Ammonium removal efficiency of biochar-based heterotrophic nitrifying bacteria immobilization body in water solution
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Abstract
In order to explore the performance of biochar-based microbial immobilization body in ammonium removal from water and potential mechanisms, a strain of heterotrophic nitrifying bacteria (HNB) was isolated from activated sludge, and the biochemical and molecular biological identification of HNB was carried out. Moreover, HNO₃-, Mg²⁺-, NaOH-, and NaOH+Mg²⁺-modified rice husk-derived biochars were prepared. Then all the five kinds of biochars, including the original biochar, were used as carriers of HNB to remove NH₄⁺-N from water. Results showed that HNB was classified as Pseudomonas, and the 72-h NH₄⁺-N removal ratio of the free bacteria reached 80.24%. Compared with biochar itself, biochar-based HNB immobilization body showed a much stronger ability to remove NH₄⁺-N, especially for NaOH- and NaOH+Mg²⁺-modified biochars. At the initial NH₄⁺-N concentration of 100 mg/L and biochar addition dose of 10 g/L, NH₄⁺-N removal ratio of NaOH- and NaOH+Mg²⁺-modified biochar-based HNB immobilization bodies reached 57.78% and 58.35% after 5 h, and reached 88.66% and 90.93% after 48 h respectively, which were obviously higher than the original, HNO₃- and Mg²⁺-modified biochar-based HNB immobilization bodies. The phenomenon resulted from significantly higher bacteria adsorption ability of NaOH- and NaOH+Mg²⁺-modified biochars, which reached 773.75 and 941.17 nmol P/g biochar, respectively.

Keywords: Ammonium removal, Biochar, Heterotrophic nitrifying bacteria, Immobilization body
1. Introduction

In recent years, the widespread application of chemical fertilizers has accelerated the accumulation of nitrogen in farmland, which in turn affects natural water bodies. Studies have shown that about 20% of nitrogen enters rivers and lakes through surface runoff, causing nitrogen pollution in surface waters [1]. Among them, NH$_4^+$-N is an important factor of the malodorous water bodies. Its degradation in water is a global environmental problem and has attracted more and more attention.

In order to address the serious problem of NH$_4^+$-N pollution, microbial immobilization technology (MIT) instead of the traditional suspension biotechnology emerges remarkably depending on its excellent performance in wastewater treatment [2]. MIT typically employs the physical, chemical, or biological methods to agglomerate free microbial cells into particles that are fixed or immobilized onto a particular material [3]. MIT can separate the treated water from microbial immobilization body readily, and enable immobilized microbes to be reused and remain active for a certain period of time [4]. Therefore, MIT as a potential biological wastewater treatment technology showed bright prospect. Meanwhile, the selection of microbial immobilization carriers has become a research hotspot. Dong et al. [5] indicated that polyurethane immobilized nitrifying bacteria could achieve complete nitrification even at low ammonia concentrations, and they were more insensitive to temperature and pH than the suspended nitrifying bacteria. Yu et al. [6] studied the performance of heterotrophic nitrification-aerobic denitrifying bacteria immobilized on polyvinyl alcohol-alginate, Fe$_3$O$_4$ nanoparticles, and bacterial cellulose, respectively. Their results showed that the bacterial cellulose-based bacteria immobilization body achieved 77.22% sewage total nitrogen removal. However, the
above-mentioned bacteria immobilization carriers generally face problems such as low mechanical strength and high price.

Biochar (BC) is produced by pyrolysis of the organic agricultural waste under the condition of limited oxygen, which is stable, difficult to dissolve in water, and with high carbon content [7, 8]. In recent years, scientists have found that BC has the characteristics of cost-effectiveness, various sources of feedstock and particular physical and chemical properties, such as large specific surface area and high porosity, which provide a favorable habitat for bacteria and enhance the pollutant removal efficiency and is very suitable as a microbial immobilization carrier [9, 10]. Many literatures have shown that BC is an ideal microbial immobilization carrier. Zhang et al. [11] demonstrated that the immobilization of Corynebacterium variabile HRJ4 with wood chips BC showed higher degradation of total petroleum hydrocarbons up to 78.9% after 7-d incubation as compared to the free bacteria. Abu Talha et al. [12] found that the degradation of Congo red dye of coconut shell BC-based immobilization body was significantly higher than that of free cells. The result was mainly due to the large specific surface area of coconut shell BC, which dramatically increased the contact area between dye and microbes and the active site on the BC, which can help the microbes to capture the contaminant in bulk solution. Moreover, previous studies showed that acid or alkali modifications can enhance NH$_4^+$-N adsorption capacity of BCs [13-15]. The increased active sites on modified BCs could facilitate the catchment of NH$_4^+$-N from bulk solution which was further degraded by the bacteria immobilized on the surface of BC and thus renewing the pores for subsequent adsorption. However, there is little study about modified biochar-based bacteria immobilization bodies on NH$_4^+$-N removal from water, and the underlying mechanism for different performances of BC-
based bacteria immobilization bodies, especially for the carriers of acid, alkali or metal ions
modified BCs, to remove NH$_4$$^+$-N in water is not very clear.

