Biology and Fertility of Soils

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

--Manuscript Draft--

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Abstract:	Effectiveness of Biodiesel Co Product (BCP) in decreasing N leaching from an acidic soil (pH 3.7), effects on greenhouse gas emissions and N functional genes following surface application (0-6 cm depth) and complete mixing (0-18 cm depth) of 1.5 mg BCP-C g -1 soil was investigated in a 35 day laboratory lysimeter experiment. The BCP additions significantly decreased AOA and AOB gene copy numbers, especially from the surface BCP application . Both methods therefore inhibited nitrification and decreased N leaching. Microbial biomass N and C significantly increased following both types of BCP incorporation, particularly with surface mixing. BCP increased nifH genes with both applications. Surface application of BCP produced higher emission rates of N 2 O and CO 2 than complete mixing. Based upon (nirS + nirK)/ nosZ ratios, more N 2 O emissions, caused by denitrification, came from the surface application than complete mixing, in support of the gaseous measurement of N 2 O. However, complete mixing was more effective than surface BCP application in decreasing N leaching: 2.14% of 15 N fertilizer in the leachate from complete mixing, compared to 51% following surface application, and 68% without BCP addition. These findings demonstrate that complete mixing was more effective than surface BCP application in decreasing N leaching and gaseous losses. We conclude that BCP is an effective and biologically safe method to prevent nitrate leaching in this acidic Chinese

	soil.
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Response for editor

1) Q. Please delete L. 16-18 because they are general sentences. Please begin with "Effectiveness of Biodiesel Co Product (BCP) in decreasing"

A. Thanks. Done. See lines 20-21.

2) Q. L. 26. Conceptual mistake: N fixation transforms N_2 in ammonium-N and then in amino acids whereas microbial N immobilization is the microbial process trasforming ammonium N in organic N!!!!!! As suggested by reviewer 1 you have not determined N fixation!!!!

A. Yes, Thanks for comments, we changed it: BCP increased *nifH* genes in both applications. See line 27.

3) Please do not use the term of microbial biomass to indicate soil microbial communities. Microbial biomass is the size of soil microbiome. L. 83, "soil microbial community was"; see also L. 308;

A. Yes, Thanks for comments. We changed to microbial community. See lines 84 and 332.

4) The policy of the journal is guided by editorials and position/opinion papers. In the case of extraction of DNA from soil and its characterization we follow what reported by Vestergaard et al (2017) Biol Fertil Soils 53:479-484 and Scholer et al (2017) Biol fertil Soils 53:485-489. They have suggested to carry our negative controls because kits and solutions are often DNA-polluted;

A. Thanks, we extracted DNA by following the methods of Vestergaard et al (2017) Biol Fertil Soils 53:479-484 and Scholer et al (2017) Biol fertil Soils 53:485-489. ‘All DNA samples were diluted to give between 10000 and 100000 reads per

sample, as suggested by Schöler et al. (2017) and Vestergaard et al. (2017)'.

See lines173-175.

5) L. 310-311, be careful, heavy metals form hydroxydes, which are insoluble, by increasing pH. This is another inactivation mechanism

A. We have deleted it. See lines 330-333: Some studies found that the metabolic functions of the soil microbial community may be impaired at lower soil pH, directly via proton toxicity, or by increased availability of toxic metals, such as Al (Sanders 1983; Han et al. 2007).

6) L. 329, you have not data to state that nitrification rate was lower than urea hydrolysis; for example, you have not a direct measurement of urea hydrolysis;

A. Yes, thanks. We have deleted it. See lines 339-340: Urea application increased nitrification in our soil without BCP (Fig. 4b), which indicates that acid-tolerant nitrifers exist in acidic soils and have high activity.

7) L. 333, the decrease of pH decreases nitrification as you have reported at L. 337-338. Please do not report contradictory sentences. Bacteria and thus AOB are inhibited under acidic conditions;

A. Thanks, we have deleted the sentence. We have changed discussion: see lines 339-346. Urea application increased nitrification in our soil without BCP (Fig. 4b), which indicates that acid-tolerant nitrifers exist in acidic soils and have high activity. Increasing soil pH can promote nitrification and induce nitrate accumulation in some acidic soils (De Boer et al. 1996; SteMarie and Pare 1999; De Boer and Kowalchuk 2001; Zhang et al. 2017). BCP increased soil pH in our study (Fig. S5) but we found that BCP significantly decreased AOA and AOB amoA genes (Figs. 7a and 7b). This suggests that BCP potentially inhibited the

growth of microorganisms bearing AOA and AOB genes, it may contain biological nitrification inhibitors (Sarr et al. 2020).

8) L. 344-345, 375-376, be careful nitrate immobilization can only occur in soil if ammonium concentrations are low (there is an important paper by Rice and Tiedje published in the 1980s);

A. Yes, we changed it. See lines 361-375: The immobilization of NO_3^--N may be inhibited by concentrations of NH_4^+ as low as 0.1 μg NH_4^+-N g^{-1} soil (Rice and Tidje 1989). However, the accumulation of microbial biomass N in response to BCP proceeded despite low exchangeable NH_4^+-N in the soil (Fig. 4). This suggests that the quality of C (soil organic matter vs. BCP) is more important for NO_3^--N immobilisation than the concentration of exchangeable NH_4^+-N (Shen et al. 2021). Cheng et al. (2017) also found that NO_3^- immobilization is increased by the addition of simple organic substrates at concentrations above 0.5 mg C g^{-1} soil. The amount of BCP we used was 1.5 mg C g^{-1} which was consistent with this. Burger and Jackson (2003) also found high NO_3^- immobilization rates in neutral soils (pH=6.8 and 6.5) with low NH_4^+-N concentrations (around 1 μg N g^{-1} soil). Heterotrophic microbes assimilated less NH_4^+ than NO_3^-, probably because NH_4^+ concentrations were low and competition by nitrifiers was apparently strong. This suggests that BCP caused strong competition for NH_4^+ between nitrifiers and N immobilizers in our soils, causing NO_3^- to be more available to microbes. Previous studies also reported that fungi prefer NO_3^- than to $NH_4^+$and exchangeable NO_3^- was taken up by fungi (Marzluf 1997; Zhu et al. 2013).

9) Citations can be listed either by the alphabetical order or by the publication year. However, the two systems can not be mixed as you have done. Please list

citations by the alphabetical order and check the text carefully;

A. Thanks. We have checked it.

10) L. 391-393, another cause of nitrification inhibition may be the presence of the so called biological nitrification inhibitors in the BCP. I suggest reading Sarr et al (2020) Biol Fertil Soils 56:145-166;

A. Thanks, we change to This suggests that BCP potentially inhibited the growth of microorganisms bearing AOA and AOB genes, it may contain biological nitrification inhibitors (Sarr et al. 2020). See lines 344-346.

11) L. 404 another conceptual mistake: microorganisms bearing genes can grow and not gene can grow;

A. Thanks, we change to This suggests that BCP potentially inhibited the growth of microorganisms bearing AOA and AOB genes, it may contain biological nitrification inhibitors (Sarr et al. 2020). See lines 344-346.

12) I suggest deleting table 1 and icluding the content in the revised text as a sentence "The main soil properties were: pH..; microbiomas biomass C..; etc.

A. We have deleted Table 1. See lines 115-118: The soil is classified as a Ultisols sandy sand soil, the main soil properties were: pH 3.71, 8.2% clay, 5.8% silt, 86% sand, 0.21 g kg^{-1} total N, 2.9g kg^{-1} total C, 13.6 C/N, 250±0.63 μg g^{-1} biomass C, 49.43±6.27 μg g^{-1} biomass N, 2.98±0.22 nmol g^{-1} ATP.

These are my specific comments:

There is a manuscript dealing with BCP under revision and including

Redmile-Gordon and you Phil, as co-authors, to be cited in this manuscript.

A.Thanks, we have cited it. See lines 325, 365. While we will put it in the reference when it be accepted.

Please add "microbial "before "biomass" at L. L. 24, 119, 122, 131, 191, 192, 193, 194, 197 (twice), 198, 199 (twice), 201, 202, 207, 213, 214, 215, 216, 217, 303, 304, 315, 317, 426, 648;

A. We have added it. See 26, 121, 124, 134, 204, 205, 206, 208, 211, 212 (twice), 213 (twice), 214, 216(twice), 217, 219, 223, 229, 230, 231, 232, 233, 316, 323, 326, 327, 469,766.

L. 30-31, "18 cm); 2.14% of 15N fertilizer was in the leachate from the complete mixing, compared";

Done. See lines 32-33.

Do not indent L. 41 ("Nitrogen" and not "...Nitrogen"), 112, 129, 158, 190, 220, 234, 264, 284, 304, 365;

Done. See lines 42, 113,132, 169, 203, 236, 250, 279, 298, 317, 431.

L. 48 "Tokuda and Hayatsu 2001, 2004" are not included in the list of references; the same for the citations at L. 56, 60-61, 98, 352;

Done. See Pages 32, 28, 23, 32, 33

L. 52, "Liu and Yang 2012";

Done. See line 53.

L. 56, "minimize N leaching";

Done. See line 56.

PLease delete numbers at L. 94, 123, 126, 157;

Done. See lines 95, 125, 128,168.

L. 108, ") and involved in the N cycling.";

See line 109.

Delete numbers before headings and subheadings; see L. 110, 111, 128, 177, 188, 189, 219, 233, 263, 283, 302, 303, 364, 422;

Done. See lines 111, 112, 131,190, 201, 202, 235, 249, 278, 297, 315, 316, 430, 465

L. 150, 220, 221, 222, 223, 380, 381, 666, pleasae add "exchangeable" before ammonium because you have determined this pool and not fixed ammonium;

Done. See lines 154, 236,238, 363, 365, 408, 784

L. 168, "Gaby and Buckley 2012";

Done. See line 181.

L. 234, "The total NH4+-N concentrations in leachates";

Done. See line 250.

L. 307, "byosynthesis (";

Done. See line 320.

L. 316, "found microbial ATP";

Done. See line 327.

L. 318, "Joergensen and Mueller (";

Done. See lines 329.

