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ORIGINAL PAPER

Biochar addition affected the dynamics of ammonia oxidizers and nitrification in microcosms of a coastal alkaline soil

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Abstract Biochar amendments have frequently been reported to alter microbial communities and biogeochemical processes in soils. However, the impact of biochar application on bacterial (AOB) and archaeal ammonia oxidizers (AOA) remains poorly understood. In this study, we investigated the responses of AOB and AOA to the application of biochar derived from cotton stalk at rates of 5, 10, and 20 % by weight to a coastal alkaline soil during a 12-week incubation. The results showed that the *amoA* gene of AOB consistently outnumbered that of AOA, whereas only the AOA *amoA* gene copy number was significantly correlated with the potential ammonia oxidation (PAO) rate ($P<0.01$). The significant decrease of PAO rates in biochar treatments occurred after incubation for 4–6 weeks, which were distinctly longer than that in the control (2 weeks). The PAO rates were significantly different among treatments during the first 4 weeks of incubation ($P<0.05$), with the highest usually in the 10 % treatment. Biochar application significantly increased the abundance of both nitrifiers in the 4 weeks of incubation ($P<0.05$). Biochar amendment also decreased AOA diversity, but increased AOB diversity, which resulted in different community structures of both nitrifiers ($P<0.01$), as shown by the differences between the 5 % biochar and the control treatments. We conclude that biochar application generally enhanced the abundance and altered the composition of ammonia oxidizers; the rate of biochar application also affected the rate and dynamics of nitrification, and the risk for increasing the alkalinity and N leaching of the studied soil was lower with a lower application rate.

Keywords Ammonia oxidation · *amoA* gene · Microbial community · Nitrogen cycle · Coastal alkaline soil

Introduction

There has been an increasing interest in the use of biochar to improve soil fertility and to increase soil carbon (C) sequestration as a strategy to tackle global climate change, as well as for enhancing other ecosystem services (Lehmann 2007). Biochar added to soils can alter the physicochemical properties of soils (e.g., soil pH, organic C content, and cation exchange capacity) (Lehmann 2007; Chan and Xu 2009; Novak et al. 2009) and nutrient cycling (Clough and Condron 2010; Anderson et al. 2011), including the emission of greenhouse gases such as N_2O, CO_2, and CH_4 (Cheng et al. 2012; Yu et al. 2013; Wu et al. 2013).

The mechanisms driving microbial processes responsible for some of the biochar-induced physicochemical changes are still poorly understood despite recent advances (Lehmann et al. 2011). The biochar-rich terra preta (meaning black earth in Portuguese) has distinctly different microbial communities as compared with adjacent, non-terra preta soils (O'Neill et al. 2009; Grossman et al. 2010; Taketani and Tsai 2010). In addition, biochar addition has been reported to increase bacterial diversity, but it decreases archaeal and fungal diversity (Kim et al. 2007; Jin 2010; Taketani and Tsai 2010), as well as

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to increase bacterial phylogenetic diversity in both corn rhizosphere and bulk soils (Jin 2010). A clear differentiation in the structure of root-associated bacterial community between the biochar-amended and control plots was demonstrated using the analysis of denaturing gradient gel electrophoresis and terminal restriction fragment length polymorphism of 16S rRNA gene fragments (Kolton et al. 2011). Biochar amendment could also impact the abundance or biomass of bacteria and arbuscular mycorrhizal fungi (Birk et al. 2009; Warnock et al. 2010; Matsubara et al. 2002; Khodadad et al. 2011). However, most of these studies characterized the phylogenetic composition of microbial communities using genetic markers such as 16S rDNA or internal transcribed spacer, from which limited functional information can be obtained. The responses of the composition and size of functional microbial communities in the soil to biochar application have been rarely studied.

Ammonia oxidation is the first and rate-limiting step of nitrification in the nitrogen (N) cycle. It was only recently recognized that, in addition to the ammonia-oxidizing bacteria (AOB) belonging to monophyletic β- and γ-proteobacteria, the ammonia-oxidizing archaea (AOA) play an important role in the ammonia oxidation process in various environments (Kowalchuk and Stephen 2001; Francis et al. 2007; Chen et al. 2008). By using molecular approaches targeting the *amoA* gene, a functional gene encoding the α-subunit of ammonia monooxygenase which catalyzes the first step in ammonia oxidation, community composition, and abundance of these two functional groups (AOA and AOB) in the soil has been studied. It has been demonstrated that both AOB and AOA are key players in ammonia oxidation in agricultural soils, but their relative importance has been variable. For example, in general, AOA were found to be more abundant than AOB in soils of different origins (Leininger et al. 2006; He et al. 2007; Chen et al. 2008), while AOB might outnumber AOA in certain agricultural soils (Wu et al. 2011). The AOB functionally dominated ammonia oxidation in an agricultural soil, despite that AOA was numerically more abundant (Jia and Conrad 2009). Recent studies suggest that pH is a key factor shaping the community composition of both ammonia oxidizers (Nicol et al. 2008). However, while most work studied on acidic or neutral agricultural soils (Lehtovirta et al. 2009; Di et al. 2009; Yao et al. 2011; He et al. 2012; Zhang et al. 2012), few have studied the community composition of ammonia oxidizers in alkaline soils (Shen et al. 2008; Xia et al. 2011).

Coastal soils in China are often alkaline, sandy, and of low fertility, but hold a great potential to increase grain production thus further ensure China's food security. Recently, the Bohai Barn Project was launched for sustainable use of coastal land in four provinces off the Bohai Sea, and reclamation of saline–alkaline soils to increase soil fertility and agricultural production is one of the targets. To this end, biochar application could be one of the solutions because of its capability to adsorb ammonia and nitrate (Asada et al. 2006; Saleh et al. 2012; Eldridge et al. 2010; Taghizadeh-Toosi et al. 2012; Spokas et al. 2012) and reduce nitrate leaching and improve N fertilizer use efficiency (Eldridge et al. 2010; Spokas et al. 2012). Two concerns on the application of biochar in alkaline soils are the risks in increasing the alkalinity and nitrification activity and thus nitrate leaching because higher nitrification rates generally lead to N leaching, thus undermine the benefit of fertilization. However, little is known about the dynamics and activities of AOA and AOB, two microbial groups involved in ammonia oxidation (and thus nitrate production), in biochar-amended alkaline soils.