Therefore, the study prepared and characterized the HNO$_3$-, Mg$^{2+}$-, NaOH-, and
NaOH+Mg$^{2+}$-modified rice husk-derived BCs firstly. Then the immobilized bodies of the
heterotrophic nitrifying bacteria (HNB) screened from a wastewater treatment plant, based on all
the five kinds of BCs (including the original one), were prepared by using the adsorption method,
and NH$_4$$^+$-N removal efficiency of the BC-based HNB immobilized bodies and the potential
mechanism were explored. The study will serve as a theoretical basis for the large-scale
wastewater treatment engineering application of BC-based microbial immobilization.

2. Material and Methods

2.1. Enrichment and Isolation of Heterotrophic Nitrifying Bacteria

Heterotrophic nitrifying bacteria (HNB) were enriched from the activated sludge collected from
the oxidation ditch of Xi'an Wastewater Treatment Plant No. 3. HNB enrichment experiment was
operated by adding 20 mL supernatant of the active sludge to 180 mL heterotrophic nitrification
liquid medium (HNLM) (g/L, sodium citrate 4.32, (NH$_4$)$_2$SO$_4$ 0.548, KH$_2$PO$_4$ 1.50,
Na$_2$HPO$_4$$·$12H$_2$O 10.50, MgSO$_4$$·$7H$_2$O 0.20; microelement solution 2 mL/L, pH 7.0~7.5;
 microelement solution (g/L, ZnSO$_4$ 2.2, FeSO$_4$$·$7H$_2$O 3.0, CaCl$_2$ 5.5, MnCl$_2$$·$4H$_2$O 5.0,
 CuSO$_4$$·$5H$_2$O 1.6, CoCl$_2$$·$6H$_2$O 1.6, (NH$_4$)$_6$Mo$_7$O$_2$$·$4H$_2$O 1.1); pH 7.0~7.5), and then the mixture
was incubated on the shaker (30$^\circ$C, 170 r/min) for 3 d. After clarification, the supernatant was
collected and inoculated into HNLM at the ratio of 1% (v/v) and incubated for another 3 d (30$^\circ$C,
170 r/min), and this step was repeated for three times with the same manner. HNB enrichment experiment was manipulated with 6 replicates.

Pure HNB strains were gained through the plate smearing and repeated plate streaking method. The enriched HNB suspension was diluted with sterilized water for $10^{-7}$~$10^{12}$ times, and then 1 mL inoculum was spread evenly on the heterotrophic nitrification solid medium (HNSM) (the same nutrient content as HNLM but with agar (20 g/L) addition). In all, 36 Petri dishes were gained and transferred to a biochemical incubator (30°C) for about 4 d. Then the single colony (usually occurred at the $10^{-11}$ and $10^{-12}$ dilution gradients) was selected for repeated plate streaking separation.

To purify the isolated HNB, the selected single colony was streaked on HNSM using a sterilized inoculating loop and incubated at 30°C for about 4 d. The streaking manipulation was carried out about 3 times based on the previous streaked plate until all the colonies on the surface of the culture-medium were clear and without mixed and associated bacteria. In all, three pure HNB strains (designated as Strain-II, S2-1, and S2-2) were isolated, and they were saved on the agar slant culture-medium with HNB specificity at 4°C.

### 2.2. Identification of the Isolated HNB

#### 2.2.1. Biochemical identification

To explore NH$_4^+$-N degradation dynamics of the three isolated HNB strains, they were inoculated into 200 mL HNLM (initial NH$_4^+$-N 116.18 mg/L) and incubated on a shaker (170 r/min) at 30°C for 72 h, respectively. The thoroughly mixed suspension (4 mL) was withdrawn from the conical flask at an interval of 8 h during the incubation period. The concentration of
NH₄⁺-N, NO₃⁻-N, NO₂⁻-N, and total nitrogen (TN) was determined after the suspension was filtered through a 0.22 µm MF-Millipore Membrane. NH₄⁺-N, NO₃⁻-N, and NO₂⁻-N concentrations were determined by a colorimetric method using UV/Vis spectrophotometer (752, Jinghua Instruments, Shanghai) at the wavelength of 420, 220/275, and 540 nm, respectively. TN was determined by the alkaline potassium persulfate oxidation method [16]. Considering the potential function of heterotrophic nitrification and aerobic denitrification of Strain-II, just Strain-II was used for further study. The NH₄⁺-N degradation performance of the other two strains was shown in Fig. S1 of the Supplementary Materials (SM).

2.2.2. Molecular biological identification

Bacterium genomic DNA was extracted by using the Ezup column-type bacterium genomic DNA extraction Kit (Sango Biotech, Shanghai). Genomic DNA was subject to PCR amplification of 16S rDNA gene with the universal primers 27F (AGT TTG ATC MTG GCT CAG) and 1492R (GGT TAC CTT GTT ACG ACT T) [17]. PCR product was purified before sequencing using SanPrep column-type DNA gel extraction Kit (Sango Biotech, Shanghai) and then sequenced by automated DNA sequencer 3100 DNA Analyzer from Applied Biosystems using BigDye Terminator v1.1 cycle sequencing Kit by 1/2 reaction system (Applied Biosystems, USA).

Further, the sequence obtained was aligned manually with published sequences in the NCBI database using CLUSTALW multiple sequence alignment program. Phylogenetic analyses were conducted using MEGA version 4.0 and the neighbor-joining method was used to calculate
the distances and to construct a phylogenetic tree. The 16S rDNA gene sequence of Strain-II has been submitted to the GenBank database under accession number MK621209.

