Coupled steel slag and biochar amendment correlated with higher methanotrophic abundance and lower CH_4 emission in subtropical paddies

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Abstract Aerobic methanotrophs in paddies serve as methane (CH_4) filters and thereby reduce CH_4 emissions. Amending soil with waste products can mitigate CH_4 emissions in crops, but little is known about the impacts of amendments with steel slag and biochar on the populations and activities of aerobic methanotrophs in rice cropland. We used Real-time quantitative PCR detecting system (qPCR) and high-throughput sequencing to determine the effects of slag and biochar amendments on CH_4 emission, abundance and community structure of methanotrophs, and the relationships between soil properties and the abundance and community composition of methanotrophs during the rice growing season in both early and late paddies. Soil salinity and pH were significantly higher for an amendment with both slag andbiochar than the control in both the early and late paddies, and pH was significantly higher for a slag amendment in the late paddy. Cumulative CH_4 emission was lower for the slag and slag+biochar amendments than the control in early paddy by - 34.1%. Methanotrophic abundance was three- and six-fold higher for the slag + biochar amendment than the control in the early and late paddies (p<0.05), respectively. The abundance of different groups of methanotrophs varied among the treatments. The relative abundance of *Methylosarcina* was higher for the slag amendment than the control, and the relative abundance of *Methylomonas* was lower for biochar, and slag +biochar amendments than the control. The relative abundance of *Methylocystis* was higher for the slag and slag+biochar amendments than the control in the early paddy, and the relative abundance of *Methylocystis* was higher for the slag, biochar, and slag +biochar amendments in the late paddy. Univariate and multivariate analyses indicated that the higher abundance of

methanotrophic bacteria for the slag and slag+biochar amendments was correlated with soil pH, salinity, soil organic carbon (SOC), and C:N ratio, and the relative abundances of *Methylocystis, Methylomonas*, and *Methylosarcina* were associated with the effective mitigation of CH_4 emission in the paddies. A discriminant general analysis indicated that the total population of methanotrophs was larger for the slag+biochar amendment than the control and that this effect was only weakly correlated with changes in the soil properties, demonstrating that this effect on the size and species composition of methanotrophic soil populations was mostly associated with a direct effect of the slag+biochar amendment.

Keywords Slag · biochar · greenhouse gases · methanotrophs · paddies

58 **Introduction**

59 Methane is the second most important greenhouse gas after carbon dioxide,
60 contributing approximately 18% of anthropogenic radiative forcing (Bridgham et al.
61 2013). Even small changes of CH_4 concentrations in the atmosphere contribute
62 substantially to global warming, because the global-warming potential of CH_4 is 25-
63 fold higher than that of CO_2 (Bridgham et al. 2013; Lee et al. 2014). CH_4 is an important
64 greenhouse gas due to its geophysical properties, such as its atmospheric residence time
65 of 12.4 years and instantaneous forcing of 1.37×10^5 W m^{-2} ppb^{-1} (IPCC 2014). Rice
66 paddy fields, which are cultivated worldwide on 155 million ha, contribute about 5-19%
67 of the annual atmospheric CH_4 emissions and are considered the most important
68 anthropogenic source of CH_4 (Ma et al. 2010).

69 CH_4 emission from paddies are governed primarily by two microbial processes,
70 CH_4 production (by methanogens) and CH_4 oxidation (by methanotrophs) (Wang et al.
71 2014; Nguyen et al. 2015). Methanogens are a group of strict anaerobic microorganisms
72 that produce CH_4 using CO_2 or acetate as the final electron acceptor and hydrogen as
73 an electron donor and are phylogenetically affiliated with the phylum Euryarchaeota of
74 the domain Archaea (Woese et al. 1990). Methanotrophs, though, use CH_4 as the main
75 carbon source and can metabolize both aerobically and anaerobically. Aerobic
76 methanotrophs belong to the Proteobacteria and Verrucomicrobia taxa. The former can
77 be broadly divided into two physiological and phylogenetic groups: type I
78 (Gammaproteobacteria) and type II (Alphaproteobacteria) methanotrophs (Sharp et
79 al.2014).

Some practices of agricultural management (e.g. water management and addition of straw compost) have been recommended for reducing CH_4 emissions from paddies (Pandey et al 2014; Wang et al. 2014; Nguyen et al. 2015). Biochar amendment may reduce CH_4 emissions mostly by inhibiting methanogenic activity or increasing CH_4 oxidation associated with an increase in soil aeration (Liu et al. 2011; Dong et al. 2013; Han et al. 2016). Increasing the abundance of methanotrophs or decreasing the *mcr*A/*pmo*A ratio can also reduce CH_4 emissions (Feng et al. 2012; Han et al. 2016). The application of biochar may therefore decrease CH_4 emissions, and the structure of biochar can provide a suitable environment for bacterial CH_4 oxidation, but little attention has been paid to the influence of biochar amendments on methanotrophic diversity in paddies.

Steel slag is a waste product from the pyro-metallurgical processing of various ores. Interest in finding uses for slag has been steadily increasing, because large volumes, on the order of hundreds of millions of tonnes, of this waste are produced annually worldwide (Piatak et al. 2015). Steel slag contains high concentrations of electron acceptors, such as active and free oxides of iron, and can effectively lower CH_4 emissions from temperate paddies (Ali et al. 2008a, 2009). The steel slag should increase the soil redox status, and thus we should expect effects on methane production and oxidation processes (Wang et al. 2018). The addition of steel slag and/or biochar to soil could influence CH_4 emissions by affecting the physicochemical properties of the soil and thus the microorganisms that emit and metabolize CH_4 (Ali et al. 2008b; Wang et al. 2015). However, these effects remain largely unknown

By using molecular approaches such as polymerase chain reactions (PCRs) targeting methane monooxygenase (pMMO) genes (Lau et al. 2013; Lima et al., 2014; Lüke et al. 2014; Yun et al. 2015) we aimed to : (1) determine the abundance and community structures of methanotrophs and the relationships between soil properties and the abundance and community composition of methanotrophs during the growing season in both early and late paddies, and (2) analyze the relationship between methanotrophs and CH_4 emission. The results from this study may provide insights into the effects of waste amendments on soil methanotrophic communities and the subsequent effects on CH_4 emissions. In-depth knowledge of these relationships should be particularly important for providing a theoretical and practical basis to control CH_4 emissions in paddies.

Material and Methods

Study site and experimental design

The field experiment was carried out in the Wufeng Agronomy field of the Fujian Academy of Agricultural Sciences, Fujian, southeastern China (Fig. 1). We studied the effect of the application of steel slag, biochar, and slag + biochar on CH_4 emissions and methanotrophs during the early paddy season (16 April to 16 July, Hesheng 10 cultivar) and the late paddy season (25 July to 6 November, Qinxiangyou 212 cultivar) in 2015. Air temperature and humidity during the study period are shown in Fig. S1. Our study site has a "*Warm and humid climate*" according with Köppen (1936) classification. The physicochemical properties in the top 15 cm were shown in Wang et al. (2014, 2015).

The management of the paddies (including plowing, water management, and fertilization) was typical for subtropical paddies in China (Zhang et al. 2013; Wang et al. 2015), more detail was shown in the Wang et al. (2015).

The experimental field plots were laid out in a randomized block design, with triplicate plots (10 m^2) for each of the four treatments (including a control). The experiment tested the following treatments in a completely randomized block design: 1) control; 2) steel slag; 3) biochar; and 4) slag + biochar. We applied 8 Mg ha^{-1} of both steel slag and biochar. The selected steel slag was provided in granular form by Fujian Jinxing iron company and was composed mainly of CaO (34.9%), SiO_2 (40.7%), and Fe_2O_3 (4.8%). This represents C (56.6%), N (1.4%), P (1.0%) and K (1.8%) on dry weight. Rice straw was heated at 600 °C to produce biochar. The chemical compositions of both amendments are presented in Table S1. All control and amended plots received the same amount of water and same mineral fertilizer and urea. More detail seen in Wang et al. (2015).