In this study, soil nutrients, the community compositions, and activities of ammonia oxidizers in a coastal saline soil were investigated during a 12-week incubation after the application of a biochar derived from cotton stalk and an ammonium-based fertilizer. We hypothesized that (1) biochar application would decrease the nitrification rate and the greater the biochar application rate, the lower the nitrification rate, and (2) biochar application would increase soil pH and change the community composition and abundance of both AOA and AOB communities.

Materials and methods

Field site, soil sampling, and experiment design

The soil used for the experiment was collected from a sandy loam in the south coastal plain of Laizhou Bay (37°03′1.8″N, 119°33′21.2″E), a part of the Yellow River Delta. The climate is temperate, with an average annual rainfall of 600 mm and a mean annual temperature of 12 °C. The sampling field has been planted with maize (*Zea mays* L.) for years. The original soil was slightly alkaline (pH 8.0), but non-saline (0.52‰, with an electrical conductivity of 0.2 mS cm^{-1}), a median grain size of 71.3 μm, total organic C content of 840 mg kg^{-1}, organic N of 90 mg kg^{-1}, ammonium of 16.18 mg kg^{-1}, nitrite of 0.78 mg kg^{-1}, and nitrate of 4.3 mg kg^{-1} dry soil.

After removing plant residuals on the soil surface, soil samples from the top layer (0–20 cm) were collected from five randomly selected locations in the field and were pooled, ground, and then sieved (<2 mm) for a pot experiment.

The biochar used in this study was purchased from Shandong Dongxin New Energy Company (Jinan, China). The biochar was made from pyrolysis of cotton stalks at a temperature around 650 °C, and the air-dried biochar was characterized for chemical contents (*w*/*w*) using a CE 440 CHN analyzer (Exeter Analytical); it had the following properties: moisture content, 4.43 %; ash content, 11.23 %; volatile matter, 13.94 %; fixed C, 70.40 %; hydrogen, 2.21 %; and

total sulfate, 0.31 %. Biochar was ground to 2-mm size before being carefully mixed with the soil.

Since the content of biochar naturally produced by wildfires in the soil has been estimated to be 15–35 % in temperate forest and prairie soils (DeLuca and Aplet 2008; Skjemstad et al. 2002), four treatments with biochar application rates of 0, 5, 10, and 20 % (*w*/*w*) (thereafter referred to as CK, BC5, BC10, and BC20, respectively), replicated three times, were set up in a temperature-controlled glasshouse (20–26 °C). Biochar-amended soils (1.5 kg oven dry equivalent) were packed into plastic pots (18.5 cm i.d. and 15.0 cm tall), and a 50-mL nutrient solution (0.75 mmol L^{-1} $(NH_4)_2SO_4$, 0.3 mmol L^{-1} KH_2PO_4, and 0.7 mmol L^{-1} K_2SO_4) was applied to each pot. The soil moisture content of all pots was then adjusted to 60 % of field capacity using deionized water. The pots were placed in a shallow tray and regularly watered to maintain water content throughout the experiment. The pots were pre-incubated for a week; thereafter, soil samples (0–10 cm) were collected from the pots by coring for chemical and molecular biological analysis at weeks 1, 2, 4, 6, 8, and 12.

Analyses of chemical properties and potential ammonia oxidation activity

For each sample, soil pH, dissolved inorganic N, and potential ammonia oxidation (PAO) rate were analyzed. Soil pH was determined with a soil-to-$CaCl_2$ (0.01 mol L^{-1}) ratio of 1:5 (*w*/*v*), and concentrations of nitrate (NO_3^--N), nitrite (NO_2^--N), and ammonium (NH_4^+-N) in the soil were extracted with 2 mol L^{-1} KCl (Mulvaney 1996) and determined by an autoanalyzer (Seal, Germany). Measurement of the PAO activity followed the perfusion method (Killham 1987). Briefly, 5.0 g fresh soil was added to a 100-mL Erlenmeyer flask and then 20 mL substrate containing 1 mmol L^{-1} ammonium sulfate and 0.1 mL sodium chlorate (1.5 mol L^{-1}); the samples were then incubated for 5 h at 24 °C on a rotating shaker (140 rpm) under aerobic conditions. The concentration of nitrite was determined immediately with a sulfanilamide colorimetric procedure, with absorbance measured at 520 nm (Rider and Mallon 1946).

DNA extraction, PCR amplification, cloning, and sequencing

About 0.8~1.0 g of soil sample was used for DNA extraction using the UltraClean Soil DNA Kit (MoBio, Carlsbad, CA) according to the manufacturer's instructions. DNA concentrations were measured using a spectrophotometer Nanodrop 2000c (Thermo Fisher, USA).

Primers Arch-*amoA*F and Arch-*amoA*R for AOA (Francis et al. 2005) and *amoA*-1F and *amoA*-2R for AOB (Rotthauwe et al. 1997) were employed for PCR amplifications of *amoA* genes and subsequent cloning. The 25-μL PCR reaction mixture contained 1× PCR buffer, 2.5 mmol L^{-1} $MgCl_2$, 0.2 mmol L^{-1} deoxynucleoside triphosphates, 0.4 μmol $L^{-1}$of each primer, 0.675 U of Taq DNA polymerase, and 1 μL of DNA template. The thermocycling steps were as follows: initial denaturation for 5 min of at 94 °C, denaturation for 1 min at 94 °C, elongation for 60 s at 72 °C, and a final extension of 10 min at 72 °C; with annealing for 60 s at 53 °C and 35 cycles for AOA; and 10 touchdown cycles of annealing for 45 s at 62 °C (−0.5 °C per cycle), followed by another 25 cycles with annealing for 45 s at 57 °C for AOB.