2.3. Immobilization of the Isolated HNB by Biochar

2.3.1. Biochar preparation

The biochar (BC) used for microbial immobilization was purchased from the company of Kansai (Japan), which was produced from rice husk at the carbonization temperature of 500°C. Besides the original rice husk-derived BC, there were four kinds of modified rice husk-derived BC, i.e. HNO$_3$-, Mg$^{2+}$-, NaOH-, and NaOH+Mg$^{2+}$-modified BCs, were also used as carriers for microbial immobilization. The detailed information about the method of making modified BC was shown in SM. pH and electrical conductivity (EC) of the original and modified BCs were determined with a BC to water ratio of 1:15 (g:mL) using a pH meter (Mettler Toledo Delta 320) and an EC meter (DDS-307A, Shanghai Instrument Science), respectively. The surface charge distribution of BC was characterized by pH$_{pzc}$, which was determined by the same method as Čerović et al. [18]. The specific surface area, total pore volume, and mean pore size of BC were determined by a surface area analyzer (Quadrasorb SI). BC surface oxygen-containing functional groups were determined by the Boehm titration method [19].

2.3.2. Biochar-based HNB immobilization body preparation

The BC-based HNB immobilization body was prepared by adsorption method using original and modified BCs, respectively. The saved Strain-II was inoculated into 200 mL LB liquid medium (g/L: tryptone 10.0, yeast extract 5.0, NaCl 10.0, pH 7.0~7.5), and incubated on a shaker (30°C,
170 r/min) for about 24 h until the OD$_{600}$ of the suspension reached 1.0 (OD$_{600}$, the absorbance at 600 nm). After centrifuged at 12,000 r/min for 3 min, the bacteria were washed by resuspension in sterilized water and centrifuging for another 2 times. Finally, the bacteria were resuspended in 200 mL sterilized water, which was called the seed solution. The prepared BC (2.0 g) was put into a sterilized conical flask containing 200 mL seed solution, and incubated on a shaker (30°C, 150 r/min) for 24 h to facilitate the bacteria immobilizing onto the surface of BC. After that, BC possessing immobilized bacteria was filtered and rinsed with sterilized water, which was named as BC-based HNB immobilization body.

2.4. Performance of Biochar-based HNB Immobilization Body and Biochar on NH$_4^+$-N Removal

To explore NH$_4^+$-N removal ability of the original and modified BC-based HNB immobilization bodies, 2.0 g BC-based HNB immobilization body was added in 300 mL conical flask containing 200 mL HNLM (the initial concentration of NH$_4^+$-N in HNLM was adjusted at 100, 200 and 300 mg/L, respectively). Then the conical flasks were put on a shaker (30°C, 170 r/min) for 48 h. The thoroughly mixed suspension of 1 mL was sampled from the conical flask at 1/6, 0.5, 1, 2, 5, 8, 12, 18, 24, 31, 39, and 48 h during the incubation. The concentration of NH$_4^+$-N was determined after the suspension was filtered through a 0.22 µm MF-Millipore Membrane. NH$_4^+$-N concentration was determined by the colorimetric method as mentioned before.

The highest NH$_4^+$-N removal efficiency that NaOH- and NaOH+Mg$^{2+}$-modified BC-based HNB immobilization bodies showed inspired us to explore how about NaOH- and NaOH+Mg$^{2+}$-modified BC adsorption in NH$_4^+$-N removal. Then the NH$_4^+$-N adsorption kinetics
experiment of the original and modified BCs was carried out. The original or modified BC (2.0 g) was put into a conical flask which contained 200 mL (NH₄)₂SO₄ solution with the initial NH₄⁺-N concentrations of 100, 200 and 300 mg/L, respectively. In all, there were 15 treatments (5 adsorbents × 3 initial concentrations), and each treatment was carried out with three replicates. Then the conical flasks were incubated on a shaker (30℃, 170 r/min) for 5 h. According to our previous study [20], the adsorption of NH₄⁺-N by BC would reach equilibrium for about 5 h. Then 10 mL suspension was sampled and filtrated through a 0.45 µm MF-Millipore Membrane, and the concentration of NH₄⁺-N was determined by the same colorimetric method.

2.5. Determination of Microbial Amount Adsorbed by Original and Modified Biochars

To explore the difference of microbial adsorption ability of the original and modified BCs, microbial amount adsorbed by BCs was determined. The number of living microbes could be indicated by the phospholipid content of cell membrane. The phospholipid analytical technique was used to evaluate the number of microbes attached to the surfaces of BC [21]. The HNB immobilization body based on 0.1 g BC was added to a sterilized conical flask containing phospholipid extract solution (10 mL chloroform, 20 mL methanol, and 8 mL water, with the volume ratio of 1:2:0.8). After shaking for 10 min vigorously and standing still for 12 h, 10 mL chloroform and 10 mL water were compensated making the chloroform: methanol: water volume ratio being right on 1:1:0.9, and then the suspension was standing still for 12 h again. The chloroform containing the phospholipid in the deep layer of the mixture was transferred to a digestion tube, and the chloroform was evaporated in a water bath at 70℃. After digested by 4
mL potassium persulfate (5%, 5 g/100 mL) at 120°C for 30 min, the extracted phospholipid (nmol P/g BC) was determined by a colorimetric method at the wavelength of 700 nm.