There are too may pencileld comments on page 12 and thus I can not list all comments here. I am attaching the scannerised copy of the page as a file;

Thanks for this. Done.

L. 356, "Ritz and Griffith";

Done. See line 383.

L. 384, "between abundances of AOA genes";

Done. See line 349.

Delete commas in the citations, see L. 415; please check carefully all text;

Done. See L. 463. Check it.

L. 418-419, "immobilization. However,..decreased abundances of ammonia";

Done. See lines 477-479. We moved it to conclusion.

L. 430, "nitrifier growth";

Done. See line 474.

References

Please, please list them according to the alphabetical order;

Thanks, we improved it.

L. 450, "Adv Soil Sci volume:113-142; the same at L. 531-532

Done. See line 492; 604.

L. 484, please include editoris, "In...(Eds) Practices";

Done. See line 543.

L. 493, "39:1468"; please delete the number of the issue at L 551 (15:), 562, 629;

Done. See lines 552, 631, 653, 743.

L. 564, "In...(Eds) Global";

Done. See line 647.

L. 572, pleaase delete the comma fter the family name and write "Brookes PC (2014).";

Done. See line 649.

L., 577, "Geoderma volume:259-";

Done. See line 653.

Are the references at L. 585, 598, 609, 612, 631 cited in the text?

Yes, L. 585, 598 and 631 in lines 62, 329, 54. We have deleted L.609 and 612.

L. 623, "Plant Soil"

Done. See 736.

Reviewers' comments:

Reviewer #1: Comments on BFSO-D-20-00790

Shen et al. report the effect of biodiesel co-product (BCP) on soil N transformation by nitrification-denitrification or N fixation activity by soil microbes in acidic soil for tea production. The authors used the small-scale lysimeters with 350 g of soil (fig. 1) and compared the treatments between BCP application to the surface (0-6 cm) and complete mixing of BCP to the soil (0-18 cm) with two types of control. The author assessed the N transformation following the N application by measuring NO3 reaching, N2O emission, and qPCR targeting nitrification (bacterial/archaeal amoA), denitrification (nirS, nirK, nosZ), and N fixation (nifH).

The major findings of this work are, the BCP addition can enhance the activity of soil microbes, which can immobilize the N and prevent the NO3 leaching. Complete mixing (T3) is most effective to prevent the NO3 leaching, while the effect of surface application (T4) was very much limited (Fig. 5d). The experimental design is clear enough and the dataset seems to be valuable. However, the data interpretation and discussion section are very much descriptive and not exciting enough. Most of the discussion section seems to be only the repetition of the description of the results. The authors should try to explain what happened in each treatment more. Therefore, this reviewer can recommend the manuscript to be published in Biology and Fertility in Soils after modification according to the comments below.

Thanks for your comments. We have improved our manuscript as requested

Major comments

It would be much helpful for the readers to make another figure which summarizes the fate of the added N. It should be more comprehensive to include the NO_3 reaching, NH_4 reaching, N_2O emission, and remained N, to show the

whole N balance in each column. Then some figures like Figure 6 or 5c and d will be unneeded.

A. Thanks for your comments. We have incorporated the figure of the whole N balance in each column as new Fig.6. And we deleted 5c and d, and moved the Figures 6 to SI.

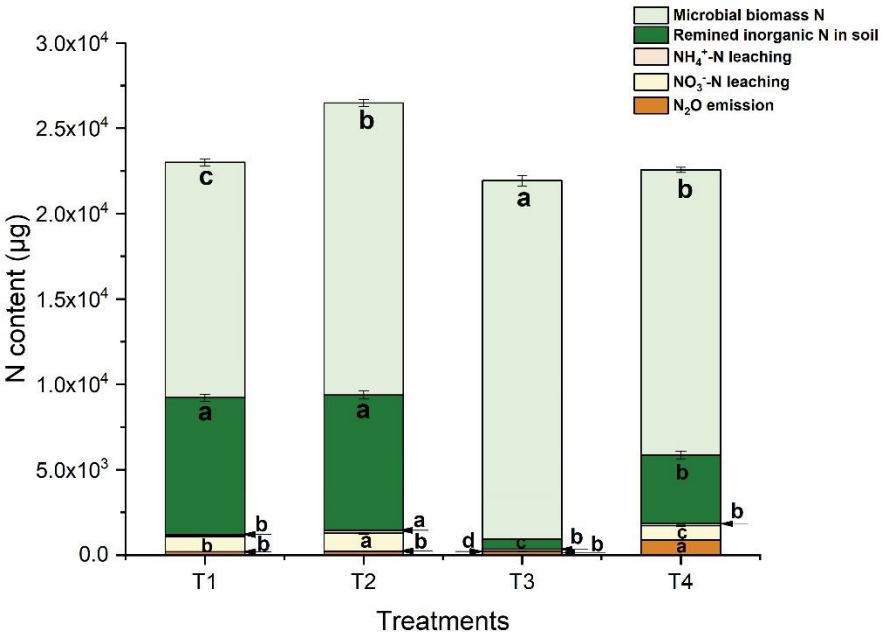

Fig. 6 The fate of N after 35-day application of urea and/or BCP. Error bars represent standard errors of the means (n = 3). Different lower case letters indicate significant differences among different treatments, which were determined by an one-way ANOVA by a Tukey test for post-hoc comparison at $P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

BCP addition seems to have several effects on N cycling, one is N immobilization by N uptake by the microbe, others are lowering nitrification or enhancing the denitrification and loss as N_2O or N_2 into the atmosphere. The authors should try to partition these effects and summarize the whole N transformation in each column more. The discussion section should be substantially revised from this point of view.

A. Thanks for your comments. We have rewritten the discussion. Hope it meet your request. We have added Fig.6.

NO_3 dropped significantly in the T4(0-6) treatment (Fig. 4b), which explains well the high N_2O emission in this treatment (Fig. 3), but I could not find any sentence to state this. The authors claim that the rapid decline of NO_3 was immobilized by soil microbe and it was not consumed by denitrification (L375-376), but I do not see appropriate data presentation which supports this interpretation. The authors should show the denitrification activity data with the acetylene inhibition method, otherwise, the fate of NO_3 in these treatments are not clear at all. Again, try to explain what happened in each treatment in the discussion section and avoid just the repetition of your results.

A. Thanks for your comments. We have changed this section (See lines 359-378).

The authors provide CH_4 emission without qPCR data of mcrA while providing nifH abundance data without N_2 fixation activity. These things should be presented together otherwise the data interpretation can be poor. Try to explain why CH4 emission dropped only in T4 on day 10 while others did not.

A. Thanks for your comments. Here, in our manuscript, we reported the effect of biodiesel co-product (BCP) on soil N transformation by nitrification-denitrification by soil microbes in acidic soil for tea production. Our main interest was N transformation, so we didn't provide qPCR data of mcrA. To make it clear, we put the CH_4 and CO_2 emission into SI, we also have decreased discussion section of CH_4 emission in the discussion: See lines 395-405: We have revised the section to: *The highest CH_4 emission rate in T4 treatment from day 0-5 (Fig. S6b). This suggests that higher labile C caused higher demand for O_2: leading to increased CH_4 emissions. After day 5, CH_4 emission rate in T4 treatment greatly decreased, suggesting that labile C may be depleted because of higher rate of CO_2 emission before day 5.*

Similarly, we provided nifH abundance data without N_2 fixation activity. nifH abundance is not the main point. We have rewriten it: See lines 455-464: *The nifH gene abundance is strongly associated with the N_2 fixation rate in soils with low available N (0.5 μg N g^{-1}) (Lindsay et al. 2010). The abundance of nifH genes (Fig. 8d) in treatment T4 (0-6) was significantly higher than other treatments on day 5. It decreased on day 35 but remained higher than in treatments T1 and T2. The copy number of nifH genes in treatment T3 was significantly higher than in the other treatments on day 35. This suggests that the surface application T4 (0-6 cm) of BCP maintained increased nifH genes throughout the incubation, while the mixed application T3 (0-18 cm) increased the nifH genes after the BCP was exhausted, as increasing substrate C availability increases biological N_2 fixation (Orr et al. 2012; Chen et al. 2019), which has high energy requirements, supplied by BCP (Mortenson 1964; Silsbury 1977; De Luca et al. 2002).*

Minor comments

L62 this sentence requires citation

Thanks. Done. See line 62.

L92 abbreviations AOA and AOB need clarification

Done. See line 93.

L142-144 15N-urea should be applied to 0-18 cm to make it same with (iii) but it seems to be absent between 6-7 cm

Thanks for this, here, it is the same as 0-18cm. 0-6cm+7-18cm=0-18cm. So to make it clear we changed to (iv) Treatment 4(T4) ^{15}N-urea mixed 0-18 cm: surface application of BCP (4500 µg g-1 soil) 0-6cm depth T4 (0-6); 7-18 cm sampling depth T4 (7-18). See line 145.

L155-156 composition of BCP should be presented

Done. We have put it in Table 1. See line 161.

L163-165 citation needed for the primer sets

Done, in the Table S2.

L167 nosZ

Done, Thanks, see line 180.

L227 I do not see any plot for T3(7-18) in Fig. 4b

Sorry, we made a mistake. We change to T4 (7-18). See line 236.

General comments on the discussion section: Overall, the discussion section is not exciting with poor data interpretation, especially nitrogen fixation and nifH gene RE. Discuss more and try to explain what happened in each treatment, which should not be only the repetition of the description of the results. I see many descriptive sentences that can be just a repeat of the results section. Avoid repetitive sentences as much as you can.

A: Thanks for your comments. We have improved our manuscript. And we have shortened it.

L400-406 it would be better to add the correlation analysis to explain the relationship between (*nirS*+*nirK*)/*nosZ* (presented in Table 2) and N_2O emission like you did for between NO_3 and amoA gene abundance, which will help the

readers understanding.

Fig 4b: I do not see any T3 plots (for both depth). Were they all under detection limit?