In order to characterize the community composition of AOA and AOB, clone libraries of both archaeal and bacterial *amoA* genes were constructed for the BC5 and the CK that had been incubated for 8 weeks. The samples at this time point were selected because of the relatively stable and active status of the communities of ammonia oxidizers, which was indicated by the relatively stable *amoA* gene abundances and the lowest level of ammonium (see Figs. 1b and 4). The PCR products of the triplicate were pooled, purified with a TIANgel Midi Purification Kit (TIANGEN, China), cloned into the pTZ57R/T vector (Fermentas, USA), and transformed into competent *Escherichia coli* DH5α. Positive clones were identified by PCR amplification with the universal primer pairs M13F/M13R and screened by restriction fragment length polymorphism (RFLP). Endonucleases *MspI* and *RsaI* (Fermentas, USA) were used for digesting AOB-amoA gene PCR products and *HhaI* and *RsaI* for AOA-*amoA* gene PCR products at 37 °C separately for 30 min. Around 100 clones were screened in each clone library, and three clones of each RFLP types were randomly selected and sent to the Sangon Company for sequencing with ABI 3730 (Sangon Biotech, China). The newly obtained sequences have been deposited in GenBank under the accession number KF179386–KF179517.

Quantification using real-time quantitative PCR

The primer pairs *amoA*-1F/*amoA*-2R (491 bp) and Arch-*amoA*-for/Arch-*amoA*-rev (256 bp) were used for quantifying bacterial and archaeal *amoA* genes, respectively (Rotthauwe et al. 1997; Tourna et al. 2008). The real-time quantitative PCR assay was based on the fluorescence intensity of the SYBR Green dye, and reactions for each sample were carried out in an ABI 7500 fast real-time PCR system (Applied Biosystems, USA). The quantification was performed in a total volume of 25 μL, using SYBR Green/ROX qPCR Kit (Fermentas, USA), 0.4 μmol L^{-1} of each primer, and 1 μL soil DNA. Bovine serum albumin was added to reach final concentrations of 0.4 ng μL^{-1}. Standard curves for the AOB and AOA were obtained using serial dilutions of linearized plasmids (pTZ57R/T, Fermentas, USA) containing the target *amoA* gene amplified from sequenced environmental clones

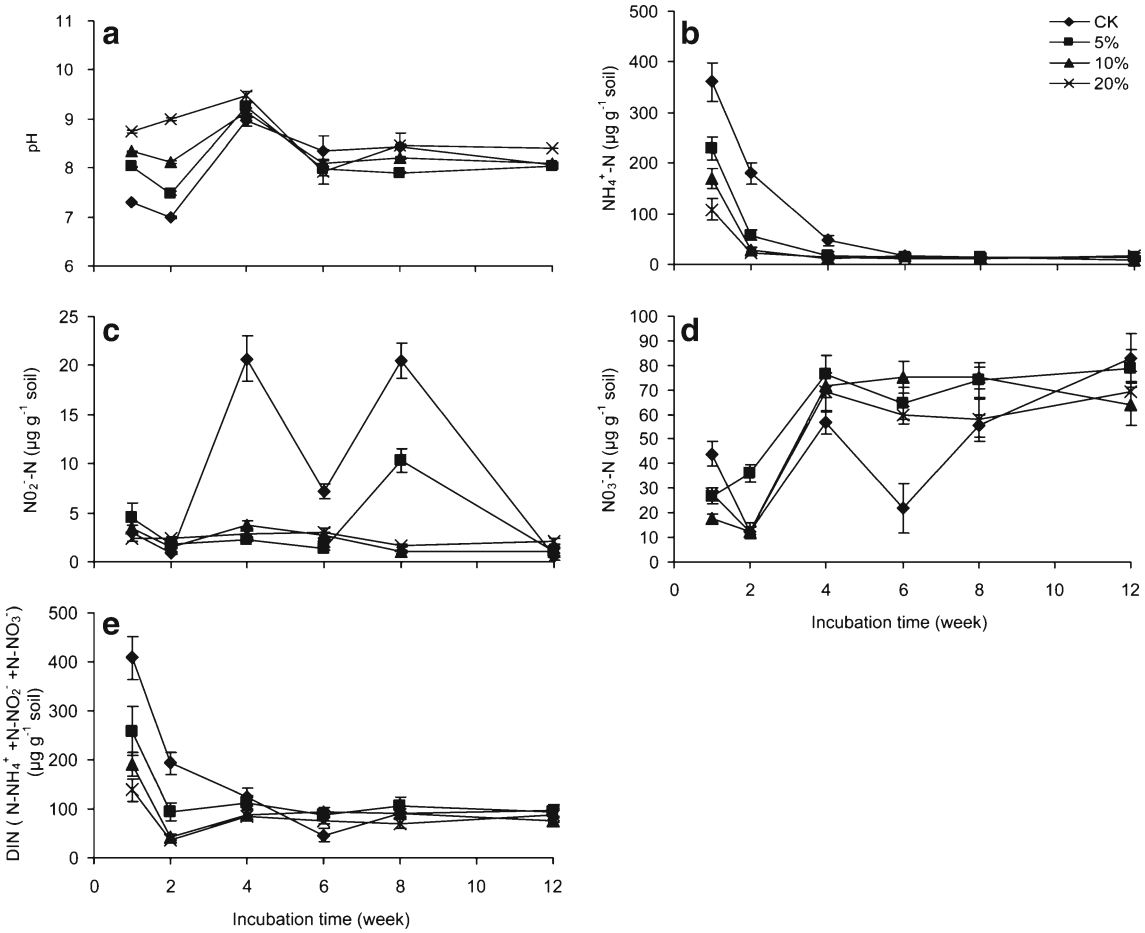

Fig. 1 Dynamics of pH (**a**), NH_4^+-N (**b**), NO_2^--N (**c**), NO_3^--N (**d**), and dissolved inorganic nitrogen (DIN) (e) in soils amended with 5, 10, and 20 % biochar and the control (*CK*) over a 12-week incubation. *Error bars* represent standard errors ($n=3$)

($R^2 \geq 0.99$ for both standard curves). The PCR amplification efficiencies were 100 % for AOA and 89 % for AOB.

Phylogenetic and statistical analysis

DNA sequences were examined and edited by the BioEdit software (Hall 1999). Phylogenetic trees were constructed by MEGA 4.1 (Tamura et al. 2007) with the neighbor-joining methods, and bootstrap resampling analysis for 1,000 replicates was performed to estimate the confidence of the tree nodes. Operational taxonomic units (OTUs) for community analysis were defined by a 2 % difference in nucleotide sequences, as determined using the furthest neighbor algorithm in DOTUR (Schloss and Handelsman 2005). Rarefaction analysis, Shannon index (H), and Simpson index (D) were also calculated in DOTUR.