2.6. Data Analysis
The variance between any triplicate measurements in this study was smaller than 5%, and the average value ± standard deviation was reported (n = 3). Variance analysis (one-way ANOVA) was examined by the protected LSD multiple range test (p < 0.05) using the software Statistica 6.0. The graphing was performed using OriginPro 8.0 software.

3. Results and Discussion
3.1. Biochemical and Molecular Identification of Heterotrophic Nitrifying Bacteria (HNB)
The isolated single colony was inoculated into the heterotrophic nitrification liquid medium (HNLM), and the nitrifying ability of Strain-II was identified by measuring the dynamic changes of NH$_4^+\text{-N}$, NO$_3^-$-N, NO$_2^-$-N, and TN concentration in the medium. The results indicated that NH$_4^+\text{-N}$ decreased from 116.18 to 49.75 mg/L during 0~24 h, and the average degradation rate was 2.77 mg/(L·h). However, a slow NH$_4^+\text{-N}$ degradation rate (0.56 mg/(L·h)) was observed during 24~72 h, and NH$_4^+\text{-N}$ reduced to 22.96 mg/L at the end of incubation. TN decreased from 116.18 to 91.37 mg/L during the whole incubation, and the average degradation rate was 0.35 mg/(L·h). NO$_3^-$-N and NO$_2^-$-N were always stayed at the relatively low level, with the maximum value of 9.80 and 1.37 mg/L, respectively (Fig. 1). In all, the isolated Strain-II has the heterotrophic nitrification ability.
The neighbor-joining phylogenetic tree of 16S rDNA gene of the isolated Strain-II was shown in Fig. 2. The homology comparison with the nucleic acid sequences in the GenBank showed that the isolated Strain-II had a closer evolutionary distance (similarity 99%) with Pseudomonas sp. strain SMCC B0310 (AF500620). So, the isolated strain was named as Pseudomonas sp. Strain-II (MK621209). The genus Pseudomonas has been recorded frequently possessing the ability of heterotrophic nitrification [22, 23].

![Image of phylogenetic tree]

**Fig. 1.** The dynamics of inorganic nitrogen and total nitrogen concentrations in the enrichment liquid medium with HNB specificity.

![Image of nitrogen dynamics graph]

**Fig. 2.** Neighbor-joining phylogenetic tree of 16S rDNA gene of the heterotrophic nitrifying bacterium *Pseudomonas* sp. Strain-II isolated from the active sludge (bootstrap values (100 iterations) greater than 50% are shown).
Table 1. The Physicochemical Properties of the Original and Modified Rice Husk-derived Biochars (BCs)

<table>
<thead>
<tr>
<th></th>
<th>Original BC</th>
<th>HNO₃-modified BC</th>
<th>Mg²⁺-modified BC</th>
<th>NaOH-modified BC</th>
<th>NaOH+Mg²⁺-modified BC</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH (BC/water ratio, 1 g:15 mL)</td>
<td>8.78 ± 0.27 a</td>
<td>4.30 ± 0.30 b</td>
<td>7.78 ± 0.62 c</td>
<td>9.33 ± 0.37 a</td>
<td>10.20 ± 0.30 d</td>
</tr>
<tr>
<td>EC (mS/m)</td>
<td>25.00 ± 1.67 a</td>
<td>45.13 ± 2.21 b</td>
<td>17.97 ± 1.47 c</td>
<td>23.27 ± 2.46 ad</td>
<td>21.13 ± 1.55 cd</td>
</tr>
<tr>
<td>pH&lt;sub&gt;pzc&lt;/sub&gt;</td>
<td>5.77 ± 0.15 a</td>
<td>2.67 ± 0.31 b</td>
<td>6.13 ± 0.65 a</td>
<td>8.87 ± 0.57 c</td>
<td>8.13 ± 0.76 c</td>
</tr>
<tr>
<td>Carboxyl (mmol/g)</td>
<td>0.288 ± 0.040 a</td>
<td>0.954 ± 0.185 b</td>
<td>0.279 ± 0.138 a</td>
<td>0.018 ± 0.000 c</td>
<td>0.047 ± 0.000 c</td>
</tr>
<tr>
<td>Carbonyl (mmol/g)</td>
<td>0.536 ± 0.157 a</td>
<td>0.536 ± 0.157 a</td>
<td>0.770 ± 0.157 ab</td>
<td>0.047 ± 0.000 c</td>
<td>0.047 ± 0.000 c</td>
</tr>
<tr>
<td>Phenolic hydroxyl (mmol/g)</td>
<td>0.171 ± 0.110 a</td>
<td>0.224 ± 0.052 a</td>
<td>0.225 ± 0.086 a</td>
<td>0.022 ± 0.045 b</td>
<td>0.082 ± 0.096 ab</td>
</tr>
<tr>
<td>Total acidic oxygen-containing functional group (mmol/g)</td>
<td>0.996 ± 0.245 a</td>
<td>2.084 ± 0.220 b</td>
<td>1.039 ± 0.210 a</td>
<td>0.797 ± 0.160 a</td>
<td>0.789 ± 0.097 a</td>
</tr>
<tr>
<td>Specific surface area (m&lt;sup&gt;2&lt;/sup&gt;/g)</td>
<td>53.77</td>
<td>85.54</td>
<td>88.80</td>
<td>160.92</td>
<td>191.28</td>
</tr>
<tr>
<td>Total pore volume (cm&lt;sup&gt;3&lt;/sup&gt;/g)</td>
<td>0.0435</td>
<td>0.0649</td>
<td>0.0648</td>
<td>0.1238</td>
<td>0.1392</td>
</tr>
<tr>
<td>Mean pore size (nm)</td>
<td>3.24</td>
<td>3.03</td>
<td>2.92</td>
<td>3.08</td>
<td>2.91</td>
</tr>
</tbody>
</table>