Measurement of CH_4 flux

Static closed chambers were used to measure CH_4 emissions during the rice growing season (Datta et al. 2013). More detail seen the Wang et al. (2015). We deployed three replicate chambers in each treatment. A wooden boardwalk was built for accessing the plots to minimize disturbance of the soil during gas sampling. Gas flux was measured weekly in all chambers. The sampling CH_4 concentrations in the headspace air samples were determined by gas chromatography (GC) using a stainless steel Porapak Q column

(2 m long, 4 mm outside diameter, 80/100 mesh) (Shimadzu GC-2010, Kyoto, Japan). More detail of the gas sampling and concentrations determination were shown in Wang et al. (2015).

Measurement of soil properties

Soil samples were collected from the 0-15 cm layer in triplicate for each treatment using a soil sampler during the elongation stage (Wang et al. 2014; Wang et al. 2018). We only collected one time in the elongation period, i.e. at mid period of rice growth, when the water and fertilizer management was also at the average level between the beginning of the rice transplantation and the rice ripening period. This sampling time was the sampling time also used in previous studies (Wang et al. 2018). The samples were immediately stored in sterile bags in ice coolers and transported to the laboratory. Subsamples were then immediately processed for DNA extraction. The remaining soil was stored at 4 °C until the analysis of physical and chemical properties.

Soil properties, such as pH was measured with a pH/temperature meter (IQ Scientific Instruments, Carlsbad, USA), and salinity was measured using a 2265FS EC meter (Spectrum Technologies Inc., Paxinos, USA). Soil water content was measured by weighing the soil before and after drying at 105 °C to a constant weight (Barton et al. 2013). Soil organic carbon (SOC) and TN concentrations were determined using a Vario Max Elemental Analyzer (Elementar Scientific Instruments, Hanau, Germany).

DNA extraction and PCR amplification

When we determined the amount of DNA we use three repeats per plot. However, when

we determined the microbe structure we only used the mixed into 0.5-g composite samples per plot, thus following the method applied in several previous studies (Wang et al. 2018). Total genomic DNA was extracted from these samples using an E.Z.N.A ™ Soil DNA Kit (Omega, USA). The DNA quality and concentration were assessed by 1× TAE agarose gel (1%) electrophoresis and spectrophotometric analysis using a NanoDrop 1000 spectrophotometer (Thermo Scientific Technologies Inc., Waltham, USA).

The *pmo*A genes of methanotrophs were amplified by PCR using the primer pair A189F (5′-GGN GAC TGG GAC TTC TGG-3′) and mb661R (5′-CCG GMG CAA CGT CYT TAC C-3′)(May et al. 2018). Amplification was performed in a final volume of 25 μL containing 2.5 μL of 10× PCR buffer, 2.5 μL of 2.5 mM dNTPs, 0.25 μL of Taq polymerase (5 U μL^{-1}) (Takara, Japan), 0.5 μL of each primer (final concentration 0.3 μM), and 20 ng of extracted DNA. The PCR program had an initial denaturation at 94 °C for 3 min followed by 30 cycles of denaturation at 94 °C for 45 s, annealing at 55 °C for 1 min, and extension at 72 °C for 90 s, with a final extension at 72 °C for 10 min. Amplified PCR products were purified with a PCR clean-up kit (Sangon Inc., Shanghai, China) and stored at -20 °C for further analysis.

MiSeq Sequence processing and analysis

All PCR products were sequenced by Novogene Corporation, Beijing, China. Index sequences were trimmed, aligned to the SILVA database (Quast et al. 2013), screened, and filtered by the mothur pipeline (Schloss et al. 2009). The sequences were taxonomically classified using the training set, version 9, of the Ribosomal Database

191 Project (Cole et al. 2009), followed by the removal of non-archaeal/bacterial sequences
192 based on the taxonomic classification. Diversity indices and operational taxonomic
193 units (OTUs) at 97% identity with *pmo*A were estimated using mothur
194 (https://www.mothur.org/). The *pmo*A sequences have been deposited in the GenBank
195 database (https://www.ncbi.nlm.nih.gov/bioproject/PRJNA445632/) under accession
196 number SRR6901783. Data can be obtained from the Biosample database
197 (https://www.ncbi.nlm.nih.gov/biosample), accession number SAMN 08794436.
198
199 Quantitative analysis of the methanotrophs by real-time PCR
200 Methanotrophic abundance was determined by qPCR using *pmo*A-targeted primers
201 A189F/mb661R in triplicate 20-μL reaction mixtures containing SYBR green Master
202 Mix (Sangon Inc., Shanghai, China). The reaction mixture contained 1× Master Mix
203 (Sangon Inc., Shanghai, China), 200 nM of each primer, ten-fold diluted DNA
204 extractions, and double-distilled H_2O to a final volume of 20 μL. Real-time quantitative
205 PCR detecting system (qPCR) was carried out with the protocol for target groups as:
206 denaturation at 95 °C for 3 min followed by 45 cycles of denaturation at 95 °C for 15
207 s, annealing at 57 °C for 20 s, and plate reading at 83 °C. Standard curves were obtained
208 with serial dilutions of plasmid DNA containing the target genes. The data were
209 analyzed using LightCycler 480 Software Setup (Roche Inc., Shanghai, China).
210
211 Statistical analysis
212 The sequencing data were processed as described by Caporaso et al. (2010) and Wang

et al. (2017). A one-way analysis of variance (ANOVA) was conducted to test the differences in soil physicochemical properties and methanotrophic abundances among the treatments in both crop seasons. All statistical analyses used SPSS Statistics 17.0 (IBM SPSS Inc., Chicago, USA).

We also performed multivariate statistical analyses. We used a principal component analysis (PCA) to determine the overall differences of soil salinity, pH, water content, TN content, C:N ratio, SOC content, bulk density, CH_4 emission, and methanotrophic gene abundance among treatments in the early and in late paddies. We conducted one-way ANOVAs with Bonferroni post hoc tests of the scores of the first PC axis to determine differences among the treatments. We then used a general discriminant analysis (GDA) to determine the overall differences of soil salinity, pH, water content, TN content, C:N ratio, SOC content, bulk density, CH_4 emission, and methanotrophic gene abundance among treatments using the combined data from the two paddy seasons. These analyses also assessed the component of the variance due to the paddy season (early and late) as an independent categorical variable. Discriminant analyses consist of a supervised statistical algorithm that derives an optimal separation between groups established a priori by maximizing between-group variance while minimizing within-group variance (Raamsdonk et al. 2001). GDA is thus an appropriate tool for identifying the variables most responsible for the differences among groups while controlling the component of the variance due to other categorical variables, the two paddy seasons (early and late) in this study. The GDAs were performed using Statistica 8.0 (StatSoft, Inc., Tulsa, USA).

Results

Soil physicochemical properties in the paddies

The soil physicochemical properties for the treatments and paddy fields are shown in Table 1. Soil salinity and pH were significantly higher ($p<0.05$) in the slag + biochar treatment than the control in both the early and late paddies, and pH was significantly higher ($p<0.05$) in the slag treatment than the control in the late paddy.

Cumulative CH_4 emission in paddies

Total cumulative CH_4 emission varied among the treatments in both the early and late paddies (Fig. 2). Cumulative CH_4 emission was lower in the slag and slag + biochar amendments than the control in both the early and late paddies, by 10.2 and 34.1% and 14.9 and 33.5%, respectively. Cumulative CH_4 emission for the biochar treatment, however, was 7.6% higher in the early paddy field but 43.7% lower in late paddy relative to the control.