A. Thanks for your comments. However, the relationship between soil NO_3 and amoA gene abundance was made in two separated depths.While the N_2O was collected from the top of the column, only have one result in each column, and the (nirS+nirK)/nosZ was analyzed from two depths, so the N_2O and ratios cannot be matched. The T3 plots in Fig. 4b were overlaped with T4 (0-6). We have added an explanation in the legend of figure 4 See lines 785: ***Fig. 4*** *The changes in soil exchangeable* NH_4^+ *(a) and* NO_3^- *(b) at the different incubation times (T3 plots of* NO_3^- *were overlapped with T4 (0-6)).*

Reviewer #2: This manuscript was shown that the application of Biodiesel Co-Product (BCP) clearly suppressed the outflow of nitric acid, which has the unique and interesting viewpoint and contains important information. I principally believe that this research data is very important for the agricultural fields in an acidic tea soil. It is important to judge the data based on statistically significant differences. If the author's focus is on the microbial flora, it is not enough to simply carry out the data analysis to investigate the changes of community structure. It is necessary to examine deeply on what that change means in this manuscript.

This text contains some concerns. This manuscript is inadequate in interpretation and assessment of relevance, and many typographical errors are found.

1) Is it considered that there are no microorganisms in BCP?

A. Sorry, we have added this detail. See line 159. No microorganisms were detected in BCP after heating (90℃) for 2 hours.

2) Please show the gas analysis method and DON measurement method in the section "Materials and Methods" of the text.

A. Thanks for these. See lines 165-167.

3) The authors continuously sampled from the lysimeters (Lines128-149). Is there a risk of soil disturbance? To avoid doubts of the reader, it is necessary to describe the detailed method.

A. Thanks for this, here we used destructive sampling, New, intact columns were used at each sampling date. See Lines 152-153. So it will not have the risk of soil disturbance.

4) Please show the chemical properties of the BCP used in this study. Maybe, BCP include glycerol, salts of fatty acids, methylesters, so on (Lines 62-67). Table 2 shows only the contents of total C and total N.

A. This is now given in Table 1. See line 162.

In addition, this manuscript has some parts that need to be improved as below;

Lines329-330: This sentence is unclear. In soil?

A. Yes, in soil, we have changed this part. See line 359.

Lines340-347: The author's claim was shown about "the immobilization of soil NO_3^- and NH_4^+". Please mention in relation with pH, immobilization, and microbial community structure in acidic soil.

A. Thanks for the comments. We have added more information about the immobilization of soil NO_3^-. See lines 359-378.

Lines390-393: "BCP inhibited AOA and AOB genes", What do you mean?

A. Sorry, we change to BCP inhibited the growth of microorganisms bearing AOA and AOB genes. See lines 344-345.

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

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Abstract

Effectiveness of Biodiesel Co Product (BCP) in decreasing N leaching from an acidic soil (pH 3.7), effects on greenhouse gas emissions and N functional genes following surface application (0-6 cm depth) and complete mixing (0-18 cm depth) of 1.5 mg BCP-C g^{-1} soil was investigated in a 35 day laboratory lysimeter experiment. The BCP additions significantly decreased AOA and AOB gene copy numbers, especially from the surface BCP application. Both methods therefore inhibited nitrification and decreased N leaching.

Microbial biomass N and C significantly increased following both types of BCP incorporation, particularly with surface mixing. BCP increased *nifH* genes with both applications. Surface application of BCP produced higher emission rates of N_2O and CO_2 than complete mixing. Based upon (*nirS*+*nirK*)/*nosZ* ratios, more N_2O emissions, caused by denitrification, came from the surface application than complete mixing, in support of the gaseous measurement of N_2O. However, complete mixing was more effective than surface BCP application in decreasing N leaching: 2.14% of ^{15}N fertilizer in the leachate from complete mixing, compared to 51% following surface application, and 68% without BCP addition. These findings demonstrate that complete mixing was more effective than surface BCP application in decreasing N leaching and gaseous losses. We conclude that BCP is an effective and biologically safe method to prevent nitrate leaching in this acidic Chinese soil.

Key words: Biodisel Co-Product; ^{15}N-urea; Nitrogen leaching; N_2O; N-related functional genes; (*nirK*+*nirS*)/*nosZ*

Introduction

Nitrogen (N) is one of the most important nutrients for plant growth. However, losses of N derived from extensive applications of chemical fertilizers are a major source of eutrophication on a global scale, causing decreased quality of ground and surface waters, serious economic problems, and damage to aquatic and soil-based ecosystems (Norse 2005; Williams et al. 1997). In China, approximately 300 million rural residents lack access to safe drinking water because of agricultural pollution (Liu and Yang 2012). Nitrogen addition, in

both mineral and organic fertilizers, may be applied at rates as high as 450-1000 kg N ha^{-1} y^{-1} to Chinese tea plantations (Tokuda and Hayatsu 2001, 2004; Xue et al. 2006; Li et al. 2013). Urea (46% N) is the most commonly used N fertilizer in China and especially in tea plantations. High fertilizer N applications, especially urea, may cause excess residual N in soil, which can increase the risk of nitrate leaching and nitrous oxide (N_2O) emissions, and soil acidification (Xue et al. 2006; Zhu et al. 2011; Hirono and Nonaka 2012; Liu and Yang 2012; Zhu et al. 2014). Therefore, to alleviate the contamination of groundwater by nitrate N (NO_3^--N) derived from tea fields, it is necessary to have better management of N, such as proper fertilizer application rates and incorporation of residues, to immobilize N and minimize N leaching (Morita et al. 2002). Although this is less effective than using cover crops (Justes et al. 1999), their use is often inconvenient, due, for example, to adverse Spring weather conditions. Nitrification inhibitors can also be effective in decreasing nitrate leaching and N_2O emissions (Menendez et al. 2012), as nitrate-N is preferred over N_2O as a terminal electron acceptor and N_2O evolution can increase whenever NO_3^--N supply is greater than the reducing demands of the denitrifiers (Swerts et al. 1996).

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

There are several major types of liquid biofuels, including biodiesel, bioethanol and

pyrolysis bio-oil. In 2018, 2.6 M barrels of biofuels per day, dominated by the USA and Brazillian markets, comprised about 87 % of global production. The EU and Chinese shares were 5% and 3% respectively (Mizik et al. 2020). By 2050, biofuels are predicted to comprise 27% of the world's liquid fuel supply (Guo et al. 2020). Based on the projections of OECD and the FAO, by 2027 the USA will still be the main producer. While its market share will decline to 46%, Brazil's will increase to 25%, and China's will reach 8% (OECD 2020). This suggests that there will be increased BCP produced in China. Biofuel production is instrumental in improving energy security by decreasing foreign oil imports and promoting renewable energy resources (Prasad et al. 2020).

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

The application of BCP to soil as a substrate for the native soil microbial community was previously found to be 99% effective in immobilizing inorganic N in near neutral soils and preventing N leaching losses from the plough layer (Redmile-Gordon et al. 2014). The BCP application also increased soil exocellular polysaccharides (EPS) and protein synthesis. Therefore, biodiesel has considerable potential for improving N use-efficiency and limiting the environmental damage caused by 'leaky' agriculture (Redmile-Gordon et al. 2015). Increasing labile C availability, by adding BCP, will also increase biological N_2 fixation (Orr et al. 2012; Chen et al. 2019).

Soil nitrification is a two-step process, where ammonia is first oxidized to nitrite by ammonia oxidizing archaea (AOA) and ammonia oxidizing bacteria (AOB), then converted to nitrate by nitrite-oxidizing bacteria (NOB). The AOA generally make a much greater contribution than AOB to ammonia oxidation in acidic soils (Li et al. 2018). Denitrification occurs under anaerobic conditions where oxygen is limited (Luo et al. 1999). During denitrification, the nitrate is successively reduced to N_2O or N_2 by heterotrophic denitrifiers (Liu et al. 2019). Nitrite reductase is encoded by the *nirS* and *nirK* gene and N_2O reductase is encoded by the *nosZ* gene (Avrahami and Bohannan 2010; Conrad 1996; Wrage et al. 2001; Xu et al. 2017). The *nifH* gene has the ability to fix atmospheric N_2 (Zehr et al. 2003).

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

Materials and Methods and

Soil sampling and analyses

The soil was sampled from the surface layer (0-20 cm depth) of a tea field from Meijiawu tea

region (30°21′N, 120°10′E), Hangzhou, Zhejiang Province, China by collecting 12 of 25 cm diameter cores and bulking. The soil is classified as a Ultisol sandy. The main soil properties were: pH 3.71, 8.2% clay, 5.8% silt, 86% sand, 0.21 g kg^{-1} total N, 2.9g kg^{-1} total C, 13.6 C/N, 250±0.63 μg g^{-1} microbial biomass C, 49.43±6.27 μg g^{-1} microbial biomass N, 2.98±0.22 nmol g^{-1} ATP. The pH was determined using a 1: 2.5 soil: water ratio, and total C and N contents by an elemental analyzer (Elementar Analysensysteme Gmb H., Germany). All measurements were done immediately before leaching except the gaseous emissions. Soil microbial biomass C (biomass C) was determined by fumigation extraction, and microbial biomass C was calculated from: Biomass C = 2.22 Ec, where Ec = [(organic C extracted from fumigated soil) - (organic C extracted from non-fumigated soil)] (Vance et al. 1987; Wu et al. 1990). Soil microbial biomass N (biomass N) measured in the same extracts as microbial biomass C by fumigation extraction (KEc= 0.45) (Brookes et al. 1985). Soil adenosine 5′-triphosphate (ATP) was extracted from soil by ultrasonics (Jenkinson and Oades 1979) and determined as described by Redmile-Gordon et al. (2011), with three replicates of moist soil containing 3.0 g oven dry soil. ATP in the soil extracts blanks and standards (0–100 pmol 50 μl^{-1}) were measured with a luminometer (Glomax 96. Promega, USA) using the firefly luciferin-luciferase reagent.

