sustainability

Article

Biochar Amendment in Vermi-Wetland for Enhancing Nitrification during Excess Sludge Recycling

Ting Bai [1,†], Gratien Twagirayezu [2,3,4,†], Zhen Wang [1], Hui Xia [2,*], Chunlei Sang [2], Kui Huang [2] and Hongguang Cheng [3]

[1] Gansu Research Institute of Chemical Industry Co., Ltd., Lanzhou 730070, China; baiting860926@163.com (T.B.)
[2] School of Environmental and Municipal Engineering, Lanzhou Jiaotong University, Lanzhou 730070, China; tgratien0@gmail.com (G.T.)
[3] State Key Laboratory of Environmental Geochemistry, Institute of Geochemistry, Chinese Academy of Sciences, Guiyang 550002, China
[4] University of Chinese Academy of Sciences, Beijing 100049, China
* Correspondence: xiahui@mail.lzjtu.cn; Tel./Fax: +86-09314938027
† These authors contributed equally to this work.

Abstract: Vermi-wetland is a sustainable technology for recycling excess sludge in small-town areas. Although biochar (BC) amendment into the vermi-wetland could considerably boost the effectiveness of treating sludge, its impact on the nitrogen transformation in vermi-wetland remains unclear. Hence, this study aimed to explore the mechanism and performance of BC amendment into the vermi-wetland for enhancing nitrogen transformation during excess sludge recycling. The semi-aquatic plant *Acorus calamus* and the earthworm *Eisenia fetida* were planted in the designed vertical vermi-reactor, with corncob BC added to the upper and lower layers of one vermi-reactor, in comparison with the vermi-reactor without BC. The vermi-reactor with BC significantly lowered ($p < 0.05$) ammonia nitrogen (NH_4^+-N) and nitrite nitrogen (NO_2^--N) in the effluent by 1.63 and 4.85-fold, respectively, and increased considerably nitrate nitrogen (NO_3^--N) in the effluent by 1.5-fold. The numbers of ammonia-oxidizing bacteria (AOB) and archaea (AOA) in the vermi-reactor with BC were greatly enriched by 6 and 1.42-fold, compared with their counterparts ($p < 0.05$). Moreover, *nirS* and *nirK* gene copies in the vermi-reactor with BC were considerably improved ($p < 0.05$) by 2.03 and 1.82-fold, respectively. BC significantly enhanced the growth of earthworms by 6.92-fold and promoted plant growth by 1.28-fold. In addition, the AOB members like *Nitrosomonas* and *Nitrosospira* and the AOA members like *Crenarchaeota* and *Thaumarchaeota* cohabited in BC. Overall, these results suggest that a vermi-reactor amended with BC could enhance the nitrification processes of excess sludge, thereby improving the treatment performance of vermi-wetland.

Keywords: ammonia oxidation; biochar; earthworms; vermicomposting; sludge treatment; wetland plant

check for updates

Citation: Bai, T.; Twagirayezu, G.; Wang, Z.; Xia, H.; Sang, C.; Huang, K.; Cheng, H. Biochar Amendment in Vermi-Wetland for Enhancing Nitrification during Excess Sludge Recycling. *Sustainability* **2023**, *15*, 16551. https://doi.org/10.3390/su152416551

Academic Editor: Antonio Caggiano

Received: 27 October 2023
Revised: 25 November 2023
Accepted: 27 November 2023
Published: 5 December 2023

1. Introduction

Recently, the construction of wastewater treatment facilities has progressively increased due to the rapid development of small cities and rural regions, resulting in an enormous amount of excess sludge. Nevertheless, excess sludge treatment and disposal have emerged as a worldwide concern due to a significant quantity of water and unstable organic compounds [1]. However, if sludge is well managed and treated with proper procedures, it can be repurposed and utilized beneficially in various farming activities, contributing to sustainable agricultural practices and resource optimization. The typical methods for disposing of excess sludge include anaerobic digestion, incineration, landfilling, composting, and thermal drying. These techniques require consolidation and dehydration before sludge disposal, which incurs high costs [2]. Therefore, these excess sludge treatment and disposal options are not cost-effective in underdeveloped and developing nations, especially in rural regions and smaller cities. In this regard, a sustainable and cost-effective approach that can transform organic matter using microorganisms and certain abiotic species is a better choice for excess sludge recycling [1,3]. Thus, integrating excess sludge treatment and disposal is imperative to generate usable effluent for farming.

A vermi-wetland is a plant-based vermi-filter that efficiently achieves the integrated treatment and disposal of sewage sludge through the cooperation of wetland vegetation, worms, and bacteria [1]. Due to its cost-effectiveness and efficient treatment performance, this strategy is an economically, environmentally, and sustainable option in rural regions and small cities. Unlike the vermi-filter, the activities of vermi-bacteria are influenced by the actions of earthworms, including feeding, burrowing, and secretion of casts and mucus [4,5]. Plants in wetlands significantly transport microbial populations, activities, and communities as they cleanse pollutants, emit oxygen, and act as breeding grounds for microorganisms [6]. As an eco-friendly approach, vermi-wetland operates better than the traditional vermi-filter technique for treating sludge [1]. In a vermi-wetland, chemical oxygen demand (COD) and total solids (TS) removal rate during excess sludge treatment can exceed 80 and 60%, respectively [1,7]. However, the nitrogen transformation in vermi-wetland is not currently known. Therefore, investigating its transformation processes has great significance.

BC is a relatively enduring carbon-rich substance created at exceedingly elevated temperatures, possessing numerous benefits due to having an outstanding level of carbon concentration, a broad specific surface area, substantial adsorption capability, and exceptional durability [8]. Hence, it is extensively utilized in agriculture, environmental protection, and energy generation [9]. It also speeds up organic matter degradation during vermicomposting of sludge [3,10] and enhances soil fertilization [11]. BC is used in water treatment, wastewater recycling [12], and soil remediation [8]. BC can significantly enrich the microbial community and often increase its variety in the soil, benefiting plant development [13] and improving agricultural yields. BC can enhance the quality of effluent by boosting plant growth, earthworm biomass, and growth rate during excess sludge treatment [3]. BC was used in vermi-wetland to promote the removal efficiency of organic solids in the vermi-wetland during the recycling of excess sludge [3]. BC was employed in a vermi-wetland to enhance the removal efficiency of organic solids during excess sludge recycling [3]. However, the impact of BC on the nitrification process in constructed wetlands during excess sludge recycling needs to be fully elucidated.