One-way analysis of variance (ANOVA), pairwise t test, and calculation of Spearmen's correlation coefficient (ρ) were performed using SPSS (SPSS Inc., Chicago, IL, USA). LIBSHUFF in MOTHUR (Schloss et al. 2009) was used to statistically compare the structure of *amoA* gene libraries of the CK and BC5 treatments.

Results

Biochar effects on soil pH, inorganic N, and PAO activity

Soil pH showed a similar temporal pattern during the incubation in all treatments. The pH increased to and peaked at approximately 9 after 4 weeks of incubation in all treatments (Fig. 1a). The addition of 20 % biochar resulted in generally higher pH, which were significantly higher than other three treatments at the beginning (the first 2 weeks) and end of the study (week 12) (t test, $P<0.05$; Fig. 1a).

The concentrations of NH_4^+-N decreased rapidly during the first 4 weeks in all treatments (Fig. 1b). Furthermore, during the first 2 weeks, the concentrations of NH_4^+-N in the CK were 2–3-fold higher than these in the biochar treatments (t test, $P<0.01$). This was also reflected in the rapid increase of NO_3^--N concentrations, which peaked (55–

76 mg kg^{-1} soil) after 4 weeks in all treatments. The concentration of NO_2^--N in biochar treatments generally maintained at a low level (0.5–4.9 mg kg^{-1} soil) during the entire incubation, in contrast to the significantly higher levels (7–21 mg kg^{-1} soil) in the CK during the middle stage of the incubation (*t* test, $P<0.01$). Correspondingly, the concentrations of NO_3^--N were lower in the CK than in the biochar treatment during the same period (Fig. 1c, d). The NO_3^--N concentrations in the BC10 and BC20 and the CK increased after 2 weeks of incubation (Fig. 1d). For the concentrations of all the three inorganic N species, no significant differences were found among the BC5, BC10, and BC20 at most time points during the incubation (ANOVA, $P>0.05$). The concentrations of dissolved inorganic nitrogen decreased to around 80 mg kg^{-1} soil in all treatments (Fig. 1e).

A unimodal pattern of the dynamics of PAO activities was observed in all treatments during the 12-week incubation. The PAO rate reached a plateau between weeks 2 and 4 in BC10 and BC20 and between weeks 2 and 6 in the CK and then significantly decreased (Table 1; $P<0.05$). In contrast, the PAO rate of CK peaked in weeks 1 and 2, which was earlier than in the biochar treatments. During the early stages (weeks 1–4) of incubation, the PAO rates were significantly different among treatments ($P<0.05$), with the highest PAO rates observed in the BC10 treatment (Table 1), which were about two and two to five times higher than in other treatments and the CK, respectively. No significant differences of PAO rates were detected from treatments after the samples were incubated for 6 weeks ($P=0.15$) or 12 weeks ($P=0.11$).

Biochar effects on community structures of ammonia oxidizers

In order to characterize in detail the community composition of ammonia oxidizers at a relatively stable but also active stage, four *amoA* gene clone libraries were constructed for AOA and AOB in the CK and BC5 treatment for samples incubated for 8 weeks when ammonium was almost depleted and *amoA* copy numbers were relatively stable. Rarefaction curves of AOA and AOB clone libraries indicate that most of OTUs have been recovered (Fig. 2). A total of 39 AOA *amoA* OTUs were recovered, with 26 from the CK and 21 from the biochar sample and 8 OTUs in common (Table 2). The phylogenetic tree showed that all detected AOA belonged to the group I.1 of the phylum Thaumarchaeota (formerly Crenarchaeota). Compared with the CK, the relative abundances of AOA group I.1b-related OTUs increased and group I.1a-related OTUs decreased in the biochar treatment (Fig. 3a). Similarly, the group I.1a-related OTU numbers decreased from 14 to 6, and the group I.1b-related OTU numbers increased from 12 to 15, resulting in significantly decreased Shannon indices (3.33, 95 % CI=2.18–2.48 in the CK vs. 2.94, 95 % CI=2.68–3.20 in the biochar treatment), but intact Simpson indices (0.04) after 8 weeks of incubation after biochar amendment (Table 2).

In contrast, Shannon index of AOB significantly increased from 2.15 (95 % CI=1.93–2.37 in the CK) to 2.86 (95 % CI=2.69–3.05 in the biochar treatment), and Simpson index significantly decreased from 0.08 (CK) to 0.04 (biochar treatment) after biochar amendment (Table 2). There were more OTUs (22) detected in the BC5 than in the CK (16), of which six OTUs were in common (Fig. 3b). All AOB-*amoA* gene sequences obtained belonged to β-proteobacteria and grouped into a range of clusters. Interestingly, cluster 3a.2 and its related OTUs dominated, and virtually no sequences from other (*Nitrosospira* and *Nitrosomonas* like) clusters 1 to 12 were detected in the CK. The BC5, however, had more OTU (eight vs. three) and higher relative abundance of the *Nitrosospira briensis*-related group (Fig. 3b). The structure differences of bacterial ($P<0.001$) or archaeal *amoA* gene libraries ($P=0.005$) between the CK and BC5 were statistically significant when analyzed using LIBSHUFF.

Table 1 Analysis of variance for the effects of incubation time and biochar application rate on potential ammonia oxidation rates (mean ± standard error, mg N kg^{-1} 5 h^{-1} soil)

Treatment	Rate at each measurement					*F* statistic	*P* value	Periodic mean rate	
	Week 1	Week 2	Week 4	Week 6	Week 12			Weeks 1–4	Weeks 2–4
CK	2.57±0.14 abA	3.15±0.59 bA	1.29±0.24 cB	1.38±0.31 acB	0.30±0.04 bC	16.59	**0.001**	2.19±0.37 b	2.03±0.51 c
BC5	2.16±0.85 abAB	3.65±0.63 bA	3.25±0.25 bA	3.24±1.44 aA	0.51±0.10 aB	4.51	**0.041**	3.05±0.35 b	3.40±0.26 b
BC10	4.04±0.57 aB	6.47±0.76 aA	6.96±0.60 aA	2.52±0.43 abB	0.30±0.05 bC	26.89	**<0.001**	5.82±0.55 a	6.71±0.45 a
BC20	1.24±0.45 bB	3.88±0.28 bA	4.05±0.61 bA	1.12±0.09 bcB	0.35±0.03 abB	19.72	**<0.001**	2.95±0.56 b	3.98±0.35 b
F statistic	5.26	5.77	26.25	2.56	2.78			24.692	11.380
P value	**0.041**	**0.045**	**<0.001**	0.151	0.110			**<0.001**	**<0.001**

