
1 **Effects of soil incorporation depth of Biodiesel Co Product (BCP)**
2 **additions on N leaching losses and on genes involved in soil nitrogen**
3 **cycling in an acidic Chinese tea soil**

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19 **Abstract**

20 Effectiveness of Biodiesel Co Product (BCP) in decreasing N leaching from an acidic soil
21 (pH 3.7), effects on greenhouse gas emissions and N functional genes following surface
22 application (0-6 cm depth) and complete mixing (0-18 cm depth) of 1.5 mg BCP-C g⁻¹ soil
23 was investigated in a 35 day laboratory lysimeter experiment. The BCP additions
24 significantly decreased AOA and AOB *amoA* gene copy numbers, especially from the surface
25 BCP application. Both methods therefore inhibited nitrification and decreased N leaching.
26 Microbial biomass N and C significantly increased following both types of BCP

27 incorporation, particularly with surface mixing. BCP increased *nifH* genes with both
28 applications. Surface application of BCP produced higher emission rates of N₂O and CO₂
29 than complete mixing. Based upon (*nirS+nirK*)/*nosZ* ratios, more N₂O emissions, caused by
30 denitrification, came from the surface application than complete mixing, in support of the
31 gaseous measurement of N₂O. However, complete mixing was more effective than surface
32 BCP application in decreasing N leaching: 2.14% of ¹⁵N fertilizer in the leachate from
33 complete mixing, compared to 51% following surface application, and 68% without BCP
34 addition. These findings demonstrate that complete mixing was more effective than surface
35 BCP application in decreasing N leaching and gaseous losses. We conclude that BCP is an
36 effective and biologically safe method to prevent nitrate leaching in this acidic Chinese soil.

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38 **Key words:** Biodisel Co-Product; ¹⁵N-urea; Nitrogen leaching; N₂O; N-related functional
39 genes; (*nirK+nirS*)/*nosZ*

40

41 **Introduction**

42 Nitrogen (N) is one of the most important nutrients for plant growth. However, losses of N
43 derived from extensive applications of chemical fertilizers are a major source of
44 eutrophication on a global scale, causing decreased quality of ground and surface waters,
45 serious economic problems, and damage to aquatic and soil-based ecosystems (Norse 2005;
46 Williams et al. 1997). In China, approximately 300 million rural residents lack access to safe
47 drinking water because of agricultural pollution (Liu and Yang 2012). Nitrogen addition, in
48 both mineral and organic fertilizers, may be applied at rates as high as 450-1000 kg N ha⁻¹ y⁻¹

49 to Chinese tea plantations (Tokuda and Hayatsu 2001, 2004; Xue et al. 2006; Li et al. 2013).
50 Urea (46% N) is the most commonly used N fertilizer in China and especially in tea
51 plantations. High fertilizer N applications, especially urea, may cause excess residual N in
52 soil, which can increase the risk of nitrate leaching and nitrous oxide (N₂O) emissions, and
53 soil acidification (Xue et al. 2006; Zhu et al. 2011; Hirono and Nonaka 2012; Liu and Yang
54 2012; Zhu et al. 2014). Therefore, to alleviate the contamination of groundwater by nitrate N
55 (NO₃⁻-N) derived from tea fields, it is necessary to have better management of N, such as
56 proper fertilizer application rates and incorporation of residues, to immobilize N and
57 minimize N leaching (Morita et al. 2002). Although this is less effective than using cover
58 crops (Justes et al. 1999), their use is often inconvenient, due, for example, to adverse Spring
59 weather conditions. Nitrification inhibitors can also be effective in decreasing nitrate leaching
60 and N₂O emissions (Menendez et al. 2012), as nitrate-N is preferred over N₂O as a terminal
61 electron acceptor and N₂O evolution can increase whenever NO₃⁻-N supply is greater than the
62 reducing demands of the denitrifiers (Swerts et al. 1996).

63 Biodiesel Co-Product (BCP) has been previously tested as a way of decreasing N
64 leaching (Redmile-Gordon et al. 2014). It is produced as a byproduct during the conversion
65 of waste vegetable or animal cooking oils to biodiesel. It contains many residues from the
66 processing of biodiesel, including a water-soluble mixture of glycerol, salts of fatty acids,
67 methylesters, mono- and di-glycerides, potassium (or sodium) hydroxide, methanol and water
68 (Redmile-Gordon et al. 2015).

69 There are several major types of liquid biofuels, including biodiesel, bioethanol and
70 pyrolysis bio-oil. In 2018, 2.6 M barrels of biofuels per day, dominated by the USA and

71 Brazillian markets, comprised about 87 % of global production. The EU and Chinese shares
72 were 5% and 3% respectively (Mizik et al. 2020). By 2050, biofuels are predicted to
73 comprise 27% of the world's liquid fuel supply (Guo et al. 2020). Based on the projections of
74 OECD and the FAO, by 2027 the USA will still be the main producer. While its market share
75 will decline to 46%, Brazil's will increase to 25%, and China's will reach 8% (OECD 2020).
76 This suggests that there will be increased BCP produced in China. Biofuel production is
77 instrumental in improving energy security by decreasing foreign oil imports and promoting
78 renewable energy resources (Prasad et al. 2020).

79 Glycerine is the largest component of BCP. It has numerous uses, such as medical and
80 pharmaceutical preparations and as a food preservative. The use of BCP to prevent N
81 leaching losses has not yet been investigated in acidic tea soils but BCP production is in
82 excess of current use (Luo et al. 2016). With this further proposed use of BCP to decrease N
83 leaching, the cost of biodiesel production could decrease (Haas et al. 2006).

84 The application of BCP to soil as a substrate for the native soil microbial community
85 was previously found to be 99% effective in immobilizing inorganic N in near neutral soils
86 and preventing N leaching losses from the plough layer (Redmile-Gordon et al. 2014). The
87 BCP application also increased soil exocellular polysaccharides (EPS) and protein synthesis.
88 Therefore, biodiesel has considerable potential for improving N use-efficiency and limiting
89 the environmental damage caused by 'leaky' agriculture (Redmile-Gordon et al. 2015).
90 Increasing labile C availability, by adding BCP, will also increase biological N₂ fixation (Orr
91 et al. 2012; Chen et al. 2019).

92 Soil nitrification is a two-step process, where ammonia is first oxidized to nitrite by

93 ammonia oxidizing archaea (AOA) and ammonia oxidizing bacteria (AOB), then converted
94 to nitrate by nitrite-oxidizing bacteria (NOB). The AOA generally make a much greater
95 contribution than AOB to ammonia oxidation in acidic soils (Li et al. 2018). Denitrification
96 occurs under anaerobic conditions where oxygen is limited (Luo et al. 1999). During
97 denitrification, the nitrate is successively reduced to N₂O or N₂ by heterotrophic denitrifiers
98 (Liu et al. 2019). Nitrite reductase is encoded by the *nirS* and *nirK* gene and N₂O reductase is
99 encoded by the *nosZ* gene (Avrahami and Bohannan 2010; Conrad 1996; Wrage et al. 2001;
100 Xu et al. 2017).

101 Here, the BCP was either applied to the soil surface (0-6 cm depth) or incorporated into
102 soil to plough layer depth (7-18 cm depth) in a lysimeter study, using a tea soil supplied with
103 ¹⁵N labeled urea (5.18 atom % excess). The two methods of incorporation were chosen to
104 represent two different BCP incorporation practices in agricultural soils. The aim was to
105 determine the different N leaching losses and greenhouse gas emissions following the two
106 methods of BCP addition. The work was designed : 1) to test if differences in incorporation
107 of BCP affected soil nitrate immobilization and leaching; 2) to study the effect of the two
108 application methods on greenhouse gas emissions; and 3) the responses of functional genes
109 (AOA *amoA*, AOB *amoA*, *nirK*, *nirS*, *nosZ*, *nifH*) involved in N cycling.

110

111 **Materials and Methods and**

112 **Soil sampling and analyses**

113 The soil was sampled from the surface layer (0-20 cm depth) of a tea field from Meijiawu tea
114 region (30°21'N, 120°10'E), Hangzhou, Zhejiang Province, China by collecting 12 of 25 cm

115 diameter cores and bulking. The soil is classified as a Ultisol sandy. The main soil properties
116 were: pH 3.71, 9.2% clay, 9.8% silt, 81% sand, 0.21 g kg⁻¹ total N, 2.9g kg⁻¹ total C, 13.6 C/N,
117 250±0.63 µg g⁻¹ microbial biomass C, 49.43±6.27 µg g⁻¹ microbial biomass N, 2.98±0.22
118 nmol g⁻¹ ATP. The pH was determined using a 1: 2.5 soil: water ratio, and total C and N
119 contents by an elemental analyzer (Elementar Analysensysteme Gmb H., Germany). All
120 measurements were done immediately before leaching except the gaseous emissions. Soil
121 microbial biomass C (biomass C) was determined by fumigation extraction, and microbial
122 biomass C was calculated from: Biomass C = 2.22 Ec, where Ec = [(organic C extracted from
123 fumigated soil) - (organic C extracted from non-fumigated soil)] (Vance et al. 1987; Wu et al.
124 1990). Soil microbial biomass N (biomass N) measured in the same extracts as microbial
125 biomass C by fumigation extraction (KEc= 0.45) (Brookes et al. 1985). Soil adenosine
126 5'-triphosphate (ATP) was extracted from soil by ultrasonics (Jenkinson and Oades 1979) and
127 determined as described by Redmile-Gordon et al. (2011), with three replicates of moist soil
128 containing 3.0 g oven dry soil. ATP in the soil extracts blanks and standards (0–100 pmol 50
129 µl⁻¹) were measured with a luminometer (Glomax 96. Promega, USA) using the firefly
130 luciferin-luciferase reagent.