It is apparent that AOB and AOA drive the ammonia-oxidizing process, a rate-limiting phase in nitrification processes [14,15]. Most studies have focused on the dispersion and role of oxidizers of oxidizers (AOA and AOB) in diverse ecosystems [16,17]. For instance, mounted evidence indicates that the AOA dominates the soil ecosystems, displaying a greater quantity and a richer diversity of communities than the AOB [18]. However, previous studies have found that the AOB members are the predominant contributors to nitrification in the composting systems [19,20] and constructed wetland systems [21] compared to AOA members. Even though the detailed habitats and functions of AOA and AOB in ecological systems have been contested, the dominant status difference between AOA and AOB influences the quality of excess effluent sludge still needs to be well explored. In addition, the influences of BC on microbial nitrogen removal in vermi-wetland during excess sludge recycling remain unexplored. Therefore, adding BC into a vermi-reactor for investigating microbial nitrogen removal during excess sludge treatment is highly appreciated.

The goal of the present study was to scrutinize the influence of BC on nitrification in the vermi-reactor during excess sludge recycling and to clarify the mechanism of nitrogen transformation in the vermi-reactor. Herein, vermi-reactors without and with BC were separately installed. *Acorus calamus* and the earthworm *Eisenia fetida* were inoculated in these vermi-reactors, with corncob BC added to the upper and lower layers of one vermi-reactor to compare their recycling performance. In addition, the adsorption processes on BC were also determined. To determine the quantities of *nirS* genes, *nirK* copies, and *amoA* gene copies, qPCR was used. High-throughput sequencing technology was also used to analyze AOA and AOB communities that carry the *amoA* genes.

2. Materials and Methods

2.1. Materials

Eisenia fetida earthworm species were chosen due to their potential for surviving in adverse and poisonous environments [22], with higher efficiency during excess sludge recycling [1]. Plants and earthworms were cultivated in the laboratory for six months to adapt to the sludge environment before the experiment. The corncob BC with a diameter of 600 mm was purchased from the Jincheng Company in Dalian, China. Table S1 shows the characteristics of BC used in this study. Sludge vermicompost was utilized as vermi-bedding for the development of plants and earthworms. The properties of vermicompost are shown in Table S2. About 3–5 mm of medical stone and 12–16 mm of ceramsite were acquired from the Heping flower market in Lanzhou, China. Before being amended in vermi-reactor, they were cleansed separately by immersing them in piped water for 24 h. Vermi-reactors were prepared using buckets of polyethylene plastic with a few holes at the bottom for draining the effluent. Excess sludge was taken within 2 days at the Qilihe sewage treatment facility in the Anning District of Lanzhou, China. Simultaneously, it was fed to the vermi-reactors and sampled for quality analysis. Table S3 illustrates the characteristics of fresh sludge.

2.2. Experimental Set-Up

In this study, two identical vermi-reactors were constructed based on the prior test of four vermi-reactors with different fixed BCs. In a control reactor without BC, the top layer of the vermi-reactor was filled with 10 cm in the height of vermicompost, the middle layer contained 15 cm ceramsite, and the lower layer consisted of 5 cm of medical stone (Figure 1a). The vermi-reactor lower layer was equipped with an effluent collection system. A similar vermi-reactor was placed on the side and filled with 10 cm of vermicompost mixed with 3% granulated BC (Figure 1b). A 5 cm layer of BC was placed below the medical stone in the middle of the vermi-reactor. In each vermi-reactor, 100 *E. fetida* worms and two strains of *A. calamus* plants were introduced [1]. About 600 mL of fresh excess sludge was simultaneously fed onto the vermicompost layer of each vermi-reactor every two days for 32 days. Effluent samples were collected on the second day after each feeding. All vermi-reactors were well operated for 32 days at room temperature between 18 and 28 °C. However, at the end of the experiment, earthworms and plants were separately reaped from the vermi-reactor and then cleansed with fresh water to get an accurate measurement. Subsequently, all vermi-reactors were dissected to acquire internal samples from every stratum. The collected samples were placed in a fridge at 20 °C for DNA investigation, whereas the remaining samples were air-dried to analyze their physicochemical characteristics.

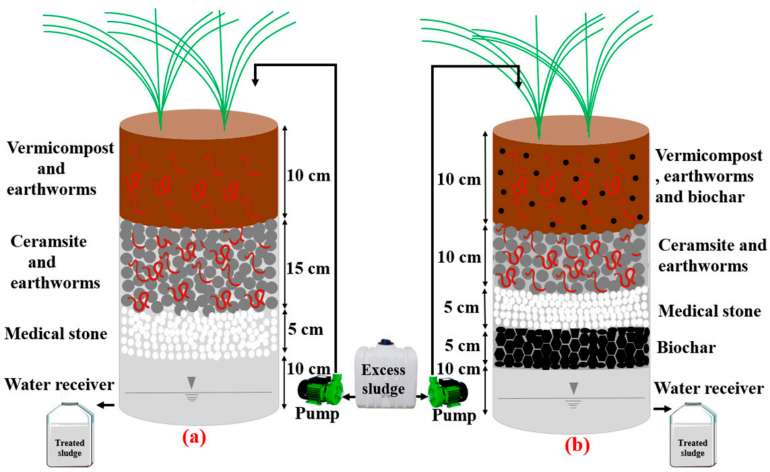

Figure 1. Schematic diagram of the vermi-reactor without (**a**) and with (**b**) biochar.

2.3. Methods

2.3.1. Physicochemical Properties Analysis

Three separate analyses were performed on each sample. Based on the analysis techniques for Chinese municipal sewage (CJ/T 221-2005) [23], physicochemical properties were assessed. pH and EC were measured using pH meters (PHS-3C, Leici, Shanghai, China) and electrical conductivity (DJS-1, Leica, Wetzlar, Germany). The excess sludge sample (50 mL) was filtered through a fiber filter membrane to assess TS using an oven at 105 °C for 8 h. The levels of NH_4^+-N and COD in the sludge were measured using a multi-parameter water-quality analysis device (CNPN–7SII, ChenNuo, Taizhou, China) and patent chemicals bought from the ChenNuo Company, China. Organic matter (OM) content was assessed by subjecting sludge to 650 °C for 2 h in the oven. Dissolved organic carbon (DOC) was measured by passing a mixed solution via a 0.45 μm membrane (Jinteng, Tianjin, China) and analyzing it with a carbon-nitrogen analyzer. The potassium persulfate digestion method with an alkaline solution was employed to ascertain the total nitrogen (TN) concentration. NO_3^--N and NO_2^--N were quantified using ultraviolet spectrophotometry at 220, 270, and 540 nm. A multi-parameter water-quality analysis device (CNPN–7SII, ChenNuo, Taizhou, China) was utilized to assess the quantity of total organic carbon (TOC).

