

Original Research

Effects of H₂O₂-modified Rice Husk-Derived Biochar on Nitrification and N₂O Emission from Water by an Isolated Heterotrophic Nitrifying Bacterium

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Abstract

In order to explore the effect of biochar on nitrification and N₂O emission from water, the indoor incubation experiment was carried out after incorporating the pristine biochar (BC) or H₂O₂-modified rice husk-derived biochar (20% H₂O₂-BC and 30% H₂O₂-BC) combined with the screened heterotrophic nitrifying bacterium into the simulated wastewater. The dynamic changes of inorganic nitrogen contents, pH, bacterial amount, and N₂O emission of the incubation system were analyzed. Results showed that compared with BC, the amount of acidic oxygen-containing functional group of H₂O₂-modified biochars increased by 24.24%~26.98%, while the amount of alkaline oxygen-containing functional group, pH, and the specific capacitance significantly decreased by 22.16%~27.29%, 2.54~2.68, and 15.90%~30.94%, respectively. H₂O₂-modified biochars significantly inhibited nitrification driven by heterotrophic nitrifying bacterium compared with BC. Moreover, the 16-h cumulative N₂O emission of the treatments with 20% H₂O₂-BC or 30% H₂O₂-BC addition (0.0516 and 0.0525 μg N₂O/100 mL solution, respectively) were significantly higher than that with BC addition (0.0355 μg N₂O/100 mL solution). Correlation analysis showed that the cumulative N₂O emission of the treatments with 20% H₂O₂-BC or 30% H₂O₂-BC addition was significantly positively correlated with NH₂OH concentration in incubation system ($r = 0.455$ and 0.497 , respectively), and showed no significant correlation relationship with NO₂⁻-N ($p < 0.05$).

Keywords: biochar, H₂O₂-modification, nitrification, N₂O emission

Introduction

Ammonia nitrogen is an important pollutant that causes water body black and odorous. Ammonia nitrogen pollution control has always been a hot spot in environmental studies. Ammonia oxidation is the rate-limiting step of nitrification and the main pathway of ammonia transformation [1]. N_2O is a potent greenhouse gas and can destroy the ozone layer [2]. The main production pathways of N_2O during nitrification are hydroxylamine (NH_2OH) oxidation and NO_2^- reduction [3-4].

Biochar, a carbon-rich material, prepared by pyrolysis of the plant biomass and other organic waste under the condition of oxygen free and high temperature (300~800°C) [5-6]. Biochar was characterized by a highly aromatic carbon structure, alkalinity (pH>7), and large specific surface area [7]. It has been widely used in environmental remediation and agricultural waste recycling [8]. The effects of biochar on soil nitrification and N_2O emission has been paid much more attention. The promotion and inhibition effect of biochar on soil N_2O emission coexisted, and the potential mechanism was also unclear [9-12]. However, there were few studies focusing on the effect of biochar on nitrification and N_2O emission from water. Considering that water has better homogeneity than soil, we speculated that the research in water could be more conducive to reveal the underlying mechanisms.

As the carrier of electron transfer, biochar is beneficial to redox reactions, and such characteristic of biochar has been reported by many other studies [13-14]. However, it was rarely reported that the effect of biochar's electrochemical property on nitrification and N_2O emission driven by heterotrophic nitrifying bacterium (HNB). H_2O_2 -modification can increase the amounts of carboxyl group and acidic oxygen-containing functional group of biochar [15-16] and decrease the electrical conductivity and the specific capacitance of biochar [14,17]. Moreover, considering that H_2O_2 -modification could induce less exogenous substances into the incubation system, the H_2O_2 oxidation method was used to prepare the modified rice husk-derived biochar.

Based on the preparation and characterization of 20% or 30% H_2O_2 -modified rice husk-derived biochars, the pristine and modified biochars were mixed with the simulated wastewater containing the screened HNB, *Pseudomonas putida* strain-N3, respectively. The dynamic changes of inorganic nitrogen concentrations, pH, bacterial amount, and N_2O emission during incubation were determined. The study aimed to explore the effects of pristine and H_2O_2 -modified biochars on nitrification and N_2O emission from water and the potential mechanisms.