131 **Experimental design**

132 After collection, the soils were sieved moist < 5 mm, soil moisture was adjusted to 40% of
133 water holding capacity (WHC) then the soils were incubated at 25 °C for 7 days prior to
134 determination of microbial biomass C and ATP. The soil was then added to soil columns (24
135 cm in length, 6 cm diameter). Twelve lysimeters were prepared, 3 lysimeters per treatment
136 (Fig. 1). Each lysimeter contained moist soil equivalent to 350 g oven-dry soil, and was

137 supplied with 80 μg urea N g^{-1} soil at 5.18% ^{15}N atom excess when required. The treatments
138 (all sampled 0-6 and 7-18 cm depth) were:

- 139 (i) Treatment 1 (T1) Control (no treatment): 0-6 cm sampling depth T1 (0-6)
140 and 7-18 cm sampling depth T1 (7-18)
- 141 (ii) Treatment 2 (T2) ^{15}N -urea addition only: 0-6 cm sampling depth T2 (0-6)
142 and 7-18 cm sampling depth T2 (7-18)
- 143 (iii) Treatment 3 (T3) BCP (1.5 mg C g^{-1} soil) and ^{15}N -urea mixed 0-18 cm: 0-6
144 cm sampling depth T3 (0-6); 7-18 cm sampling depth T3 (7-18)
- 145 (iv) Treatment 4 (T4) ^{15}N -urea mixed 0-18 cm: surface application of BCP (4.5
146 mg C g^{-1} soil) 0-6 cm depth T4 (0-6); 7-18 cm sampling depth T4 (7-18)

147 The same total amounts of BCP were applied to treatments T3 and T4.

148 After the treatments were applied, soil moisture was adjusted to 50% WHC. The soils were
149 leached at day 5, 10, 20, 35 with distilled water (100 ml). After each leaching had stopped,
150 the tops of the lysimeters were sealed with rubber stoppers for 24 hours to collect the gases
151 evolved from the soils. At each sampling time, three replicates of each treatment were
152 sampled from 0-6 cm depth and 7-18 cm depths. Destructive sampling was used in this
153 experiment, New, intact columns were used each sampling date. Soil inorganic N
154 (exchangeable NH_4^+ and NO_3^-) were extracted with 0.5 M K_2SO_4 (soil: solution ratio 1:4) and
155 measured by a flow injection analyzer (SAN^{++} , Skalar, Netherlands). Total ^{15}N and atom
156 percent ^{15}N in the leachates and soils were determined by isotope ratio mass spectrometry.
157 Total soil ^{15}N on day 5 soil was determined before leaching. Soil DNA was extracted at days
158 5 and 35 (See below). Biodiesel Co-Product was made in the laboratory from waste vegetable

159 cooking oil. It was first purged of excess methanol by heating to 90 °C for 2 h. Before
160 application, BCP was prepared in water and adjusted to pH 8 by adding 1 M HCl dropwise
161 (Redmile-Gordon et al. 2014). The organic constituents of BCP were determined as described
162 by Redmile-Gordon et al. (2015). The main components were glycerol (73%), potassium
163 soap (11.7%), volatile organics (11.7%) and potassium hydroxide (2.4%). A methane
164 conversion furnace, flame ionization detector (FID), and electron capture detector (ECD)
165 were used for the determination of the CO₂, CH₄, and N₂O, respectively (Wang et al. 2017).
166 Dissolved organic C (DOC) and N (DON) were determined using a TOC-TN analyzer
167 (Shimadzu, Japan). Dissolved organic N was calculated from: [dissolved total N (DON)
168 minus (NH₄⁺-N + NO₃⁻-N)].

169 **DNA extraction and quantitative PCR (qPCR) analysis**

170 The soil DNA was isolated from moist soils (0.5g oven-dry) using the FastDNASpin Kit for
171 soil (MP Biomedicals, Santa Ana, CA, USA) according to the manufacturer's instructions.
172 The DNA purity and concentrations were determined with a Nanodrop spectrophotometer
173 (NanoDrop Technologies, Wilmington, DE, USA) and the DNA quality was checked by gel
174 electrophoresis and stored at -20°C. All DNA samples were diluted to give between 10000
175 and 100000 reads per sample, as suggested by Schöler et al. (2017) and Vestergaard et al.
176 (2017).

177 The primers and conditions used for qPCR are shown in Table S2. The primer pairs
178 Arch-amoAF/Arch-amoAR were used for the qPCR of the AOA *amoA* genes, and AOB
179 *amoA* genes were quantified by the primers of amoA-1F/amoA-2R. The qPCR was carried
180 out using a Roche Light Cycler 480 Real-Time PCR Machine (Roche Applied Science). The

181 *nirS*, *nirK* and *nosZ* genes of quantitative PCR analysis were determined as described by Di
182 et al. (2014). The *nifH* gene of quantitative PCR analysis was described by Gaby and Buckley
183 (2012). Each 20 μ l PCR reaction contained 10 μ l SYBR Premix Ex Taq (TaKaRa, Dalian,
184 China), with 400 μ l nM of each primer. 1 μ l of DNA template was added and the final volume
185 was adjusted with Milli-Q water. Plasmids were extracted from the representative clones
186 containing each target gene, and ten-fold serial dilutions of the plasmid DNA with the known
187 gene abundance were used as the standard curve. The plasmid concentrations were measured
188 using a Nanodrop® ND-2000 UV-vis and the standard copy numbers were calculated. The
189 amplification efficiencies were 91% to 99% with the R^2 values ranging between 0.997 and
190 0.999.

191 **Laboratory analysis and data analysis**

192 The percent recovery of the applied urea- ^{15}N was calculated according to Cabrera and Kissel
193 (1989): $\text{N recovery (\%)} = p(c-b) / f(a-b) * 100$
194 where p = mols of N in leachate and soil samples, f = mols of N in urea applied, c =
195 $\text{atom\%}^{15}\text{N}$ abundance in leachate samples, a = $\text{atom\%}^{15}\text{N}$ abundance in the urea, b =
196 $\text{atom\%}^{15}\text{N}$ abundance in the leachate samples without added urea.

197 All statistical analyses were determined by Origin 9.0 and SPSS 21.0 software. One-way
198 ANOVA was used to analyze the treatment effects. Differences with values of $P < 0.05$ were
199 considered to be statistically significant. All analytical data are the means of triplicate
200 determinations.

201

202 **Results**

203 Soil microbial biomass and ATP

204 Properties of the field sampled soil are presented above. The BCP additions significantly
205 increased microbial biomass C in treatment T3 at both depths and T4 (0-6) (Fig. 2a). The
206 greatest increase was with treatment T4 (0-6) where microbial biomass C was 655 μg
207 biomass C g^{-1} soil on day 5. Thus, by this time, microbial biomass C in treatment T4 (0-6)
208 had more than doubled compared to the other treatments. However, by day 35, while
209 microbial biomass C in treatment T4 (0-6) was higher than the other treatments, the
210 difference between them was very much less compared to previous sampling days, although
211 still significant (Fig. 2a).

212 Changes in microbial biomass N in the different treatments closely followed those of
213 microbial biomass C (Fig. 2b). Again, microbial biomass N was greater following both BCP
214 additions, with the greatest microbial biomass N contents in treatment T4 (0-6). Microbial
215 biomass N in treatment T4 (0-6) was about 75 $\mu\text{g g}^{-1}$, and as with microbial biomass C, it
216 declined until day 35. Overall, there was a highly significant linear correlation between
217 microbial biomass N and microbial biomass C ($R^2=0.96$) (Fig. S1b), However, there were
218 differences in mean microbial biomass C/N ratios in the different treatments. The highest
219 ratio was in treatment T4 (7-18) at 7.70, followed with treatment T1 (7-18) at 7.50, and then
220 treatment T2 (0-6) with a ratio of 7.07. The microbial biomass C/N ratios with BCP only
221 were 5.20, 5.70 and 6.87 in the T3 (0-6), T3 (7-18) and T4 (0-6) treatments respectively. The
222 ratios in the T1 (0-6) and T2 (7-18) were 7.07 and 7.0 respectively in the two different depths.
223 (Fig. S1).

224 There was a close overall linear relationship between soil ATP and soil microbial

225 biomass C ($R^2=0.96$) (Fig. S1a). However, there were also significant differences between
226 treatments. The soil ATP concentrations in treatments T3 (0-6) (4.71 nmol g^{-1}) and T3 (7-18)
227 (4.90 nmol g^{-1}) were higher than in treatment T4 (0-6) (4.33 nmol g^{-1}) during the incubation
228 with a maximum on day 5 (Fig. 2c). There were also significant differences between
229 microbial biomass ATP concentrations ($\mu\text{mol ATP g}^{-1}$ biomass C) (Fig. S1a). The lowest
230 concentration was $9.32 \mu\text{mol ATP g}^{-1}$ microbial biomass C in treatment T4 (0-6 cm) followed
231 by T3 (7-18) with $11.97 \mu\text{mol ATP g}^{-1}$ microbial biomass C. The concentrations in treatment
232 T3 (0-6), at $12.44 \mu\text{mol ATP g}^{-1}$ microbial biomass C was higher than in the others. Those in
233 treatments T2 (0-6) and (7-18) were 11.85 and $10.78 \mu\text{mol ATP g}^{-1}$ microbial biomass C
234 respectively, and $12.23 \mu\text{mol}$ and $11.94 \mu\text{mol ATP g}^{-1}$ microbial biomass C in treatment T1
235 (0-6) and (7-18) respectively (Fig. S1a).