BC5, BC10, and BC20, treatments with biochar application rates of 5, 10, and 20 %, respectively. Two treatments sharing no lowercase letters or data of two time points sharing no uppercase letters indicate significant differences ($P<0.05$). *P* values<0.05 are highlighted in bold

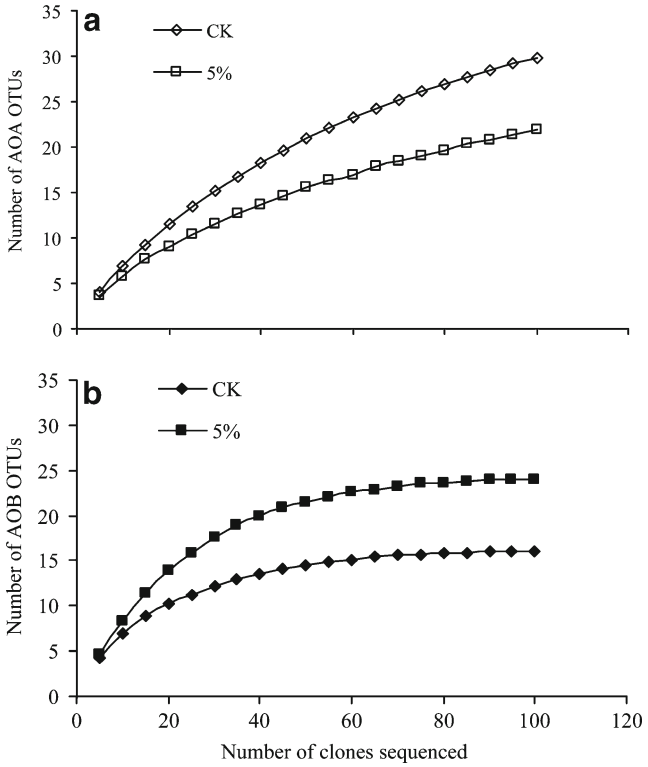

Fig. 2 Rarefaction curves for clone libraries of ammonia-oxidizing archaea (**a**) and ammonia-oxidizing bacteria (**b**) generated from the 5 % biochar-amended treatment and the control (*CK*) after 8 weeks of incubation

Biochar effects on the abundance of ammonia oxidizers

The abundance of the AOA-*amoA* genes ranged between 4.3×10^3 and 4.8×10^4 copies g^{-1} soil in the CK and between 6.6×10^2 and 4.2×10^4 copies g^{-1} soil the in biochar treatments. Abundance of AOA in the CK peaked after 2 weeks of incubation and then decreased to the lowest level after 12 weeks (Fig. 4a). In the biochar treatments, however, AOA *amoA* gene abundance reached the maximum in the first week, and then leveled off, except a rebound to 2.3×10^4 copies g^{-1} soil in the BC5 at the end of the incubation, which was significantly higher than the CK and other biochar treatments (*t* test, $P<0.05$; Supplementary Table 1).

Abundances of AOB-*amoA* genes were 2- to 903-fold that of AOA-*amoA* genes in all samples and incubation periods (Fig. 4c). In contrast to the generally decreasing trend of AOA-*amoA* gene with incubation time, the abundance of AOB generally showed an increasing trend, even though there were marked fluctuations during the incubation. AOB abundances in both CK and BC10 and BC20 peaked after 6 weeks of incubation, whereas the maximum abundance of AOB in the BC5 appeared in the fourth week. The abundances of AOB in the biochar treatments were significantly higher than these in the CK at the later stages ($P<0.05$; Supplementary Table 1), except that the abundance became significantly lower in the BC20 at the end of the incubation (Fig. 4b).

Table 2 Summary of ammonia-oxidizing bacteria (AOB) and ammonia-oxidizing archaea (AOA) *amoA* gene clone libraries

	CK	5 % biochar
No. of clones selected for screening		
AOB	105	105
AOA	102	102
No. of operational taxonomic units		
AOB	16	22
AOA	26	21
Shannon (*H*)		
AOB	2.15 (1.93–2.37)	2.86 (2.69–3.05)
AOA	3.33 (2.18–2.48)	2.94 (2.68–3.20)
Simpson (*D*)		
AOB	0.08±0.00	0.04±0.00
AOA	0.04±0.00	0.04±0.00

Shannon and Simpson diversity indices are means with 95 % confidence interval in brackets

Relationships between *amoA* copy numbers, PAO activity, and soil physicochemical properties

For samples with biochar, the PAO rates were weakly and positively correlated with the log copy numbers of AOA *amoA* gene ($\rho=0.39$, $P<0.01$), but not with AOB *amoA* ($\rho=-0.15$, $P=0.20$; Table 3). The ammonium concentration was correlated positively with AOA ($\rho=0.47$, $P<0.01$), but negatively with AOB ($\rho=-0.52$, $P<0.01$); the nitrate concentration was correlated negatively with AOA ($\rho=-0.46$, $P<0.01$), but positively with AOB ($\rho=0.87$, $P<0.01$). When only the samples from the CK were considered, the positive relationship between AOA abundances and PAO rates became even stronger ($\rho=0.94$, $P<0.01$), and log copy numbers of the AOA were negatively correlated with pH ($\rho=-0.74$, $P<0.05$).

Discussion

Effects of biochar amendment on soil pH and potential ammonia oxidation activity

This microcosm study for the first time studied the effects of biochar application on the dynamics of the physicochemical properties, the community composition, and activity of ammonia oxidizers in a coastal alkaline soil (initial pH 8.0). Previous studies showed that the biochar amendment generally increases soil pH (Chan et al. 2007), and thus, biochar application in acidic soils might be desirable (Major et al.