2.3.2. DNA Extraction and Real-Time PCR

The DNeasy PowerSoil® Kit (Qiagen, Hilden, Germany) was utilized to extract DNA from a 0.25 g thawed sample without modification. Table S4 provides a comprehensive list of PCR primers and reaction conditions suitable for real-time qPCR using the TP700 instrument from Takara, Osaka, Japan. However, each template was amplified three times. The primer and PCR reaction conditions were determined based on Huang et al. [24] approach. For the PCR reaction, mixtures of 25 μL were formed using 12.5 μL of TB Green II (Takara, Beijing, China), 0.5 μL of forward and reverse primers for every 10 μM, 0.1 μL of DNA template, and 10.5 μL of DNA-free water. Plasmids carrying target genes were utilized for quantitative PCR. The following steps comprise the preparation process: the target gene is identified after PCR amplification; it is then recovered and purified using gel cutting; and last, it is attached to the pMD20-T vector by T-A cloning (Takara, Japan). The cells were then converted into *E. coli* DH5α competent cells and kept in a Luria-Bertani solid medium for 16 h. The positive white clones were screened for enrichment in culture. A Plasmid kit (Takara, Japan) was utilized to extract Plasmid DNA content detected by Nanodrop (Thermo, Waltham, MA, USA), which was converted to the copy number using the following equation [25]:

$$C\ (\text{ng}/\mu\text{L}) \times 6.022 \times 10^{23}/\text{N} \times 660\ (\text{g/mol}) \times 10^{9}\ (\text{ng/g})$$

In this context, C denotes the plasmid concentration (ng/μL), N represents the plasmid length (number of nucleotides), and 660 signifies the average mass of a single base pair of double-stranded DNA. The amplification efficiency was controlled between 90% and 110%.

2.3.3. High-Throughput Sequencing

To replicate the 16S rDNA gene sequence, barcode bases along with the 515F (5′-GTGCCAGCCGCGGTAA-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′) primers were utilized. All PCR amplifications were performed using enzymes from the Phusion® High-Fidelity PCR Master Mix (New England Biolabs, Ipswich, MA, USA). The initial denaturation step was performed for 1 min at 98 °C. Next, 30 cycles of denaturation for 10 s at 98 °C, annealing for 30 s at 50 °C, and elongation for 30 s at 72 °C were carried out. The last extension step was carried out for 5 min at 72 °C. The findings were acquired through 2% agarose gel electrophoresis and subsequently cleansed using Gene J.E.T. Gel Extraction Kit from Thermo Scientific. Qubit @ 2.0 Fluorometer (Thermo Scientific, Waltham, MA, USA) and the Agilent Bioanalyzer 2100 were utilized to evaluate the overall quality of libraries. Subsequently, the Illumina TruSeq DNA PCR-Free Library Preparation Kit (Illumina, San Diego, CA, USA) was employed to create sequencing libraries. After that, the original DNA fragment was combined using version 1.2.7 of the Flash software to acquire the unprocessed tag data. Version 1.8.0 of the QIIME software was used to remove the low-quality sections of bases and sequences to generate efficient tags. Perl scripts were employed to evaluate α and β diversity within samples. Using a similarity criterion of 97%, the script "Pick de novo otus.py" categorized all relevant tags into OTUs. The RDP classifier correctly identified the representative sequence for each functional taxonomic category. Data from the sequencing experiment has been uploaded to the NCBI database under the alignment identifier PRJNA716490.

2.4. Statistical Analysis

An independent Sample *t*-test (SPSS 26.0) was employed to compare each layer of the vermi-reactors and determine if there were any statistically significant differences ($p < 0.05$) for specific parameters. The mean ± standard deviation (SD) was utilized to analyze the results conducted in triplicate. CANOVA 4.5 software performed a redundancy analysis to identify relationships between removal rates and sampling days between vermi-reactors. Origin 8.0 was used to generate all of the graphs. MEGA 11 software created a phylogenetic analysis of AOB amoA and AOA amoA gene sequences.

3. Results and Discussion

3.1. Effect of Earthworms and Plants Biomass for Promoting AOA and AOB

Earthworms in the vermicompost and ceramsite layers of the vermi-reactor with BC grew much faster than those in control, increasing by 2.02 and 11.8-fold, respectively ($p < 0.01$) (Figure S1). This shows that BC in the vermi-reactor can boost the development of earthworms, especially in the ceramsite layer. This may be because of BC characteristics that are agreeable with ceramsite materials. This is consistent with a previous study that indicated that BC could enhance the growth of earthworms during dewatered sludge vermicomposting [10]. Typically, earthworms promote soil nitrification by increasing the quantity of AOB and AOA in the soil [21], indicating that BC could benefit the growth of earthworms.

As depicted in Figure S2, vermi-reactor with BC significantly boosted plant growth by 1.28-fold compared to the control group ($p < 0.001$). This result is consistent with previous research that added BC to horizontal subsurface flow constructed wetlands to increase the *Typha latifolia* growth rate [26]. The fast growth of wetland plants is attributed to their ability to absorb nutrients from the sludge, which earthworms have transformed into a usable form. A recent study demonstrated that macrophytes increased the abundance of ammonia oxidizers in the sediment around their roots compared to that in the unvegetated residue [27]. In general, BC can boost the development of earthworms and wetland plants, leading to more AOA and AOB in the vermi-reactor and ultimately improving treatment effectiveness. Overall, this demonstrates that adding BC to the vermi-reactor can enhance the growth of earthworms and wetland plants, resulting in increased quantities of AOA and AOB. Ultimately, this contributes to improved treatment effectiveness.