Note: data in the same row followed by the same letter are not significantly different at p<0.05 level of probability; mean ± SD (n=3). The data of specific surface area, total pore volume, and mean pore size refer to the mesopore (2-50 nm) of BC.

3.2. Effects of Different Modification Methods on Biochar Physicochemical Properties

The physicochemical properties of the original and modified rice husk-derived biochars (BCs) were shown in Table 1. BC’s inherent characteristics decided its suitability as a microbial immobilization carrier. The pH of HNO₃- and Mg²⁺-modified BCs (4.30 ± 0.30 and 7.78 ± 0.62, respectively) was significantly lower than original BC (8.78 ± 0.27), while the pH of NaOH+Mg²⁺-modified BC (10.20 ± 0.30) was significantly higher than original BC (p < 0.05). The pH<sub>pzc</sub> of HNO₃-modified BC (2.67 ± 0.31) was significantly lower than original BC (5.77 ± 0.15), while the pH<sub>pzc</sub> of NaOH- and NaOH+Mg²⁺-modified BCs (8.87 ± 0.57 and 8.13 ± 0.76, respectively) was significantly higher than original BC (5.77 ± 0.15).
respectively) was significantly higher than original BC (p < 0.05). Though the total acidic oxygen-containing functional group amount of NaOH- and NaOH+Mg$^{2+}$-modified BCs (0.797 ± 0.160 and 0.789 ± 0.097 mmol/g, respectively) were lower than original BC (0.996 ± 0.245 mmol/g), the difference was not significant (p < 0.05). The total alkaline oxygen-containing functional group amount of NaOH-modified BC (1.317 ± 0.142 mmol/g) was significantly higher than the original BC (0.787 ± 0.175 mmol/g). Moreover, the total alkaline oxygen-containing functional group amount of NaOH+Mg$^{2+}$-modified BC (1.015 ± 0.126 mmol/g) was also higher than the original BC, but the difference was not significant (p < 0.05). Compared with original BC, all the four kinds of modification methods increased the specific surface area of the BCs with different extent. The specific surface area of the HNO$_3$-, Mg$^{2+}$-, NaOH-, and NaOH+Mg$^{2+}$-modified BCs was 1.59, 1.65, 2.99, and 3.56 times higher than the original BC, respectively.

HNO$_3$ modification significantly (p < 0.05) increased BC carboxyl, carbonyl, and total acidic oxygen-containing functional group amounts with the greatest extent among all the four modification methods. The acidic oxygen-containing functional group of HNO$_3$-modified BC released H$^+$, making the lowest value of pH$_{pzc}$ [24]. Moreover, the corrosion effect of HNO$_3$ on BC pore walls led to increased specific surface area and total pore volume [25]. However, the acidic oxygen-containing functional group has a definite antibacterial effect, and the H$^+$ released into the solution may cause bacteria activity decrease [26].

NaOH and NaOH+Mg$^{2+}$ modification significantly increased the pH and pH$_{pzc}$ of BC, which resulted from the neutralization of biochar acidic oxygen-containing functional group with alkali. Shim et al. [25] showed that NaOH modification decreased the carboxyl amount of
activated carbon, rendering increased \( \text{pH}_{\text{pzc}} \), and inferred that the amount of acidic oxygen-containing functional group was the major factor controlling \( \text{pH}_{\text{pzc}} \). A similar result was further explored by Zhou et al. [24]. The specific surface area of BC was linked to its pore structure closely. \( \text{NaOH} \) and \( \text{NaOH}+\text{Mg}^{2+} \) modification made the biochar specific surface area increase by 1.99 and 2.56 times compared with the original BC, respectively, which may result from the etching effect of \( \text{NaOH} \) on BC pores [27].

