-occurrence networks. We focused on the abundant microbial

279 phylotypes (with average relative abundance > 0.01% for bacteria and fungi) for network construction.

280 Nodes with Pearson correlations greater than 0.70 and $p < 0.05$ were retained. Network visualization

281 between microbial taxa and ecological clusters of microbial phylotypes were conducted and identified

282 by Gephi software. To obtain the keystone species of each network, a *Zi-Pi* plot series was constructed

283 to determine the role of each OTU. According to Deng et al. (2012), the plot includes (a) peripheral

284 nodes ($Z \leq 0.25$, $P \leq 0.62$). (b) module hubs ($Z > 0.25$, $P \leq 0.62$), (c) connectors ($Z \leq 0.25$, $P > 0.62$)

285 and (d) network hubs ($Z > 0.25$, $P > 0.62$). From an ecological perspective, OTUs in module hubs,

286 connectors and network hubs may be regarded as the microbial keystone taxa of the network systems

287 (Deng et al., 2015).

288 To construct the relationship between fertilization, soil function, and microbial traits, two

289 heatmaps were constructed in this study (Origin 2022). The first heatmap was constructed to reveal the

290 associations between cropland properties with microbial module comminuties. And another heatmap

291 was constructed to reveal the associations between microbial traits and fertilizers, soil properties,

292 greenhouse emissions and ecosystem multifunctionality. The random forest algorithm was performed in

293 the R package (4.0.2) "RandomForest" to estimate the importance predictors of soil properties and

294 microbial traits on ecosystem multifunctionality.

295 **3. Results**

296 **3.1 Cropland properties**

297 Data collection after a continuous 4-year in situ field experiment under different N input levels

298 revealed changes in cropland traits (Fig. 1). In terms of soil fertility, compared with the N-limitation

299 treatments (PK and 0.5N+PK), the SOC and total P contents were increased significantly by the N+PK

300 and 0.75N+PK treatments (Fig. 1a, c) ($P < 0.05$), while there were no significant changes in the total N

301 content (Fig. 1b). After straw decomposition (Fig. 1d), the amounts of straw C (Fig. 1e) and N (Fig. 1f)

302 released showed different responses to varying N fertilizer input levels. Generally, N-rich treatments

303 (N+PK and 0.75N+PK) significantly increased the straw decomposition rate and achieved higher

304 amounts of straw C and N release than the N-limitation treatments ($P < 0.05$). However, there was no

305 significant difference between N+PK and 0.75N+PK. Microbial biomass C and N content, as well as

306 associated enzyme activity were changed after different N fertilizer application rates (Fig. 1g, h, i and j).

307 The MBC (Fig. 1g) and MBN (Fig. 1h) contents were significantly higher in the N-rich treatments than

308 in the other treatments. However, the highest cellulase activity was observed in the 0.75N+PK

309 treatment, which was significantly higher than that in the other treatments (Fig. 1i) ($P < 0.05$), and the

310 N-acetyl-D-glucosaminidase activity decreased with the reduction in N application (Fig. 1j).

311 For greenhouse gas emissions, with the decrease in N fertilizer application levels, CO_2 and N_2O

312 emissions gradually decreased (Fig. 1k, l). No significant difference was observed in CH_4 emissions

313 under the different fertilization treatments (Fig. 1m). In addition, the N fertilizer levels also had a

314 strong influence on maize yields and aboveground biomass (Fig. 1n, o). The 0.75N+PK treatment

315 achieved the highest multifunctionality index (0.61), followed by N+PK (0.32), 0.5N+PK (-0.34) and

316 PK (-0.59) (Fig. 1p).

317 However, although the 0.75N+PK treatment increased the straw N release amount and may meet

318 the requirements for plant growth, the total N input was still dominated by inorganic N input (Fig. S1).

319 Therefore, the N released from the straw cannot offset the deficiency of N fertilizer. Additionally,

320 contrasting N fertilizer input levels significantly changed the stoichiometry of C, N and P (Fig. S2).

321 Notably, the 0.75N+PK treatment significantly increased the C:N ratio compared with the 0.5N+PK

322 and PK treatments ($P < 0.05$). The lowest C:N ratio was shown for the 0.5N+PK treatment (Fig. S2a).

323 The N:P and C:P ratios showed no significant difference regardless of nutrient excess or limitation (Fig.

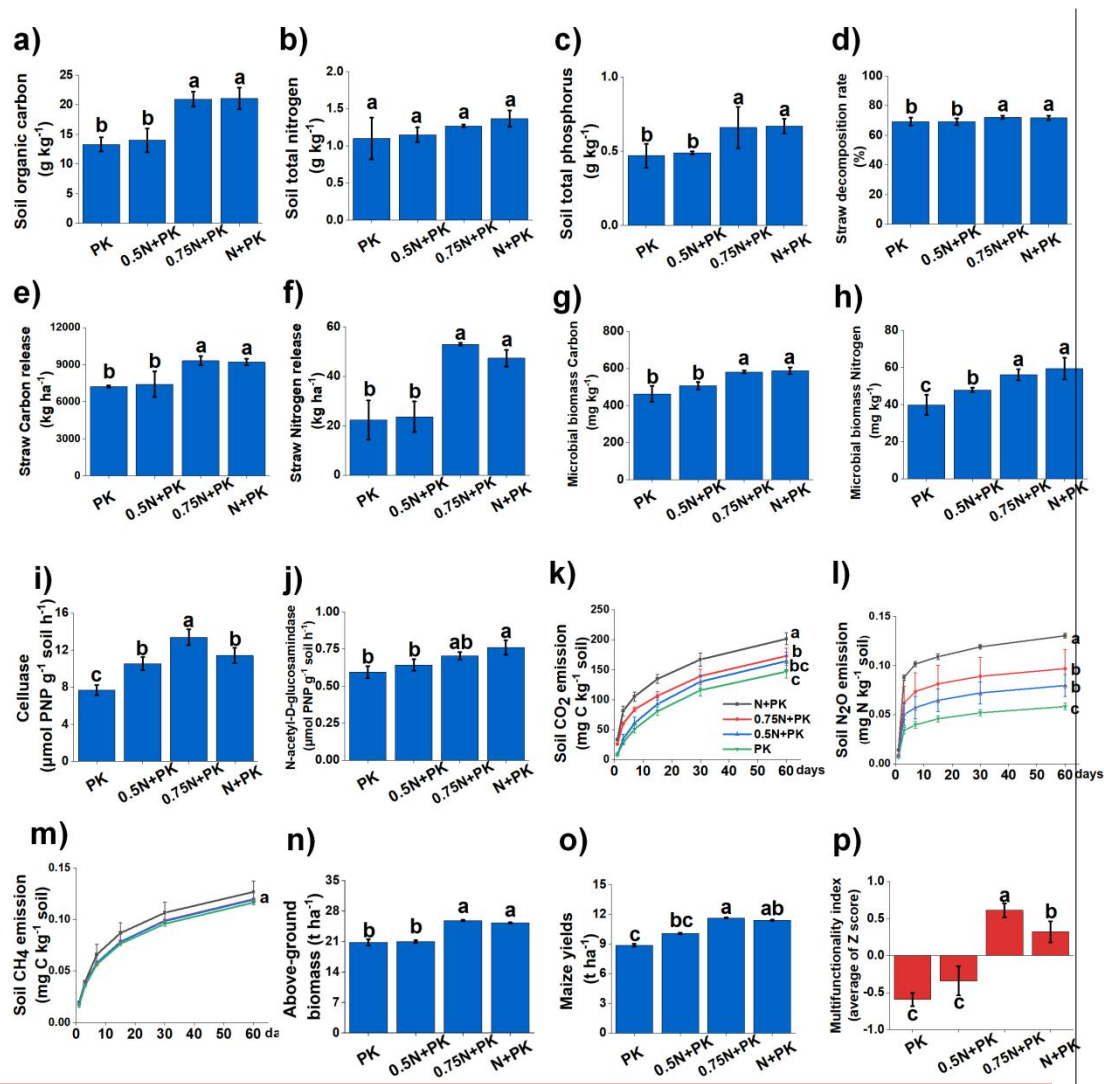
324 S2b and c).