Experimental design

After collection, the soils were sieved moist < 5 mm, soil moisture was adjusted to 40% of water holding capacity (WHC) then the soils were incubated at 25 ℃ for 7 days prior to determination of microbial biomass C and ATP. The soil was then added to soil columns (24 cm in length, 6 cm diameter). Twelve lysimeters were prepared, 3 lysimeters per treatment

(Fig. 1). Each lysimeter contained moist soil equivalent to 350 g oven-dry soil, and was supplied with 80 µg urea N g^{-1} soil at 5.18% ^{15}N atom excess when required. The treatments (all sampled 0-6 and 7-18 cm depth) were:

(i) Treatment 1 (T1) Control (no treatment): 0-6 cm sampling depth T1 (0-6) and 7-18 cm sampling depth T1 (7-18)

(ii) Treatment 2 (T2) ^{15}N-urea addition only: 0-6 cm sampling depth T2 (0-6) and 7-18 cm sampling depth T2 (7-18)

(iii) Treatment 3 (T3) BCP (1.5 mg C g^{-1} soil) and ^{15}N-urea mixed 0-18 cm: 0-6 cm sampling depth T3 (0-6); 7-18 cm sampling depth T3 (7-18)

(iv) Treatment 4 (T4) ^{15}N-urea mixed 0-18 cm: surface application of BCP (4.5 mg C g^{-1} soil) 0-6 cm depth T4 (0-6); 7-18 cm sampling depth T4 (7-18)

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

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

5 and 35 (See below). Biodiesel Co-Product was made in the laboratory from waste vegetable cooking oil. It was first purged of excess methanol by heating to 90 °C for 2 h. Before application, BCP was prepared in water and adjusted to pH 8 by adding 1 M HCl dropwise (Redmile-Gordon et al. 2014). The organic constituents of BCP were determined as described by Redmile-Gordon et al. (2015) and details are provided in Table 1. A methane conversion furnace, flame ionization detector (FID), and electron capture detector (ECD) were used for the determination of the CO_2, CH_4, and N_2O, respectively (Wang et al. 2017). Dissolved organic C (DOC) and N (DON) were determined using a TOC-TN analyzer (Shimadzu, Japan). Dissolved organic N was calculated from: [dissolved total N (DON) minus (NH_4^+−N + NO_3^-−N)].

DNA extraction and quantitative PCR (qPCR) analysis

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

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

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

Laboratory analysis and data analysis

The percent recovery of the applied urea-^{15}N was calculated according to Cabrera and Kissel (1989): N recovery (%) = p(c-b) / f(a-b) * 100

where p = mols of N in leachate and soil samples, f = mols of N in urea applied, c = atom%^{15}N abundance in leachate samples, a = atom%^{15}N abundance in the urea, b = atom%^{15}N abundance in the leachate samples without added urea.

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

Results

Soil microbial biomass and ATP

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

Changes in microbial biomass N in the different treatments closely followed those of microbial biomass C (Fig. 2b). Again, microbial biomass N was greater following both BCP additions, with the greatest microbial biomass N contents in treatment T4 (0-6). Microbial biomass N in treatment T4 (0-6) was about 75 μg g^{-1}, and as with microbial biomass C, it declined until day 35. Overall, there was a highly significant linear correlation between microbial biomass N and microbial biomass C (R^2=0.96) (Fig. S1b), However, there were differences in mean microbial biomass C/N ratios in the different treatments. The highest ratio was in treatment T4 (0-6) 6.48, followed with treatment T3 (7-18) at 5.48, and then treatment T3 (0-6) with a ratio of 5.20. The microbial biomass C/N ratios with urea only were 4.74, 4.78 and 5.10 in the T2 (0-6), T2 (7-18) and T4 (7-18) treatments respectively. The ratios in the control soils (T1) were 4.79 and 5.01 respectively in the two different depths. (Fig. S1).

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

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

Soil inorganic N

There was a distinct peak in soil exchangeable NH_4^+-N at day 5 in T2 (0-6); (7-18) and T4 (7-18). The highest concentration was with treatment T4 (7-18), at about 4.3 mg exchangeable NH_4^+-N g^{-1} soil. By day 10, soil exchangeable NH_4^+-N had declined to relatively similar levels in all treatments to between about 1.5 to 2.0 mg exchangeable NH_4^+-N g^{-1} soil. However, the smallest concentrations were consistently with treatment T3 at around 1.5 mg kg^{-1} soil (Fig. 4a).

Soil NO_3^--N concentrations in treatments T3 (0-18) and T4 (0-6) were close to zero by day 5 and remained so throughout the 35-day incubation. In contrast, the concentrations in treatment T2 (0-6; 7-18), and T4 (7-18) increased, reaching a maximum at day 5 with about 33, 32 and 31 mg kg^{-1} respectively, then remained at approximately these concentrations until

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

The effect of BCP on nitrogen leaching

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

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

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

by T3, with 16.5 mg, then T2 and T1 with 11.2 mg and 10.6 mg respectively (Fig. S5).

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

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

Functional gene shifts

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

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

in treatment T3 was significantly lower than in treatments without BCP on day 5, while both of them increased on day 35. In contrast, the abundance of *nifH* gene in treatment T4 (0-6) was highest at day 5, followed by treatment T4 (7-18). At day 35, the *nifH* genes in treatment T3 were significantly higher than in the other treatments by 7 times. Also, they were still significantly higher in treatment T4 when compared with T1 and T2 (Fig. 8d). The lowest [*nirK*+*nirS*]/[*nosZ*] ratios were with treatment T4 (0-6) on day 5 (2.40) and 35 (2.24). The highest ratio was with treatment T4 (7-18) (4.36) on day 5 (Table 2).

The effects of BCP on Greenhouse Gas emissions

The rate of nitrous oxide (N_2O) emissions was largest in treatment T4. It rapidly increased from day 0 to day 5, and reached 485 μg m^{-2} h^{-1} at day 5. It then decreased to 98 μg m^{-2} h^{-1} at day 10 and 14.5μg m^{-2} h^{-1} at day 20. However, the rates of other treatments were similar and remained stable throughout, from around 40 μg m^{-2} h^{-1} to 14 μg m^{-2} h^{-1} (Fig. 3).

Carbon dioxide (CO_2) emissions from treatments T3 and T4 also showed a similar pattern from day 0 to 20. The peak of CO_2 emission rate occurred on day 5, declined to day 20 then remained stable at about 99 mg m^{-2} h^{-1} until the end of the incubation time. The peak emission rate in treatment T4 (951 mg m^{-2} h^{-1}) was higher than in treatment T3 (727 mg m^{-2} h^{-1}). Before the rate of CO_2 emission from treatment T2 stabilized, it decreased from 84 mg m^{-2} h^{-1} to around 35 mg m^{-2} h^{-1} during the first 5 days. There was a decline in treatment T1 from day 0 to day 35 (86 to 29 mg m^{-2} h^{-1}) (Fig. S6a).

The emission rates of CH_4 increased slightly from day 0 to 5, afterwards, it halved in all treatments by the end of the experiment. The differences in the rates between treatments T4> T1>T3>T2 at 69, 67, 65.8 and 66.5 μg m^{-2} h^{-1} respectively were not significant by day 5.

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

Discussion

Changes in microbial biomass and ATP concentrations

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

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

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

Soil inorganic N and N leaching

Urea application increased nitrification without BCP (Fig. 4b), which indicates that acid-tolerant nitrifers exist in acidic soils and have high activity. Increasing soil pH can promote nitrification and induce nitrate accumulation in some acidic soils (De Boer et al. 1996; SteMarie and Pare 1999; De Boer and Kowalchuk 2001; Zhang et al. 2017). BCP increased soil pH in our study (Fig. S5) but we found that BCP significantly decreased AOA and AOB *amoA* genes (Figs. 7a and 7b). This suggests that BCP potentially inhibited the growth of microorganisms bearing AOA and AOB genes, as it may contain biological nitrification inhibitors (Sarr et al. 2020). The abundance of AOA *amoA* genes was significantly higher than AOB *amoA* genes (Figs. 7a and 7b), which is consistent with other findings (Herrmann et al. 2012; Sarr et al. 2020). There was also a linear relationship between abundances of AOA genes and NO_3^--N concentrations in the soils. This is supported by the findings of others that although AOA and AOB have the same functions, AOA, rather than AOB dominates in acid soils (pH<4.9) (Leininger et al. 2006). Therefore, AOA generally makes the greater contribution to ammonia oxidation in acid soils (Li et al. 2018; Yao et al. 2011). On day 5 the lowest AOB gene number was in treatment T4 (7-18), which suggests that the surface addition of BCP consumed much O_2 in the surface causing anaerobic conditions in T4 (7-18). By day 35, the copy number of AOB in the BCP treatment T3 (7-18)

was higher than in other treatments. while the AOA copy number in the BCP treatments were still lower than in the others. This indicates BCP addition inhibited the growth of microorganisms having AOA longer than those with AOB genes.

Addition of BCP greatly decreased the soil NO_3^--N concentrations (Fig. 5b). The lowest amounts of NO_3^--N leached in treatment T3 were less than in T4, compared to T1 and T2 i.e. No BCP. The immobilization of NO_3^--N may be inhibited by concentrations of NH_4^+ as low as 0.1 µg NH_4^+-N g^{-1} soil (Rice and Tidje 1989). However, the accumulation of microbial biomass N in response to BCP proceeded despite low exchangeable NH_4^+-N in the soil (Fig. 4). This suggests that the quality of C (soil organic matter vs. BCP) is more important for NO_3^--N immobilization than the concentration of exchangeable NH_4^+-N (Shen et al. 2021). Cheng et al. (2017) also found that NO_3^- immobilization is increased by the addition of simple organic substrates at concentrations above 0.5 mg C g^{-1} soil. The amount of BCP we used was 1.5 mg C g^{-1} which was consistent with this. Burger and Jackson (2003) also found high NO_3^- immobilization rates in near neutral soils (pH 6.8 and 6.5) with low NH_4^+-N concentrations (around 1 µg N g^{-1} soil). Heterotrophic microbes assimilated less NH_4^+ than NO_3^-, probably because NH_4^+ concentrations were low and competition by nitrifiers was apparently strong. This suggests that BCP caused strong competition for NH_4^+ between nitrifiers and N immobilizers in our soils, causing NO_3^- to be more available to microbes. Previous studies also reported that fungi prefer NO_3^- than $NH_4^+$and exchangeable NO_3^- was taken up by fungi (Marzluf 1997; Zhu et al. 2013). The application of BCP to the plough layer (23 cm) in a high pH soil was 99% effective in NO_3^- immobilization thus preventing its loss during winter (Redmile-Gordon et al. 2014). which was similar to findings of Ritz and

Griffith (1987) and Park et al. (2006).