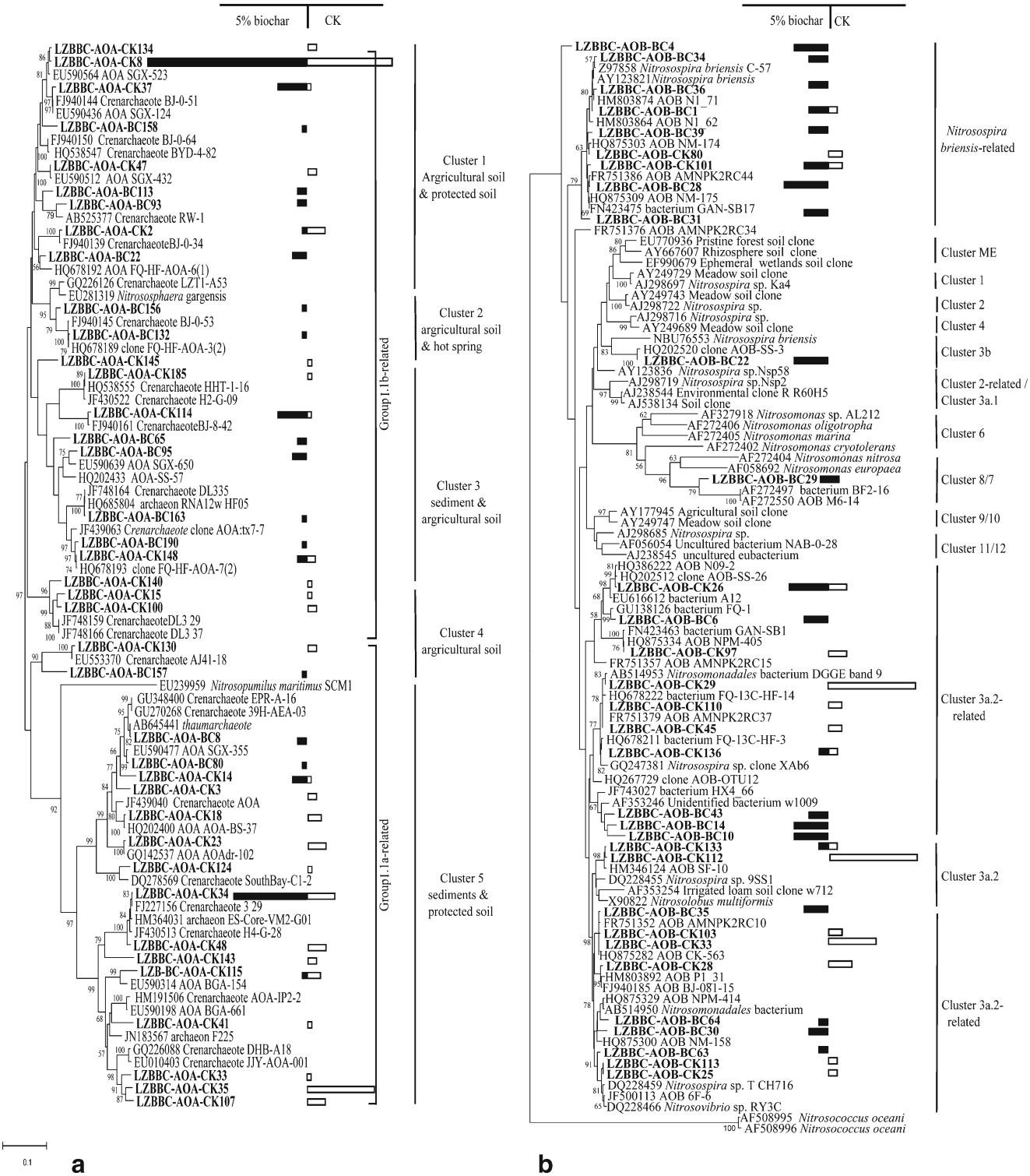

Fig. 3 Neighbor-joining trees showing the phylogenetic positions of archaeal (**a**) and bacterial *amoA* (**b**) gene sequences derived from the clone libraries of the control (*CK*) and the 5 % biochar treatment. Relative abundances of *amoA* genes in the treatment (*solid bar*) and in the CK (*open bar*) are shown on scale. Newly obtained sequences are highlighted in *bold*. The *scale bar* represents one nucleotide substitution per ten sites

2010; Jeffery et al. 2011). However, the effect of biochar application on alkaline soils is much less studied and understood. In our study, pH values varied in all samples during the incubation, but eventually reached a similar value of 8.1 in the CK, BC5, and BC10 treatments, indicating that biochar amendments with low rates do not significantly impact the