325

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.

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

N fertilizer input levels had marked impacts on the abundances of fungi and bacteria (Table S3).

The highest fungal abundance was observed in the 0.75N+PK treatment, which was significantly

higher than that in the other treatments ($P < 0.05$). The N+PK treatment significantly increased

335   bacterial abundance compared with the PK treatment ($P < 0.05$), while there were no obvious

336   differences among the N+PK, 0.75N+PK and PK treatments. The ratios of fungi to bacteria also

337   showed contrasting responses to N fertilization (Table. S3). The 0.75N+PK treatment significantly

338   increased the ratio of fungi to bacteria compared with the other treatments ($P < 0.05$), and the lowest

339   ratio of fungi to bacteria was found in the PK treatment.

340   **Table 1 The abundances of genes encoding ellulose-degrading enzymes**

341   **across different N fertilizer level treatments after straw return**

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

342   The results show means ± standard deviations (n = 3). Different lowercase letters after values

343   indicate significant differences between each treatment, $P < 0.05$. N+PK, straw return plus

344   regular inorganic N-P-K fertilizers; 0.75N+PK, straw return plus regular inorganic P-K with

345   25% N fertilizer reduction ; 0.5N+PK, straw return plus regular inorganic P-K with 50% N

346   fertilizer reduction; PK, straw return plus regular inorganic P-K without N fertilizer.

347

348   N fertilizer input levels led to changes in the expression levels of genes encoding

349   cellulose-degrading enzymes (Table 1). The N-rich treatments achieved higher fungal *cbhI* and

350   bacterial *GH48* gene abundance than the N-limitation treatments. In contrast, the highest *cbhI* gene

351   abundance was shown in the 0.75N+PK treatment, while the highest *GH48* gene abundance was shown

352 in the N+PK treatment. Compared with the PK treatment, the ratio of the fungal *cbhI* gene to the

353 bacterial *GH48* gene increased significantly under the 0.75N+PK treatment ($P < 0.05$).

3.3 Co-occurrence network analysis of the microbial community

355 Regarding fungal alpha diversities, there were no significant differences in the Chao1 index across

356 treatments. The N+PK treatment significantly increased fungal richness compared with the PK

357 treatment ($P < 0.05$) (Table S4). In addition, the PK treatment resulted in lower bacterial richness than

358 the other treatments ($P < 0.05$). No significant difference was observed in the bacterial Chao1 index

359 across treatments (Table S4). NMDS plots showed that diverse N input levels significantly changed the

360 fungal (Fig. S3a) and bacterial communities (Fig. S3b) ($P < 0.05$).

361 We further conducted network analysis to identify co-occurrence patterns between specific

362 microbial taxa (Fig. 2). The cooccurrence network was aggregated into smaller coherent modules that

363 were examined to determine important module-trait relationships. The present network comprised 1963

364 nodes (composed of 1520 bacterial taxa and 443 fungal taxa) and 62206 edges with 52.49% positive

365 associations (Fig. 2a). The results showed four dominant ecological modules (1-4) that strongly

366 co-occurred within the multitrophic network, which contributed 86.10% of the whole network. Among

367 the four modules, bacteria accounted for the highest proportion in each module, contributing more than

368 70% of the total (Fig. 2b). The percentage of edges linking bacteria to bacteria (B-B) was higher than

369 that linking fungi to fungi (F-F) and bacteria to fungi (B-F). The highest proportion of B-B (80.32%)

370 was found in Module 3, while the highest proportion of B-F (32.66%) and F-F (6.00%) was found in