Materials and Methods

Biochar Preparation and Characterization

The rice husk-derived biochar (BC) used in the experiment was purchased from Dalian Jiucheng Products Co. Ltd., which was passed through a 100-mesh sieve before use. The biochar has a pH of 7.98 ± 0.05 , a point of zero charge (pH_{pzc}) of 7.13 ± 0.09 , an electrical conductivity of 48.80 ± 1.31 mS/m, a specific capacitance of 5.85 ± 0.29 F/g, an acidic oxygen-containing functional group of 0.656 ± 0.012 mmol/g, and an alkaline oxygen-containing functional group of 0.546 ± 0.013 mmol/g. H_2O_2 -modified BC was prepared as follows: 20% or 30% H_2O_2 (250 mL) was added into the Erlenmeyer flask which was pre-filled with 5 g BC. After shaking for 12 h (25°C, 170 r/min), the mixture was filtered and rinsed with deionized water until the pH of the filtrate achieved a constant. Finally, the biochars were dried and stored for later use, which were recorded as 20% H_2O_2 -BC and 30% H_2O_2 -BC, respectively.

The pH of biochar (biochar-water ratio 1 g:15 mL) was measured by a pH meter (Mettler Toledo Delta 320). The pH_{pzc} was measured by the titration method. The types of functional groups on the surface of biochar were measured by the Fourier transform infrared spectrometer (PerkinElmer Spectrum Two) and the scanning range is 400~4000 cm^{-1} . The oxygen-containing functional group amount was determined by the Boehm titration method [18]. The cyclic voltammetry curve of biochar was tested by the electrochemical workstation (CHI 760E), and the following formula was used to calculate the specific capacitance (F/g) of biochar [19].

$$C = \frac{I}{f \cdot m} \quad (1)$$

Where, I is the average cathodic current, A; f is the scan rate, V/s; and m is the mass of biochar on the glassy carbon electrode, g.

Incubation Experiment of HNB with Biochar Addition

In order to explore the effect of biochar on nitrification by the enriched and screened HNB, 0.3 g biochar (BC, 20% H_2O_2 -BC or 30% H_2O_2 -BC) and HNB cell collected from 15-mL liquid culture medium were added into a 50-mL centrifuge tube containing 30-mL simulated wastewater (Table 1) [20], which was incubated in the dark for 48 h (25°C, 170 r/min). The three treatments were designated as HNB+BC, HNB+20% H_2O_2 -BC and HNB+30% H_2O_2 -BC, respectively. All samples were run in triplicate.

Table 1. Composition of the simulated wastewater.

Composition	Concentration (mg/L)
NH ₄ Cl	38.2
Glucose	275
MgSO ₄ ·7H ₂ O	25
FeSO ₄ ·7H ₂ O	2.5
MnSO ₄	2.5
NaCl	125
K ₂ HPO ₄	52
pH	7.0

HNB cell was prepared as the following procedures. The pre-saved HNB strain (*Pseudomonas putida* strain-N3, MN602471) [21] was selected from the slant culture medium, and then it was inoculated into 400 mL LB liquid culture medium (g/L, tryptone 10, yeast extract 5, NaCl 10) and cultivated to OD₆₀₀ = 1.0 (25°C, 170 r/min). After dividing the liquid culture medium into 15 or 50 mL, respectively, the HNB cell was collected by centrifuging the liquid culture medium followed by washing the centrifugated deposit with sterile water twice.

The water samples were taken at 1/6, 0.5, 1, 2, 5, 8, 16, 24, and 48 h during incubation, respectively. After filtering through a 0.45-μm membrane, the concentrations of NH₄⁺-N, NO₂⁻-N, and NO₃⁻-N were determined by the colorimetric method at the wavelength of 420, 220/275, and 540 nm, respectively [22]. NH₂OH concentration was measured according to the method reported by Tian [23]. The pH and HNB amount (mL⁻¹) in the water samples were also measured.