Methanotrophic abundance in the paddies

Methanotrophic *pmo*A copy number for both the early and late paddies were shown in Fig. 3. Methanotrophic *pmo*A abundance in the control, slag, biochar, and slag + biochar treatments in the early paddies were 2.84×10^5, 1.35×10^5, 2.50×10^5 and 1.44×10^6 g^{-1} dry soil, respectively. The *pmo*A copy number was 52.5 and 12.1% lower in the slag and biochar treatments, respectively, but about three-fold higher in the slag + biochar

257 treatment, than the control. The copy number was significantly higher (p<0.05) in the
258 slag + biochar treatment than the control. The *pmo*A copy numbers in the late paddy
259 field were 1.32×10^5, 5.63×10^4, 1.91×10^5, and 9.88×10^5 g^{-1} dry soil in the control, slag,
260 biochar, and slag + biochar treatments, respectively. The copy number was 57.6% lower
261 in the slag treatment but 43.5% and six-fold higher in the biochar and slag + biochar
262 treatments, respectively, than the control. The *pmo*A copy number was significantly
263 higher (p<0.05) in the slag + biochar treatment than the control in both the early and
264 late paddies. The copy number was also significantly higher (p<0.05) in the early than
265 the late paddy for the control and slag treatments.
266
267 Relationships between methanotrophic abundance and environmental factors
268 The relationships between the abundance of methanotrophs and environmental factors
269 in both the early and late paddies were shown in Table 3. The *pmo*A copy numbers in
270 both the early and late paddies were strongly positively correlated with soil salinity, pH
271 and, SOC content (Table 3). But CH_4 emission was not strongly correlated the soil
272 parameters, indicating that CH_4 emissions were not directly affected by the
273 physicochemical parameters(Table 3).
274
275 Analysis of alpha and beta diversity of pmoA in the paddies
276 The microbial communities in the paddies were analyzed based on *pmo*A gene
277 sequences using high-throughput sequencing. The number of sequences, coverage,
278 number of OTUs, and ecological indices are summarized in Table 2. More than 98%

279 coverage was obtained for all samples, with the number of OTUs ranging from 612 to
280 2537. The heatmap in Fig. 4 shows the abundance cluster of the top 50 ranking species
281 of methanotrophs. The number of unclassified bacterial genera in the paddies ranged
282 from 1.63 to 62.97%. Most methanotrophic species in the paddies belonged to
283 *Methylocystis*, *Methylogaea*, and *Methylococcus*, representing 17.26-79.35% of the
284 total.
285
286 Methanotrophic community composition in the paddies
287 High-throughput sequencing of *pmo*A was used to investigate changes in the
288 composition of the methanotrophic communities in both the early and late paddies (Fig.
289 5). Approximately 24.40-53.36% of the *Proteobacteria* sequencing reads for both the
290 early and late paddies were classified as type I methanotrophs, including
291 *Methylosarcina*, *Methylogaea*, *Methylomonas*, *Methylococcus*, *Methylomicrobium*,
292 *Methylobacte*r, *Methylocaldum*, *Methylovulum*, and *Methylomarinum*, and
293 approximately 12.63-63.00% of the sequencing reads were classified as type II
294 methanotrophs, including *Methylocystis* and *Methylosinus*. *Methylocystis* (12.26-
295 59.63%), *Methylosarcina* (6.52-22.69%), *Methylogaea* (3.24-22.27%), and
296 *Methylomonas* (2.48-18.09%) were generally dominant in both the early and late
297 paddies.
298
299 PCA and GDA of CH_4 emission, methanotrophs, and physicochemical parameters
300 The PCA (both in early and in late paddy) found that the amendments containing slag

(alone or particularly with biochar) were associated with larger methanotrophic populations and with higher soil pH, salinity, and TN and SOC contents and lower CH_4 emissions (Fig.6, 7). The GDA supported these results. We must, however, stress that all treatments were significantly separated (Table S2) and that overall soil traits, CH_4 emissions, and methanotrophic abundance differed the most in the slag + biochar treatment (Fig. 8). The GDA found that methanotrophic abundance and soil pH to some extent were most responsible for these differences among the treatments (Table S3).

Discussion

Understanding the ecology of methanotrophs and their abundance and taxonomic composition under different waste amendments is crucial for controlling CH_4 emissions in paddies. Our results provided clear evidence that amendments with slag and slag + biochar decreased CH_4 emissions in both the early and late paddies, an effect correlated with the increase in the methanotrophic abundance, specially for the relative abundances of *Methylocystis, Methylomonas*, and *Methylosarcina* that were associated with the effective mitigation of CH_4 emission in the paddies. The various treatments, however, were associated with different soil properties. The effect of the treatments was especially important in changing methanotrophic abundance and community composition, evidence that the change of methanotrophic abundance and community composition was due mainly to the components of the treatments, mainly slag.

Effects of slag amendment on the abundance and community structure of

methanotrophs

The application of slag inhibited total CH_4 emissions from the paddy fields, consistent with previous reports by Singla et al. (2015) and Wang et al. (2015). The slag amendments in our study slightly increased soil salinity, because slag usually contains a specific suite of salt components (K, Ca, Mg, and Fe) that are important for soil fertility (Ali et al. 2008b). Slag contains large amounts of chemically reactive iron oxide, which increases soil salinity (Ali et al. 2008b). Our results, though, also strongly suggest that the slag amendments increased soil pH, as also reported by Lee et al. (2012), due to the calcium and iron contents in the slag (Susilawati et al. 2015). Methanotrophic abundance, however, was not higher in the slag amendments than the control, perhaps because slag contains iron that can reduce CH_4 emissions. High levels of electron acceptors suppress CH_4 emission (Susilawati et al. 2015). The higher soil salinity and pH due to amendment with slag may not decrease CH_4 emissions by increasing the abundance of methanotrophs, but by changing the community composition or structure or the abundance of methanogens.

Slag amendment increased the relative abundance of *Methylosarcina* compared with the control (Fig. 5). *Methylosarcina* is a type I methanotroph dominant in paddies(Lee et al. 2014). The growth of *Methylosarcina* requires a pH of 5.0-9.0 (Wise et al., 2001), so slag amendments with near-neutral pHs (6.90-6.99) may increase the relative abundance of *Methylosarcina* by increasing soil pH. Furthermore, iron is an important element for methanotrophs (Mohanty et al., 2014), because methane monooxygenase, a di-iron protein complex, uses iron as a transition metal (co-factor)

345 at the active site (Guallar et al. 2002), so slag containing iron oxide may inhibit CH_4
346 emissions by increasing soil pH and thereby increasing the relative abundance of
347 *Methylosarcina*. The iron in slag fertilizers may also act as an electron acceptor,
348 suppressing CH_4 emission by decreasing methanogenic activity (Jackel et al. 2000).
349
350 Effects of biochar amendment on CH_4 emission associated with changes of the
351 abundance and community structure of methanotrophs
352 The biochar amendment decreased the total CH_4 emission, but without increasing
353 methanotrophic abundance, during the entire study period compared to the control,
354 highly consistent with previous studies (Castaldi et al. 2011; Zhang et al. 2012). Biochar
355 application can reduce CH_4 emissions (Feng et al. 2012; Wang et al. 2012) and has
356 increased rice yield up to 28% (Zhang et al. 2010, 2012; Wang et al. 2012) by increasing
357 methanotrophic abundance, which would decrease CH_4 emission (Zhang et al. 2010).
358 The impact of biochar on CH_4 emission may be due to the biochar components and the
359 influence of biochar on soil physicochemical properties. The SOC content and the C:N
360 ratio were higher in the biochar and slag + biochar treatments than the control. The
361 highly aromatic chemical structure of biochar provides an organic carbon with higher
362 biochemical activity and thermal stability, allowing the biochar to be preserved in the
363 environment for a long time. The addition of biochar to soil may therefore improve soil
364 stability. The reduced CH_4 emissions from paddies with added biochar are likely due to
365 a lack of substrate (CO_2) availability (Liu et al. 2011; Chang et al. 2012). Kim et al.
366 (2017) reported that biochar inhibited methanogenesis by increasing soil aeration and

oxygen availability. The slightly higher pHs due to the alkaline properties of biochar, though, may contribute to the inhibition of CH_4 emissions by changing methanotrophic community structure. The abundance of *Methylomonas* was lower in the slag, biochar, and slag + biochar treatments than the control. The growth of *Methylomonas* requires a pH of 5.0-9.0 and is optimal at pHs of 6.5-7.0 (Lu et al. 2016). Amendments with slag, biochar, or slag + biochar with higher pHs (6.46-7.41) may thus not be suitable for the growth and proliferation of this group. Members of the genus *Methylomonas* require higher oxygen and CH_4 concentrations for CH_4 oxidation than other methanotrophs (Reim et al. 2012). A reduction in the relative abundance of *Methylomonas* by altering soil pH may thus be a mechanism to suppress CH_4 emission under waste amendments.