3.3. Performance of Biochar-based HNB Immobilization Body on \( \text{NH}_4^+ \)-N Removal

\( \text{NH}_4^+ \)-N removal dynamics of the original and modified BC-based HNB immobilization bodies was explored within 48 h under the initial \( \text{NH}_4^+ \)-N concentration of 100, 200, and 300 mg/L. The results showed that \( \text{NH}_4^+ \)-N removal ratio of the original, \( \text{HNO}_3^- \), \( \text{Mg}^{2+}^- \), \( \text{NaOH}^- \), and \( \text{NaOH}+\text{Mg}^{2+}^- \)-modified BC-based HNB immobilization bodies during 48 h was 42.20\%, 45.73\%, 55.58\%, 88.66\%, and 90.93\%, respectively at the initial \( \text{NH}_4^+ \)-N concentration of 100 mg/L. Moreover, at the initial \( \text{NH}_4^+ \)-N concentration of 200 mg/L, \( \text{NH}_4^+ \)-N removal ratio was 33.60\%, 45.59\%, 51.40\%, 76.40\%, and 78.67\%, respectively, and at the initial \( \text{NH}_4^+ \)-N concentration of 300 mg/L, \( \text{NH}_4^+ \)-N removal ratio was 24.92\%, 25.77\%, 28.80\%, 45.04\%, and 47.06\%, respectively. \( \text{NaOH}+\text{Mg}^{2+}^- \)-modified BC-based HNB immobilization bodies possessed the strongest ability to remove \( \text{NH}_4^+ \)-N from water, while the original BC-based HNB immobilization bodies possessed the weakest ability to remove \( \text{NH}_4^+ \)-N either with the initial \( \text{NH}_4^+ \)-N concentration of 100, 200 or 300 mg/L. On the other hand, as the initial \( \text{NH}_4^+ \)-N concentration increased from 100 to 300 mg/L, \( \text{NH}_4^+ \)-N removal ratio of the original and modified BC-based HNB immobilization bodies was decreased (Fig. 3(a), (c), (e)).
NH₄⁺-N removal rate of the original, HNO₃-, Mg²⁺-, NaOH-, and NaOH+Mg²⁺-modified BC-based HNB immobilization bodies reached the peak at about 5 h after incubation either with the initial NH₄⁺-N concentration of 100, 200 or 300 mg/L. The high-efficiency period of NH₄⁺-N removal of the immobilization bodies occurred from 1 to 8 h, and the NH₄⁺-N removal rate tended to be zero at 24 h. Moreover, among all the five kinds of BCs, NaOH- and NaOH+Mg²⁺-modified BC-based HNB immobilization bodies showed the maximum NH₄⁺-N removal rate (100 mg/L: 15.64 and 15.20 mg/(L∙h); 200 mg/L: 25.73 and 26.63 mg/(L∙h); 300 mg/L: 17.82 and 17.43 mg/(L∙h)) (Fig. 3(b), (d), (f)).

NH₄⁺-N removal rates of bacteria immobilization body in water solution varied considerably under various initial NH₄⁺-N concentration, immobilization method, and microorganism species. Yu et al. [28] indicated the complex of modified walnut shell biochar and Pseudomonas stutzeri strain XL-2 showed the maximum average NH₄⁺-N removal rate of 4.40 mg/(L∙h), and approximately 96.34%-98.73% of NH₄⁺-N was removed in a sequencing batch reactor inoculating with the complex of strain XL-2 and biochar. Compared with our study, the relatively low NH₄⁺-N removal rate may result from the low initial NH₄⁺-N input. Khan et al. [29] observed stable nitritation over 300 days with NH₄⁺-N loading rate of 45.83 mg/(L∙h) in a sequencing batch reactor filled with cell-immobilized polyethylene glycol pellets and found NH₄⁺-N removal rates were more than 43.54 mg/(L∙h). The entrapping method of bacteria immobilization used in the study made a relatively higher microorganism concentration than the adsorption method used in our study. Moreover, the microorganism species harbored in the immobilization body also played an important role in nitrogen removal rate. For example, Qiao et al. [30] carried out co-immobilization of partial nitrifying and anammox biomass to achieve
single-stage autotrophic nitrogen removal, and they found that the total nitrogen removal rate reached 70.42 mg/(L·h) in a continuous experiment with nitrogen loading rate up to 91.67 mg/(L·h).

Fig. 3. NH₄⁺-N removal dynamics of the original and modified biochar-based HNB immobilization bodies under the initial NH₄⁺-N concentration of 100 (a, b), 200 (c, d), and 300 (e, f) mg/L.
3.4. Biochar-based HNB Immobilization Body Performed Better to Remove NH$_4^+$-N than Biochar Itself

After 5 h incubation, the removal ratio of NH$_4^+$-N by BC-based HNB immobilization bodies was significantly higher than BC itself. Under the condition of loading Pseudomonas sp. Strain-II, NH$_4^+$-N removal ratio ranged from 28.47% to 58.35%, 24.00% to 54.27%, and 13.07% to 25.52% at the initial NH$_4^+$-N concentration of 100, 200, and 300 mg/L, respectively, with the order of NaOH+Mg$^{2+}$-modified BC > NaOH-modified BC > Mg$^{2+}$-modified BC > HNO$_3$-modified BC > original BC. However, under the condition of unloading Pseudomonas sp. Strain-II, NH$_4^+$-N removal ratio ranged from 4.74% to 20.43%, 11.58% to 27.10%, and 4.37% to 13.44% at the initial NH$_4^+$-N concentration of 100, 200, and 300 mg/L, respectively, with the order of NaOH+Mg$^{2+}$-modified BC > NaOH-modified BC > original BC > HNO$_3$-modified BC > Mg$^{2+}$-modified BC (Table 2).