371 Module 4 (Fig. 2c).

372

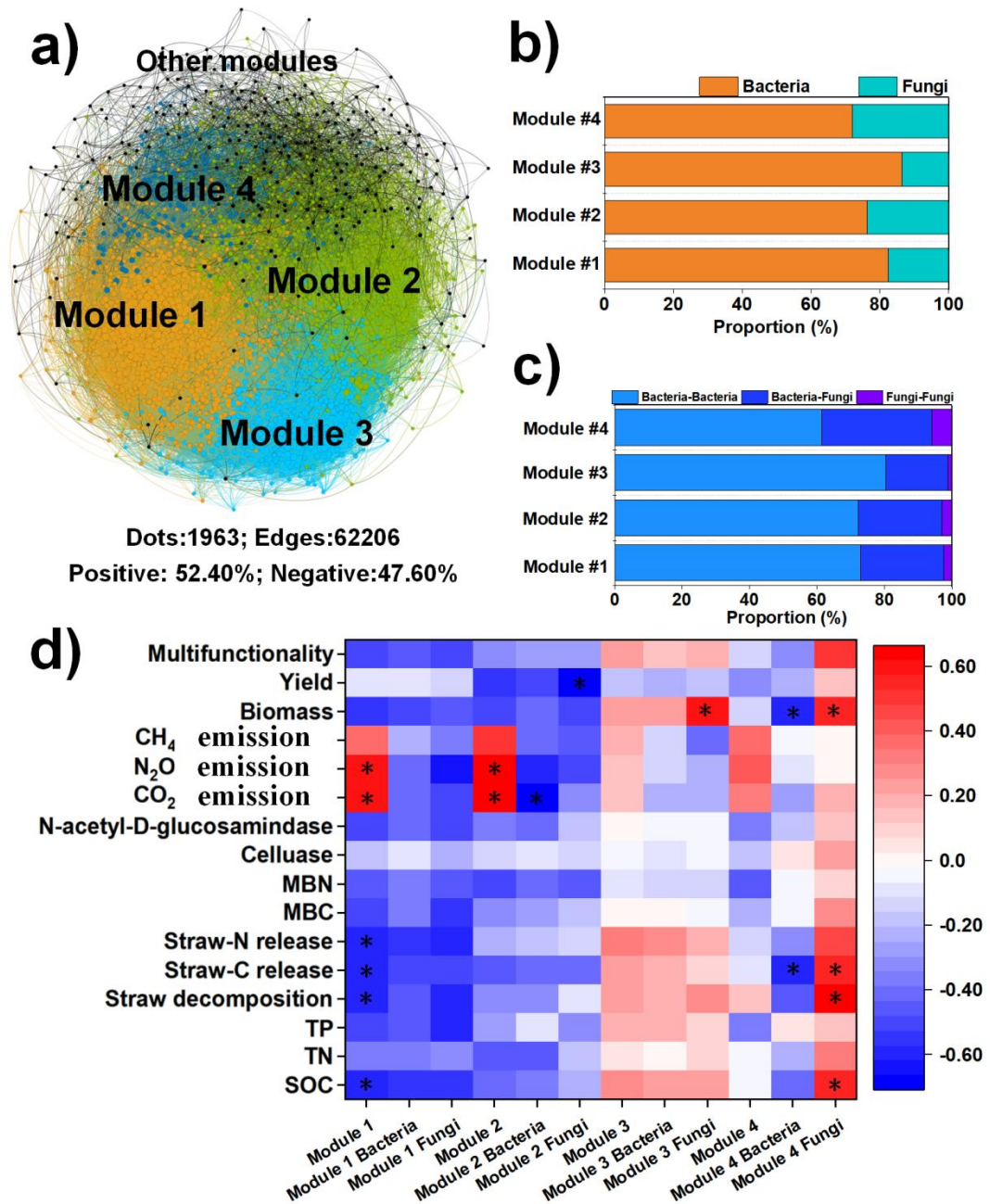



Fig. 2 The relationships of microbial module communities with cropland traits. Multitrophic

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

378   major ecological modules (c). Links between the specific module communities with cropland

379   traits (d). * indicates significance at $P < 0.05$. Abbreviations: SOC, soil organic carbon; C: N, the

380   ratio of the SOC content to the total N content; N: P, the ratio of the total N content to the total P

381   content.

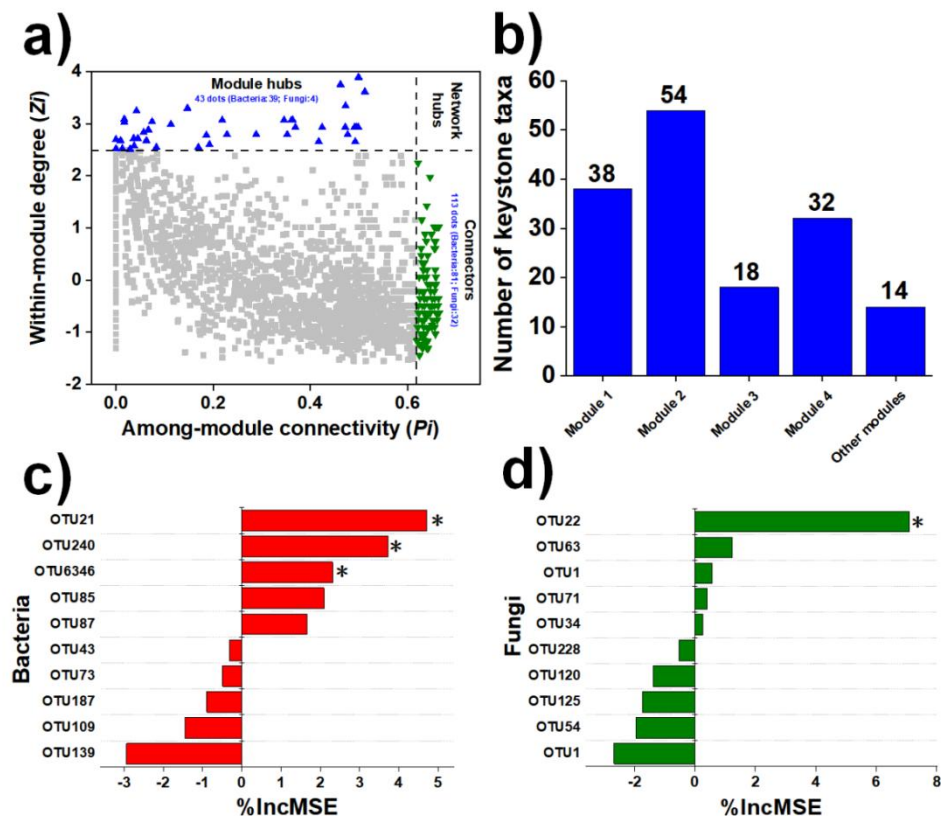
382   Individual nodes represented different roles in the microbial network based on the intramodule

383   connectivity Zi and the intermodule connectivity Pi. ZP plots were constructed to identify the

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

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

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

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

388

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

390   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.

3.4 Linkage between microbial traits and soil ecosystem multifunctionality

The heatmap showed the close correlations of N input (fertilizer and straw return) with soil

stoichiometry and microbial traits (Fig. 4). Overall, the N input level, straw biomass and C:N ratio

upregulated the abundance of genes encoding cellulose-degrading enzymes. In addition, N input was

positively correlated with bacterial abundance, while a significant correlation was observed between

straw biomass and the N input level. The random forest model was also used to identify abiotic and

biotic attributes correlated with soil ecosystem multifunctionality (Fig. 5). The model explained

83.89% of the variance in ecosystem multifunctionality. The results indicated that the N input level,

straw biomass and soil C:N ratio were the most prominent abiotic factors correlated to the ecosystem

multifunctionality index, while some biotic factors, such as the abundance of genes encoding

cellulose-degrading enzymes, significantly correlated to the ecosystem multifunctionality index.

Moreover, to clarify the potential main specific drivers of cropland traits, correlations between the

microbial physiological traits and soil properties were determined to illuminate the role of the

microbial community in soil ecosystem multifunctionality (Fig. 2d). The results indicated that the

particular microbial module community was significantly correlated with cropland traits. The

communities of modules 1 and 2 and the fungal community in module 4 showed potential in cropland

traits (Fig. 2d). Specifically, significant correlations were observed between the SOC content, straw

decomposition, straw C/N release, CO_2/N_2O emissions and the module 1 community; the module 2

community was positively correlated with greenhouse gas emissions (except for CH_4); and the fungal

community in module 4 was positively correlated with the SOC content, straw decomposition, straw

C/N release and straw biomass. Furthermore, the bacterial and fungal communities belonging to

module 2 and the fungal community belonging to module 3 were significantly correlated with CO_2

emission, maize yield and straw biomass.