Wang et al. (2012), however, found that CH_4 emission in paddy fields increased significantly when amended with biochar. The addition of biochar in flooded paddies increases the substrate supply and creates a favorable environment for methanogenic activity (Kögel-Knabner et al. 2010; Lehmann et al. 2011). The labile components of biochar can decompose and become the predominant source of substrates for methanogens, particularly in the early stages of the rice growing season (Knoblauch et al. 2008). The effects of biochar amendment on CH_4 emission are thus inconsistent among studies, and the underlying mechanisms may vary with soil type, agricultural management, and origin of the biochar (Lehmann et al. 2011).

Effects of the slag + biochar amendment on CH_4 emission associated with changes of methanotrophic abundance and community structure

389 Our study demonstrated that the three amended treatments lowered total CH_4 emissions
390 to different extents relative to the control treatment. Slag + biochar was the best
391 amendment for suppressing total CH_4 emissions in early paddy field. Methanotrophic
392 abundance was significantly higher in the slag + biochar treatment than the control,
393 biochar, and slag treatments, likely because biochar can improve soil permeability and
394 the granular texture of slag can further enhance the ability of soil to supply oxygen.
395 Slag and biochar contain K, Ca, Mg, and Fe, which strongly increase CH_4 oxidation,
396 which would increase the abundance of methanotrophs in the slag + biochar treatment.
397 CH_4 emission was inversely correlated with the abundance of methanotrophs, so the
398 increase in methanotrophic abundance would likely decrease in CH_4 emissions under
399 the slag + biochar treatment. The relative abundance of *Methylocystis* in the early paddy
400 was higher in the slag and slag + biochar treatments than the control, especially in the
401 slag + biochar treatment where the abundance was about 183% higher. The three
402 amended treatments increased the relative abundance of methanotrophs in the late
403 paddy. *Methylocystis* can form resting cells, surviving on multi-carbon compounds and
404 using CH_4 at both high and low concentrations (Ho et al. 2013). Some type II
405 methanotrophs (*Methylocella*, *Methylocapsa*, and *Methylocystis*) have recently been
406 characterized as facultative methanotrophs able to conserve energy for growth on multi-
407 carbon compounds such as acetates, pyruvate, succinate, malate, and ethanol (Esson et
408 al. 2016). The slag + biochar treatment in our study also decreased the relative
409 abundance of *Methylomonas*. Bacterial diversity may be affected by SOC and N
410 contents (Chan et al. 2006). We also found that the community composition of

methanotrophs was significantly correlated with SOC content and the C:N ratio (Fig. 6), suggesting that the combination of slag and biochar, with its high SOC content, may provide a rich substrate for CH_4 synthesis, which would further change methanotrophic community structure. *Methylocystis* has a low minimum threshold concentration (Michaelis constant, Km) for CH_4 oxidation (Lee et al. 2014). A lower Km is associated with a higher affinity of enzymes and substrates and stronger CH_4 oxidation. Increasing the abundance of CH_4-oxidizing bacteria would likely have a critical impact on CH_4 reduction (Kima et al. 2017). Increasing the relative abundance of *Methylocystis* may therefore reduce CH_4 emission. In conclusion, the slag + biochar treatment likely increased the oxygen content of the soil, which would increase the oxidation of CH_4 by methanotrophs, leading to lower CH_4 emissions.

Conclusions and final remarks

Our results indicated that soil amendment with both slag and biochar significantly increased soil salinity and pH in both the early and late paddies and that amendment with slag significantly increased pH in the late paddy. The slag and slag + biochar treatments decreased the cumulative CH_4 emission compared to the control in both the early and late paddies, by 10.2 and 34.1% and 14.9 and 33.5%, respectively. The abundance of methanotrophs in the slag + biochar treatment significantly increased methanotrophic abundance about three- and six-fold in the early and late paddies (p<0.05), respectively. The slag treatment increased the relative abundance of *Methylosarcina*, and biochar, slag + biochar treatments decreased the relative

abundance of *Methylomonas*, relative to the control. The slag and slag + biochar treatments increased the relative abundance of *Methylocystis* in the early paddy, and all three amended treatments increased the relative abundance of *Methylocystis* in the late paddy, relative to the control. The application of both slag and biochar provided the best overall results, increasing soil pH, salinity, SOC content, and the C:N ratio associated with methanotrophic abundance and the relative abundances of *Methylocystis*, *Methylomonas*, and *Methylosarcina*, which may effectively mitigate CH_4 emissions in paddies.

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641 **Tables**

642 **Table 1** Physicochemical properties for the amended and control plots in the early and late paddy fields

Paddy field	Treatment	Salinity ($dS \cdot m^{-1}$)	pH	Bulk density ($g \cdot cm^{-3}$)	Water content (%)	Soil organic carbon ($mg \cdot g^{-1}$)	Total Nitrogen ($mg \cdot g^{-1}$)	C/N
Early	Control	0.20±0.03 b	6.33±0.06 b	1.01±0.03 a	59.51±1.60 a	16.96±0.25 a	2.02±0.04 a	8.42±0.08 a
	Slag	0.22±0.03 b	6.99±0.23 ab	1.03±0.01 a	57.92±0.14 a	17.45±0.56 a	2.05±0.07 a	8.51±0.01 a
	Biochar	0.20±0.02 b	6.54±0.08 ab	1.06±0.04 a	56.03±1.95 a	17.91±1.09 a	2.01±0.01 a	8.91±0.58 a
	Slag + biochar	0.57±0.04 a	7.41±0.46 a	1.08±0.02 a	54.69±1.30 a	22.72±5.40 a	2.08±0.09 a	10.76±2.05 a
Late	Control	0.23±0.03 b	6.08±0.22 b	1.05±0.05 a	51.34±2.64 a	15.70±1.24 a	1.93±0.14 a	8.13±0.05 a
	Slag	0.31±0.04 b	6.90±0.03 a	1.04±0.04 a	52.78±3.05 a	16.14±0.55 a	1.97±0.06 a	8.18±0.07 a
	Biochar	0.30±0.02 b	6.46±0.04 ab	1.11±0.01 a	48.40±0.68 a	20.94±3.47 a	2.11±0.08 a	9.84±1.21 a
	Slag + biochar	0.47±0.05 a	7.21±0.39 a	1.08±0.02 a	50.52±1.06 a	21.61±3.78 a	2.12±0.06 a	10.12±1.50 a

643 Different letters within a column indicate significant differences among the treatments at $p<0.05$.

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645

646 **Table 2** Methanotrophic diversity for the treatments in the growth stage for the early and late paddy fields

Paddy field	Treatment	Sequences	Alpha diversity				
			OTUs	Chao1	ACE	Shannon	Coverage (%)
Early	Control	43989	1929	2328	2396.45	5.08	98.82
	Slag	50324	2098	2500	2650.87	5.31	98.86
	Biochar	84218	2433	2692	2802.28	5.62	99.44
	Slag + biochar	18683	612	940	1244.10	3.52	98.57
Late	Control	42203	2020	2337	2435.33	5.09	98.82
	Slag	26199	1336	1741	1734.17	5.04	98.47
	Biochar	31884	1762	2153	2245.01	5.17	98.39
	Slag + biochar	35717	2537	2811	2971.21	5.36	98.29