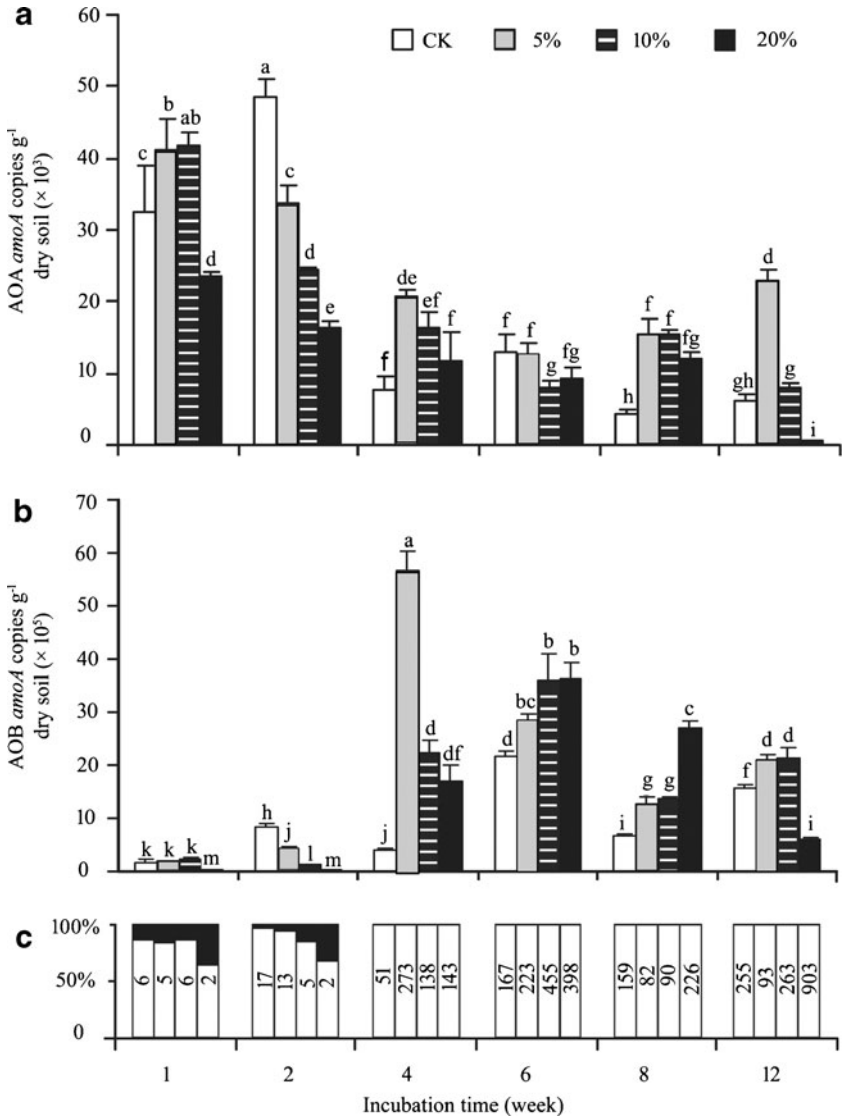

Fig. 4 Copy numbers of archaeal (**a**) and bacterial (**b**) *amoA* genes in the control (*CK*) and the soils amended with 5, 10, and 20 % biochar during 12 weeks of incubation. Relative percentages and ratios between archaeal (*solid bar*) and bacterial (*open bar*) amoA copy numbers were shown in (**c**). *Error bars* represent standard errors ($n=3$). Significant differences (*t* test, $P<0.05$) of copy numbers among the treatments are denoted with different *lowercase letters*

Table 3 Spearman's correlation between log *amoA* gene copy numbers and physiochemical factors and activities

	pH	NH_4^+	NO_2^-	NO_3^-	PAO
Samples with biochar					
AOB	−0.04	−0.52**	−0.03	0.87**	−0.15
	$n=54$	$n=54$	$n=54$	$n=54$	$n=45$
AOA	−0.06	0.47**	0.15	−0.46**	0.39**
	$n=54$	$n=54$	$n=54$	$n=54$	$n=45$
Samples without biochar					
AOB	−0.09	−0.59**	−0.37	−0.14	−0.30
	$n=18$	$n=18$	$n=18$	$n=18$	$n=15$
AOA	−0.74**	0.76**	−0.35	−0.76**	0.94**
	$n=18$	$n=18$	$n=18$	$n=18$	$n=15$

PAO potential ammonia oxidation rate, *AOB* ammonia-oxidizing bacteria, *AOA* ammonia-oxidizing archaea

*$P<0.05$; **$P<0.01$

pH of the alkaline soil. Therefore, application of biochar as a soil amendment in a wide range of soil types, including soils with low alkalinity, is possible.

Our study showed that the coupling between dynamics of ammonium and nitrate in soils and the activity of ammonia oxidation was time dependent. After 1 week of incubation, the concentrations of NH_4^+-N in the biochar treatments were significantly lower than these in the CK, with the lowest in the BC20 treatment (Fig. 1b). However, the substantial "loss" of NH_4^+-N in the treatments during the first week can hardly be caused by the microbial transformation processes because the PAO rates in the BC5 and BC20 treatments were not significantly different from or lower than that in the CK. Recent studies demonstrated that, by using a 2.0 mol L^{-1} KCl extraction solution, the adsorbed NH_4^+ could hardly be released from a biochar derived from peanut stalk (Saleh et al. 2012) but with over 90 % recovered from a biochar produced

from greenwaste (Eldridge et al. 2010), suggesting that the release of the adsorbed NH_4^+ may be biochar dependent. A reasonable explanation for the loss of NH_4^+in this study is NH_4^+ adsorption by the biochar derived from cotton stalk (Asada et al. 2006; Taghizadeh-Toosi et al. 2012; Spokas et al. 2012). It should be noted that the NH_4^+ adsorption by the biochar should not affect our measurement of PAO in this study because the ammonium adsorption by the positive charges is exchangeable and thus available to nitrifiers, and that the PAO was calculated based on the difference of ammonia oxidation product (i.e., nitrite), for which the ion adsorption should be low due to its negative charge, and hence not significantly affected by ion adsorption.

The application of biochar to the alkaline coastal soil led to substantial increases of nitrification during weeks 3 and 4 (Table 1), which corresponds to the sharp increases of nitrate concentrations measured with KCl extraction during the same period. This result is consistent with previous studies on forest soils where nitrifications were enhanced by biochar addition (Berglund et al. 2004; Ball et al. 2010). Nevertheless, during this microbe-active stage, the PAO rate was the highest in the 10 % biochar treatment, but was not affected by biochar application rate, suggesting that the enhancement of nitrification in the soil was not a linear function of biochar application rate. The relatively low PAO in the 20 % biochar treatment can be attributed to the increased NH_4^+ adsorption capability after biochar application, which limited the availability of ammonia to nitrifiers (as discussed above) and to ammonia volatilization. Alternatively, when a biochar with high C/N ratio is added to the soil, net immobilization of inorganic N can occur and a temporary reservoir of organic N could be created (DeLuca et al. 2009), which may, in turn, temporarily reduce the ammonia oxidation activity in the 20 % biochar treatment.

AOB dominated nitrifier populations, but nitrification was positively correlated with AOA abundance

The AOB outnumbered the AOA in all treatments and the CK, with a substantial increase after 2 weeks of incubation (Fig. 4b). Our study thus presents one of a few examples of AOB dominance (Wu et al. 2011) because AOA have been shown to be more abundant than AOB in soils in most other studies (Leininger et al. 2006; He et al. 2007; Chen et al. 2008). The predominance of AOB in the alkaline soil as was shown in this study thus supports the notion that the ratio of AOA to AOB abundance decreases with an increased pH (Nicol et al. 2008; Prosser and Nicol 2008; Erguder et al. 2009; Bru et al. 2011). In contrast, AOA *amoA* gene copy numbers were significantly higher than those of AOB *amoA* genes in an alkaline sandy loam with pH ranging from 8.3 to 8.7 (Shen et al. 2008). This indicates that pH was not the only factor determining the relative abundance of AOA and AOB in soils.