N₂O Emission Determination

In order to explore the effect of biochar on N₂O emission during nitrification driven by the selected HNB, 1.0 g biochar (BC, 20% H₂O₂-BC or 30% H₂O₂-BC) and HNB cell collected from 50-mL liquid culture medium were added into a 280-mL culture flask containing 100-mL simulated wastewater (Table 1). The mouth of the culture flask was sealed with a rubber stopper, and a three-way valve with good air tightness was installed on the rubber stopper for gas sample collection. The 9 culture flasks (3 treatments × 3 replicates) were incubated in the dark (25°C, 170 r/min). 20-mL gas sample was collected with a syringe through the three-way valve after injecting 20-mL air into the headspace of the culture flask with three times of push-and-pull at 1/6, 0.5, 1, 2, 5, 8, and 16 h, respectively. N₂O concentration was determined by a gas chromatograph (SP-3420A), and the cumulative N₂O emission was calculated as μg N₂O/100 mL solution.

Data Analysis

Microsoft Excel 2010 was used for data calculation and statistics. Origin 8.5 was used for graphing and equation fitting. SPSS Statistics 22 (IBM, New York, USA) was used for variance analysis and multiple comparison (One-way ANOVA).

Results and Discussion

Effect of H₂O₂-modification on the Properties of Rice Husk-Derived Biochar

Compared with BC, the carboxyl and acidic oxygen-containing functional group amounts of 20% H₂O₂-BC and 30% H₂O₂-BC significantly increased, while the amount of alkaline oxygen-containing functional group, pH, point of zero charge (pH_{pzc}), electrical conductivity, and the specific capacitance of 20% H₂O₂-BC and 30% H₂O₂-BC significantly decreased (*p*<0.05) (Table 2).

The reason for the phenomenon that the pH and pH_{pzc} of H₂O₂-modified biochars were significantly lower than BC was that H₂O₂ is weakly acidic, which can neutralize the alkaline oxygen-containing functional group and enhance the acidic oxygen-containing functional group growth on the surface of biochar. In particular, the significantly increased carboxyl could lower the pH_{pzc} of biochar. The oxidation of biochar by H₂O₂ destroyed the aromatic ring structure of biochar and weakened its aromaticity [13,16]. Therefore, the conductivity of 20% H₂O₂-BC and 30% H₂O₂-BC decreased by 87.40% and 88.01% compared with BC, respectively. Moreover, the specific capacitance of biochar was also significantly impaired after H₂O₂-modification. The results were consistent with the characteristics of the biochars prepared at different pyrolysis temperatures reported by Chen et al. [17].

Fourier transform infrared (FTIR) spectrum can distinguish the type of functional group on the surface of biochar. The FTIR spectra of BC, 20% H₂O₂-BC, and 30% H₂O₂-BC were shown in Fig. 1. The characteristic absorption peaks of the three kinds of biochar were almost the same, which occurred at the wavenumbers of 3432, 1605, 1105, and 800 cm⁻¹, indicating the presence of -OH, C=O, C-O, and C-H functional groups, respectively. Compared with BC, the absorption intensity of 30% H₂O₂-BC at all the wavenumbers were enhanced, indicating the amount of -OH, C=O, C-O, and C-H increased after 30% H₂O₂-modification.

H₂O₂-modified Biochars Inhibited Nitrification Driven by HNB

NH₄⁺-N concentration of the incubation system decreased during 0~16 h, while NH₂OH, NO₂⁻-N, and NO₃⁻-N concentrations increased during 0~8 h. Moreover, the pH showed a decreasing trend during the whole incubation period (0~48 h). All the results

Table 2. Properties of rice husk-derived biochar before and after H₂O₂-modification.

	pH	pH _{pzc}	Carboxyl	Lactone	Phenolic hydroxyl (mmol/g)	Acidic oxygen-containing functional group	Alkaline oxygen-containing functional group	Electrical conductivity (mS/m)	Specific capacitance (F/g)
BC	7.98±0.05a	7.13±0.09a	0.595±0.016a	0.025±0.013a	0.036±0.015a	0.656±0.012a	0.546±0.013a	48.80±1.31a	5.85±0.29a
20% H ₂ O ₂ -BC	5.44±0.08b	4.46±0.08b	0.763±0.011b	0.013±0.012a	0.039±0.006a	0.815±0.006b	0.425±0.012b	6.15±0.10b	4.92±0.03b
30% H ₂ O ₂ -BC	5.30±0.13b	4.22±0.07c	0.760±0.013b	0.026±0.014a	0.048±0.009a	0.833±0.005c	0.397±0.005c	5.85±0.12b	4.04±0.02c

Note: data in the same column followed by the same letter have no significant difference at $p < 0.05$; mean±standard deviation ($n = 3$); pH_{pzc}: point of zero charge.