236 **Soil inorganic N**

237 There was a distinct peak in soil exchangeable $\text{NH}_4^+\text{-N}$ at day 5 in T2 (0-6); (7-18) and T4
238 (7-18). The highest concentration was with treatment T4 (7-18), at about 4.3 mg
239 exchangeable $\text{NH}_4^+\text{-N g}^{-1}$ soil. By day 10, soil exchangeable $\text{NH}_4^+\text{-N}$ had declined to
240 relatively similar levels in all treatments to between about 1.5 to 2.0 mg exchangeable
241 $\text{NH}_4^+\text{-N g}^{-1}$ soil. However, the smallest concentrations were consistently with treatment T3 at
242 around 1.5 mg kg^{-1} soil (Fig. 4a).

243 Soil $\text{NO}_3^-\text{-N}$ concentrations in treatments T3 (0-18) and T4 (0-6) were close to zero by
244 day 5 and remained so throughout the 35-day incubation. In contrast, the concentrations in
245 treatment T2 (0-6; 7-18), and T4 (7-18) increased, reaching a maximum at day 5 with about
246 33 , 32 and 31 mg kg^{-1} respectively, then remained at approximately these concentrations until

247 the end of the incubation. Soil NO_3^- -N concentrations in treatments T2 (0-6), T2 (7-18),
248 T1(0-6) and T1(7-18) were 21.99, 20.44, 19.14 and 19.52 mg kg^{-1} respectively, followed by
249 treatment T4 (7-18) with 14.79 mg kg^{-1} (Fig. 4b).

250 **The effect of BCP on nitrogen leaching**

251 The total NH_4^+ -N concentrations in leachates from treatments T1 to T4 were 103, 171, 103,
252 and 118 μg respectively (Fig. 6). The amount of NH_4^+ -N leached from treatment T3 was
253 significantly lower than from treatments T2 and T4, and was the same as in T1 (Fig. 6).
254 Except for treatment T1, the maximum amount of NH_4^+ -N leached was on day 20.

255 The NO_3^- -N leached from the four treatments were 1031, 1060, 20, and 840 μg ,
256 respectively (Fig. 6). The amount from treatment T3 was significantly lower than from all
257 other treatments, followed by treatment T4, and was maximal with treatment T2 (Fig. 6). The
258 NH_4^+ -N leaching from treatment T3 was negligible after day 5 and remained stable until the
259 end of the leaching period. In treatment T2 and T4, NO_3^- -N leaching levels decreased by day
260 5, remaining at this low level throughout (Fig. 6). The recovery of ^{15}N from the labeled urea
261 in the leachate from T3 was 2.14%, which was significantly lower than from T2 (68%) and
262 T4 (51%) (Fig. S2b). The mixing treatment (treatment T3) was therefore more effective than
263 the surface application (T4) at decreasing N leaching. Treatment T3 decreased NO_3^- -N
264 leaching 4 times more than from treatment T4, and 5 times more than from T2 (urea only)
265 (Fig. 6).

266 Similarly, the amount of dissolved organic N (DON) came from treatment T3 (2.1 mg)
267 and was highest in treatment T2 at 35.5 mg and with similar amounts of DON in treatments
268 T1 and T4. The largest amount of leached DOC was from treatment T4 at 29.2 mg, followed

269 by T3, with 16.5 mg, then T2 and T1 with 11.2 mg and 10.6 mg respectively (Fig. S5a).

270 The maximum leaching of NO_3^- -N and NH_4^+ -N occurred at different times. The maximum
271 leaching of NO_3^- -N was on day 5 in all treatments except treatment T3 as mentioned above
272 (Fig. 5b). The leaching of NH_4^+ -N was at a maximum on day 20 except from T1, with a
273 maximum on day 10 (Fig. 5a). The biggest leaching loss was from treatment T2.

274 On day 5, before leaching commenced, the percentage recoveries of ^{15}N in soils (Fig. S2a)
275 were all similar, and nearly 100%. On day 35, the highest rate of ^{15}N recovery was from
276 treatment T3 (0-6) at 96.4%, followed by T3 (7-18) (88.7%) and T4 (0-6) (71.7%). Only
277 23.7%, 17.7% and 23.3% of added ^{15}N remained in the soil treatments T2 (0-6), T2 (7-18)
278 and T4 (7-18) treatments respectively.

279 **Functional gene shifts**

280 The abundance of the AOA *amoA* genes were significantly higher than those of the AOB
281 *amoA* genes (Figs. 7a and 7b). The BCP additions significantly decreased the abundance of
282 AOA *amoA* genes on day 5 and day 35 ($P < 0.05$). The abundance of AOB *amoA* genes in the
283 BCP treatments were significantly lower than those in treatments without BCP except for
284 treatment T4 (7-18) on day 5. However, the abundance was significantly higher in treatment
285 T3 (7-18) than in the others where there were no significant differences on day 35. The linear
286 relationship between AOA genes and NO_3^- -N concentrations ($R^2 = 0.60$; $P < 0.001$) was
287 stronger than between AOB genes and NO_3^- -N concentrations ($R^2 = 0.16$; $P < 0.01$) (Figs. 7c
288 and 7d).

289 The abundance of *nirS*, *nirK* and *nosZ* genes in treatment T4 (0-6) was significantly
290 lower than in the other treatments on day 5 and day 35 (Fig. 8). The abundance of these genes

291 in treatment T3 was significantly lower than in treatments without BCP on day 5, while both
292 of them increased on day 35. In contrast, the abundance of *nifH* gene in treatment T4 (0-6)
293 was highest at day 5, followed by treatment T4 (7-18). At day 35, the *nifH* genes in treatment
294 T3 were significantly higher than in the other treatments by 7 times. Also, they were still
295 significantly higher in treatment T4 when compared with T1 and T2 (Fig. 8d). The lowest
296 $[nirK+nirS]/[nosZ]$ ratios were with treatment T4 (0-6) on day 5 (2.40) and 35 (2.24). The
297 highest ratio was with treatment T4 (7-18) (4.36) on day 5 (Table 1).

298 **The effects of BCP on Greenhouse Gas emissions**

299 The rate of nitrous oxide (N₂O) emissions was largest in treatment T4. It rapidly increased
300 from day 0 to day 5, and reached 485 $\mu\text{g m}^{-2} \text{h}^{-1}$ at day 5. It then decreased to 98 $\mu\text{g m}^{-2} \text{h}^{-1}$ at
301 day 10 and 14.5 $\mu\text{g m}^{-2} \text{h}^{-1}$ at day 20. However, the rates of other treatments were similar and
302 remained stable throughout, from around 40 $\mu\text{g m}^{-2} \text{h}^{-1}$ to 14 $\mu\text{g m}^{-2} \text{h}^{-1}$ (Fig. 3).

303 Carbon dioxide (CO₂) emissions from treatments T3 and T4 also showed a similar
304 pattern from day 0 to 20. The peak of CO₂ emission rate occurred on day 5, declined to day
305 20 then remained stable at about 99 $\text{mg m}^{-2} \text{h}^{-1}$ until the end of the incubation time. The peak
306 emission rate in treatment T4 (951 $\text{mg m}^{-2} \text{h}^{-1}$) was higher than in treatment T3 (727 mg m^{-2}
307 h^{-1}). Before the rate of CO₂ emission from treatment T2 stabilized, it decreased from 84 mg
308 $\text{m}^{-2} \text{h}^{-1}$ to around 35 $\text{mg m}^{-2} \text{h}^{-1}$ during the first 5 days. There was a decline in treatment T1
309 from day 0 to day 35 (86 to 29 $\text{mg m}^{-2} \text{h}^{-1}$) (Fig. S6a).

310 The emission rates of CH₄ increased slightly from day 0 to 5, afterwards, it halved in all
311 treatments by the end of the experiment. The differences in the rates between treatments T4>
312 T1>T3>T2 at 69, 67, 65.8 and 66.5 $\mu\text{g m}^{-2} \text{h}^{-1}$ respectively were not significant by day 5.

313 Then, all rates declined, with the fastest decline in treatment T4, which declined steeply to
314 $31.2 \mu\text{g m}^{-2} \text{h}^{-1}$ by day 10. After day 20, CH_4 emissions from all treatments had stabilized at
315 about $33 \mu\text{g m}^{-2} \text{h}^{-1}$ (Fig. S6b).

316 **Discussion**

317 **Changes in microbial biomass and ATP concentrations**

318 Microbial biomass C and ATP concentrations were significantly higher in the BCP treatments
319 (T3 and T4 (0-6)) compared to treatments without BCP (Figs. 2a and 2c). Therefore, at least a
320 large BCP fraction was biologically available, leading to high microbial growth and activity,
321 and also stimulation of microbial biosynthesis (Redmile-Gordon et al. 2014; Zhang et al.
322 2020). High microbial C utilization is typically associated with an enhanced N demand (Brant
323 et al. 2006; Mondini et al. 2006; Schneckenberger et al. 2008), consistent with the associated
324 increase in microbial biomass N with BCP (Fig. 2). The surface addition of BCP (T4 (0-6))
325 produced the highest biomass N content, due to the highest rate of BCP addition with a high
326 C/N ratio that promoted N immobilization (Redmile-Gordon et al. 2015; Shen et al. 2021).
327 There was a linear relationship between microbial biomass C and ATP (Fig. S1a) as reported
328 by Contin et al. (2002). Shen et al. (2018) also found microbial biomass ATP had linear
329 relationships with water-hold capacity (WHC). Microbial biomass C and N also had a linear
330 relationship (Fig. S1b), which is consistent with Joergensen and Mueller (1996).