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

GHG-C emission rates (CO_2 and CH_4)

Higher labile C inputs cause higher cumulative CO_2 emissions in aerobic soils (Tsai et al. 1997; Miller et al. 2008). This is consistent with our results where the highest rate of CO_2 emission was from treatment T4, followed by treatment T3 (Fig. S6b). The higher rate of CO_2 emission was on day 5 and then sharply declined. Brant et al. (2006) found that a readily

mineralizable pool of substrate C was respired during the early stage (first 3d of incubation). The CH_4 production rate was low, because methanogens is inhibited in strongly acidic soils (Ye et al. 2012). The highest CH_4 emission rate was in T4 treatment (Fig. S6b). This suggests that the greater labile C in BCP caused a higher demand for O_2, producing anaerobic conditions. After day 5, the CH_4 emission rate in the T4 was greatly decreased, suggesting that labile C was becoming depleted.

N_2O emissions from soil

Parton et al. (1996) found that N_2O fluxes caused by nitrification were proportional to soil N turnover and that high levels of soil exchangeable NH_4^+ (> 3 mg N kg^{-1} soil) increased N_2O emission. In our soils the NH_4^+-N concentration was below 3 mg N kg^{-1} soil (Fig. 4a), so it would not affect N_2O emission. The highest rate of N_2O emission was from the T4 treatment on day 5 (Figs. 3 and 6). This suggests that the addition of high rates of BCP increases the tendency for soil anoxia, favoring the growth of denitrifiers (Beauchamp et al. 1989; Azam et al. 2002). Several studies have shown the importance of spatial and temporal soil heterogeneity in providing soil O_2 concentrations for N_2O emissions (Meyer et al. 2002; Khalil et al. 2004; Morley and Baggs 2010). Nitrification can account for 55-95% of N_2O emissions when the water filled pore space (WFPS) is between 40 and 60% (Linn and Doran 1984). In this study, the soil WHC was 50%, which is around 40%WFPS to 60%WFPS. The N_2O emissions rate was generally low (< 40 μg m^{-2} h^{-1}) in our soil except in the T4 treatment (Fig. 3). This suggests that N_2O emissions in T1, T2 and T3 are mainly derived from nitrification. The N_2O emission rate was high in treatment T4 but not in T3. This suggests that the main N_2O emission from T4 may not come from nitrification. Soil NO_3^--N

concentration rapidly declined to zero in treatments T3 and T4 (0-6), which agrees with previous work (Shen et al. 2021), indicating that NO_3^--N was immobilized by soil microbes, rather than being denitrified. Therefore, the high N_2O emission rate may come from denitrification in T4 (7-18), which will be discussed in next section. The recovery rate of ^{15}N-urea in the soils of the different treatments at day 5 was almost 100 % (Fig. S2). This suggests that volatilization loss of ^{15}N-urea was negligible before day 5. Rochette et al. (2013) previously showed virtually no urea volatilization below soil pH<6, which agrees with this finding.

Functional genes shifts

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

The labile C in BCP does not only support the activity of denitrifiers, but also has the indirect effect of causing microsite anaerobiosis, due to increased respiratory demand for O_2. It would favor the completed denitrification to N_2 in saturated soil (90%WFPS), while it

significantly stimulated N_2O emissions at 40% WPFS (Sanchez-Martin et al. 2008). In our study, the WHC of soil (50%) was lower than 90% (WFPS), indicating that BCP addition would not support complete denitrification. Higher ratios of (*nirS*+*nirK*)/*nosZ* are related to higher N_2O emissions (Guo et al. 2018). The highest gene ratio of (*nirS*+*nirK*)/*nosZ* (4.36) was in T4 (7-18) (Table 2), suggesting that the high N_2O emission rate in T4 was derived from denitrification from the 7-18 cm depth. The BCP addition would have caused more O_2 consumption in the T4 (0-6) soil surface layer, leading to decreased O_2 entering soil below this depth, (Kuang et al. 2019), which may cause anaerobic conditions in T4 (7-18). This supports the above findings (Fig. 3a). Also, the lowest ratio of (*nirS*+*nirK*)/*nosZ* was in T4 (0-6) on both day 5 and 35, suggesting that the high rate of BCP addition (T4 (0-6)) may have the potential to decrease both N leaching and N_2O emission.

The *nifH* gene abundance is strongly associated with the N_2 fixation rate in soils with low available N (0.5 μg N g^{-1}) (Lindsay et al. 2010). The abundance of *nifH* genes (Fig. 8d) in treatment T4 (0-6) was significantly higher than other treatments on day 5. It decreased on day 35 but remained higher than in treatments T1 and T2. The copy number of *nifH* genes in treatment T3 was significantly higher than in the other treatments on day 35. This suggests that the surface application T4 (0-6 cm) of BCP maintained increased *nifH* genes throughout the incubation, while the mixed application T3 (0-18 cm) increased the *nifH* genes after the BCP was exhausted, as increasing substrate C availability increases biological N_2 fixation (Orr et al. 2012; Chen et al. 2019), which has high energy requirements, supplied by BCP (Mortenson 1964; Silsbury 1977; De Luca et al. 2002).

Conclusions

Complete BCP mixing, (Treatment T3 (0-18)) was much more efficient in preventing NO_3^- N leaching than T4 (Surface application (0-6)). This is attributed to more biological activity in treatment T3 with its deeper mixed BCP application. Therefore, more fertilizer N was immobilized, as shown by increased microbial biomass C and N and decreased DON leaching losses. This suggests that Treatment T3 would also be best under field conditions. No harmful effects of BCP applications on microbial activity were observed. Although the surface application (T4) was less effective in decreasing N leaching, the high rate of application (T4 (0-6)) maybe be more effective in decreasing N leaching by inhibiting nitrifier growth. Also, it has potential in decreasing N_2O emissions by decreasing the ratio of (*nirK*+*nirS*)/*nosZ*. Field trials in a range of acidic Chinese tea soils under different climatic conditions are now required to test the efficiency and safety of BCP applications to decrease N leaching under field conditions. Finally, whether BCP addition would promote biological N_2 fixation and why it decreased the abundances of ammonia oxidizers and denitrifiers need further work.

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Legend of Figures

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

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

Fig. 3 The emssion rates of N_2O in the different treatments at different incubation times. Error bars represent standard errors of the means (n = 3). T1: control; T2: ^{15}N-urea addition only; T3: application with mixture of BCP (1500 μg g^{-1} soil) and ^{15}N-urea; T4: surface application (0-6 cm) of BCP (4500 μg g^{-1} soil) and ^{15}N-urea together with only ^{15}N-urea applied to 7-18 cm depths.

Fig. 4 The changes in soil exchangeable NH_4^+ (a) and NO_3^- (b) at the different incubation times (T3 plots of NO_3^- were overlap with T4 (0-6)). Error bars represent standard errors of the means (n = 3). T1 (0-6): 0-6 cm sampling depth of control; T1(7-18): 7-18 cm sampling depthe of control; T2 (0-6): 0-6 cm sampling depth of ^{15}N-urea addition; T2 (7-18): 7-18 cm sampling depthe of ^{15}N-urea addition; T3 (0-6): 0-6 cm sampling depth of application with mixture of BCP (1500 μg g^{-1} soil) and ^{15}N-urea; T3 (7-18): 7-18 cm sampling depthe of application with mixture of BCP (1500 μg g^{-1} soil) and ^{15}N-urea; T4 (0-6): surface application (0-6cm) of BCP (4500 μg g^{-1} soil) and ^{15}N-urea; T4 (7-18): only ^{15}N-urea applied

to 7-18 cm depths.

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

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 $P < 0.05$.

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

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

applied to 7-18 cm depths.

Figure1

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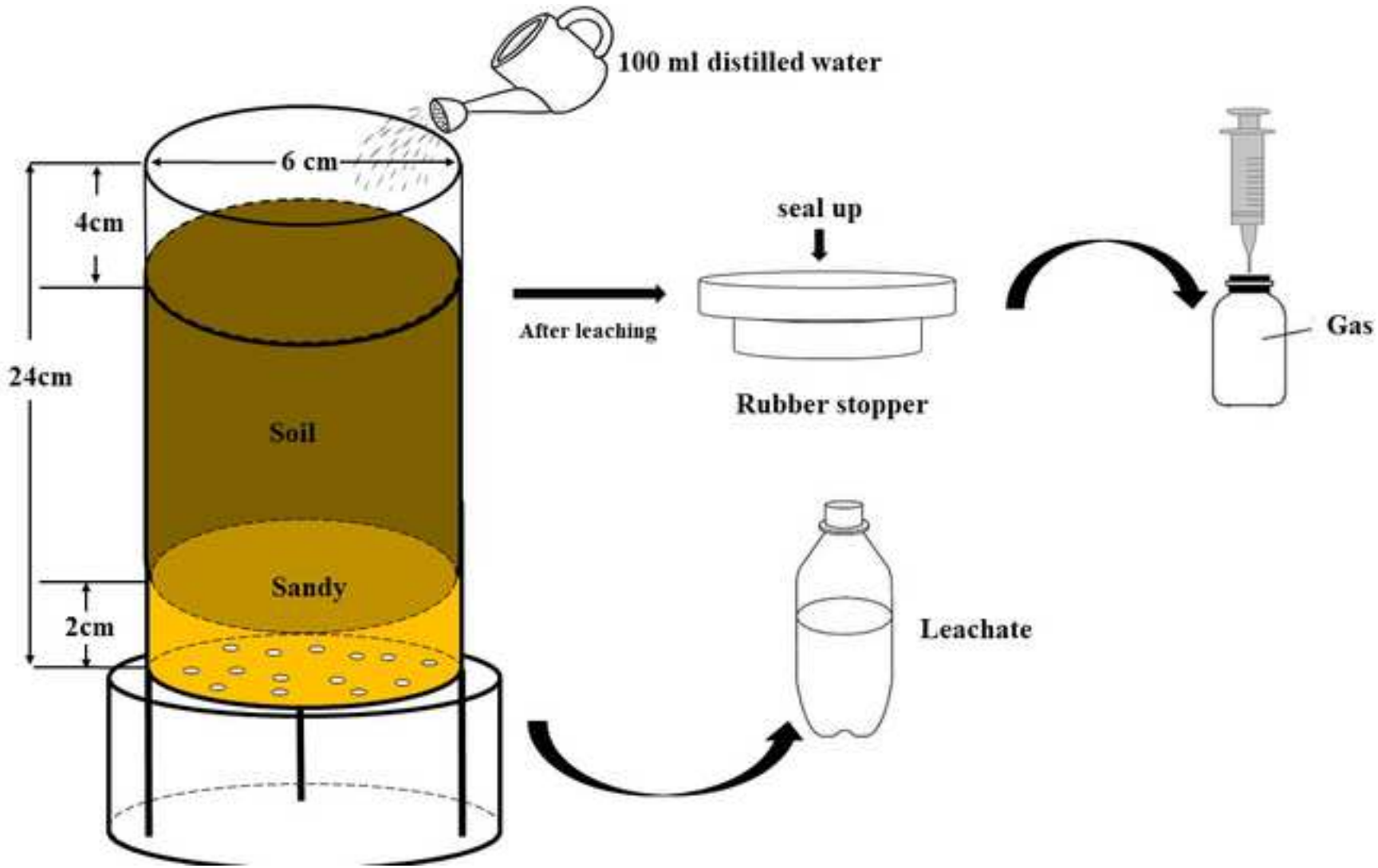