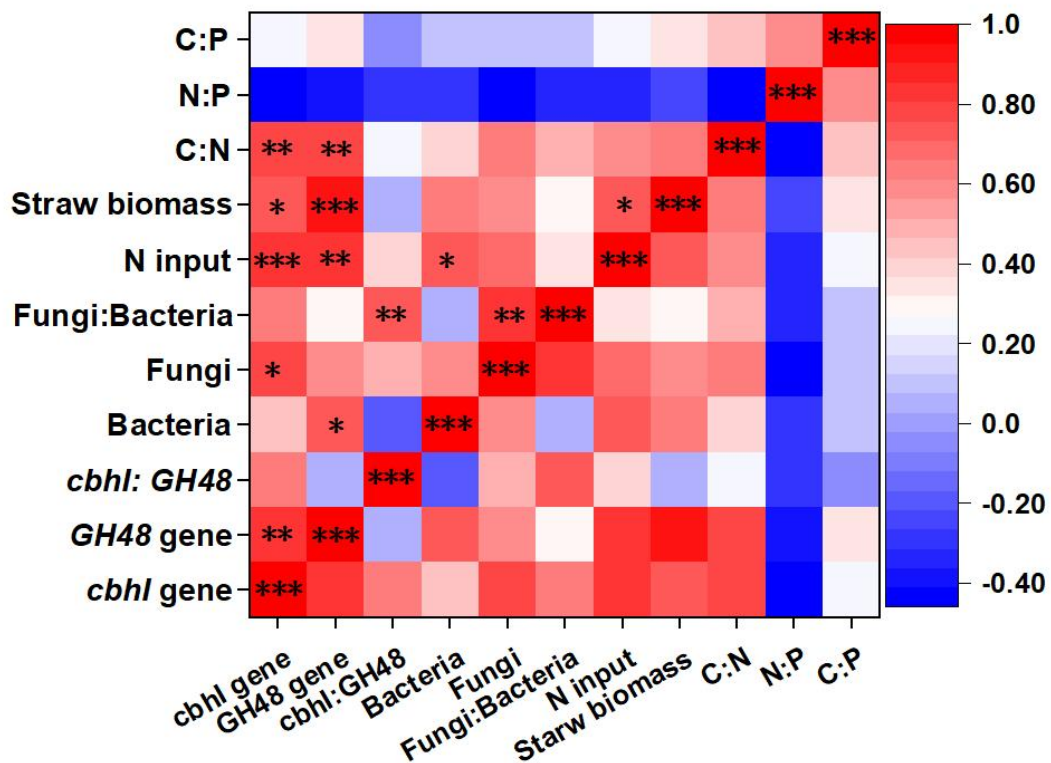



Fig. 4 Heatmap revealing the correlation coefficients between microbial traits with fertilization

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.

At the scale of microbial species, we selected the 20 keystone taxa (10 bacterial and 10 fungal

taxa) with the highest relative abundance for further analysis. The random forest models indicated that

the specific keystone taxa strongly influenced soil ecosystem multifunctionality (Fig. 3c and d).

426 Bacterial *Terrimonas* (in module 1), Myxococcales (in module 2) and *Terrimonas* (in module 3) were

427 highlighted as essential predictors of soil ecosystem multifunctionality, and fungal Lasiosphaeriaceae

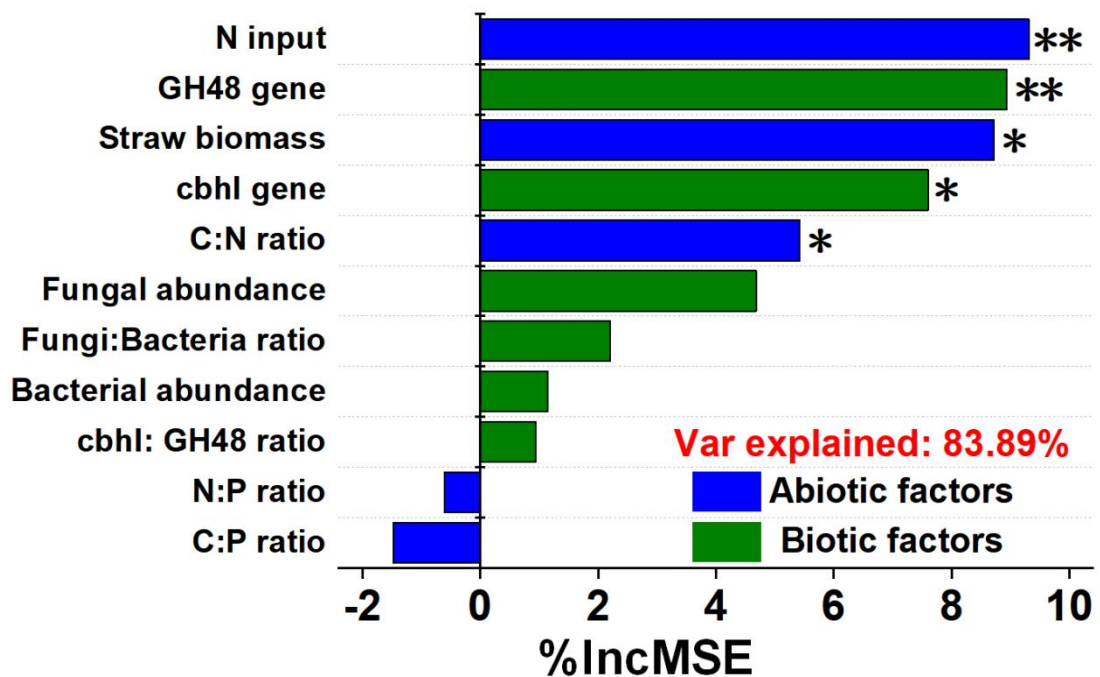
428 (module 3) was also found to be an important variable for predicting its changes. Subsequently, the

429 relative abundances of selected keystone taxa were different across different N fertilizer level

430 treatments after straw return (Table S5). The relative abundances of fungal OTU22 and bacterial

431 OTU21 were higher in the N-rich treatments than in the N-limitation treatments. Moreover, compared

432 with the N+PK treatment, the 0.75N+PK treatment increased the relative abundances of fungal OTU22

433 by 38.20% and bacterial OTU21 by 40.63%.

434

435 **Fig. 5** Contribution of abiotic and biotic variables to the soil multifunctionality index. *, ** and

436 *** indicate significance at $P < 0.05$, 0.01 and 0.001, respectively. Abbreviations: C: N, the ratio

437 of the SOC content to the total N content; N: P, the ratio of the total N content to the total P

438 content.