NaOH and NaOH+Mg$^{2+}$ modification enhanced NH$_4^+$-N adsorption ability of the rice husk-derived BC. At the initial NH$_4^+$-N concentration of 100 mg/L, NH$_4^+$-N removal ratio shift from 15.37% (original BC) to 17.10% (NaOH-modified BC) and 20.43% (NaOH+Mg$^{2+}$-modified BC) after 5 h incubation. Moreover, at the initial NH$_4^+$-N concentration of 200 and 300 mg/L, NH$_4^+$-N removal ratio of NaOH and NaOH+Mg$^{2+}$ treatments was also higher than the original BC treatment with different extent (Table 2). Vu et al. [13] also concluded that the corncob BC modified by HNO$_3$+NaOH had better NH$_4^+$-N adsorption capacity than the original and HNO$_3$-modified BCs.
The rice husk-derived BC loaded with Pseudomonas sp. Strain-II, i.e. BC-based microbial immobilization body showed a much stronger ability to eliminate NH$_4^+$-N compared with BC itself. The result indicated that besides NH$_4^+$-N adsorption, NH$_4^+$-N microbial degradation played a much more prominent role in NH$_4^+$-N removal by BC-based microbial immobilization body in our study. Moreover, among all the five materials as the carrier of Pseudomonas sp. Strain-II, NaOH- and NaOH+Mg$^{2+}$-modified BC-based microbial immobilization bodies showed the strongest ability to remove NH$_4^+$-N. The main reason for this phenomenon may be that NaOH- and NaOH+Mg$^{2+}$-modified BC possessed significantly stronger bacteria adhering ability than original, HNO$_3$- or Mg$^{2+}$-modified BCs.

**Table 2.** Removal of NH$_4^+$-N by Biochar-immobilized *Pseudomonas* sp. Strain-II and Biochar Without Loading Bacteria (170 r/min, 30°C, 5 h incubation)

<table>
<thead>
<tr>
<th></th>
<th>100 mg/L</th>
<th>200 mg/L</th>
<th>300 mg/L</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Final concentration (mg/L)</td>
<td>Removal ratio (%)</td>
<td>Final concentration (mg/L)</td>
</tr>
<tr>
<td><strong>Loading</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Pseudomonas</em> sp. Strain-II</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Original BC</td>
<td>71.53 ± 6.54</td>
<td>28.47%</td>
<td>151.99 ± 6.36</td>
</tr>
<tr>
<td>HNO$_3$-modified BC</td>
<td>70.19 ± 6.66</td>
<td>29.81%</td>
<td>141.46 ± 7.28</td>
</tr>
<tr>
<td>Mg$^{2+}$-modified BC</td>
<td>63.49 ± 4.39</td>
<td>36.51%</td>
<td>128.43 ± 6.33</td>
</tr>
<tr>
<td>NaOH-modified BC</td>
<td>42.22 ± 2.39</td>
<td>57.78%</td>
<td>95.10 ± 5.76</td>
</tr>
<tr>
<td>NaOH+Mg$^{2+}$-modified BC</td>
<td>41.65 ± 3.51</td>
<td>58.35%</td>
<td>91.46 ± 5.18</td>
</tr>
<tr>
<td><strong>Unloading</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Pseudomonas</em> sp. Strain-II</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Original BC</td>
<td>84.63 ± 1.66</td>
<td>15.37%</td>
<td>171.44 ± 1.39</td>
</tr>
<tr>
<td>HNO$_3$-modified BC</td>
<td>92.67 ± 0.17</td>
<td>7.33%</td>
<td>172.82 ± 1.21</td>
</tr>
<tr>
<td>Mg$^{2+}$-modified BC</td>
<td>95.26 ± 6.03</td>
<td>4.74%</td>
<td>176.84 ± 2.42</td>
</tr>
<tr>
<td>NaOH-modified BC</td>
<td>82.90 ± 1.47</td>
<td>17.10%</td>
<td>157.87 ± 6.85</td>
</tr>
<tr>
<td>NaOH+Mg$^{2+}$-modified BC</td>
<td>79.57 ± 0.79</td>
<td>20.43%</td>
<td>145.80 ± 1.21</td>
</tr>
</tbody>
</table>
3.5. Microbe Amount Adhering to the Surfaces of the Original and Modified Biochars

To explore the reason for the different NH$_4^+$-N removal performance of the original and modified BC-based HNB immobilization bodies, the number of living microbes adhering to the surface of the original and modified BCs were determined, which was indicated by the phospholipid content of cell membrane. After 24-h adsorption of Pseudomonas sp. Strain-II by BC, the BC-based immobilization body was isolated and its phospholipid content was determined. The result showed that all the four modification ways increased the bacteria adsorption ability, but with different extent. The phospholipid contents of the NaOH+Mg$^{2+}$- and NaOH-modified BC-based immobilization body (941.17 and 773.75 nmol P/g BC, respectively) were significantly higher than the original BC-based immobilization body (608.12 nmol P/g BC). However, there was no significant difference between Mg$^{2+}$- or HNO$_3$-modified BC-based immobilization body (642.20 and 612.70 nmol P/g BC) and the original BC (p < 0.05) (Fig. 4). The phenomenon indicated that NaOH+Mg$^{2+}$ and NaOH modification improved the ability of BC to adsorb microorganisms greatly, but not for Mg$^{2+}$ or HNO$_3$ modification methods.