The N fertilization and the high soil C/N ratio in biochar treatments could also contribute to the dominance of AOB in this study. AOA are well adapted to growth in environments with very low NH_4^+ concentrations (Martens-Habbena et al. 2009), whereas growth of AOB is favored at high NH_4^+ concentrations (Verhamme et al. 2011). The high NH_4^+ adsorption of biochar could make the amended biochar a better place for AOB, but not for AOA, as AOA can be inhibited by ammonia at high concentrations (Pratscher et al. 2011). However, we observed that the high AOB/AOA ratios were maintained during the whole experimental period, even when the NH_4^+ concentrations returned to a low level (about 20 $\mu g\ g^{-1}$ soil) after 4 weeks of incubation. This could be due to the ability of AOB in maintaining stable population size during periods of low NH_4^+ availability (Mendum et al. 1999). Furthermore, Adair and Schwartz (2008) reported that the abundance of AOB was positively correlated to soil C/N, but no correlation was detected between the AOA abundance and soil C/N. In our study, for the treatments incubated for at least 4 weeks, the ratios of AOB to AOA were generally higher in the biochar treatments (ranging between 82 and 903) than in the control (ranging between 51 and 255) (Fig. 4c), which supports that the bioavailable carbon introduced by biochar application may further enhance the dominance of AOB especially in these treatments.

Despite the low abundance of AOA *amoA* in this study, we found that there was a significant positive relationship between the abundances of AOA am*oA* genes and the potential ammonia oxidation. This may imply that nitrification was mainly driven by AOA in the alkaline soil with or without biochar amendment. However, the detected gene may not be expressed, and there may be an alternative function of *amoA* gene in both bacterial and archaeal ammonia oxidizers (Prosser and Nicol 2008). Furthermore, the AOB might function better than AOA during the PAO assay, as some AOA (e.g., *Nitrosopumilus maritimus*) may not tolerate shaking (Martens-Habbena et al. 2009). Consequently, other approaches (e.g., stable isotope tracing, specific inhibition of AOA or AOB) may be needed to verify whether AOA is the major player in nitrification of the alkaline soil amended with biochar.

Biochar amendment enhanced the abundances of ammonia oxidizers

Understanding microbial colonization and adaptation processes is essential for clarifying the effect of biochar on nitrifiers. In this study, both AOB and AOA *amoA* abundances gradually decreased with the increased biochar application rate after 2 weeks of incubation (Fig. 4a, b); however, both nitrifiers generally became more abundant in the biochar treatments after 4 weeks of incubation and that pattern was maintained for another 8 weeks, as compared with the CK. The higher

abundance of AOA and AOB in biochar treatments in the relatively "stable" stages (after 4 weeks) indicates that biochar application increased the community size of both ammonia oxidizers in the coastal alkaline soil. This is consistent with two previous studies that terra preta soils subjected to agriculture and charcoal-containing forest soils had higher numbers of archaeal and bacterial *amoA* gene copy numbers than control soils (Ball et al. 2010; Taketani and Tsai 2010). Increased retention of soil moisture, which is essential for microbial growth (Schimel et al. 2007), and adsorption of microbial populations on the biochar that has a large surface area and may thus protect microbes from predators (Ogawa 1994; Ezawa et al. 2002; Saito and Marumoto 2002; Thies and Rillig 2009) are some of the potential mechanisms contributing to the biochar effects on microbial populations.

It was remarkable to see the revival of AOA in the 5 % biochar treatment after 12 weeks of incubation. Compared with these at the fourth week, AOB *amoA* copy numbers significantly decreased at the 12th week, whereas AOA *amoA* copy numbers almost recovered to the same level (Fig. 4a, b), suggesting that the 5 % biochar treatment at the late stage of incubation provides a preferable niche for the growth of AOA. The pores inside biochar and space between soil aggregates and biochar particles could form oxygen-limited micro-environments due to consumption of heterotrophic respiration in the earlier stages, and NH_4^+ remained at low concentrations during the late stage of incubation. All these conditions should favor the growth of AOA (Martens-Habbena et al. 2009; Verhamme et al. 2011). In addition, AOA appear to be smaller than AOB in cell size (Prosser and Nicol 2008), which could also be an advantage for AOA colonizing or attaching to the biochar amended. Nevertheless, the revival of AOA did not occur in the 10 and 20 % biochar treatments, suggesting again an effect of the biochar application rate on ammonia oxidizers.

Biochar amendment affected community composition of AOA and AOB

Our study showed that the biochar amendment to the coastal saline soil favored a few AOA *amoA* phylotypes and many AOB *amoA* in various clusters (Fig. 3). In this study, both the relative abundances and OTU numbers of group I.1b-related AOA increased, whereas group I.1a-related AOA decreased in the biochar treatment as compared with the CK. This result is consistent with a previous study that described the response of these two AOA subgroups to elevated soil pH (Shen et al. 2012), indicating that the increased pH could contribute to changes in AOA community composition in biochar treatments. For the AOB community, we found that the *Nitrosospira amoA* cluster 3a.2-related OTUs dominated in the coastal alkaline soil, again in agreement with previous results from alkaline and neutral soils (Shen et al. 2012). However, the dominance of *N. briensis*-related AOB was only observed in the biochar-amended alkaline soil in this study.

Conclusions

Our study documents for the first time the influence of biochar amendment on the abundance, composition, and activities of ammonia oxidizers in alkaline soil microcosms. Application of biochar to an alkaline soil stimulated the ammonia oxidation rate in the first 4 weeks of incubation, especially in the 10 % biochar treatment. AOB *amoA* genes consistently outnumbered AOA *amoA*, but nitrification was positively correlated with AOA but not with AOB. However, the major contributor to nitrification in these soils needs to be further studied. AOA and AOB communities responded differently to biochar additions, with AOB phylotypes more adaptive than AOA in biochar-amended soils. Low biochar application rate to weakly alkaline soil did not increase the soil pH. Therefore, such low biochar application rates pose low risks for increasing the alkalinity and potential nitrification rate of the soil being treated. This has important implications for using biochar in reclamation of alkaline soils. Because different feedstock types and production procedures (e.g., pyrolysis temperature) could lead to biochar with different physiochemical properties and hence different effects on soil ecology and biogeochemistry, further study on the effects of different types of biochar is needed to systematically evaluate their effects on microbe-mediated nutrient cycling in alkaline soils.

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