439 **4. Discussion**

4.1 Effect of N fertilizer reduction on cropland traits after straw return

Soil fertility, straw decomposition, C and N release amounts, and crop productivity were mostly higher under 0.75N+PK and N+PK than other treatments, implying that better soil multifunctionality was achieved. Moreover, N+PK increased greenhouse gas emissions (Fig. 1). Higher microbial biomass C and N, as well as relevant enzyme activities, were also observed under N-rich treatments, indicating the strong positive impact of abundant N fertilizer application (Fig. 1g, h, i, j). It was reported that straw return with N fertilizer application can stimulate microbial activity and promote biomass accumulation (Treseder, 2008). The substantially increased straw decomposition and straw C and N release under N-rich treatments may be primarily attributed to the activation of microbial activity (Fig. 1d, e, f), which is consistent with previous research (Ramirez et al., 2012). Our results indicated that 0.75N+PK maintained soil fertility index net primary production compared to N+PK. This study demonstrates that 0.75N+PK has similar effects on cropland traits as N+PK. Therefore, it can be concluded that 0.75N+PK is a more efficient and effective option for improving soil functions. Moreover, 0.75N+PK may enhances N fertilizer use efficiency and stimulates microbial functioning by modulating the stoichiometry of C,N and P in the soil, which promotes soil fertility and crop yield (Liu et al., 2010). Reducing the amount of N fertilizer by more than 50% led to insufficient N input to meet the needs of both crops and microbes, resulting in a decline in soil health (Williams et al., 2013). Recent studies have also proven that rational N input can stimulate microbial ex vivo production of extracellular enzymes to accelerate straw decomposition and nutrient transformation (Chen et al., 2016). Moreover, it is well known that fungi have high nutrient utilization efficiency; thus, more straw-derived C and N would be stored in soil under N-rich treatments than under N-limited treatments (Hou et al., 2020). Rational N availability is also the premise of straw decomposition and SOM formation due to

462 the microbial "stoichiometry decomposition" theory, while the "N-mining" theory in N-limitation

463 treatments reveals that oligotrophic species (such as *K*-strategists) degrade native SOM because of the

464 lack of N fertilizer inputs (Chen et al., 2014). In this study, a 25% reduction in N fertilizer application

465 may be the threshold value for the "N-mining" and the "stoichiometry decomposition". Finally, the

466 increases in SOC, total N, and P contents and straw C and N release, as well as microbial biomass and

467 function, are commonly attributed to high aboveground biomass and maize yields (Fig. 1n, o), which

468 are favourable from the viewpoint of cropland traits.

469 However, the overuse of N inputs also causes more greenhouse gas emissions (Tang et al., 2019).

470 In the present study, greenhouse gas emissions were quantified to evaluate the ecosystem degeneration

471 under different N fertilizer input levels, which means the larger the value, the lower the soil ecosystem

472 multifunctionality (Fig. 1k, l, m). Straw return with N fertilizer addition might be the crucial driver of

473 CO_2 and N_2O emissions from agroecosystems and has been widely studied in previous literature

474 (Gregorich et al., 2005). CO_2 and N_2O emissions increased significantly compared with those under the

475 PK treatment, likely by stimulating the activity of copiotrophs when sufficient C and N substrates were

476 provided. For example, Dieleman et al. (2010) implied that an increase in CO_2 and N_2O as the amount

477 of N input increases through meta-analysis and field experiments, respectively. Qiu et al. (2019)

478 indicated that the emission of CO_2 enhanced root and mycorrhizal N uptake and increased N_2O

479 emissions, which was related to the changes in the soil denitrifier community composition in favour of

480 N_2O-producing taxa (nirK- or nirS-type). In addition, there was no difference in CH_4 emissions among

481 treatments, although contradictory results have been widely reported in previous literature (Tang et al.,

482 2019). Mapanda et al. (2011) and Liu et al. (2012) indicated that the emission of CH_4 depended highly

483 on the soil water content in maize crops, which is in line with our results. To summarize, previous

484   researches clearly demonstrated a positive correlation between CO_2 and N_2O emissions with N input.

485   So, this study unequivocally showed that N+PK emits more greenhouse gases than 0.75N+PK.

486   In summary, compared with the N+PK treatment, the 0.75N+PK treatment supported multiple soil

487   functions, including promoting soil fertility, straw nutrient release and microbial activity and

488   alleviating greenhouse gas emissions (Fig. 1p). Therefore, a reduction of 25% in chemical N fertilizer

489   input with straw return may be the appropriate regime to promote soil functions in meadow soils on the

490   Northeast China Plain.

491   **4.2 Responses of the microbial abundance and function to straw return with N fertilizer**

492   **reduction**

493   Fungal and bacterial abundances, as well as the ratio of fungi to bacteria, were sensitive to the

494   changes in the N fertilizer input levels (Table S3 and Fig. 2). Straw addition with N fertilizer input

495   supplied enough C and N for microbial metabolism, thus promoting microbial proliferation (Chen et al.,

496   2016). Generally, bacterial abundance decreased with reduced N fertilizer input. This is mainly because

497   bacteria are more sensitive to N availability than fungi, which is in line with a previous study (Ramirez

498   et al., 2020). It is worth noting that a 25% reduction of N fertilizer significantly increased fungal

499   abundance compared with regular N inputs. This result might be attributed to the negative effect of

500   excess N fertilizer (Wan et al., 2015). Moreover, Ning et al. (2020) demonstrated that the C:N ratio was

501   the pivotal factor in fungal community compositions after performing 7 long-term field experiments

502   under different fertilization conditions across China and reported a significant positive correlation

503   between them. It is well known that fungi have a strong C utilization efficiency compared to bacteria

504   (Duan et al., 2021). Therefore up-regulation of fungal abundance and lowering the ratio of bacteria to

505   fungi are crucial for straw degradation and SOC accumulation. Previous studies have shown that C:N:P

ratio of soil microbial biomass was stable at 60:7:1 (Cleveland et al., 2007) and fungal biomass C:N

ratio was higher than this ratio (nearly 15.7:1) (Zhang et al., 2017). Generally, Bahram et al. (2018)

concluded that higher C:N ratio may promote fungal abundance and decrease bacteria:fungi ratio.

Excessive N fertilizer input may reduce the soil C: N ratio, while little N fertilizer input can not meet

the growth requirements of crops and microorganisms (Ning et al., 2020). Therefore, appropriate

enhancement of soil C: N ratio can increase the ratio of fungi to bacteria, stimulate fungal function, and

promote straw degradation and SOC accumulation. Therefore, the 0.75N+PK treatment with a higher

C:N ratio (16.47) may facilitate the proliferation of microorganisms and promote an increase in

microbial abundance.

Subsequently, our results showed that N-rich treatments resulted in higher microbial

cellulose-degrading gene abundances than the PK treatment (Table 1), which demonstrated the

irreplaceable role of N inputs in straw degradation (Zhang et al., 2017). Additionally, compared with

bacterial *GH48* gene abundance, the increase in fungal *cbhI* gene abundance required adequate N

fertilizer inputs and was regulated by the soil C:N ratio, which suggests that rational N fertilizer inputs

could promote fungal function for further degradation of recalcitrant straw components (Hou et al.,

2020). Therefore, the ratio of *cbhI* gene abundance to *GH48* gene abundance was higher under

0.75N+PK than under the N-limitation treatments since the increased expression of a fungal

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

Our results indicated that 75%-100% N fertilizer could upregulate fungal and *cbhI* gene

abundances, which may lead to straw decomposition and SOC accumulation. It is therefore necessary

to further explore the potential associations between microbial traits and soil functions under diverse N

fertilizer input levels.