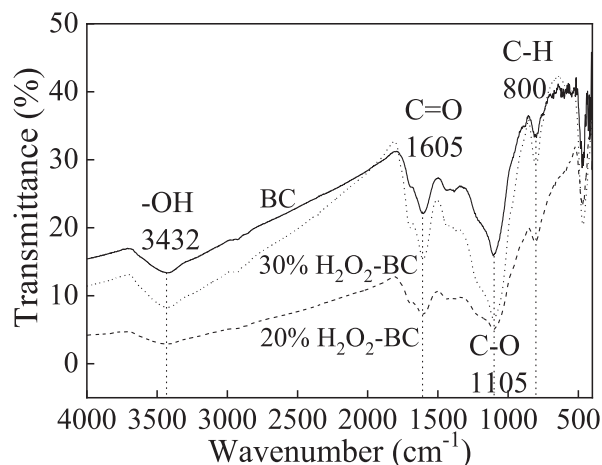


Fig. 1. Fourier transform infrared spectrum of rice husk-derived biochar before and after H₂O₂-modification.

indicated that microbial nitrification occurred with different extent during 0~8 h. However, NO₃⁻-N concentration of the incubation system decreased, while NO₂⁻-N and NH₄⁺-N concentrations increased during 16~48 h, which might be resulted from dissimilatory nitrate reduction to ammonium caused by the inadequate dissolved oxygen in the latter phase of incubation.

NH₄⁺-N concentrations of the treatments HNB+20% H₂O₂-BC and HNB+30% H₂O₂-BC (decreased from 9.70 to 1.47 mg/L and from 9.70 to 1.49 mg/L, respectively) were higher than that of the treatment HNB+BC (decreased from 9.92 to 0.47 mg/L) during 0~16 h. At the same time, NO₃⁻-N concentrations of the treatments HNB+20% H₂O₂-BC and HNB+30% H₂O₂-BC (increased from 0.24 to 1.96 mg/L and from 0.24 to 2.20 mg/L, respectively) were lower than that of the treatment HNB+BC (increased from 0.21 to 2.27 mg/L) during 0~8 h. In addition, the pHs of the treatments HNB+20% H₂O₂-BC and HNB+30% H₂O₂-BC were obviously lower than that of the treatment HNB+BC during the whole incubation period, and the amounts of HNB were also lower than that of the treatment HNB+BC during 16~48 h. The results indicated that H₂O₂-modified rice husk-derived biochars inhibited microbial nitrification of the incubation system (Fig. 2).

Why H₂O₂-modified rice husk-derived biochars inhibited microbial nitrification? Firstly, compared with BC, the pHs of 20% H₂O₂-BC and 30% H₂O₂-BC decreased by 2.54 and 2.68, respectively. Correspondingly, the pHs of the treatments HNB+20% H₂O₂-BC and HNB+30% H₂O₂-BC decreased by 0.47 and 0.54, respectively after 1/6 h incubation. Considering nitrification consumes alkalinity and 2 mol H⁺ will be produced for every 1 mol NH₄⁺ oxidation, the relatively lower pH of the incubation system was not conducive to nitrification.