3.2. Effect of BC on the Nitrification Mechanisms inside Vermi-Wetland

3.2.1. Effect of BC on the Nitrification Process during Excess Sludge Recycling

The average content of NH_4^+-N in the influent of the vermi-reactor reactor with BC was significantly lower ($p < 0.05$) than that of the reactor without BC, with removal rates of 0.81 ± 0.17 mg/L and 1.32 ± 0.38 mg/L, respectively (Figure 2a). This finding demonstrates that adding BC to the vermi-reactor could improve NH_4^+-N reduction in excess sludge. Removing NH_4^+-N from excess sludge is crucial due to the effluent risks in the environment. Notably, the effluent from wetlands that include BC may be able to fulfill the grade-III (NH_4^+-N 1 mg/L) requirements of China's national surface water guidelines (GB3838-2002) [28]. Xu et al. [29] showed that the porous structural properties of

BC contribute to its high capacity for NH_4^+-N interception. This study aligns with previous research that demonstrated the potential of BC to adsorb NH_4^+-N in an aerated vertical flow constructed wetland during wastewater treatment [30]. This proves that the pores in the BC may provide a favorable environment for the growth of microbial communities, such as ammonia-oxidizing bacteria, which could also enhance the removal of NH_4^+-N [31].

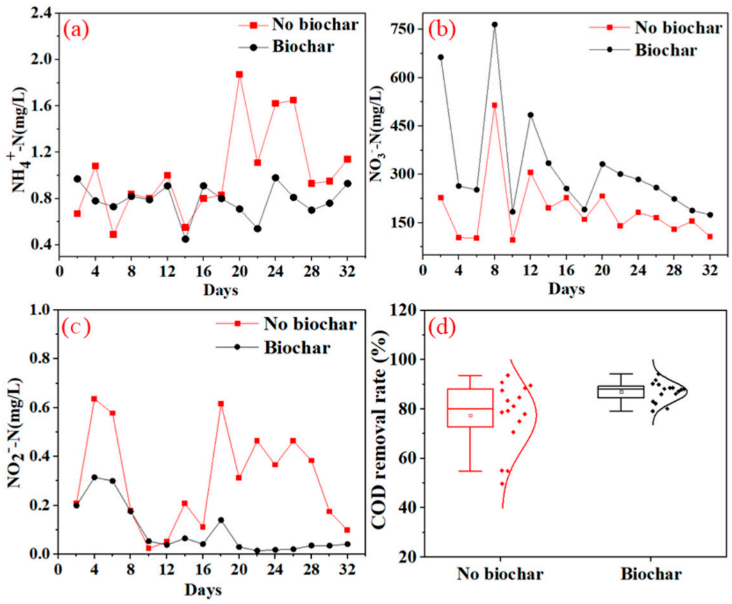

Figure 2. Change in NH_4^+-N (**a**), NO_3^--N (**b**), NO_2^--N (**c**), and the removal rate of COD (**d**) in vermi-reactor with BC and without BC during excess sludge treatment. There was a significant difference (*t*-test, $p < 0.05$) for each removal rate and after the 18th day between the two groups.

As illustrated in Figure 2b, the addition of BC to the vermi-reactor resulted in a substantial increase in nitrate-nitrogen (NO_3^--N) levels ($p < 0.05$), with 17.49 ± 20.49 mg/L and 11.61 ± 16.43 mg/L observed in the vermi-reactor with and without BC, respectively. This finding is consistent with the previous study, which displayed that the contents of NO_3^+-N elevated with increased BC application rate in the black soil [32]. In contrast, another study that examined the production and assessment of BC from wetland plants for removing nitrogen in surface flow-constructed wetlands showed reduced contents of NO_3^+-N due to BC addition [33]. This resulted from a high denitrification rate of the substrate due to the elevated carbon source supplied by BC. In addition, the higher level of nitrification may be due to the higher migration of NO_3^--N from the roots to the shoots because BC is added to the constructed wetland system, facilitating higher plant growth.

As shown in Figure 2c, the nitrite nitrogen (NO_2^+-N) content significantly decreased in the vermi-reactor with BC compared to without BC, and the values were 0.07 ± 0.08 mg/L and 0.34 ± 0.19 mg/L, respectively ($p < 0.05$). This might have happened because NH_4^+-N and NO_2^+-N are beneficial for later nitrification and plant uptake due to the substantially wider specific surface area, micropore volumes, and total pore of the BC. Before the 18th day, the NO_2^--N concentration fluctuated but remained steady afterward. Subsequently, denitrification was impeded due to an inadequate supply of carbon sources in the wetland, resulting in an evident NO_3^--N concentration buildup. Therefore, BC can potentially serve as a carbon source, promoting wetland denitrification. As shown in Figure 2d, COD removal efficiencies in the vermi-reactor without and with BC were 70.65–89.5% and 88.51–94.34%, respectively. This study demonstrated improved results compared to the recent research that revealed that the removal rate of COD could attain 36%–68% in the planted vermi-reactor during the recycling of excess sludge [1]. Another previous study also showed that the removal rate of COD might range between 48.5–53.5% in the vermi-filter during excess sludge treatment [34], which confirms that BC can improve the effectiveness of vermi-wetland. This study reinforces earlier research findings that suggested the COD removal rate can reach 94.9% when BC is added into an aerated vertical flow constructed wetland (VFCW) to recycle domestic wastewater [30].

3.2.2. Effect of Biochar on the Nitrification Process inside Vermi-Wetlands

The average NH_4^+-N amount in the layers of a reactor with BC significantly lowered ($p < 0.01$) by 1.55 times compared with those in control (Figure 3a). BC increased the adsorption of NH_4^+-N due to its porosity [33], heterogeneous surfaces, shallow channels, and composition of coarse [26]. The removal efficiency of NH_4^+-N in aerobic systems is typically dependent on nitrification. Enhancing the removal of NH_4^+-N through nitrification can be attained by increasing the dissolved oxygen content, for instance, through mechanical aeration. BC is a porous material commonly utilized as a biofilter to enhance the removal of COD and NH_4^+-N, owing to its high capacities for adsorption and microbial adhesion.