![Fig. 4. Phospholipid content of Pseudomonas sp. Strain-II immobilized by the original and modified biochars (Bars following the same letter are not significantly different at p < 0.05 level of probability, n = 3).](image-url)
Why NaOH- and NaOH+Mg$^{2+}$-modified biochar possessed prominent adsorption ability of HNB? When bacteria adhered to the surface of BC, the free bacteria and the BC hydrophobic surface attracts each other through van der Waals force, electrostatic force, etc., and then the viscous long-chain exopolysaccharide secreted by bacteria make the bacteria adhere to the surface of BC [31]. However, in our study, there are three main reasons making NaOH- and NaOH+Mg$^{2+}$-modified BC possess prominent adsorption ability of HNB.

Firstly, bacteria adsorption capacity of BC relies on the large specific surface area and the pore structure of BC [12]. Generally, a large specific surface area and a rich pore structure could enhance the adsorption capacity of biochar, which may not only help bacteria adsorb to the surface of BC, but also increase the chance of contact between bacteria and NH$_4^+$-N [12, 32]. Therefore, the NaOH- and NaOH+Mg$^{2+}$-modified BCs with the large specific surface area (2.99 and 3.56 times of original BC) facilitated the adsorption of bacteria more readily than the original, HNO$_3$- and Mg$^{2+}$-modified BCs. Secondly, Mg$^{2+}$ loaded on the surface of BC could form a large number of metal derivatives with the functional groups of BC by ion exchange or complexation. Mg$^{2+}$ derivatives formed on the surface of BC increased BC surface roughness and adsorption site, facilitating bacteria adhering [33, 34]. Thirdly, the electrostatic attraction between bacteria and BC is also a major reason why bacteria can adhere to the surface of BC [35]. Generally, the functional group on cell outer membrane of microbe has a net negative charge, which can be adsorbed by electrostatic attraction of the positively charged BC [36].

When preparing the BC-based microbial immobilization body, the original and modified BCs were immerged into the seed solution containing enriched HNB. However, the pH of the seed solution (near neutral) was lower than the pH$_{pzc}$ of NaOH- and NaOH+Mg$^{2+}$-modified BCs (8.87
± 0.57 and 8.13 ± 0.76, respectively), making the surface of NaOH- and NaOH+Mg$^{2+}$-modified 
BCs be positively charged [24,37]. And then when NaOH- and NaOH+Mg$^{2+}$-modified BCs 
contacted the bacteria with negative charge, the electrostatic attraction between them makes 
bacteria adhere to the surface of NaOH- and NaOH+Mg$^{2+}$-modified BCs.

NaOH- and NaOH+Mg$^{2+}$-modified BC-based HNB immobilization bodies performed 
better than original, HNO$_3$- and Mg$^{2+}$-modified BCs on NH$_4^+$-N removal from water. Besides the 
distinguished NH$_4^+$-N adsorption ability and microbe adsorption capacity of NaOH- and 
NaOH+Mg$^{2+}$-modified BCs, the significantly large amount of surface alkaline oxygen-containing functional group and the consequent high pH of them (9.33 ± 0.37 and 10.20 ± 0.30, 
respectively) may also play an important role in promoting ammonia oxidation. NH$_3$ is the 
substrate for ammonia-oxidizing microbes, and its concentration increases exponentially with 
increasing pH due to the deionization of NH$_4^+$ to NH$_3$ [38]. The considerable basic ions and 
groups in the region of biochar-water interface may facilitate the deionization process. On the 
other hand, nitrification could consume alkalinity, and generally high pH is conducive to 
nitrification [39]. Incidentally, oxygen is another important factor influencing nitrification. 
However, the air-permeable reaction apparatus and the constant rotation speed during incubation 
in our study could make no significant difference of oxygen concentration in the reaction system 
between treatments. In all, the alkaline property, large HNB adsorption capacity and NH$_4^+$-N 
adsorption ability of NaOH- and NaOH+Mg$^{2+}$-modified BCs render their immobilization bodies 
show excellent NH$_4^+$-N removal efficiency.
4. Conclusions

A strain of heterotrophic nitrifying bacteria (HNB) was isolated, which was classified as *Pseudomonas*, and the 72-h NH$_4^+$-N removal ratio of the free bacteria reached 80.24%. NH$_4^+$-N adsorption by the original and modified BC itself contributed little to NH$_4^+$-N removal from water solution. Compared with the BC without loading HNB, the BC-based HNB immobilization body showed a much stronger ability to remove NH$_4^+$-N, especially for the NaOH- and NaOH+Mg$^{2+}$-modified BCs. At the initial NH$_4^+$-N concentration of 100 mg/L and biochar addition dose of 10 g/L, NH$_4^+$-N removal ratio of NaOH- and NaOH+Mg$^{2+}$-modified BC-based HNB immobilization bodies reached 57.78% and 58.35% after 5 h, and reached 88.66% and 90.93% after 48 h respectively, which were obviously higher than the original, HNO$_3$- and Mg$^{2+}$-modified BC-based HNB immobilization bodies. The phenomenon resulted from the significantly ($p < 0.05$) higher bacteria adsorption ability of NaOH- and NaOH+Mg$^{2+}$-modified BCs, which reached 773.75 and 941.17 nmol P/g BC, respectively.

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Author Contributions

C.W. (associate professor) conceived and designed the experiments. C.W. (associate professor) and J.R. (master student) performed the data analyses and wrote the manuscript. X.Q. (master student) conducted the experiments. M.H. (master student) helped review and edit the
manuscript.

References