Secondly, biochar can promote electron transfer between the substrate and microorganisms as a redox active mediator, which was called "electron shuttle" [14,

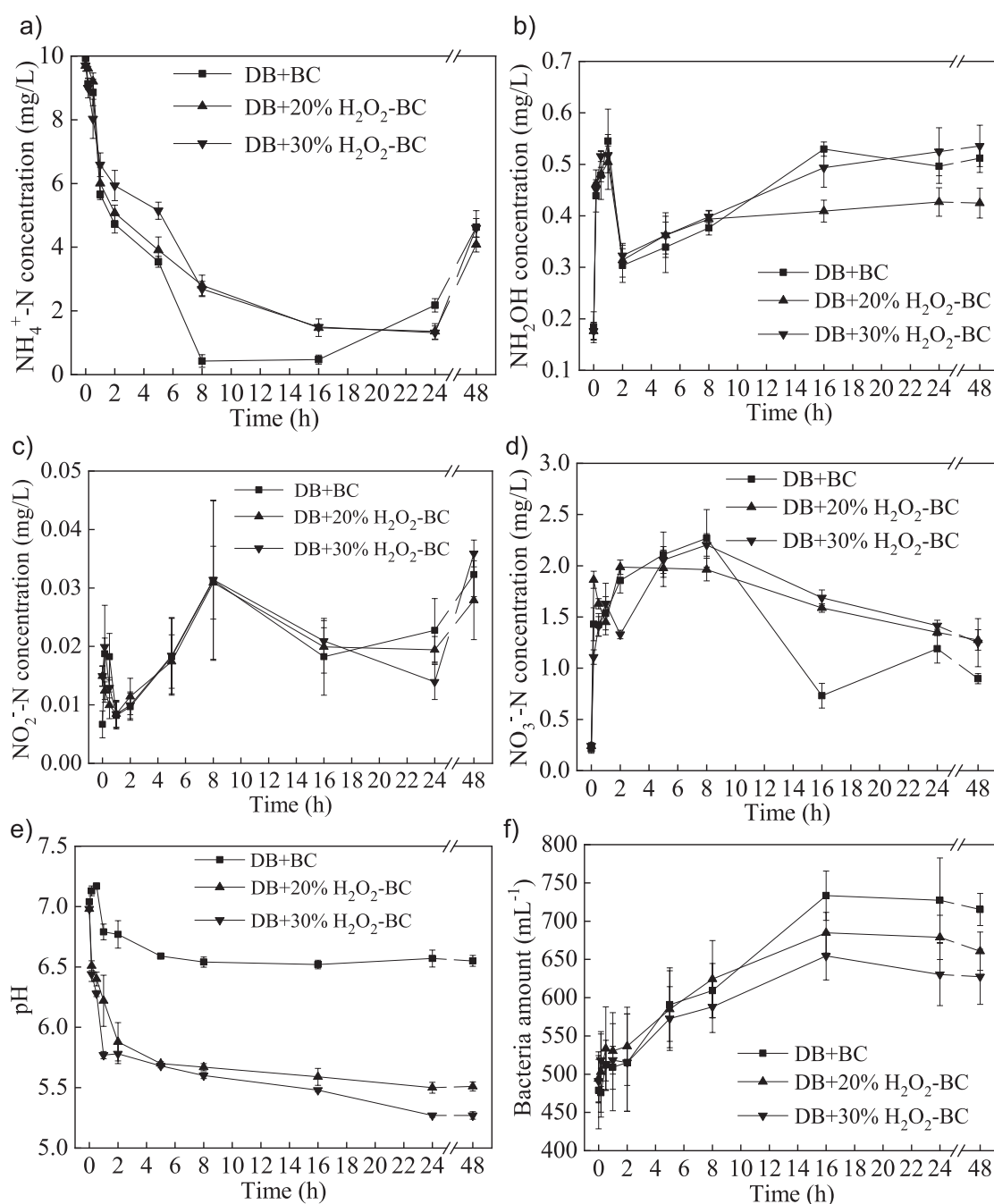


Fig. 2. Dynamic changes of NH₄⁺-N a), NH₂OH b), NO₂⁻-N c), NO₃⁻-N d) concentrations, pH e), and bacteria amount f) in the incubation system.

17, 24]. Compared with BC, the specific capacitances of 20% H₂O₂-BC and 30% H₂O₂-BC decreased by 15.90% and 30.94%, respectively, which weakened the ability of biochar as an electron shuttle and was not conducive to the electron transfer between NH₄⁺-N and the screened HNB, thereby inhibiting nitrification.