331 The BCP significantly increased soil pH ($P < 0.05$; Fig. S5). Some studies found that the
332 metabolic functions of the soil microbial community may be impaired at lower soil pH,
333 directly via proton toxicity, or by increased availability of toxic metals, such as Al (Sanders
334 1983; Han et al. 2007). Many studies have shown that increasing soil pH enhances microbial

335 activity and increases soil respiration (Kemmitt et al. 2006; Pietri and Brookes 2008).
336 Therefore, BCP, not only decreased N leaching but also has the potential to alleviate the
337 effects of fertilizer by increasing soil pH (Fig. S5), thereby increasing microbial activity (Fig.
338 2c).

339 **Soil inorganic N and N leaching**

340 Urea application increased nitrification without BCP (Fig. 4b), which indicates that
341 acid-tolerant nitrifiers exist in acidic soils and have high activity. Increasing soil pH can
342 promote nitrification and induce nitrate accumulation in some acidic soils (De Boer et al.
343 1996; SteMarie and Pare 1999; De Boer and Kowalchuk 2001; Zhang et al. 2017). BCP
344 increased soil pH in our study (Fig. S5) but we found that BCP significantly decreased AOA
345 and AOB *amoA* genes (Figs. 7a and 7b). This suggests that BCP potentially inhibited the
346 growth of microorganisms bearing genes for coding ammonia oxygenase, as it may contain
347 biological nitrification inhibitors (Sarr et al. 2020). The abundance of AOA *amoA* genes was
348 significantly higher than AOB *amoA* genes (Figs. 7a and 7b), which is consistent with other
349 findings (Herrmann et al. 2012; Sarr et al. 2020). There was also a linear relationship between
350 abundances of AOA *amoA* genes and NO_3^- -N concentrations in the soils. This is supported by
351 the findings of others that although AOA and AOB *amoA* have the same functions, AOA
352 *amoA*, rather than AOB *amoA* dominates in acid soils ($\text{pH}<4.9$) (Leininger et al. 2006).
353 Therefore, AOA generally makes the greater contribution to ammonia oxidation in acid soils
354 (Li et al. 2018; Yao et al. 2011). On day 5 the lowest AOB *amoA* gene number was in
355 treatment T4 (7-18), which suggests that the surface addition of BCP consumed much O_2 in
356 the surface causing anaerobic conditions in T4 (7-18). By day 35, the copy number of AOB

357 *amoA* in the BCP treatment T3 (7-18) was higher than in other treatments. while the AOA
358 *amoA* copy number in the BCP treatments were still lower than in the others. This indicates
359 BCP addition inhibited the growth of microorganisms having AOA *amoA* longer than those
360 with AOB *amoA*.

361 Addition of BCP greatly decreased the soil NO_3^- -N concentrations (Fig. 5b). The lowest
362 amounts of NO_3^- -N leached in treatment T3 were less than in T4, compared to T1 and T2 i.e.
363 No BCP. The immobilization of NO_3^- -N may be inhibited by concentrations of NH_4^+ as low
364 as $0.1 \mu\text{g NH}_4^+\text{-N g}^{-1}$ soil (Rice and Tidje 1989). However, the accumulation of microbial
365 biomass N in response to BCP proceeded despite low exchangeable NH_4^+ -N in the soil (Fig.
366 4). This suggests that the quality of C (soil organic matter vs. BCP) is more important for
367 NO_3^- -N immobilization than the concentration of exchangeable NH_4^+ -N (Shen et al. 2021).
368 Cheng et al. (2017) also found that NO_3^- immobilization is increased by the addition of
369 simple organic substrates at concentrations above 0.5 mg C g^{-1} soil. The amount of BCP we
370 used was 1.5 mg C g^{-1} which was consistent with this. Burger and Jackson (2003) also found
371 high NO_3^- immobilization rates in near neutral soils (pH 6.8 and 6.5) with low NH_4^+ -N
372 concentrations (around $1 \mu\text{g N g}^{-1}$ soil). Heterotrophic microbes assimilated less NH_4^+ than
373 NO_3^- , probably because NH_4^+ concentrations were low and competition by nitrifiers was
374 apparently strong. This suggests that BCP caused strong competition for NH_4^+ between
375 nitrifiers and N immobilizers in our soils, causing NO_3^- to be more available to microbes.
376 Previous studies also reported that fungi prefer NO_3^- than NH_4^+ and NO_3^- was taken up by
377 fungi (Marzluf 1997; Zhu et al. 2013). The application of BCP to the plough layer (23 cm) in
378 a high pH soil was 99% effective in NO_3^- immobilization thus preventing its loss during

379 winter (Redmile-Gordon et al. 2014). which was similar to findings of Ritz and Griffith (1987)
380 and Park and Matzner 2006.

381 Labile C additions decreased N leaching in a sandy loam soil in other lysimeter
382 experiments (Eschen et al. 2007; Chaves et al. 2008). Sucrose and glucose additions also
383 immobilized urine-N and decreased N leaching (Shepherd et al. 2010). Glucose addition also
384 significantly decreased NO_3^- -N leached from a sandy soil (Ritz and Griffith 1987). These
385 results are consistent with ours. However, sucrose and glucose are too expensive for practical
386 use, unlike BCP. The recovery rates of ^{15}N -urea fertilizer in the leachates were least in the
387 mixed application of BCP (Treatment T3) (Fig. S2b). This suggests that it is effective in
388 decreasing fertilizer N leaching losses from soil to surface and groundwaters, so decreasing
389 environmental and human health risks (WHO 1984). The maximum leaching of NO_3^- -N was
390 earlier than exchangeable NH_4^+ -N (Figs. 5a and 5b). NO_3^- -N has a diffuse single negative
391 charge over a large anion and so is more mobile than the smaller and highly positively
392 charged NH_4^+ -N ion, and it is not fixed by soil colloids (Wang 2008). Therefore, NH_4^+ -N is
393 usually adsorbed by soil exchange sites and is little leached (Mengel 1985; Di and Cameron
394 2005). Overall, these findings indicate that: i) The abundance of AOA *amoA* is higher than
395 AOB *amoA* in strongly acidic soils, ii) BCP addition inhibited the growth of microorganisms
396 bearing AOA *amoA* longer than bearing AOB *amoA* genes, and iii) BCP decreases N
397 (especially NO_3^- -N) leaching.

398 **GHG-C emission rates (CO_2 and CH_4)**

399 Higher labile C inputs cause higher cumulative CO_2 emissions in aerobic soils (Tsai et al.
400 1997; Miller et al. 2008). This is consistent with our results where the highest rate of CO_2

401 emission was from treatment T4, followed by treatment T3 (Fig. S6b). The higher rate of CO₂
402 emission was on day 5 and then sharply declined. Brant et al. (2006) found that a readily
403 mineralizable pool of substrate C was respired during the early stage (first 3d of incubation).
404 The CH₄ production rate was low, because methanogens is inhibited in strongly acidic soils
405 (Ye et al. 2012). The highest CH₄ emission rate was in T4 treatment (Fig. S6b). This suggests
406 that the greater labile C in BCP caused a higher demand for O₂, producing anaerobic
407 conditions. After day 5, the CH₄ emission rate in the T4 was greatly decreased, suggesting
408 that labile C was becoming depleted.

409 **N₂O emissions from soil**

410 Parton et al. (1996) found that N₂O fluxes caused by nitrification were proportional to soil N
411 turnover and that high levels of soil exchangeable NH₄⁺ (> 3 mg N kg⁻¹ soil) increased N₂O
412 emission. In our soils the NH₄⁺-N concentration was below 3 mg N kg⁻¹ soil (Fig. 4a), so it
413 would not affect N₂O emission. The highest rate of N₂O emission was from the T4 treatment
414 on day 5 (Figs. 3 and 6). This suggests that the addition of high rates of BCP increases the
415 tendency for soil anoxia, favoring the growth of denitrifiers (Beauchamp et al. 1989; Azam et
416 al. 2002). Several studies have shown the importance of spatial and temporal soil
417 heterogeneity in providing soil O₂ concentrations for N₂O emissions (Meyer et al. 2002;
418 Khalil et al. 2004; Morley and Baggs 2010). Nitrification can account for 55-95% of N₂O
419 emissions when the water filled pore space (WFPS) is between 40 and 60% (Linn and Doran
420 1984). In this study, the soil WHC was 50%, which is around 40%WFPS to 60%WFPS. The
421 N₂O emissions rate was generally low (< 40 µg m⁻² h⁻¹) in our soil except in the T4 treatment
422 (Fig. 3). This suggests that N₂O emissions in T1, T2 and T3 are mainly derived from

423 nitrification. The N₂O emission rate was high in treatment T4 but not in T3. This suggests
424 that the main N₂O emission from T4 may not come from nitrification. Soil NO₃⁻-N
425 concentration rapidly declined to zero in treatments T3 and T4 (0-6), which agrees with
426 previous work (Shen et al. 2021), indicating that NO₃⁻-N was immobilized by soil microbes,
427 rather than being denitrified. Therefore, the high N₂O emission rate may come from
428 denitrification in T4 (7-18), which will be discussed in next section. The recovery rate of
429 ¹⁵N-urea in the soils of the different treatments at day 5 was almost 100 % (Fig. S2). This
430 suggests that volatilization loss of ¹⁵N-urea was negligible before day 5. Rochette et al. (2013)
431 previously showed virtually no urea volatilization below soil pH<6, which agrees with this
432 finding.