4.3 Linkages of cropland properties with microbial traits

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

Numerous studies have shown that core microbiota play a vital role in maintaining the stability of soil microbial function and the complexity of microbial networks and then promoting soil nutrient cycling and other soil functions (Ghannoum et al., 2015), and keystone species may show great explanatory power in terms of specific network (or module) structure and function (Chen et al., 2019b). In the present study, *Terrimonas* (bacterial species in module 1) and *Lasiosphaeriaceae* (fungal species in module 3) were detected as the keystone taxa in influencing soil multifunctionality of the cooccurrence network (Table S5). A previous study demonstrated that straw addition significantly increased the relative abundance of *Lasiosphaeriaceae*, which implied straw decomposition ability (Song et al., 2020). Afterwards, *Lasiosphaeriaceae* was proven to promote straw-derived C and N accumulation by secreting multiple extracellular enzymes (Guo et al., 2022). Meanwhile, Sun et al.

550 (2023) revealed that *Lasiosphaeriaceae* abundance was regulated by the soil C:N ratio, especially

551 changes in mineral N. Therefore, *Lasiosphaeriaceae* can effectively promote straw degradation and

552 straw C and N release while driving the function and community of module 1, which is consistent with

553 our results (Fig. 2d). However, relatively few studies have focused on the function of *Terrimonas*, so

554 this study focused on *Chitinophagaceae*. As reported in the previous literature, straw return was the

555 main method to increase *Chitinophagaceae* abundance (Li et al., 2021). Furthermore,

556 *Chitinophagaceae* was indicated to have a strong ability to accumulate soil C and N and degrade

557 cellulose (Zhong et al., 2022), facilitating production improvement by regulating the module 3

558 community and function, which is in line with our results (Fig. 2d).

559   Overall, straw return with sufficient N fertilizer application can increase the C:N ratio and

560 stimulate microbial traits, which ultimately achieve soil ecosystem multifunctionality (Fig. 6). Straw

561 return without enough N supply cannot support soil functions due to the decomposition of native SOM

562 and the out-of-balance microbial community composition, according to the "N-mining" theory (Chen et

563 al., 2014); straw return with sufficient N application (N+PK and 0.75N+PK) can promote soil fertility,

564 straw release, microbial activity and crop productivity, which can be explained by the "stoichiometry

565 decomposition" theory (Chen et al., 2014). Meanwhile, N+PK also caused more serious ecosystem

566 degeneration, such as greenhouse gas emissions, than the 0.75N+PK treatment. Moreover, compared

567 with the N+PK treatment, the 0.75N+PK treatment increased the soil C:N ratio and stimulated

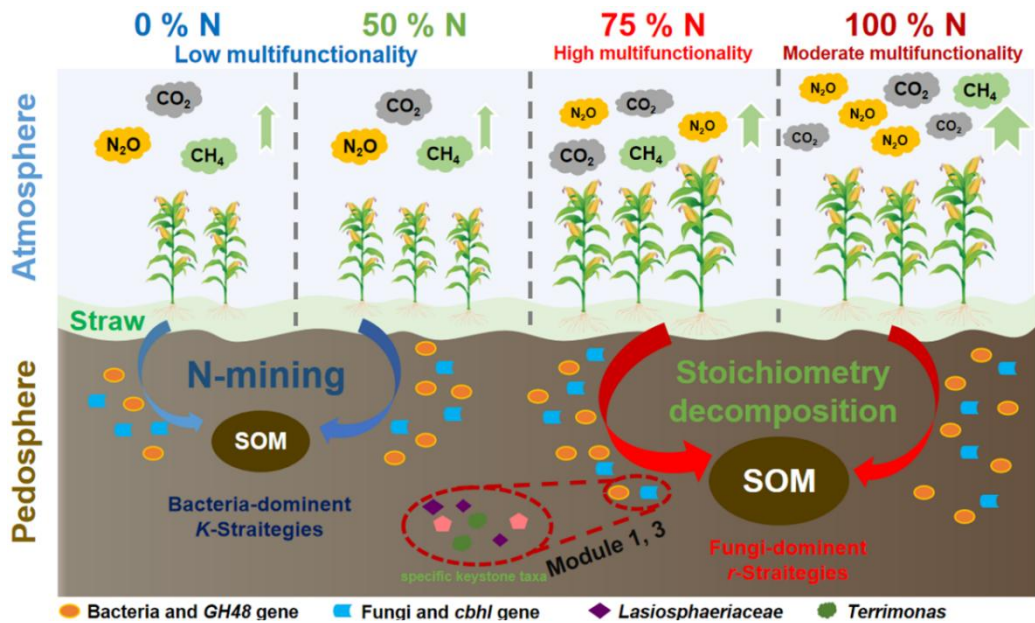
568 microbial module 1 and 3 communities function, *cbhI* gene abundance, and keystone taxa abundances,

569 which were significantly positively correlated with soil ecosystem multifunctionality. While

570 *Lasiosphaeriaceae*-driven module 1 and *Terrimonas*-driven module 3 communities may be involved in

571 maintaining soil ecosystem multifunctionality. Our study provides evidence that a 25% reduction of

572   chemical N fertilizer after straw return was the optimal agronomic measure for soil functions in

573   meadow soil on the Northeast China Plain.

574

575   **Fig. 6** A graphical sketch of the changes in cropland traits and potential microbial mechanisms in

576   response to different chemical N fertilizer application rates after straw return. N, nitrogen; SOM, soil

577   organic matter

578   **5. Conclusion**

579   Straw return combined with different chemical N fertilizer application rates significantly changed

580   cropland traits. Collectively, our work indicates that compared with the N+PK treatment, straw return

581   with a 25% reduction in chemical N fertilizer has the potential to improve soil functions by maintaining

582   soil fertility, productivity, microbial biomass and function, promoting straw decomposition and C and

583   N release and alleviating greenhouse gas emissions. The 0.75N+PK treatment achieved higher soil

584   ecosystem multifunctionality than all other treatments. In addition, the N input level, straw biomass and

585   soil C:N ratio can upregulate the abundances of the *cbhI* and *GH48* genes, which may together achieve

586   soil ecosystem multifunctionality.