Thirdly, the nature of biochar surface charge is related to both the pH_{pzc} of biochar and the original pH of the incubation solution [23]. The pH_{pzc} of BC (7.13) was higher than the original pH of the simulated wastewater (7.00), making its surface positively charged. However, the pH_{pzc} of 20% H₂O₂-BC and 30% H₂O₂-BC (4.46 and

4.22, respectively) were lower than the original pH of the simulated wastewater, making its surface negatively charged. Given that the pH_{pzc} of most bacteria was 3~4 [25], which was lower than the original pH of the simulated wastewater, the surface of bacteria would be negatively charged. Therefore, the positively charged BC could adsorb microorganisms in the incubation system by electrostatic attraction. Moreover, biochar possessed abundant bioavailable carbon, and its porous structure could provide a suitable habitat for the growth of HNB [26], thus being in favor of NH₄⁺-N degradation. However, the electrostatic repulsion between the negatively charged

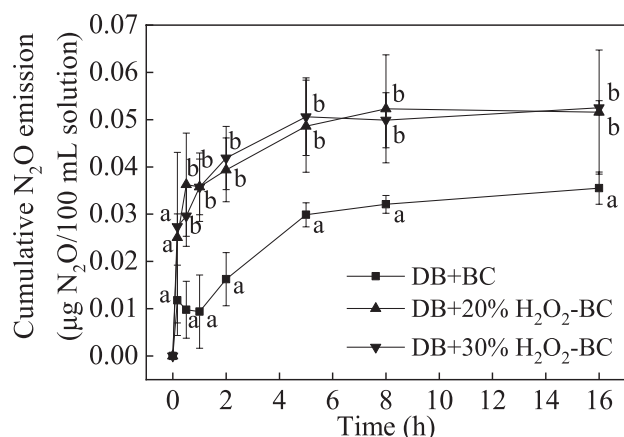


Fig. 3. Dynamic change of cumulative N₂O emission from the incubation system. Data marked with the same letter at each time point indicate no significant difference between treatments at $p < 0.05$.

20% H₂O₂-BC/30% H₂O₂-BC and the negatively charged microorganisms was not conducive to microorganism attachment to the surface of biochar and their growth would be negatively affected. Consequently, compared with the treatment with BC addition, HNB amount of the treatments with H₂O₂-modified biochar addition was obviously lower. Nitrifiers are the main players of the microbial transformation of ammonia to nitrate, and their quality affects the progress of nitrification directly [27].

H₂O₂-modified Biochars Promoted N₂O Emission during Nitrification

The cumulative N₂O emission in the headspace of culture flask increased sharply during the 0~5 h of incubation while it increased moderately during the 5~16 h. The cumulative N₂O emissions of the treatments HNB+BC, HNB+20% H₂O₂-BC, and HNB+30% H₂O₂-BC during 0~5 h were 0.0299, 0.0486, and 0.0506 µg N₂O/100 mL solution, respectively, which accounted for 84.12%, 94.21%, and 96.42% of the cumulative N₂O emission during the whole incubation period, respectively.

The cumulative N₂O emissions of the treatments HNB+20% H₂O₂-BC and HNB+30% H₂O₂-BC during 0.5~16 h were significantly higher than that of the

treatment HNB+BC, while there was no significant difference between the treatments with H₂O₂-modified biochar additions ($p < 0.05$). The cumulative N₂O emission of the treatments HNB+20% H₂O₂-BC and HNB+30% H₂O₂-BC during 0~16 h were 0.0516 and 0.0525 µg N₂O/100 mL solution, respectively, which were 1.45 and 1.48 times that of the treatment HNB+BC (Fig. 3).

Under the condition of relatively high dissolved oxygen concentration, the main pathway of N₂O production during nitrification was NH₂OH oxidation [28]. Liu et al. [29] also reported that N₂O production had closer relationship with the activity of hydroxylamine oxidase under aerobic conditions, who found that dissolved oxygen was sufficient at the initial stage of incubation, and NH₄⁺-N was rapidly oxidized to NH₂OH, resulting in NH₂OH accumulation. Further, NH₂OH was oxidized to N₂O under the catalysis of hydroxylamine oxidase. In our study, NH₂OH concentrations of the treatments with H₂O₂-modified biochar addition were higher than that of control during the incubation period of 0~8 h. Correspondingly, the cumulative N₂O emission of the treatments with H₂O₂-modified biochar addition was also significantly higher than that of control during the incubation period of 0.5~16 h. Therefore, we speculated the reason that H₂O₂-modified biochars promoted N₂O emission during nitrification could be related to NH₂OH oxidation.