433 **Functional genes shifts**

434 Gene copy numbers of *nirS* were more abundant than *nirK* in all treatments. This is consistent
435 with Kleineidam et al. (2010), who also found that *nirS* copy numbers were more abundant
436 than *nirK* copy numbers in two arable soils. The BCP addition significantly decreased the
437 copy numbers of *nirK*, *nirS* and *nosZ* genes on day 5, indicating that BCP inhibited the
438 growth of denitrifiers and therefore changed the denitrifier communities. The copy numbers
439 of *nirK*, *nirS* and *nosZ* genes in treatment T4 (0-6) were significantly lower than in other
440 treatments on day 35 while these genes copy numbers in treatment T3 increased. This
441 suggests that the high application rates of BCP (T4 (0-6)) inhibited the growth of
442 microorganisms bearing denitrification genes longer than the relatively lower rate of BCP
443 application (T3).

444 The labile C in BCP does not only support the activity of denitrifiers, but also has the

445 indirect effect of causing microsite anaerobiosis, due to increased respiratory demand for O₂.
446 It would favor the completed denitrification to N₂ in saturated soil (90%WFPS), while it
447 significantly stimulated N₂O emissions at 40% WFPS (Sanchez-Martin et al. 2008). In our
448 study, the WHC of soil (50%) was lower than 90% (WFPS), indicating that BCP addition
449 would not support complete denitrification. Higher ratios of *(nirS+nirK)/nosZ* are related to
450 higher N₂O emissions (Guo et al. 2018). The highest gene ratio of *(nirS+nirK)/nosZ* (4.36)
451 was in T4 (7-18) (Table 1), suggesting that the high N₂O emission rate in T4 was derived
452 from denitrification from the 7-18 cm depth. The BCP addition would have caused more O₂
453 consumption in the T4 (0-6) soil surface layer, leading to decreased O₂ entering soil below
454 this depth (Kuang et al. 2019), which may cause anaerobic conditions in T4 (7-18). This
455 supports the above findings (Fig. 3a). Also, the lowest ratio of *(nirS+nirK)/nosZ* was in T4
456 (0-6) on both day 5 and 35, suggesting that the high rate of BCP addition (T4 (0-6)) may have
457 the potential to decrease both N leaching and N₂O emission.

458 The *nifH* gene abundance is strongly associated with the N₂ fixation rate in soils with
459 low available N (0.5 µg N g⁻¹) (Lindsay et al. 2010). The abundance of *nifH* genes (Fig. 8d)
460 in treatment T4 (0-6) was significantly higher than other treatments on day 5. It decreased on
461 day 35 but remained higher than in treatments T1 and T2. The copy number of *nifH* genes in
462 treatment T3 was significantly higher than in the other treatments on day 35. This suggests
463 that the surface application T4 (0-6 cm) of BCP maintained increased *nifH* genes throughout
464 the incubation, while the mixed application T3 (0-18 cm) increased the *nifH* genes after the
465 BCP was exhausted, as increasing substrate C availability increases biological N₂ fixation
466 (Orr et al. 2012; Chen et al. 2019), which has high energy requirements, supplied by BCP

467 (Mortenson 1964; Silsbury 1977; De Luca et al. 2002).

468 **Conclusions**

469 Complete BCP mixing, (Treatment T3 (0-18)) was much more efficient in preventing $\text{NO}_3^- \text{N}$
470 leaching than T4 (Surface application (0-6)). This is attributed to more biological activity in
471 treatment T3 with its deeper mixed BCP application. Therefore, more fertilizer N was
472 immobilized, as shown by increased microbial biomass C and N and decreased DON
473 leaching losses. This suggests that Treatment T3 would also be best under field conditions.
474 No harmful effects of BCP applications on microbial activity were observed. Although the
475 surface application (T4) was less effective in decreasing N leaching, the high rate of
476 application (T4 (0-6)) maybe be more effective in decreasing N leaching by inhibiting
477 nitrifier growth. Also, it has potential in decreasing N_2O emissions by decreasing the ratio of
478 (*nirK+nirS*)/*nosZ*. Field trials in a range of acidic Chinese tea soils under different climatic
479 conditions are now required to test the efficiency and safety of BCP applications to decrease
480 N leaching under field conditions. Finally, whether BCP addition would promote biological
481 N_2 fixation and why it decreased the abundances of ammonia oxidizers and denitrifiers need
482 further work.

483

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487

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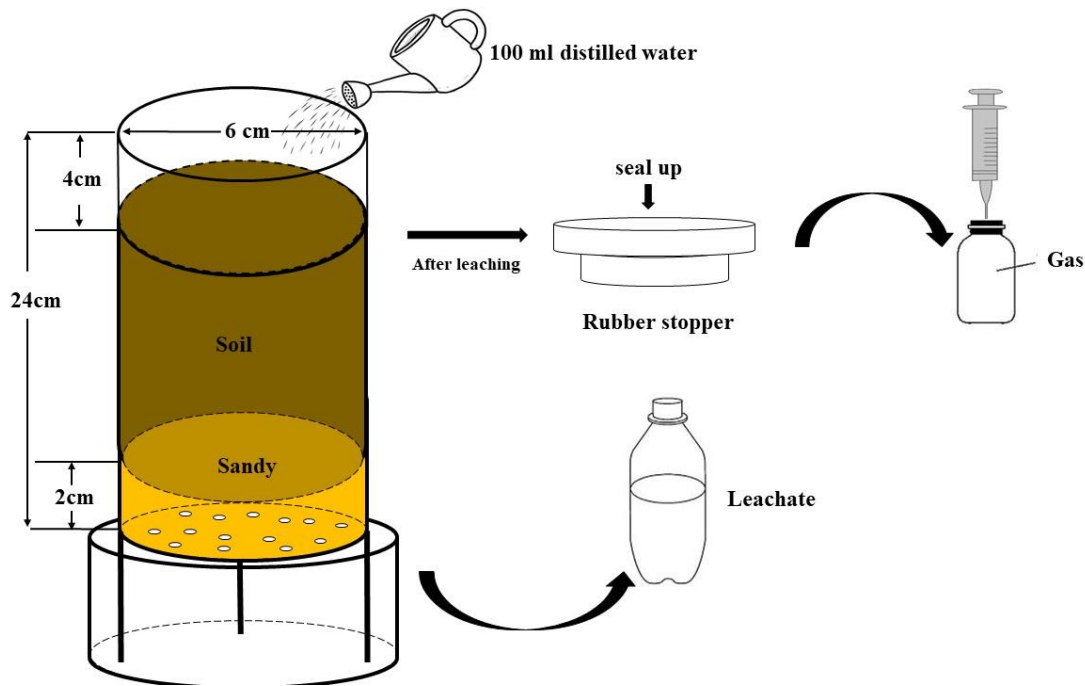
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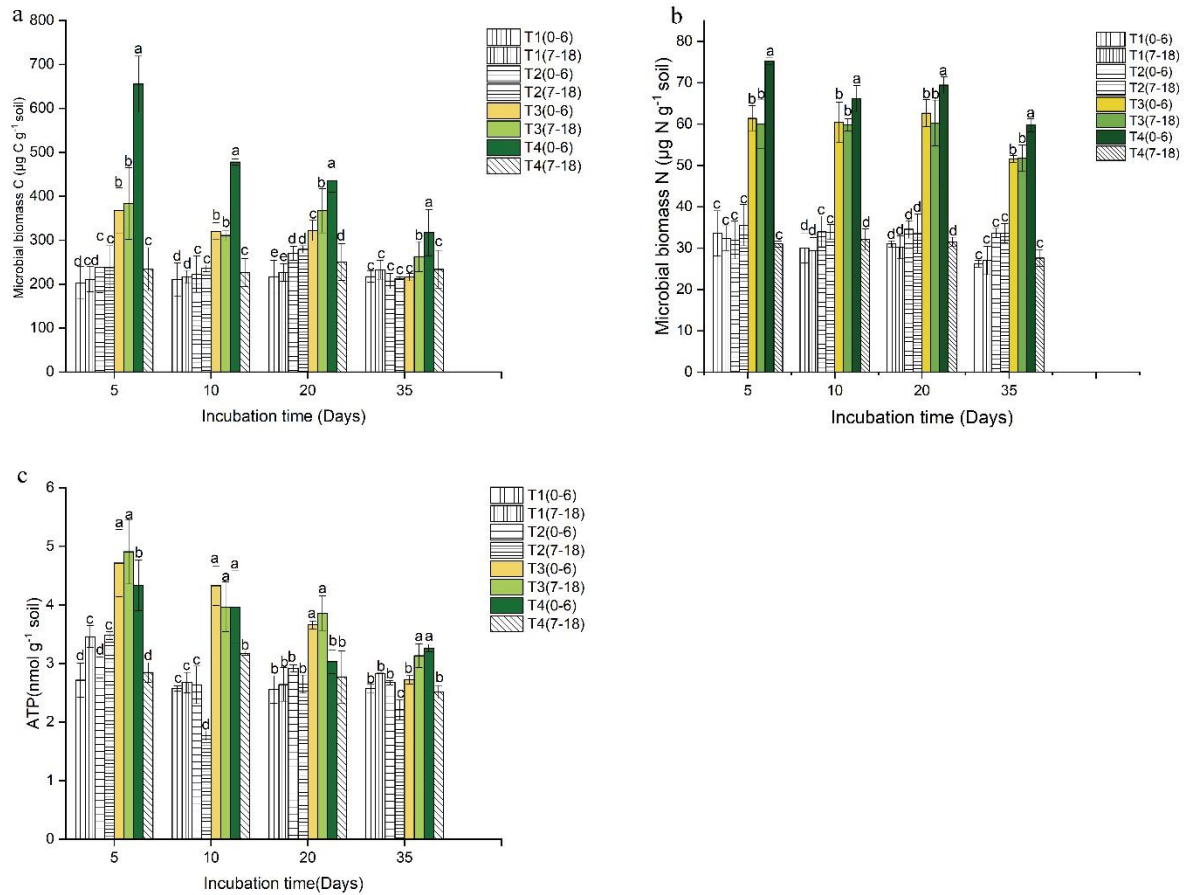
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759 **Figures**