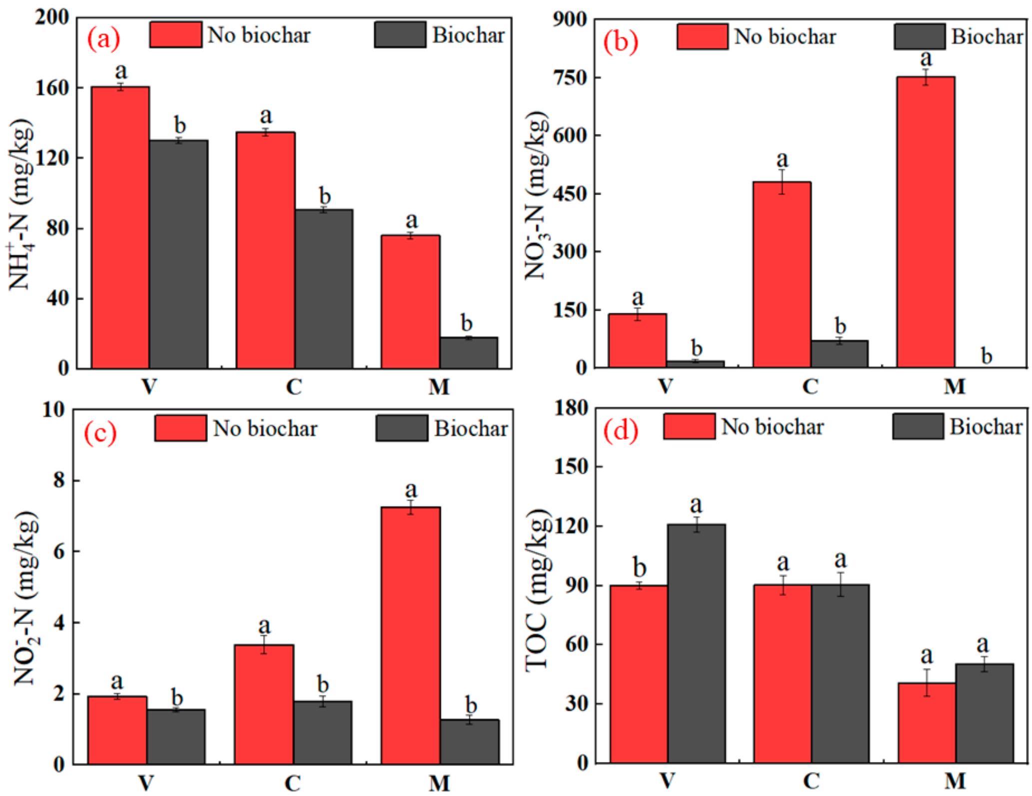

Figure 3. Transformation of ammonium nitrogen (NH_4^+-N) (**a**), nitrate-nitrogen (NO_3^--N) (**b**), nitrite nitrogen (NO_2^--N) (**c**), and total organic carbon (TOC) (**d**) in a different layer of different vermi-reactors. V, C, and M symbols indicate Vermicompost, Ceramsite, and Medical stone layers. Data are presented as means ± standard deviations (n = 3). The significant difference (HSD-test, $p < 0.05$) between the two groups is represented by the superscript letters above the error bars.

The concentration of $NO^-{}_3$-N in vermi-reactor layers with BC decreased significantly ($p < 0.01$) by 15.39-fold compared to the vermi-reactor layers without BC (Figure 3b). This finding is consistent with a recent study that reported a decline in NO_3^--N concentration with the incorporation of BC into the process of sludge composting [35]. In contrast, Zhu et al. [36] demonstrated that adding BC to the soil could enhance the transfer of NO_3^--N from the root to the shoot. Knowles et al. [37] also revealed that BC could lower NO_3^--N leaching in the soil. Therefore, the reduced NO_3^--N content after adding BC into the vermi-reactor could be related to the adsorption of NO_3^--N by BC. Compared to the vermi-reactor layers without BC, the NO_2^--N concentration in the vermi-reactor layers with BC considerably lowered by 2.71 times ($p < 0.05$) (Figure 3c). As shown in Figure 3d, the proportion of TOC in the vermi-reactor with BC was considerably enhanced by 1.20 times in comparison to the vermi-reactor without BC ($p < 0.05$). This finding is aligned with a recent study that revealed that BC raised the pH of the calcareous soil from 8.2 to 8.57, increasing TOC [38].

3.2.3. Effect of Biochar on Numbers of Nitrification Genes inside Vermi-Wetland

As illustrated in Table 1, the numbers of AOB within the vermicompost layer of the vermi-reactor exhibited a substantial increase by 1.35-fold ($p < 0.05$) when BC was added, in stark contrast to the vermi-reactor without BC. Herein, a slight increase in AOB counts was observed between the ceramsite layers ($p > 0.05$), with a 1.19-fold increase noted in the vermi-reactor with BC compared to its counterpart. This finding indicates that BC may considerably increase the number of AOBs in the vermi-reactor layers while recycling excess sludge. Typically, the population of nitrifying bacteria proliferates, leading to a significant conversion of ammonia nitrogen into nitrate nitrogen, facilitated by AOB, which converts NH_4^+-N into NO_2^--N.

Table 1. Quantitative analysis of AOA, *nirS*, AOB, and *nirK* abundance in a different layer of vermi-reactor. The letters V1, V2, C1, C2, and M indicate the vermicompost-upper layer, vermicompost-lower layer, ceramsite-upper layer, ceramsite-lower layer, and medical stone. Data are presented as means ± standard deviations (n = 3). The significant difference (HSD-test, $p < 0.05$) between the two groups is represented by the superscript letters behind the data.

Layers	**AOA/*nirS* (ng/g)**		**AOB × 10^3/*nirK* (ng/g)**	
	Without BC	**With BC**	**Without BC**	**With BC**
V	0.017 ± 0.006^b / 0.15 ± 0.03^b	0.023 ± 0.004^a / 0.33 ± 0.13^a	0.56 ± 0.39^a / 0.87 ± 0.35^b	0.94 ± 0.84^a / 1.79 ± 0.07^a
C	0.010 ± 0.005^a / 0.1 ± 0.09^b	0.012 ± 0.005^a / 0.17 ± 0.02^a	0.45 ± 0.16^b / 1.47 ± 0.82^a	0.63 ± 0.19^a / 1.74 ± 1.01^a
M	0.010 ± 0.001^b / 0.84 ± 0.07^b	0.019 ± 0.001^a / 1.71 ± 0.39^a	1.40 ± 0.26^b / 1.72 ± 0.76^b	12.71 ± 1.76^a / 3.86 ± 0.62^a
Total	0.038 ± 0.011^b / 1.09 ± 0.19^b	0.054 ± 0.010^a / 2.22 ± 1.16^a	2.41 ± 0.81^b / 4.05 ± 2.5^b	14.33 ± 1.06^a / 7.39 ± 2.06^a

AOA copies in the vermicompost layer of the vermi-reactor with BC were significantly higher by 1.68-fold than those without BC ($p < 0.05$). The abundance of AOA copies in the ceramsite layers of the vermi-reactor with BC was increased by 1.4-fold compared to those in the layers of the vermi-reactor without BC ($p < 0.05$). Moreover, AOA copies in the medical stone layer with BC showed a significant difference ($p < 0.05$) by 1.9-fold compared with their counterparts. The AOA numbers in the medical stone layer in the vermi-reactor with BC were significantly higher by 9.07 times than those in the vermi-reactor without BC ($p < 0.01$), indicating that BC could also promote the AOA numbers in vermi-reactor layers during excess sludge treatment. The increased AOA in the vermi-reactor systems with BC could result from organic matter mineralization or ammonia produced from BC. The results of this study are similar to the study that showed the enhanced quantity of AOB and AOA copies in the soil [39,40]. This indicates that the amendment of BC in the vermi-reactor could create a favorable habitat for a diverse range of autotrophic, denitrifying, and nitrifying bacteria. These bacteria can expeditiously promote anaerobic ammonia oxidation (Anammox), denitrification, and nitrification activities [41]. The number of amoA genes is primarily associated with nitrification rates as its gene products are predominantly responsible for converting NH_4^+ to NO_2^- in aerobic environments.