In addition, NO₂⁻-N concentration of all the treatments during incubation was very low, which was an order of magnitude lower than NH₂OH concentration. Yu et al. [30] also indicated that NO₂⁻ hardly accumulated during nitrification because NH₂OH did not transformed to NO₂⁻ but transformed to N₂O and N₂ and released from the incubation system. Therefore, the reason that H₂O₂-modified biochars promoted N₂O emission during nitrification could not be related to NO₂⁻ reduction.

Correlation Analysis between the Cumulative N₂O Emission and NH₂OH, NO₂⁻-N Concentration, and pH of the Incubation System

In order to explore the influence of biochar on N₂O emission during nitrification and the potential mechanisms further, the correlation relationship

Table 3. Pearson correlation coefficients between the cumulative N₂O emission and NH₂OH, NO₂⁻-N concentration, and pH of the incubation system during the 48-h incubation period.

	NH ₂ OH (mg/L)	NO ₂ ⁻ -N (mg/L)	pH	Data range
Cumulative N ₂ O emission (µg N ₂ O/100 mL solution)	0.280	0.576**	-0.795***	HNB+BC (n = 24)
	0.455*	0.353	-0.565**	HNB+20% H ₂ O ₂ -BC (n = 24)
	0.497*	0.259	-0.654**	HNB+30% H ₂ O ₂ -BC (n = 24)
	0.358**	0.337**	-0.860***	All three treatments (n = 72)

Note: *, **, and *** denoted the significant difference at 0.05, 0.01, and 0.001 level, respectively.

between the cumulative N₂O emission and the concentrations of the intermediate products of nitrification, NH₂OH and NO₂⁻-N, was analyzed. The results showed that the cumulative N₂O emission was significantly positively correlated with NH₂OH concentration both in the treatments HNB+20% H₂O₂-BC and HNB+30% H₂O₂-BC, and the correlation coefficients were 0.455 and 0.497, respectively, while there was no significant correlation relationship with NO₂⁻-N ($p < 0.05$). The correlation analysis based on the data of all the three treatments showed that the cumulative N₂O emission was significantly positively correlated with both NH₂OH and NO₂⁻-N concentration, and the correlation coefficient with NH₂OH (0.358) was higher than that with NO₂⁻-N (0.337). Furthermore, there was a significant negative correlation between the cumulative N₂O emission and the pH of incubation system ($p < 0.01$), indicating that the cumulative N₂O emission was affected by pH greatly (Table 3).

Conclusions

Compared with BC, the amount of acidic oxygen-containing functional group of H₂O₂-modified biochars significantly increased by 24.24%~26.98%, while the amount of alkaline oxygen-containing functional group, pH, and the specific capacitance significantly decreased by 22.16%~27.29%, 2.54~2.68, and 15.90%~30.94%, respectively. Compared with BC, H₂O₂-modified biochars inhibited nitrification driven by the isolated heterotrophic nitrifying bacterium (HNB), *Pseudomonas putida* strain-N3. The main reasons were that H₂O₂-modified biochars with relatively low pH made the pH of the incubation system lower, and the decreased specific capacitance of H₂O₂-modified biochars weakened the function of biochar as an electron shuttle, which was not conducive to the proliferation of heterotrophic nitrifying bacteria and nitrification. Compared with the treatment with BC addition, the cumulative N₂O emission of the treatments with H₂O₂-modified biochar addition increased significantly. Correlation analysis showed that the cumulative N₂O emission of the treatments HNB+20% H₂O₂-BC and HNB+30% H₂O₂-BC was significantly positively correlated with NH₂OH concentration in incubation system ($r = 0.455$ and 0.497 , respectively), and showed no significant correlation relationship with NO₂⁻-N ($p < 0.05$). The study will provide a theoretical reference for the application of biochar in wastewater treatment.

Acknowledgments

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Conflict of Interest

The authors declare no conflict of interest.

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