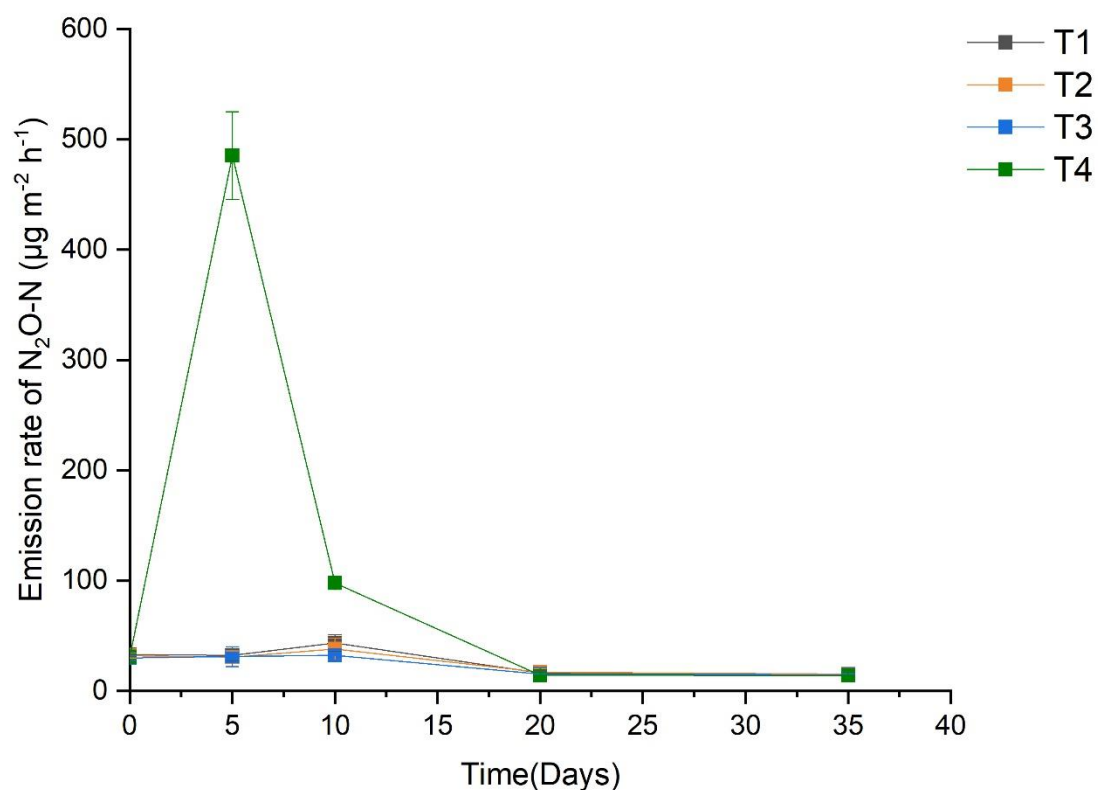
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761 **Fig. 1** The leaching column, 6 cm diameter, 24 cm length, 20 cm soil depth, (4 cm
762 headspace) .



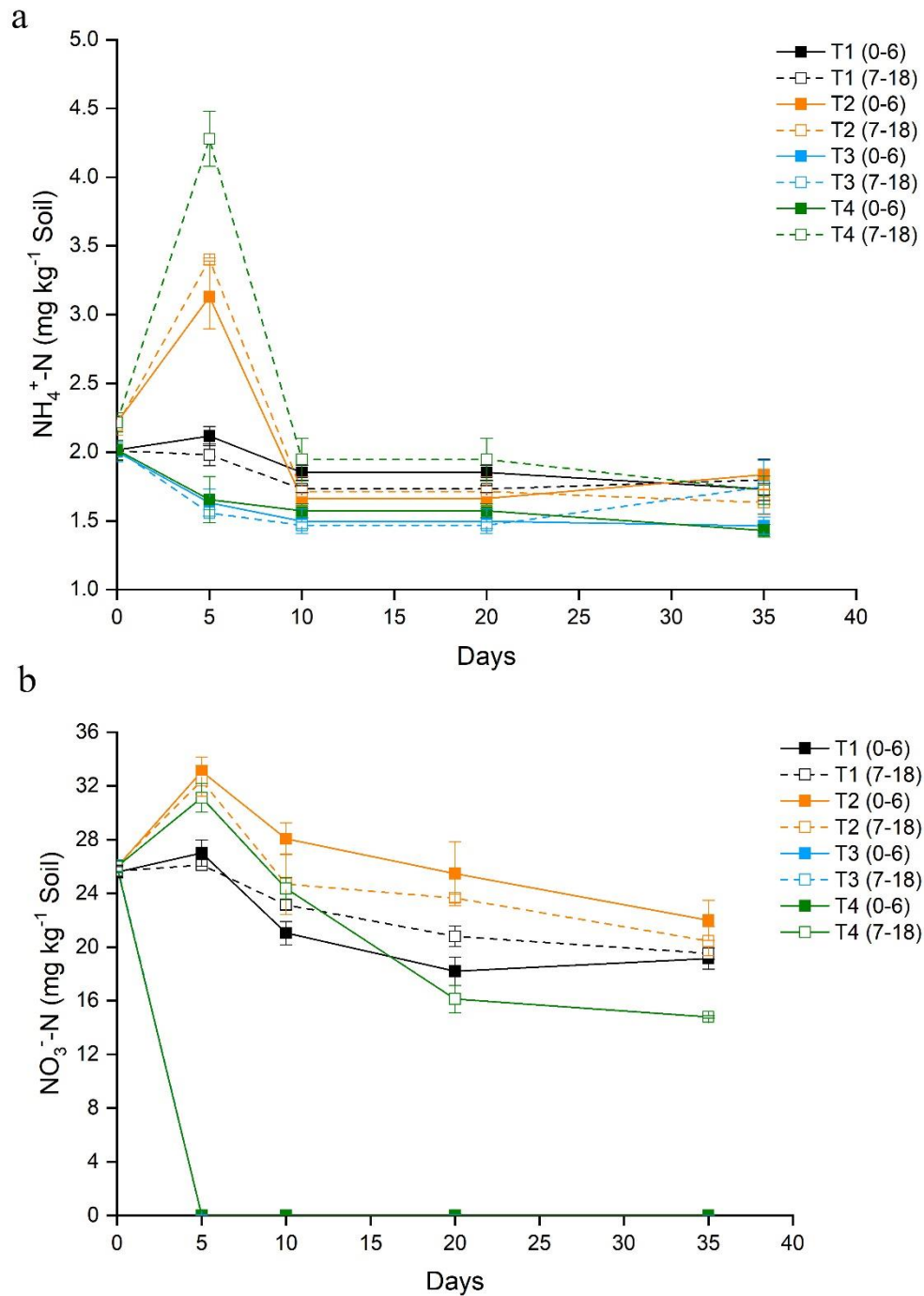
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764 **Fig. 2** The changes in microbial biomass C (a), biomass N (b) and ATP (c) in the different
 765 treatments at different incubation times. Error bars represent standard errors of the means (n
 766 = 3). Different lowercase letters indicate significant differences among different treatments
 767 within each incubation day, which were determined by a one-way ANOVA by a Tukey test for
 768 post-hoc comparison at $P < 0.05$. T1 (0-6): 0-6 cm sampling depth of control; T1(7-18): 7-18
 769 cm sampling depth of control; T2 (0-6): 0-6 cm sampling depth of ^{15}N -urea addition; T2
 770 (7-18): 7-18 cm sampling depth of ^{15}N -urea addition; T3 (0-6): 0-6 cm sampling depth of
 771 application with mixture of BCP ($1500 \mu\text{g g}^{-1}$ soil) and ^{15}N -urea; T3 (7-18): 7-18 cm
 772 sampling depth of application with mixture of BCP ($1500 \mu\text{g g}^{-1}$ soil) and ^{15}N -urea; T4 (0-6):
 773 surface application (0-6cm) of BCP ($4500 \mu\text{g g}^{-1}$ soil) and ^{15}N -urea; T4 (7-18): only ^{15}N -urea
 774 applied to 7-18 cm depths.