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651 **Table 3.** Correlations between methanotrophic abundance and environmental factors (soil salinity, pH, SOC content, TN content,

652 bulk density, water content, and CH_4 flux)

	CH_4 flux	*pmo*A	Salinity	pH	SOC content	TN content	Bulk density	Water content
CH_4 flux	1	-0.36	-0.15	-0.41	-0.45	-0.53	0.04	-0.62
*pmo*A	-0.36	1	0.90**	0.71*	0.83*	0.61	0.42	-0.11
Salinity	-0.16	0.90**	1	0.79*	0.82*	0.56	0.55	-0.35
pH	-0.41	0.71*	0.79*	1	0.64	0.57	0.24	0.02
SOM	-0.45	0.83*	0.82*	0.64	1	0.88**	0.76*	-0.35
TN	-0.53	0.61	0.56	0.57	0.88**	1	0.60	-0.22
Bulk density	0.04	0.42	0.55	0.24	0.76*	0.60	1	-0.80*
Water content	-0.62	-0.11	-0.35	0.02	-0.35	-0.22	-0.80*	1

653 * and ** indicate significant correlations at the 0.05 and 0.01 levels (2-tailed), respectively.

654

Figure legends

Fig. 1 Location of the study area and sampling sites (▲) in Fujian province, southeastern China

Fig. 2 Cumulative methane emissions for the control and treatments in paddies. Different letters above the bars indicate significant differences among the treatments at $p<0.05$

Fig. 3 Quantification of methanotrophic *pmo*A copy number for the control and treatments in the early and late paddies. Different letters above the bars indicate significant differences among the treatments at $p<0.05$

Fig. 4 Heatmap of methanotrophs for the treatments in the early and late paddies. E-SB: slag + biochar amendment in early paddy; E-B: biochar amendment in early paddy; L-S: slag amendment in late paddy; L-B: biochar amendment in late paddy; E-S: slag amendment in early paddy; L-SB: slag + biochar amendment in late paddy; E-C: control in early paddy; L-C: control in late paddy

Fig. 5 Relative abundances of methanotrophs in the treatments determined by high-throughput sequencing of the *pmo*A gene for the early (A) and late (B) paddies

Fig. 6 PCA analysis of physicochemical parameters in the treatments in the early paddy field. SOC, soil organic carbon concentration; Water (%), soil water content; [N], soil total nitrogen concentration; GA, methanotrophic gene abundance; C:N, carbon:nitrogen ratio

Fig. 7 PCA of physicochemical parameters in the treatments in the late paddy field.

SOM, soil organic carbon concentration; Water (%), soil water content; [N], soil total nitrogen concentration; GA, methanotrophic gene abundance; C:N, carbon:nitrogen ratio

Fig. 8 GDA of physicochemical parameters as independent continuous variables, waste amendment as a categorical dependent variable, and crop season (early and late) as a dependent controlling continuous variable. SOC, soil organic carbon concentration; Water, soil water content; [N], soil total nitrogen concentration; GA, methanotrophic gene abundance; C:N, carbon:nitrogen ratio

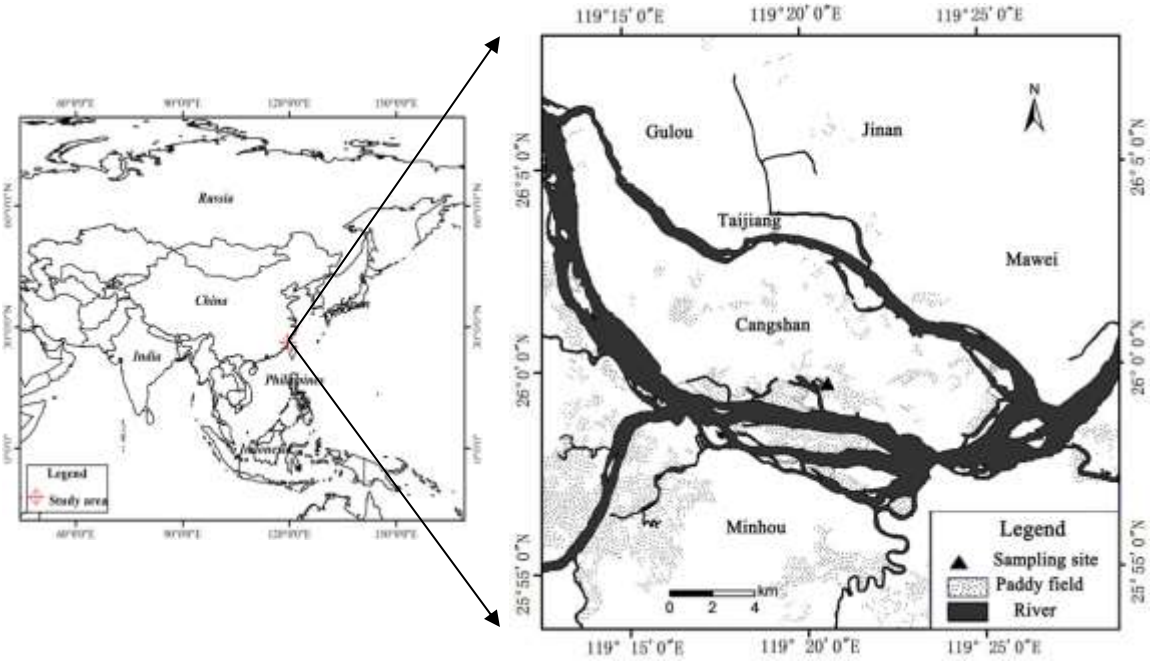
Russia
China
India
Legend
Study area
119°15′0″E
119°20′0″E
119°25′0″E
26°5′0″N
26°0′0″N
25°55′0″N
Gulou
Jinan
Taijiang
Mawei
Cangshan
Minhou
N
km
0 2 4
Legend
Sampling site
Paddy field
River

Fig. 1.

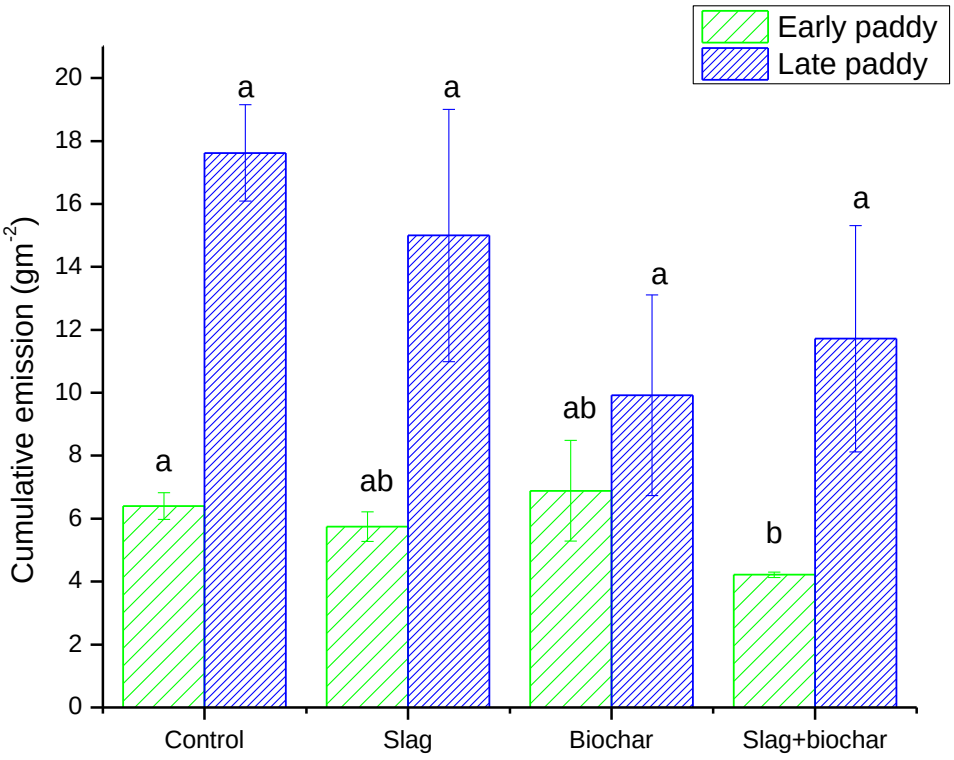
Early paddy
Late paddy
Cumulative emission (gm-2)
0
2
4
6
8
10
12
14
16
18
20
a
a
ab
a
ab
a
b
a
Control
Slag
Biochar
Slag+biochar

Fig. 2.