As detailed in Table 1, *nirS* gene copies in the vermicompost layer of the vermi-reactor with BC were significantly higher by 2.2-fold compared with *nirS* gene copies in the vermicompost lower layer without BC ($p < 0.05$). The abundance of *nirS* gene copies in the ceramsite layer of vermi-reactors was 1.7 times greater than in the vermi-reactor without BC. ($p < 0.05$). Moreover, *nirS* gene copies in the medical stone layer with BC evinced a considerable difference ($p < 0.05$) by 1.39-fold compared with its counterpart. The abundances of *nirK* gene copies in the vermi-reactor with BC were considerably higher by 2.05-fold compared with *nirK* gene copies in the vermi-reactor without BC ($p < 0.05$). The abundance of *nirK* gene copies in the ceramsite layer of the vermi-reactor with BC was 1.18 times greater than those in the vermi-reactor without BC. *nirK* gene copies in the medical stone layer with BC showed a considerable difference ($p < 0.05$) by 2.24-fold compared with their counterparts. These results suggested that adding corncob BC to the vermi-reactor promoted the abundance of nitrite reductase genes (*nirK* and *nirS*) copies. The findings of this study are consistent with previous studies that demonstrated an increase in the quantity of the *nirK* gene in the soil environment following the addition of BC [39,40].

3.3. BC Provides an Inhabitation for AOA, AOB, nirK, and nirS Genes

As shown in Figure 4, BC in the vermicompost-lower layer displayed various pores compared to other layers. BC in different layers of vermi-reactor significantly revealed different copy numbers of AOA and AOB and the *nirS* and *nirK* genes. The vermicompost-lower layer showed a significant difference ($p < 0.05$) in the amount of AOA, AOB, *nirS*, and *nirK* gene copies compared to the vermicompost-upper and BC layers. This occurred due to the movement of BC from the upper layer to the lower layer when excess sludge was sprinkled in the vermi-reactor. This result indicates that adding BC into the vermi-reactor for sludge treatment could directly influence the abundance of the nitrification gene *amoA* and denitrification genes *nirK* and *nirS* because its surfaces and pores create a favorable environment for the growth of those genes. The high number of genes might be due to the augmented pore volume and surface area within corncob BC, which enhance the development of genes [39,40]. In addition, BC should not be used to improve the number of genes on its own and should be combined with other substances, such as compost, to achieve the desired results.

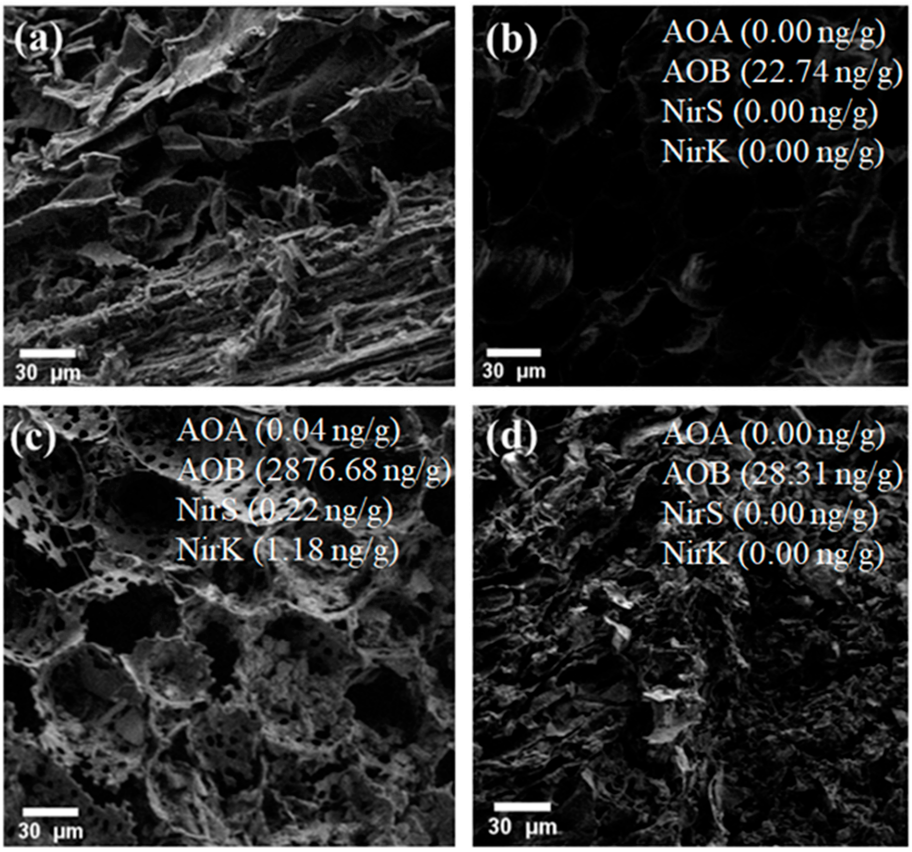

Figure 4. SEM images of corncob BC before treatment (**a**), added to the vermicompost-upper layer (**b**), added to the vermicompost-lower layer (**c**), and used as a layer in the lower layer of vermi-reactor (**d**).

As shown in Figure 5, BC had a substantial influence on the abundance of AOA, AOB, and *nirS* in the vermicomposting layers of vermi-reactor during excess sludge recycling ($0.4 \geq$ Mantel's r ≥ 0.2, $p < 0.05$). Correspondingly, there was no correlation in the ceramsite layers. However, there was a strong correlation in the medical stone layers of the vermi-reactor (Mantel's r < 0.4, $p < 0.05$). This could be attributed to the fact that the surface and porous structure of BC in the vermi-reactor serves as a habitat for the growth of bacterial genes and directly impacts microbial community structures during sludge treatment. These results demonstrate that BC could enhance nitrification in the vermi-reactor during excess sludge recycling.