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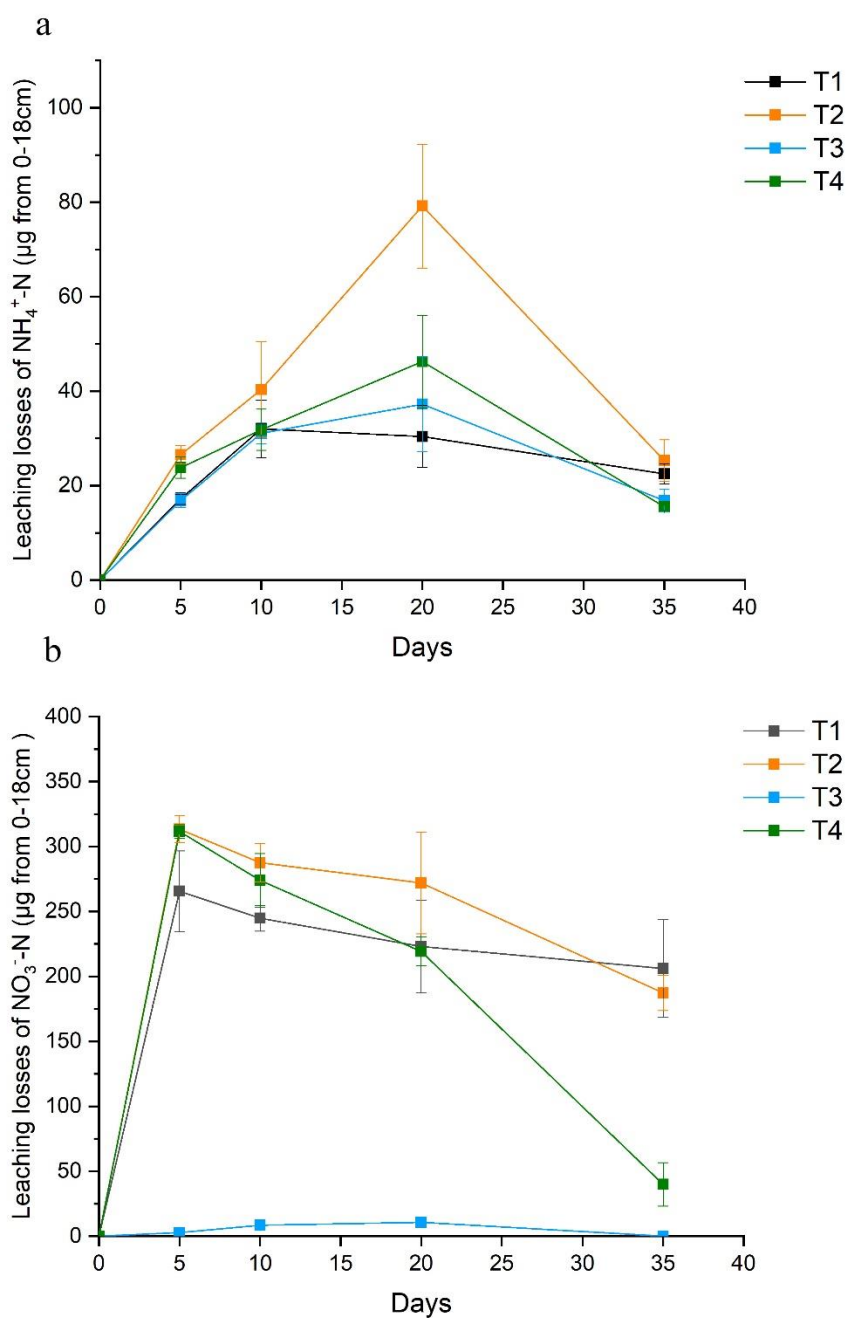
776 **Fig. 3** The emission rates of N₂O in the different treatments at different incubation times.
777 Error bars represent standard errors of the means (n = 3). T1: control; T2: ¹⁵N-urea addition
778 only; T3: application with mixture of BCP (1500 µg g⁻¹ soil) and ¹⁵N-urea; T4: surface
779 application (0-6 cm) of BCP (4500 µg g⁻¹ soil) and ¹⁵N-urea together with only ¹⁵N-urea
780 applied to 7-18 cm depths.



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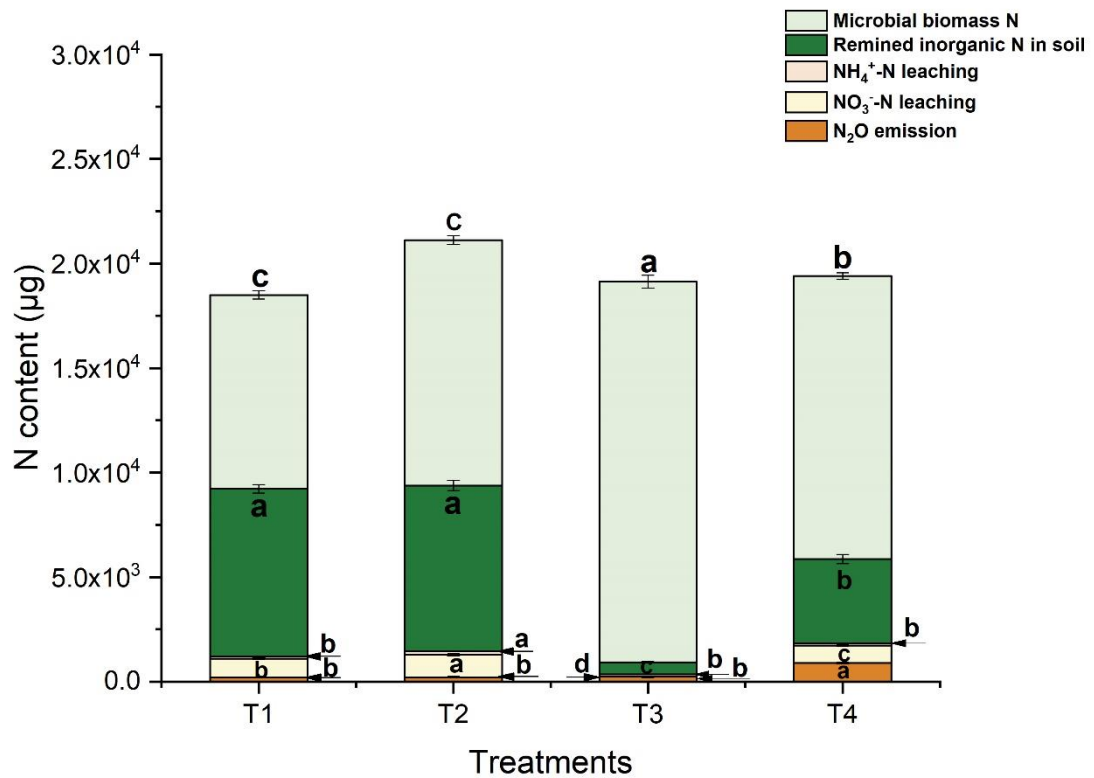
782 **Fig. 4** The changes in soil exchangeable NH_4^+ (a) and NO_3^- (b) at the different incubation
 783 times (T3 plots of NO_3^- were overlap with T4 (0-6)). Error bars represent standard errors of
 784 the means ($n = 3$). T1 (0-6): 0-6 cm sampling depth of control; T1(7-18): 7-18 cm sampling
 785 depth of control; T2 (0-6): 0-6 cm sampling depth of ^{15}N -urea addition; T2 (7-18): 7-18 cm
 786 sampling depth of ^{15}N -urea addition; T3 (0-6): 0-6 cm sampling depth of application with
 787 mixture of BCP ($1500 \mu\text{g g}^{-1}$ soil) and ^{15}N -urea; T3 (7-18): 7-18 cm sampling depth of
 788 application with mixture of BCP ($1500 \mu\text{g g}^{-1}$ soil) and ^{15}N -urea; T4 (0-6): surface
 789 application (0-6cm) of BCP ($4500 \mu\text{g g}^{-1}$ soil) and ^{15}N -urea; T4 (7-18): only ^{15}N -urea applied
 790 to 7-18 cm depths.

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793

794 **Fig. 5** The leaching amounts of NH_4^+ (a) and NO_3^- (b) in the different treatments at the
 795 different incubation times. Different letters indicate significant difference ($p < 0.05$). Error
 796 bars represent standard errors of the means ($n = 3$). Different lowercase letters indicate
 797 significant differences among different treatments, which were determined by a one-way
 798 ANOVA by a Tukey test for post-hoc comparison at $P < 0.05$. T1: control; T2: ^{15}N -urea
 799 addition only; T3: application with mixture of BCP ($1500 \mu\text{g g}^{-1}$ soil) and ^{15}N -urea; T4:
 800 surface application ($0-6 \text{ cm}$) of ($4500 \mu\text{g g}^{-1}$ soil) and ^{15}N -urea together with only ^{15}N -urea
 801 applied to $7-18 \text{ cm}$ depths.