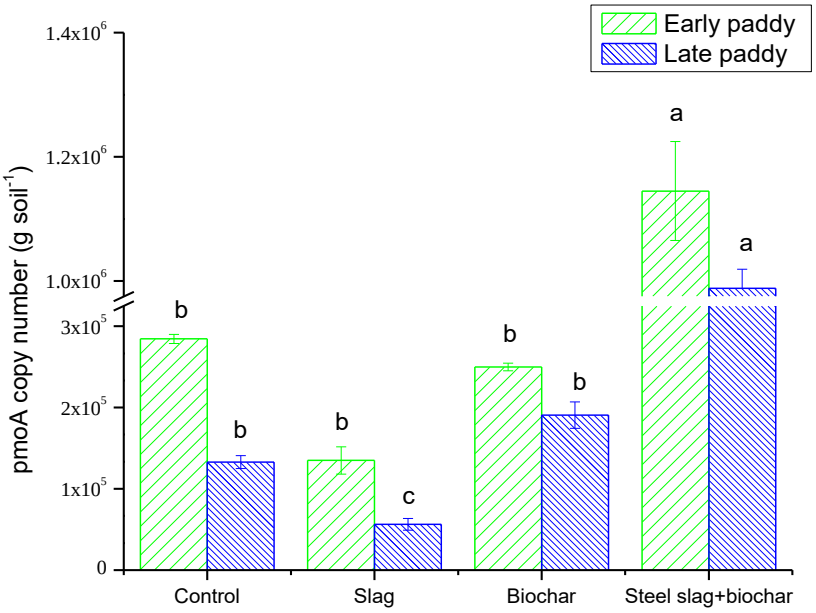
Early paddy
Late paddy
pmoA copy number (g soil-1)
1.4x10^6
1.2x10^6
1.0x10^6
3x10^5
2x10^5
1x10^5
0
a
a
b
b
b
b
b
b
c
Control
Slag
Biochar
Steel slag+biochar

Fig. 3.

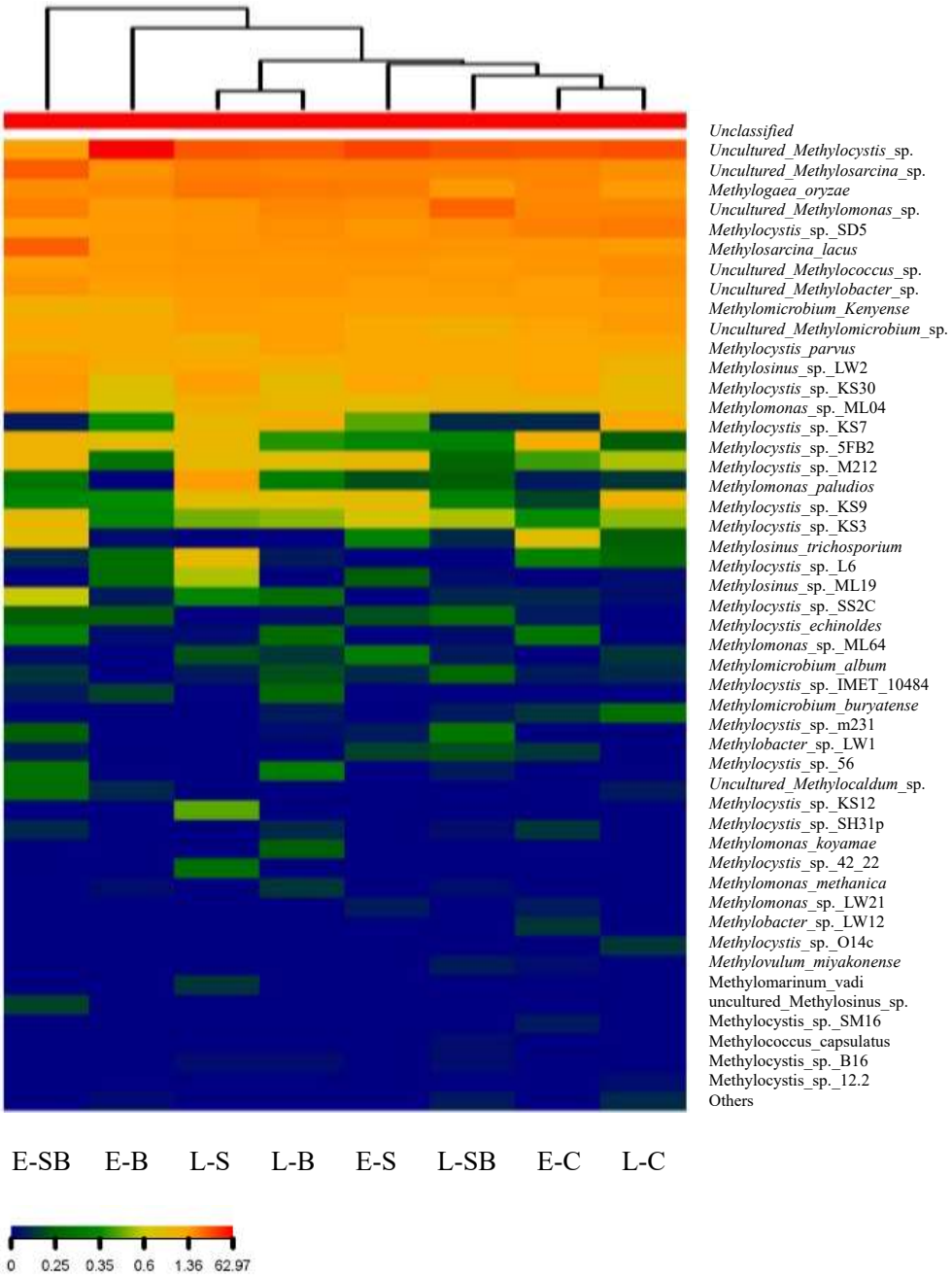
Unclassified
Uncultured_Methylocystis_sp.
Uncultured_Methylosarcina_sp.
Methylogaea_oryzae
Uncultured_Methylomonas_sp.
Methylocystis_sp._SD5
Methylosarcina_lacus
Uncultured_Methylococcus_sp.
Uncultured_Methylobacter_sp.
Methylomicrobium_Kenyense
Uncultured_Methylomicrobium_sp.
Methylocystis_parvus
Methylosinus_sp._LW2
Methylocystis_sp._KS30
Methylomonas_sp._ML04
Methylocystis_sp._KS7
Methylocystis_sp._5FB2
Methylocystis_sp._M212
Methylomonas_paludios
Methylocystis_sp._KS9
Methylocystis_sp._KS3
Methylosinus_trichosporium
Methylocystis_sp._L6
Methylosinus_sp._ML19
Methylocystis_sp._SS2C
Methylocystis_echinoldes
Methylomonas_sp._ML64
Methylomicrobium_album
Methylocystis_sp._IMET_10484
Methylomicrobium_buryatense
Methylocystis_sp._m231
Methylobacter_sp._LW1
Methylocystis_sp._56
Uncultured_Methylocaldum_sp.
Methylocystis_sp._KS12
Methylocystis_sp._SH31p
Methylomonas_koyamae
Methylocystis_sp._42_22
Methylomonas_methanica
Methylomonas_sp._LW21
Methylobacter_sp._LW12
Methylocystis_sp._O14c
Methylovulum_miyakonense
Methylomarinum_vadi
uncultured_Methylosinus_sp.
Methylocystis_sp._SM16
Methylococcus_capsulatus
Methylocystis_sp._B16
Methylocystis_sp._12.2
Others
E-SB
E-B
L-S
L-B
E-S
L-SB
E-C
L-C
0
0.25
0.35
0.6
1.36
62.97

Fig. 4.