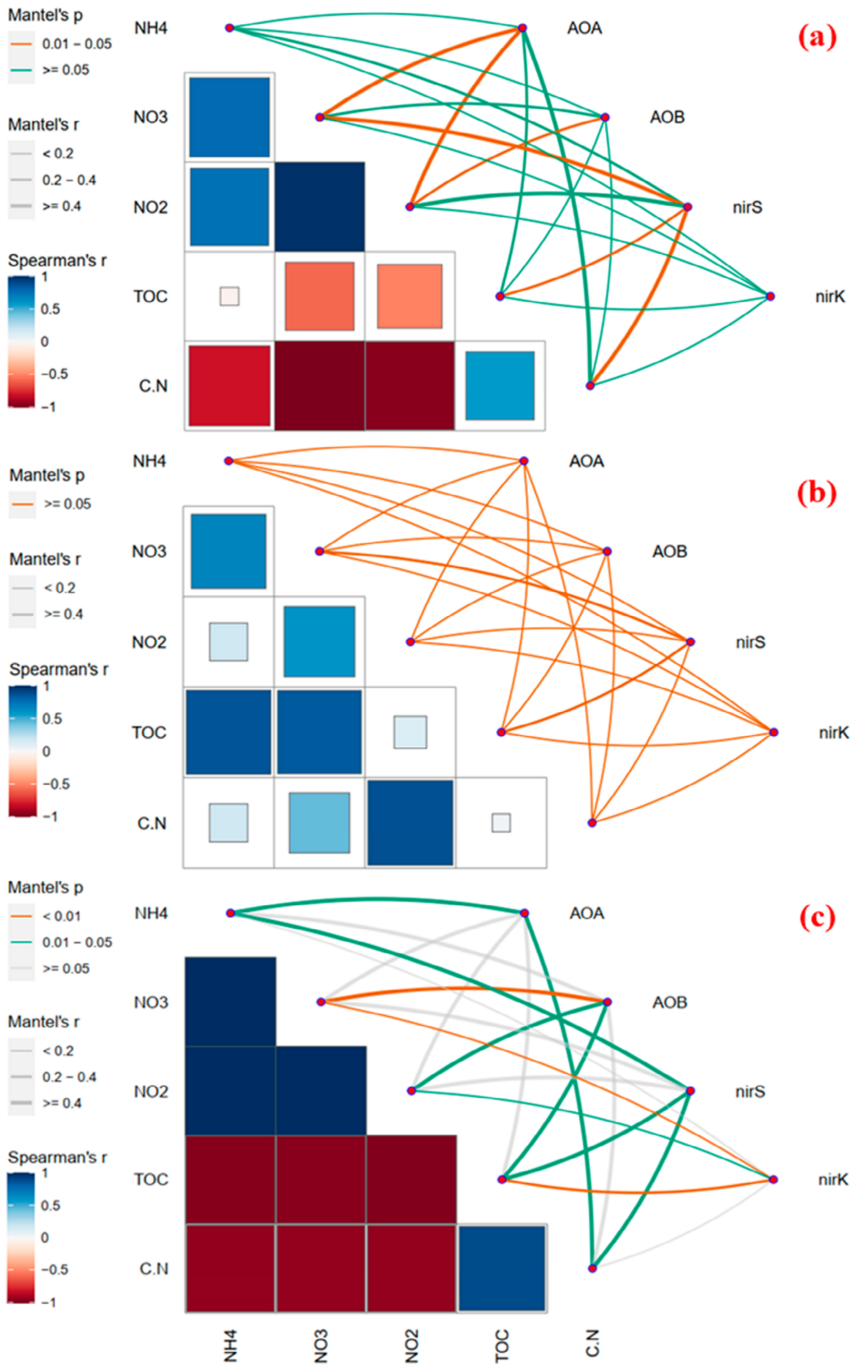

Figure 5. Mantel test of nitrification in vermicompost (**a**), ceramsite (**b**), and medical stone (**c**) layers in vermi-reactor with and without BC. Mantel's r > 0.4, $0.4 \geq$ Mantel's r ≥ 0.2, and Mantel's r < 0.2 represent strong correlation, general correlation, and slight correlation, respectively.

AOB is included in the genera *Nitrosomonas* and *Nitrosospira*, which were observed in all vermi-reactors, as shown in Figure S2. This was consistent with the study of the mechanisms between BC and compost in the soil environment [42]. Kumar et al. [43] displayed that the dominating members of AOB in the constructed wetland systems were *Nitrosospira* sp. and *Nitrosomonas* sp. during the recycling of domestic wastewater. Remarkably, the members of *Nitrosomonas* actively participate in the complete nitrification process and reside in the anaerobic wastewater systems as compared to the members of *Nitrosospira* [19].

Considering the findings highlighted in Figure S3, the AOA community component attached to the BC was divided into *Thaumarchaeota*, *Crenarchaeota*, and Unclassified AOA. Commonly, *crenarchaeota* groups are regarded as the most significant ammonium oxidizers in terrestrial ecosystems due to their numerical abundance and transcription [19,44]. Sun et al. [45] showed that several archaea amoA genes, such as '*Candidatus Nitrososphaera gargensis*', are active throughout the composting of cow manure. As deep-branch Crenarchaeota members, *Thaumarchaeota* may undertake ammonia oxidizing in either an autotrophic or heterotrophic manner utilizing various substrates [46]. The AOA number and community diversity in BC were lower than the AOB in BC. This result shows that the AOB is a predominant performer in ammonia-oxidizing in the vermi-reactor with BC. However, regarding vermicomposting systems for recycling fresh food wastes, the AOA is far more prevalent than the AOB [19]. However, the inhabitation mechanisms of AOA and AOB in BC are still unknown.