587   Furthermore, the changes in multiple cropland traits were strongly associated with microbial

588 module communities and keystone taxa. The relationships between cropland properties and microbial

589 traits were examined here to confirm that the *Lasiosphaeriaceae* driving the function and structure of

590 the module 1 community leads to the promotion of straw degradation and straw C and N release, while

591 *Terrimonas* driving the function and structure of the module 3 community probably contributes to

592 production improvement under 0.75N+PK treatment. Therefore, a 25% reduction in chemical N

593 fertilizer with straw return might be a win–win strategy that not only produces considerable ecological

594 benefits for the pedosphere and atmosphere but also reduces fertilizer expenditures in meadow soil on

595 the Northeast China Plain.

Author contributions

YD, LFW, and XHM designed the experiment; YD, HMC, ZN, WLZ, and YMW performed the measurements; YD, YMC, MXZ, and JYL analyzed the data; YD and MHC wrote the manuscript draft; YML, JYL and LFW reviewed and edited the manuscript.

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 cropland traits. 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 cropland traits (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 cropland traits 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 ellulose-degrading enzymes across different N fertilizer level treatments after straw return

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

The results show means ± 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.
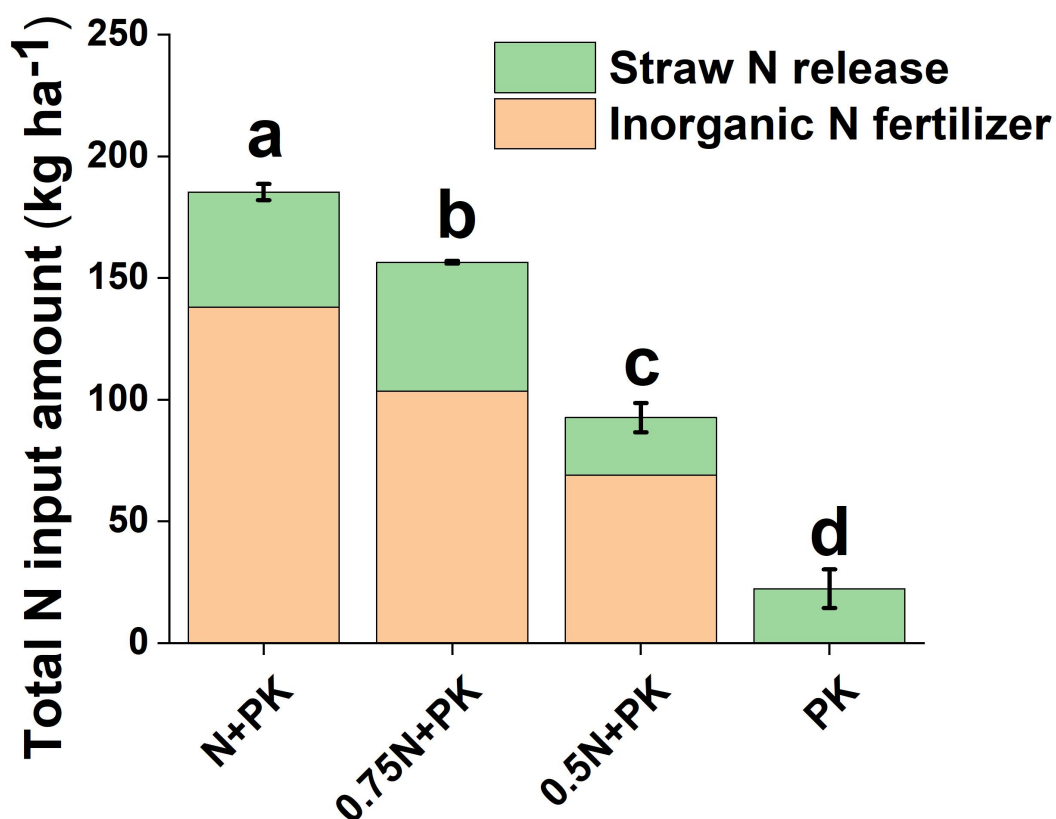
Fig. S1 The total N fertilizer input amount under different treatments. Different letters indicate significant differences at the level of $P < 0.05$. N+PK, straw return plus regular inorganic N-P-K fertilizers; 0.75N+PK, straw return plus regular inorganic P-K with 25% N fertilizer reduction; 0.5N+PK, straw return plus regular inorganic P-K with 50% N fertilizer reduction; PK, straw return plus regular inorganic P-K without N fertilizer.
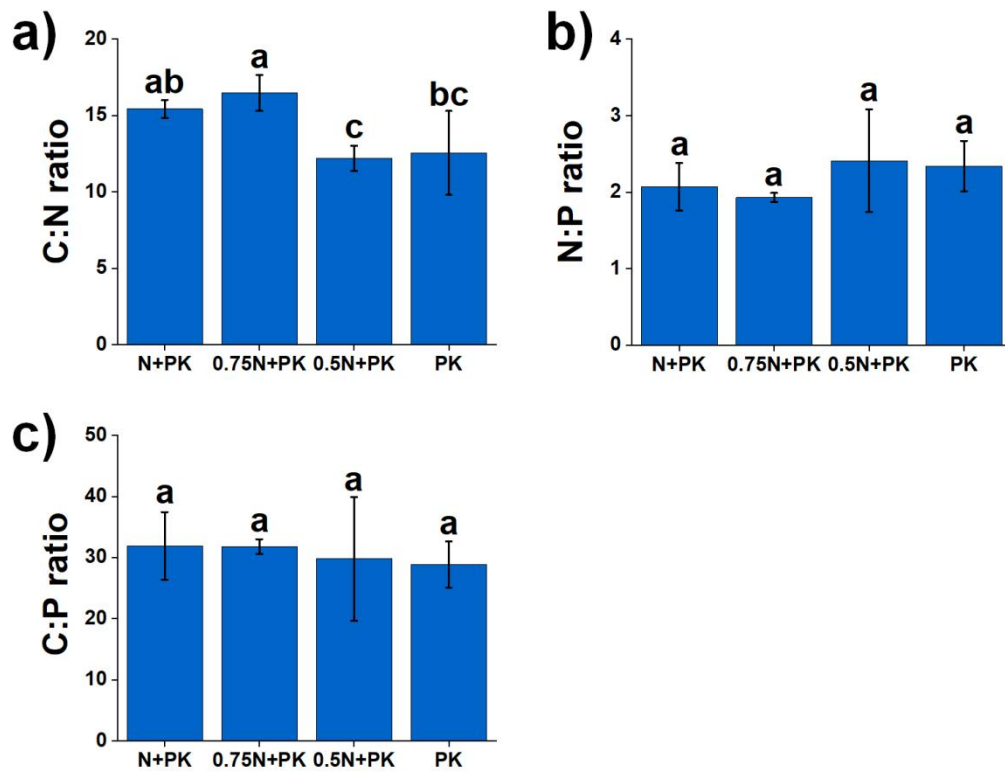
Fig. S2 The soil C:N ratio (a), N:P ratio (b) and C:P ratio (c) under different treatments. Different letters indicate significant differences at the level of p < 0.05. N+PK, straw return plus regular inorganic N-P-K fertilizers; 0.75N+PK, straw return plus regular inorganic P-K with 25% N fertilizer 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.
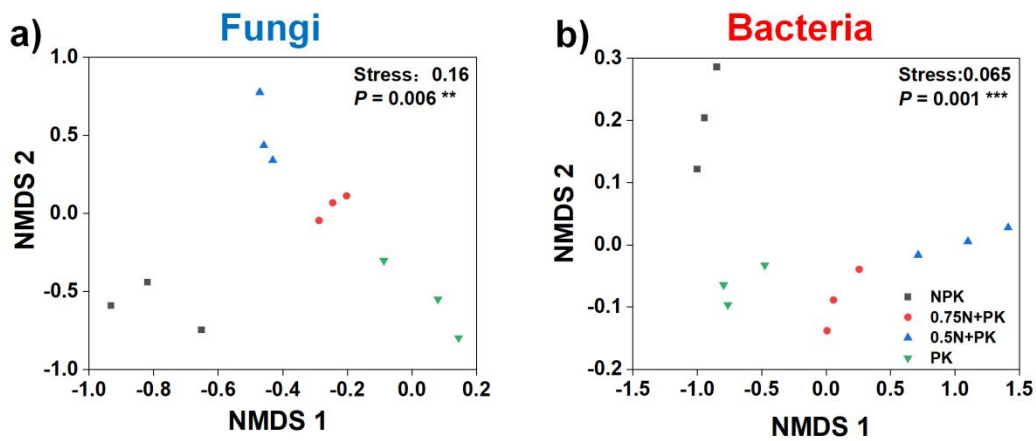
Fig. S3 Non-metric multidimensional scaling ordination showing the fungi (a) and bacteria (b) under