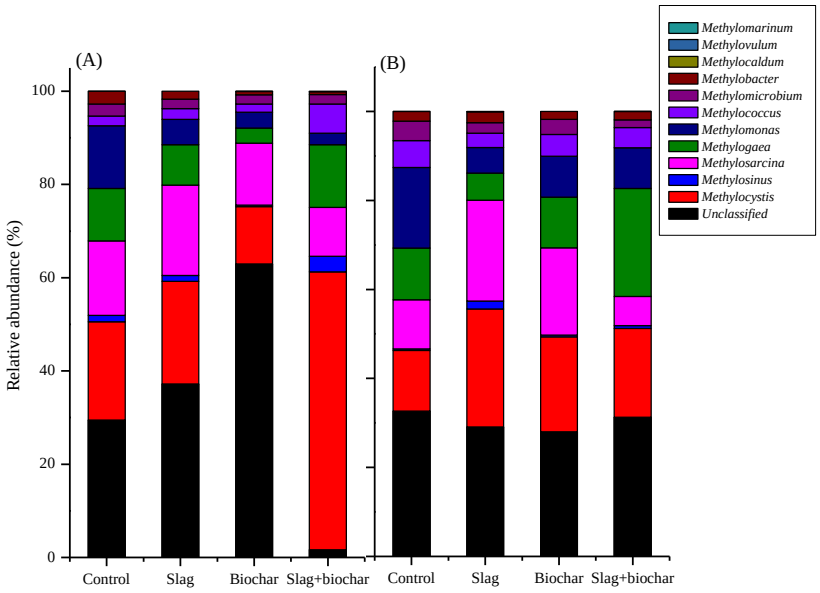
(A)
(B)
Relative abundance (%)
0
20
40
60
80
100
Control
Slag
Biochar
Slag+biochar
Control
Slag
Biochar
Slag+biochar
Methylomarinum
Methylovulum
Methylocaldum
Methylobacter
Methylomicrobium
Methylococcus
Methylomonas
Methylogaea
Methylosarcina
Methylosinus
Methylocystis
Unclassified

Fig. 5

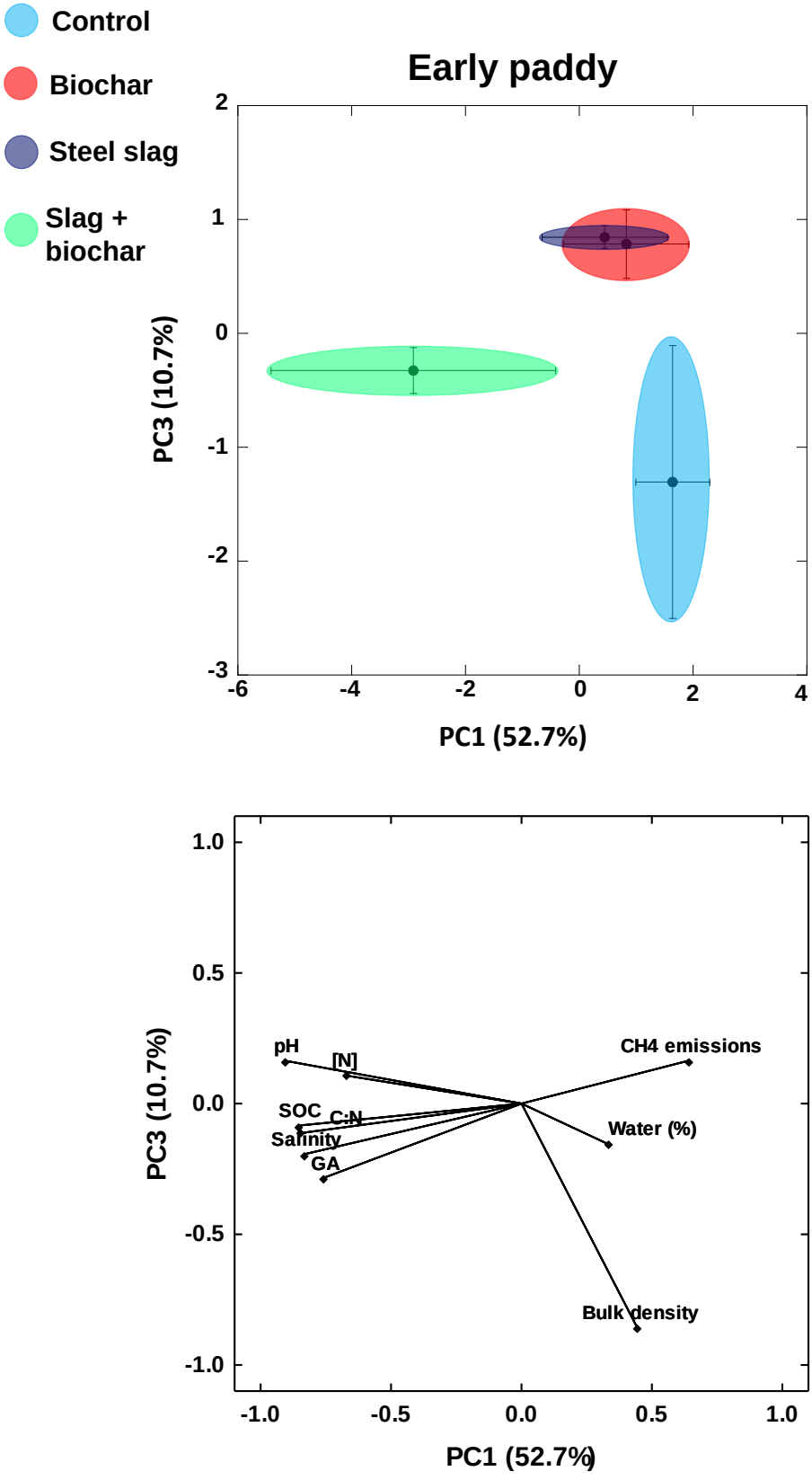
Control
Biochar
Steel slag
Slag + biochar
Early paddy
PC3 (10.7%)
PC1 (52.7%)
2
1
0
-1
-2
-3
-6
-4
-2
0
2
4
1.0
0.5
0.0
-0.5
-1.0
pH
[N]
SOC
C:N
Salinity
GA
CH4 emissions
Water (%)
Bulk density