Figure 2

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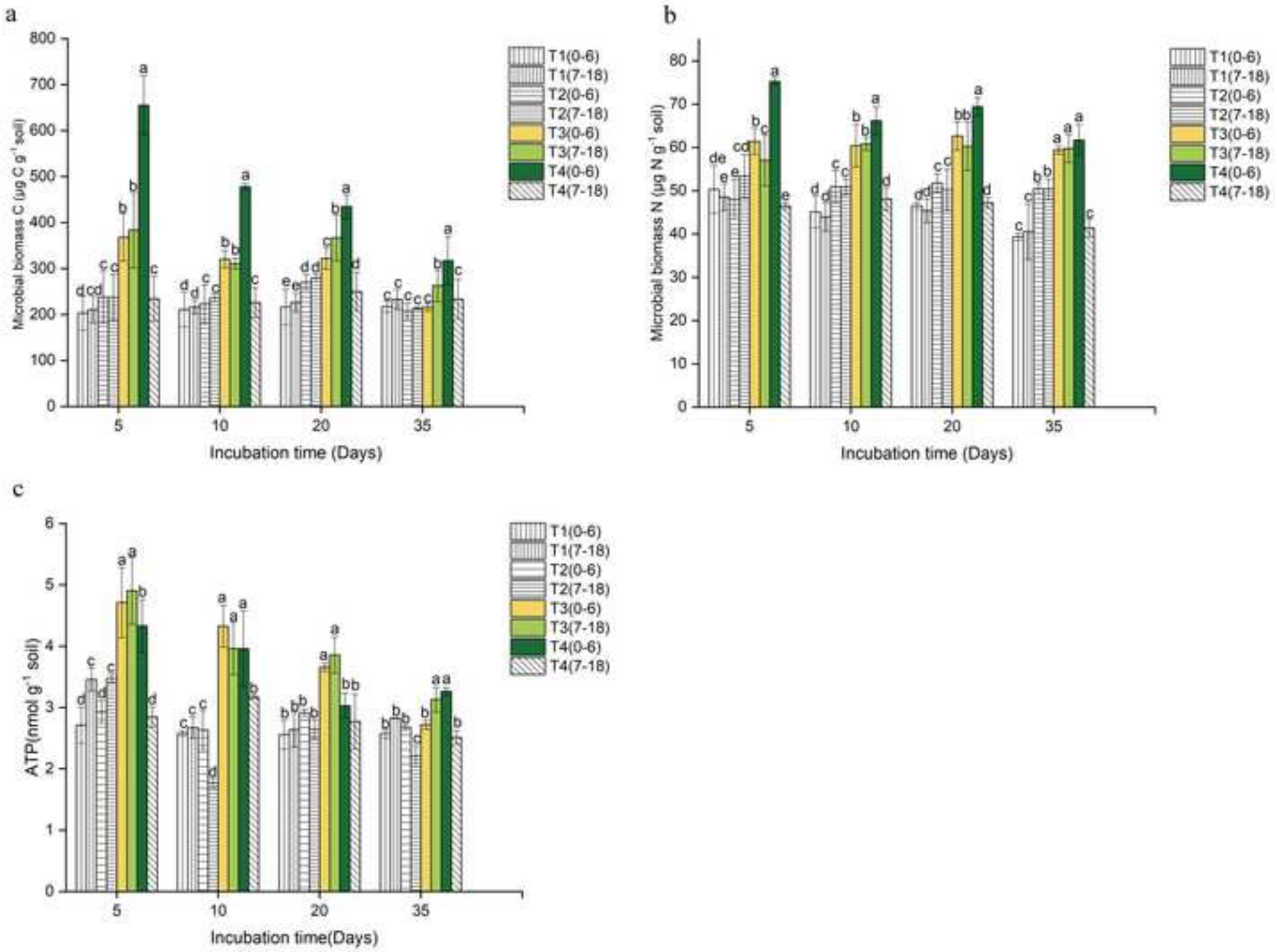

Figure 3

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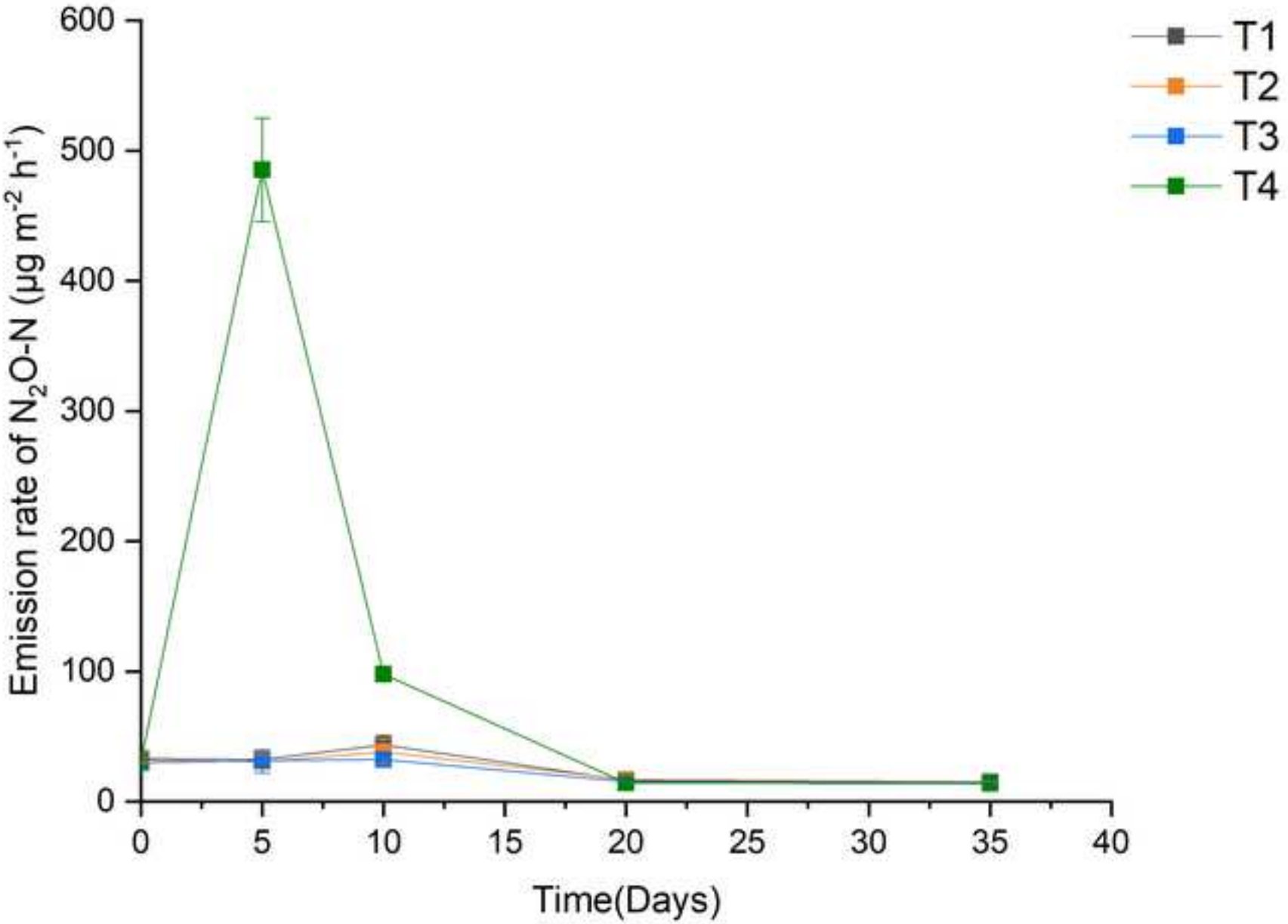