different N input levels; significant differences in sample clustering are measured by ANOSIM. 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.

Table S1 The yields and soil chemical properties under different treatments of bulk soil during the experimental process

Year	Treatment	Yield (t ha^{-1})	pH	SOC (g kg^{-1})	Total N (g kg^{-1})	Total P (g kg^{-1})
2019	N+PK	11.17±0.73 a	7.29±0.14 a	17.60±2.10 a	0.93±0.02 a	0.65±0.08 a
	0.75N+PK	10.91±0.29 a	7.31±0.20 a	15.87±3.12 ab	0.94±0.08 a	0.61±0.12 a
	0.5N+PK	9.81±0.32 b	7.39±0.18 a	14.01±1.42 b	0.89±0.10 a	0.64±0.03 a
	PK	9.93±0.39 b	7.51±0.20 a	13.58±0.15 b	0.86±0.05 a	0.55±0.27 a
2020	N+PK	11.39±0.33 a	7.27±0.10 a	17.11±1.95 a	0.93±0.07 a	0.69±0.07 a
	0.75N+PK	12.00±1.19 a	7.28±0.14 a	17.01±1.77 a	0.88±0.21 a	0.63±0.10 a
	0.5N+PK	9.88±0.84 b	7.27±0.11 a	12.45±0.16 b	0.86±0.01 ab	0.67±0.07 a
	PK	9.84±0.44 b	7.48±0.16 a	11.76±0.82 b	0.81±0.03 b	0.59±0.05 a
2021	N+PK	11.41±0.05 ab	7.25±0.21 a	21.08±1.82 a	1.37±0.11 a	0.67±0.05 a
	0.75N+PK	11.65±0.06 a	7.28±0.14 a	20.95±1.27 a	1.27±0.02 a	0.66±0.19 a
	0.5N+PK	10.08±0.08 bc	7.25±0.02 a	14.01±2.01 b	1.15±0.10 a	0.49±0.10 b
	PK	8.89±0.13 c	7.39±0.10 a	13.33±1.18 b	1.10±0.28 a	0.47±0.08 b

The results show means ± standard deviations (n = 3). Different lowercase letters after values indicate significant differences between each treatment in the same year, P < 0.05. SOC, soil organic carbon; 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.

Table S2 The basic chemical properties of initial and treated straw under different N input levels after straw return

Treatment	Straw C (g kg^{-1})	Total N (g kg^{-1})	Total P (g kg^{-1})	Total K (g kg^{-1})
Initial	485.77±25.21 a	6.72±0.36 c	2.01±0.12 a	21.00±0.13 a
N+PK	428.86±17.82 b	17.20±0.51 ab	1.36±0.11 b	1.48±0.16 bc
0.75N+PK	429.00±30.21 b	16.72±0.45 b	1.45±0.10 b	1.18±0.14 c
0.5N+PK	427.72±29.96 b	18.15±1.03 a	1.81±0.17 a	1.86±0.10 b
PK	446.36±2.42 b	18.33±0.53 a	1.88±0.35 a	1.77±0.46 b

The results show means ± standard deviations (n = 3). Different lowercase letters after values indicate significant differences between each treatment, $P < 0.05$. SOC, soil organic carbon; N, nitrogen; P, phosphorus; K, potassium; 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.

Table S3 The abundances of fungal and bacterial abundances across different N fertilizer level treatments after straw return

Treatment	Fungi abundance ($\times 10^7$ copies g^{-1} soil)	Bacteria abundance ($\times 10^7$ copies g^{-1} soil)	Fungi: Bacteria ratio
N+PK	0.63±0.16 bc	3.15±0.30 a	0.20±0.04 b
0.75N+PK	0.85±0.09 a	2.88±0.24 ab	0.30±0.05 a
0.5N+PK	0.57±0.04 c	2.87±0.42 ab	0.20±0.03 b
PK	0.39±0.05 d	2.17±0.43 b	0.18±0.02 c

The results show means ± 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.

Table S4 The fungal and bacterial alpha diversity under different N input levels after straw return

	Treatment	Chao1	Richness
Fungi	N+PK	1118.60±71.84 a	883.00±38.57 a
	0.75N+PK	1117.82±67.17 a	796.33±28.45 ab
	0.5N+PK	1063.37±84.82 a	781.00±33.87 ab
	PK	1054.50±22.29 a	772.33±27.54 b
Bacteria	N+PK	5917.52±149.48 a	4475.00±87.11 a
	0.75N+PK	5920.19±197.47 a	4396.67±27.43 a
	0.5N+PK	5881.07±152.30 a	4398.33±32.35 a
	PK	5672.76±82.25 a	4241.00±64.55 b

The results show means ± 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.

Table S5 The relative abundances of keystone taxa across different N fertilizer level treatments after straw return

Treatment	FOTU22 (Module 3)	BOTU21 (Module 1)	BOTU6346 (Module 3)	BOTU240 (Module 2)
N+PK	388.33	453.67	63.67	44.33
0.75N+PK	536.67	638	147	79
0.50N+PK	367	303.33	150	82.66
PK	109.33	421	18.33	63

The results show means (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. FOTU, the OTU in fungi; BOTU, the OTU in Bacteria.