Fig. 6

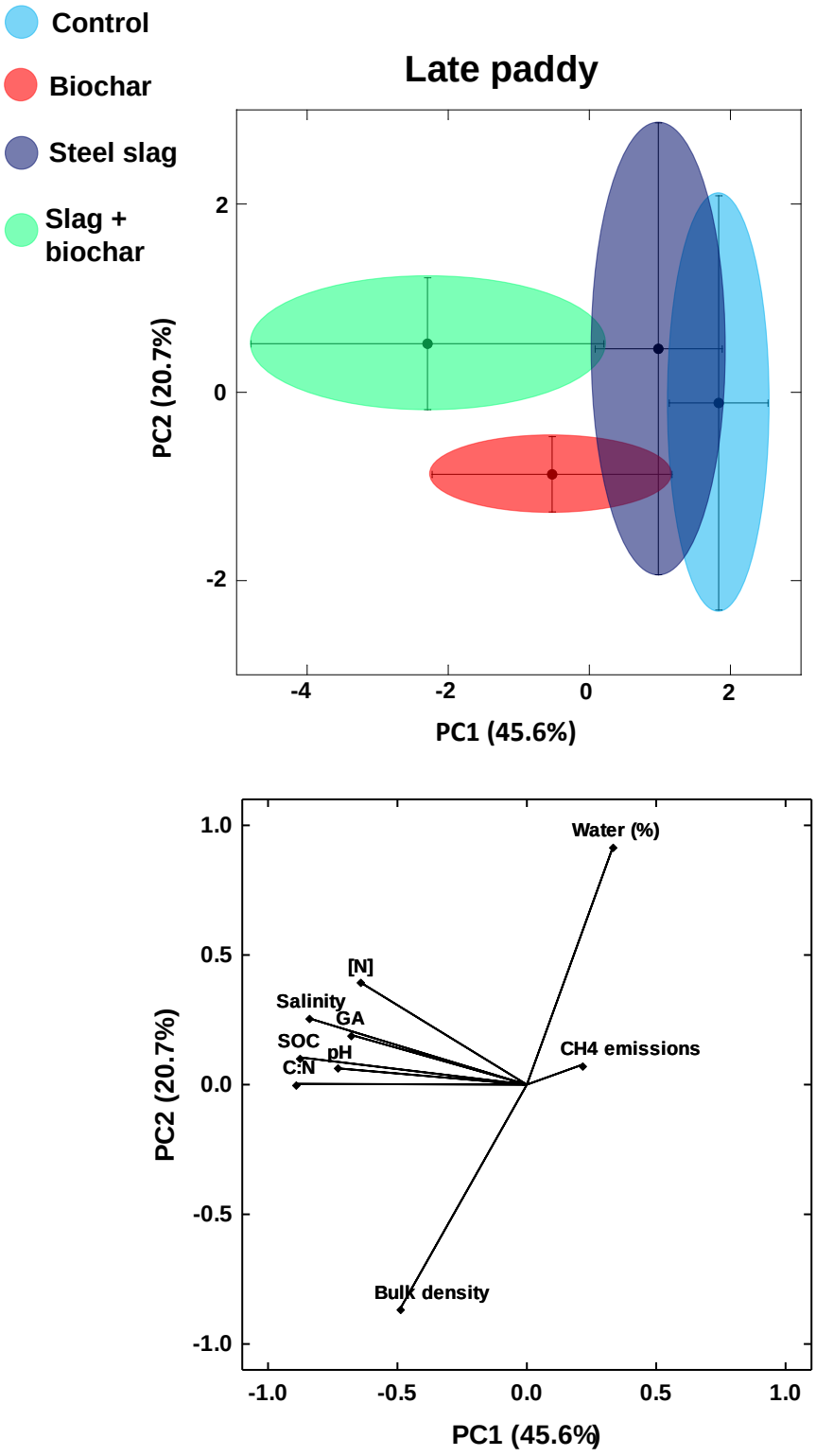
Control
Biochar
Steel slag
Slag + biochar
Late paddy
PC2 (20.7%)
PC1 (45.6%)
2
0
-2
-4
-2
0
2
Water (%)
[N]
Salinity
GA
SOC
pH
C:N
CH4 emissions
Bulk density
1.0
0.5
0.0
-0.5
-1.0
-1.0
-0.5
0.0
0.5
1.0
PC2 (20.7%)
PC1 (45.6%)

Fig. 7

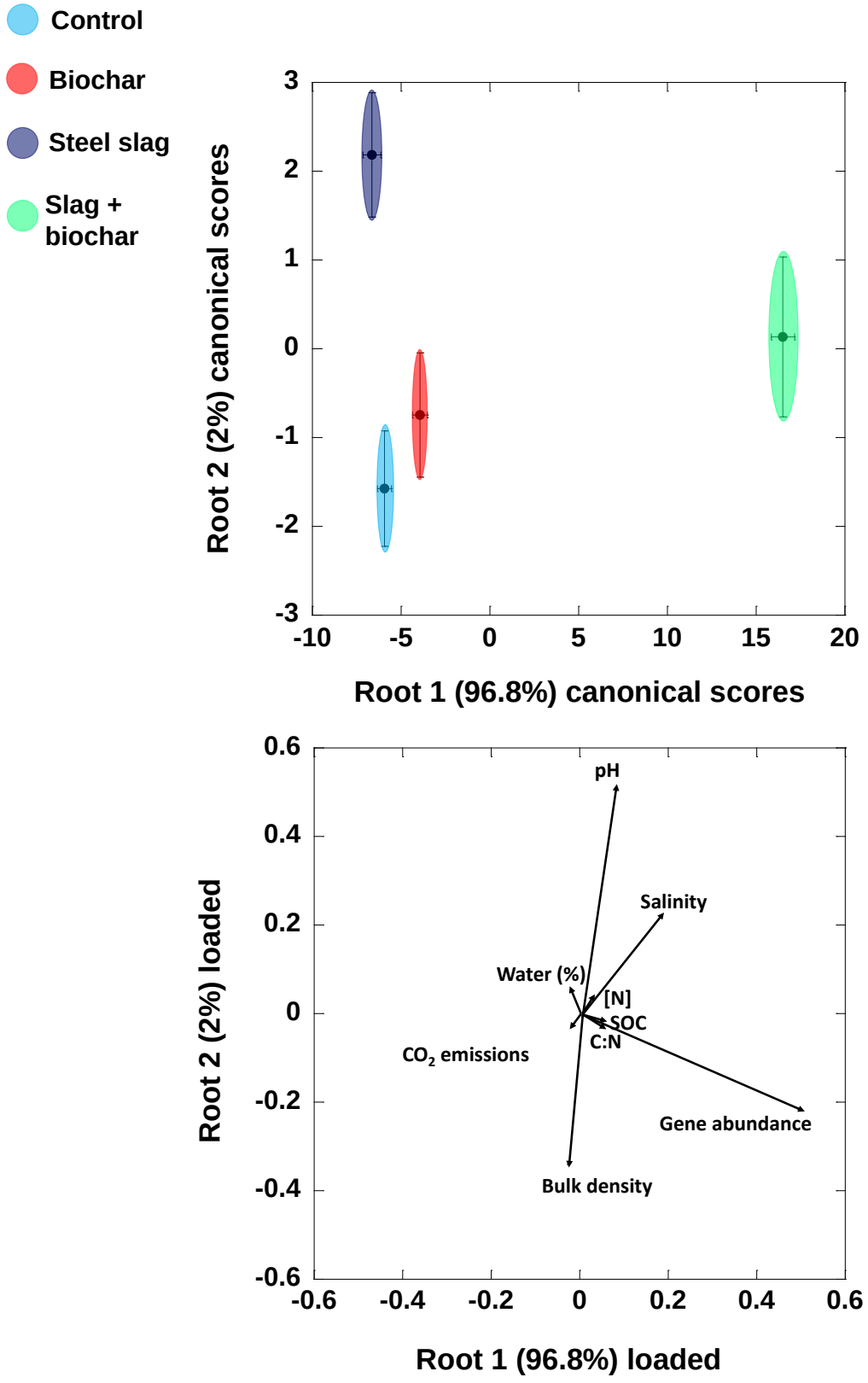
Control
Biochar
Steel slag
Slag + biochar
Root 2 (2%) canonical scores
Root 1 (96.8%) canonical scores
3
2
1
0
-1
-2
-3
-10
-5
0
5
10
15
20
Root 2 (2%) loaded
Root 1 (96.8%) loaded
0.6
0.4
0.2
0
-0.2
-0.4
-0.6
pH
Salinity
Water (%)
[N]
SOC
C:N
CO2 emissions
Gene abundance
Bulk density

Fig. 8