Figure 4

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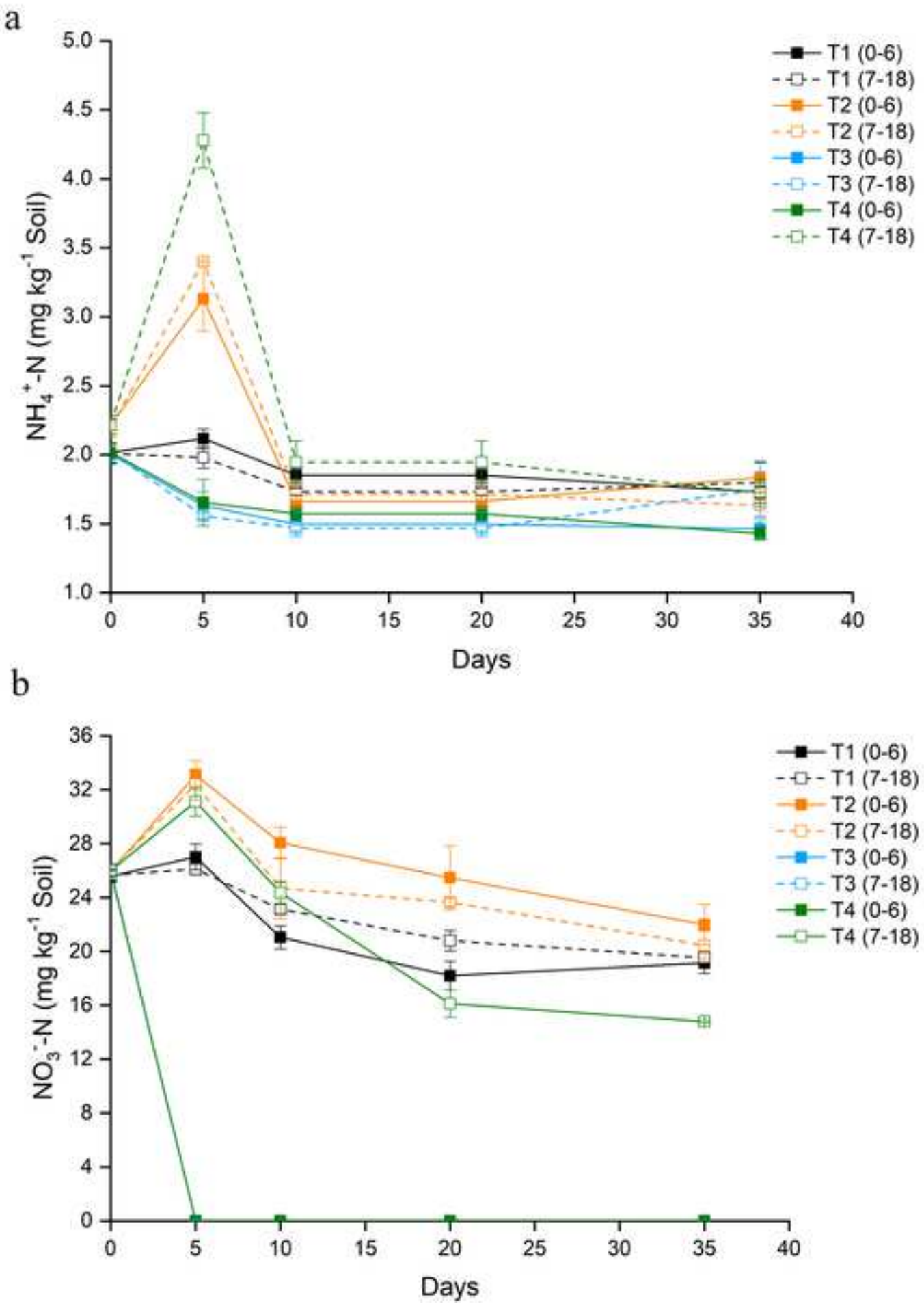

Figure 5

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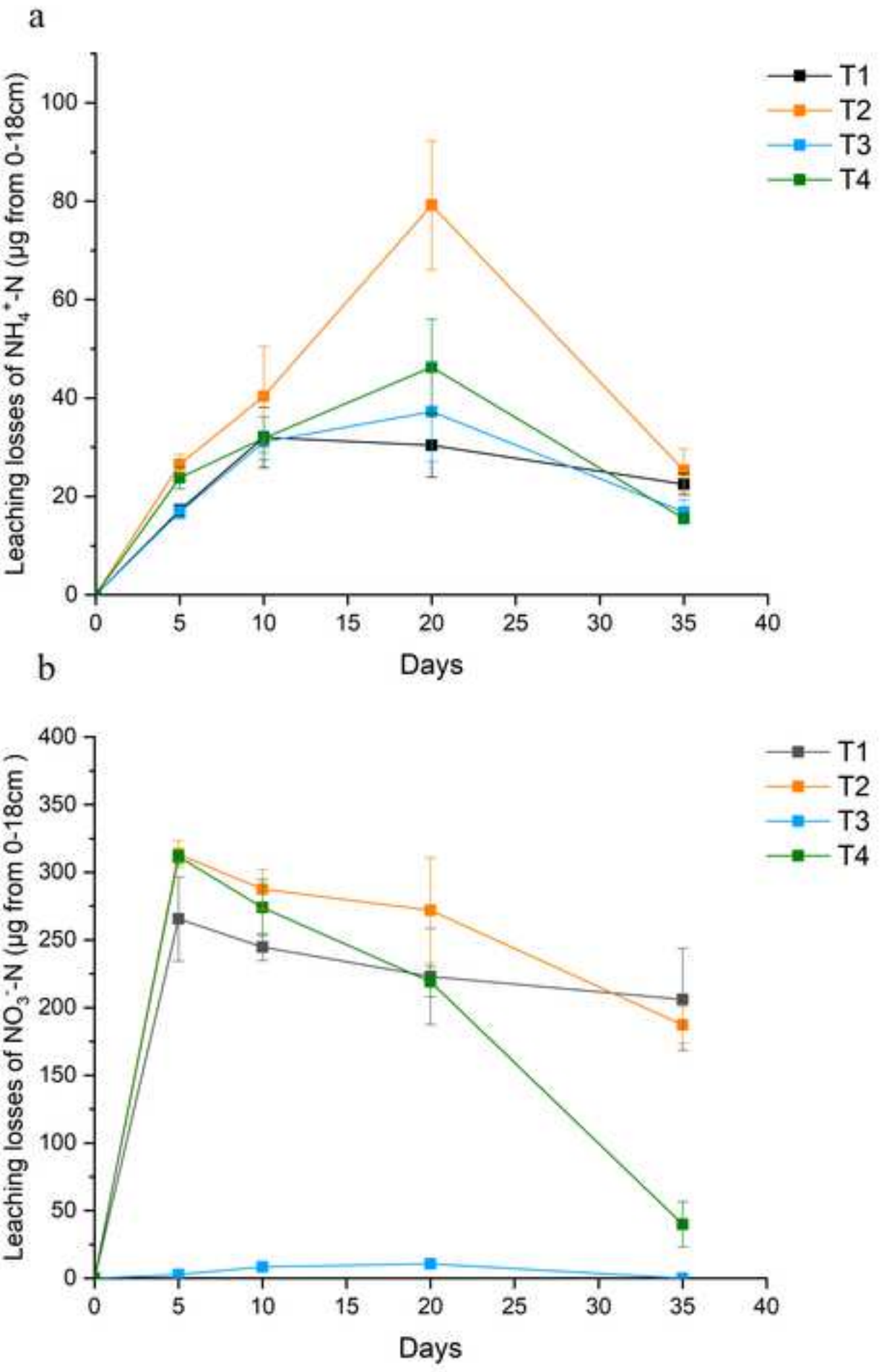

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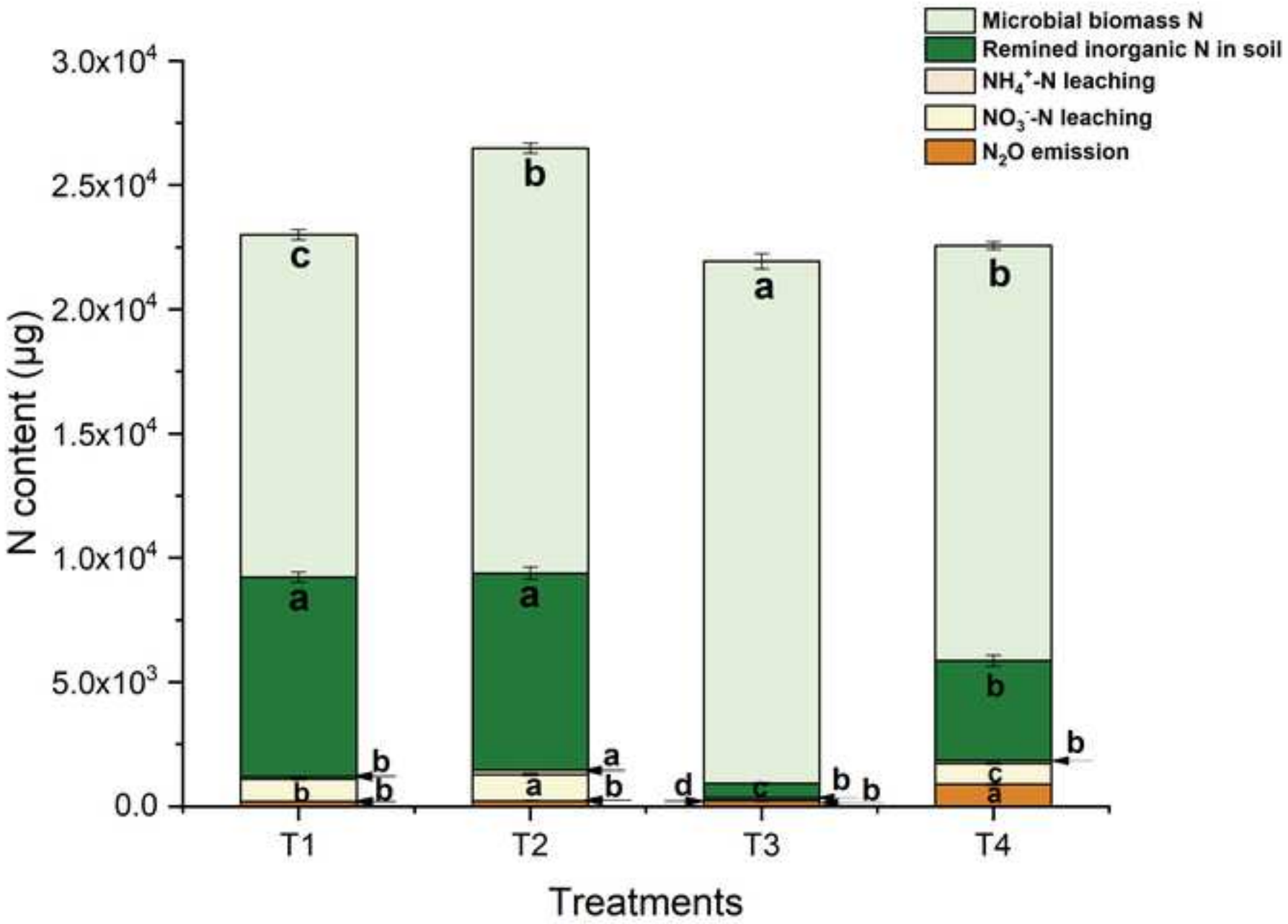