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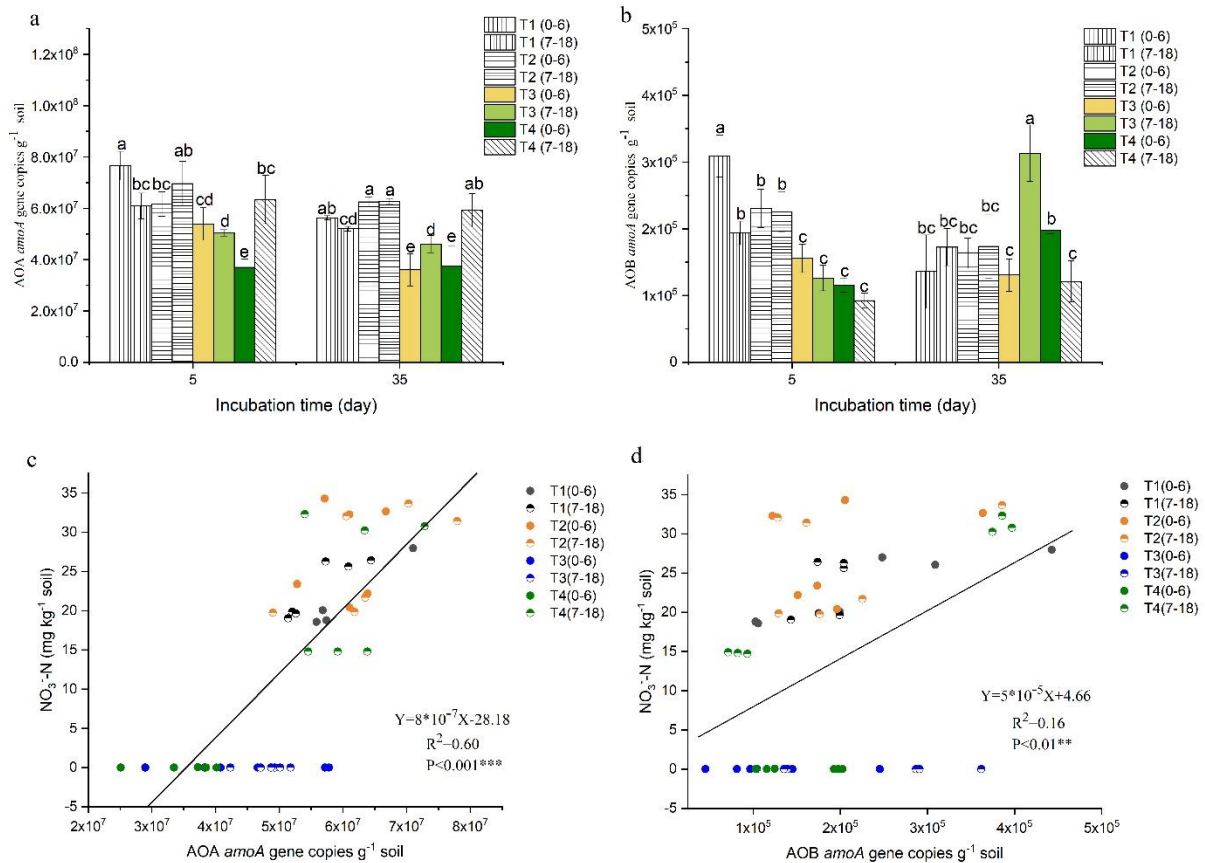
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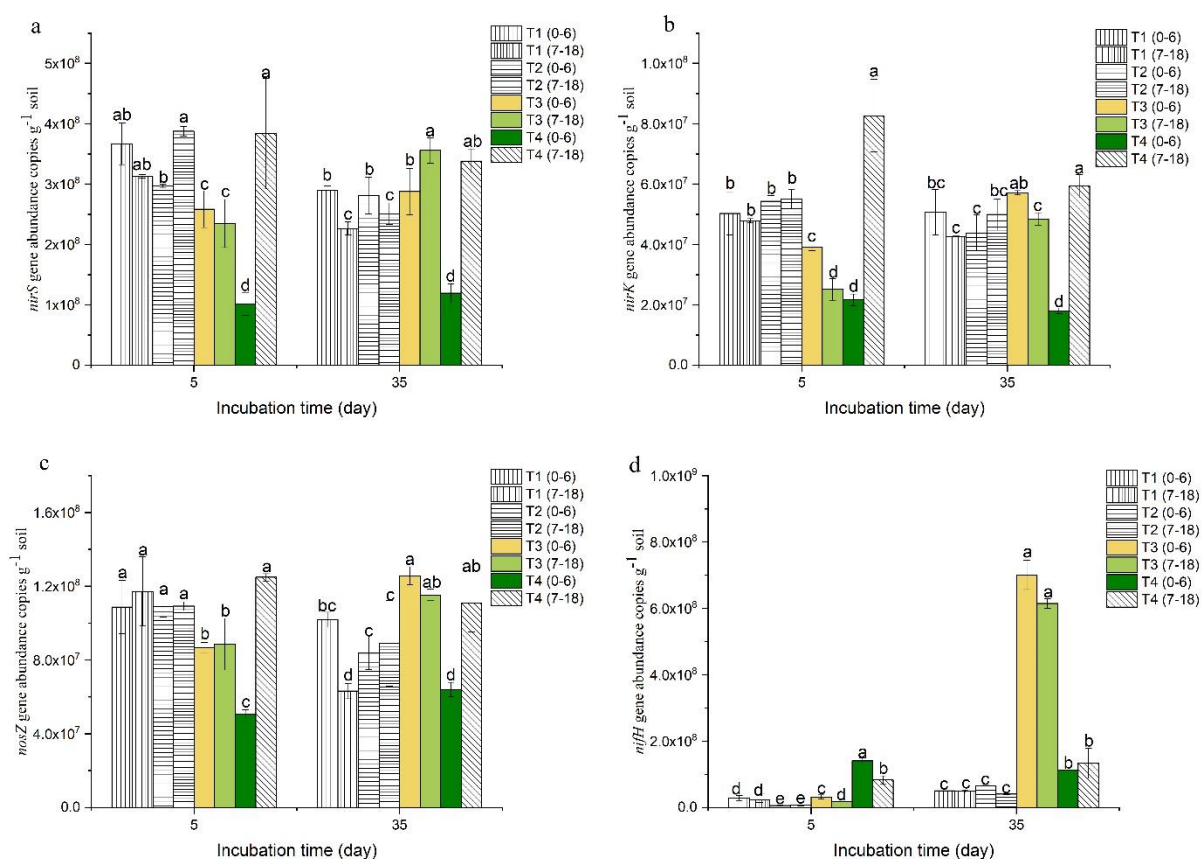
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Fig. 6 The fate of N after 35-day application of urea and/or BCP. Error bars represent standard errors of the means ($n = 3$). Different lower case letters indicate significant differences among different treatments, which were determined by an one-way ANOVA by a Tukey test for post-hoc comparison at $P < 0.05$.



807

808 **Fig. 7** The copy number of AOA (a) and AOB (b) *amoA* genes in the different treatments at
 809 day 5 and day 35. Error bars represent standard errors of the means ($n = 3$). In a and b,
 810 different lowercase letters indicate significant differences among different treatments within
 811 each incubation day, which were determined by a one-way ANOVA by a Tukey test for
 812 post-hoc comparison at $P < 0.05$. For c and d: The linear relationships between NO_3^- -N
 813 concentrations and AOA and AOB *amoA* gene copy number, respectively. T1 (0-6): 0-6 cm
 814 sampling depth of control; T1(7-18): 7-18 cm sampling depth of control; T2 (0-6): 0-6 cm
 815 sampling depth of ^{15}N -urea addition; T2 (7-18): 7-18 cm sampling depth of ^{15}N -urea
 816 addition; T3 (0-6): 0-6 cm sampling depth of application with mixture of BCP ($1500 \mu\text{g g}^{-1}$
 817 soil) and ^{15}N -urea; T3 (7-18): 7-18 cm sampling depth of application with mixture of BCP
 818 ($1500 \mu\text{g g}^{-1}$ soil) and ^{15}N -urea; T4 (0-6): surface application (0-6cm) of BCP ($4500 \mu\text{g g}^{-1}$
 819 soil) and ^{15}N -urea; T4 (7-18): only ^{15}N -urea applied to 7-18 cm depths.



820

821 **Fig. 8** The copy number of *nirS* (a), *nirK* (b), *nosZ* (c) and *nifH* (d) in the different treatments
 822 at day 5 and day 35. Error bars represent standard errors of the means (n = 3). Different
 823 lowercase letters indicate significant differences among different treatments within each
 824 incubation day, which were determined by an one-way ANOVA by a Tukey test for post-hoc
 825 comparison at $P < 0.05$. T1 (0-6): 0-6 cm sampling depth of control; T1(7-18): 7-18 cm
 826 sampling depth of control; T2 (0-6): 0-6 cm sampling depth of ^{15}N -urea addition; T2 (7-18):
 827 7-18 cm sampling depth of ^{15}N -urea addition; T3 (0-6): 0-6 cm sampling depth of
 828 application with mixture of BCP ($1500 \mu\text{g g}^{-1}$ soil) and ^{15}N -urea; T3 (7-18): 7-18 cm
 829 sampling depth of application with mixture of BCP ($1500 \mu\text{g g}^{-1}$ soil) and ^{15}N -urea; T4 (0-6):
 830 surface application (0-6cm) of BCP ($4500 \mu\text{g g}^{-1}$ soil) and ^{15}N -urea; T4 (7-18): only ^{15}N -urea
 831 applied to 7-18 cm depths.

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View Letter

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Date: 28 Mar 2021
To: "Philip Brookes" philip.brookes@rothamsted.ac.uk
From: "Paolo Nannipieri" paolo.nannipieri@unifi.it
Subject: BFSO: Your manuscript entitled Effects of soil incorporation depth of Biodiesel Co Product (BCP) additions on N leaching losses and on genes involved in soil nitrogen cycling in an acidic Chinese tea soil

Ref.:
Ms. No. BFSO-D-20-00790R2
Effects of soil incorporation depth of Biodiesel Co Product (BCP) additions on N leaching losses and on genes involved in soil nitrogen cycling in an acidic Chinese tea soil
Biology and Fertility of Soils

Dear Phil,

I am pleased to tell you that your work has now been accepted for publication in Biology and Fertility of Soils.

It was accepted on 28 March 2021.

Please Phil check carefully the proofs so as to avoid mistakes because soetimes it happens with the version showing the marked changes

Thank you for submitting your work to this journal.

With kind regards

Paolo Nannipieri
Editor-in-Chief
Biology and Fertility of Soils

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