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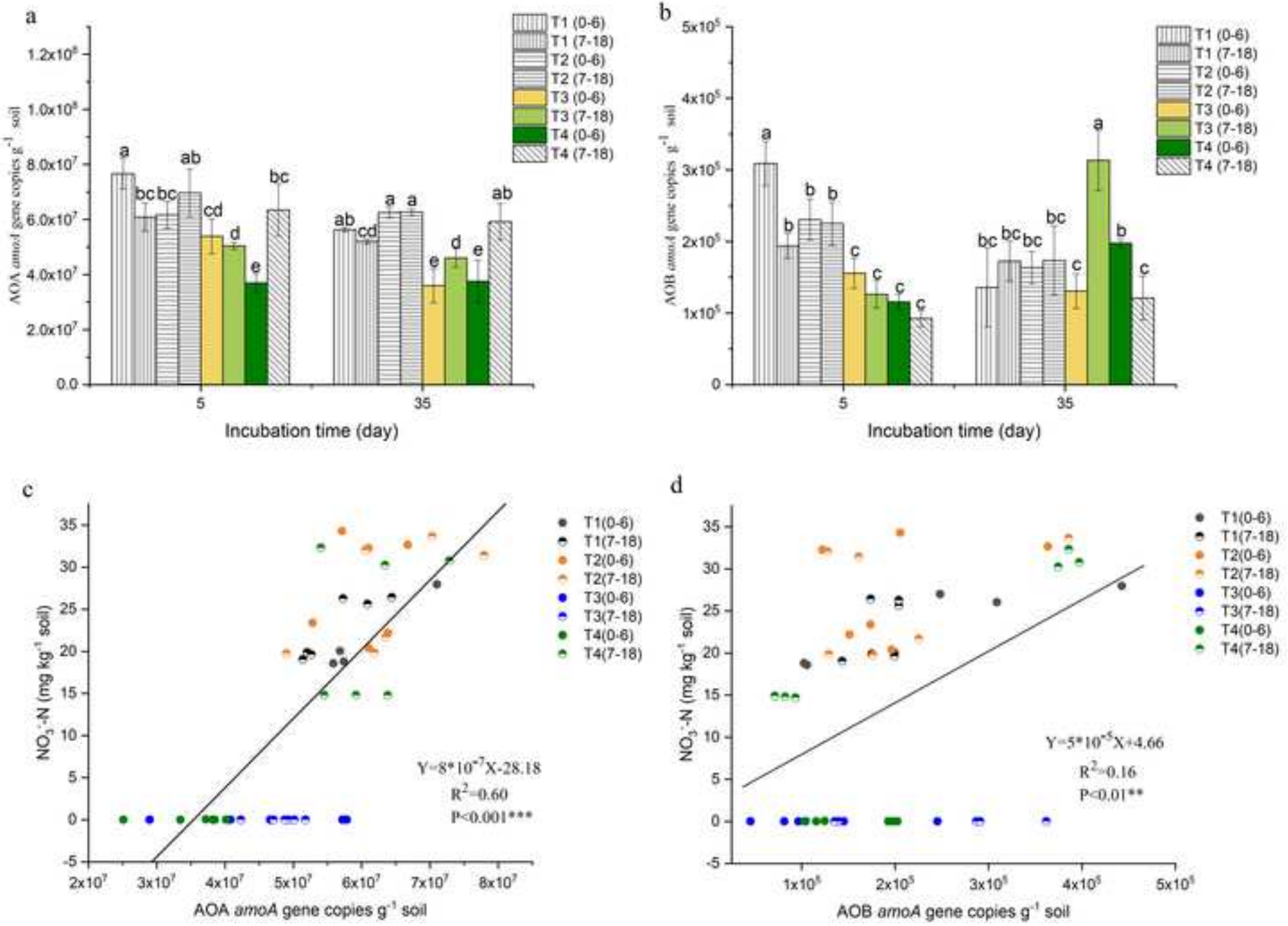

Figure 8

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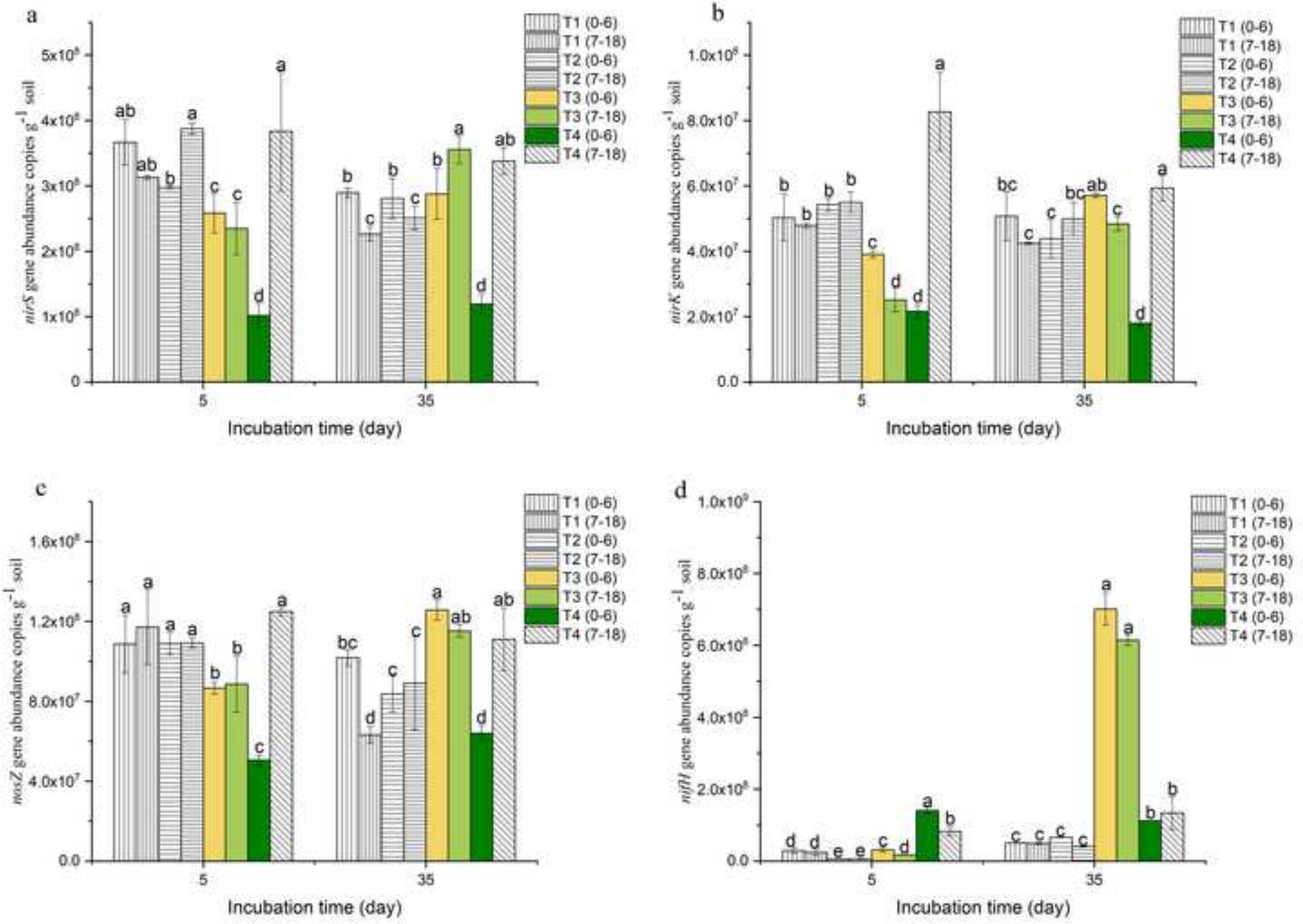

Table 1

Table 1 Properties of BCP

Biodiesel Co-Product	Potassium hydroxide (% KOH)	Potassium soap (%oleate equivalent)	Fatty acid methyl esters (FAME; %)	Volatile organics (% at 105℃)	Glycerol (%)	H_2O (%)
BCP	2.4	11.7	0.4	11.7	73	1.6

Table 2

Table 2

[*nirK*+*nirS*]/[*nosZ*] ratios in different treatments

mg C applied g^{-1} soil (as BCP-C)	Treatments	[*nirK*+*nirS*]/[nosZ] day 5	[*nirK*+*nirS*]/[*nosZ*] day 35
0	T1 (0-6)	3.66±0.02 ^{bc}	3.30±0.23^{b}
0	T1 (7-18)	3.50±0.23 ^{bc}	3.37±0.18^{ab}
0	T2 (0-6)	3.33±0.22 ^{c}	**3.68±0.13^{a}**
0	T2 (7-18)	3.80±0.06 ^{bc}	2.86±0.14^{c}
1.5	T3 (0-6)	3.40± 0.19 ^{bc}	2.57±0.34^{c}
1.5	T3 (7-18)	3.39± 0.06^{b}	3.44±0.02 ^{ab}
4.5	T4 (0-6)	**2.40± 0.28^{d}**	**2.24±0.22^{d}**
0	T4 (7-18)	**4.36± 0.41^{a}**	3.38±0.04 ^{ab}

^{abcd} Lowercase letters denote statistically significant significance at $P < 0.05$.