4. Conclusions

In summary, BC played an imperative role in nitrogen transformation in the vermi-wetland during excess sludge recycling. BC could reduce NH_4^+-N concentration by 40% and increase the NO_3^--N concentration by 50% in the effluent. Additionally, BC could enrich the AOB population by 6-fold and AOA by 1.42-fold in layers during excess sludge treatment. Furthermore, BC could enhance the abundance of *nirS* and *nirK* genes up to 2.03-fold and 1.82-fold in the layer of vermi-reactor, respectively. The application of BC in vermi-wetland resulted in a substantial 6.92-fold increase in earthworm growth and a 1.28-fold boost in plant growth. The surface and porous structure of the BC in the vermi-reactor might offer a favorable environment for bacterial genes and directly impact the microbial community structures during sludge treatment. The performance of nitrification in the vermi-reactors with BC was much better than in the vermi-reactors without BC. This study suggests that adding BC to the vermi-reactor could enhance nitrification, denitrification, and the effectiveness of treatment during excess sludge treatment.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/su152416551/s1, Table S1: Properties of corncob biochar used in the study; Table S2: properties of vermicompost used in this study; Table S3: Physicochemical properties of fresh sludge; Table S4: primer and reaction condition of PCR; Figure S1: effect of biochar on the growth rates of earthworms (a) and plants in different vermi-wetland (b); Figure S2: phylogenetic analysis of ammonia-oxidizing bacterial amoA gene sequences in the vermi-wetlands; Figure S3: phylogenetic analysis of ammonia-oxidizing archaeal amoA gene sequences in the biochar.

Author Contributions: T.B. Conceptualization, Resources, Project administration, Visualization; G.T. Methodology, Data curation, Writing—original draft, Software, Validation, Investigation; Z.W. Validation, Software, Resources; H.X. Resources, Supervision, Visualization, Validation, Funding acquisition; C.S. Formal analysis, Investigation, Visualization; K.H. Supervision, Revise—original draft; H.C. Visualization, Formal analysis, Resources. All authors have read and agreed to the published version of the manuscript.

Funding: This study was financed by the Science and Technology Program Foundation of Gansu Province (22JR5RA335).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Data will be made available on request.

Conflicts of Interest: Authors T.B. and Z.W. are employed by Gansu Research Institute of Chemical Industry Co., Ltd. The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

References

1. Huang, K.; Sang, C.; Guan, M.; Wu, Y.; Xia, H.; Chen, Y.; Nie, C. Performance and Stratified Microbial Community of Vermi-Filter Affected by *Acorus calamus* and *Epipremnum aureum* during Recycling of Concentrated Excess Sludge. *Chemosphere* **2021**, *280*, 130609. [CrossRef]
2. Zhang, Q.; Hu, J.; Lee, D.J.; Chang, Y.; Lee, Y.J. Sludge Treatment: Current Research Trends. *Bioresour. Technol.* **2017**, *243*, 1159–1172. [CrossRef]
3. Twagirayezu, G.; Huang, K.; Sang, C.; Guan, M.; Xia, H.; Li, Y. Performance and Mechanisms of Biochar for Promoting the Removal Efficiency of Organic Solids in the Vermi-Wetland during the Recycling of Excess Sludge. *J. Clean. Prod.* **2022**, *360*, 132172. [CrossRef]
4. Singh, R.; Bhunia, P.; Dash, R.R. A Mechanistic Review on Vermifiltration of Wastewater: Design, Operation and Performance. *J. Environ. Manag.* **2017**, *197*, 656–672. [CrossRef] [PubMed]
5. Samal, K.; Dash, R.R.; Bhunia, P. A Comparative Study of Macrophytes Influence on Performance of Hybrid Vermifilter for Dairy Wastewater Treatment. *J. Environ. Chem. Eng.* **2018**, *6*, 4714–4726. [CrossRef]
6. Hu, S.; Zuo, X.; Lv, Z.; He, J.; Wu, Y.; Liu, H.; Chen, Z. Drained Water Quality in Sludge Treatment Wetlands: Effects of Earthworm Densities and Plant Species. *J. Clean. Prod.* **2020**, *247*, 119128. [CrossRef]
7. Hu, S.; Lv, Z.; Zuo, X.; Liu, H.; Vymazal, J.; Chen, Z. Effects of Loading Rates and Plant Species on Sludge Characteristics in Earthworm Assistant Sludge Treatment Wetlands. *Sci. Total Environ.* **2020**, *730*, 139–152. [CrossRef] [PubMed]
8. Cheng, H.; Xing, D.; Twagirayezu, G.; Lin, S.; Gu, S.; Tu, C.; Hill, P.W.; Jones, D.L. Effects of Field-Aging on the Impact of Biochar on Herbicide Fate and Microbial Community Structure in the Soil Environment. *Chemosphere* **2023**. [CrossRef]
9. Tan, H.; Lee, C.T.; Ong, P.Y.; Wong, K.Y.; Bong, C.P.C.; Li, C.; Gao, Y. A Review On The Comparison Between Slow Pyrolysis And Fast Pyrolysis On The Quality Of Lignocellulosic And Lignin-Based Biochar. *IOP Conf. Ser. Mater. Sci. Eng.* **2021**, *1051*, 012075. [CrossRef]
10. Gong, X.; Cai, L.; Li, S.; Chang, S.X.; Sun, X.; An, Z. Bamboo biochar amendment improves the growth and reproduction of Eisenia fetida and the quality of green waste vermicompost. *Ecotoxicol. Environ. Saf.* **2018**, *156*, 197–204. [CrossRef]
11. Blenis, N.; Hue, N.; Maaz, T.M.C.; Kantar, M. Biochar Production, Modification, and Its Uses in Soil Remediation: A Review. *Sustainability* **2023**, *15*, 3442. [CrossRef]
12. Deng, Z.; Gu, S.; Cheng, H.; Xing, D.; Twagirayezu, G.; Wang, X.; Ning, W.; Mao, M. Removal of Phosphate from Aqueous Solution by Zeolite-Biochar Composite: Adsorption Performance and Regulation Mechanism. *Appl. Sci.* **2022**, *12*, 5334. [CrossRef]
13. Meng, J.; Tao, M.; Wang, L.; Liu, X.; Xu, J. Changes in Heavy Metal Bioavailability and Speciation from a Pb-Zn Mining Soil Amended with Biochars from Co-Pyrolysis of Rice Straw and Swine Manure. *Sci. Total Environ.* **2018**, *633*, 300–307. [CrossRef] [PubMed]
14. Tao, R.; Li, J.; Hu, B.; Chu, G. Ammonia-Oxidizing Bacteria Are Sensitive and Not Resilient to Organic Amendment and Nitrapyrin Disturbances, but Ammonia-Oxidizing Archaea Are Resistant. *Geoderma* **2021**, *384*, 114814. [CrossRef]
15. Coca-Salazar, A.; Richaume, A.; Florio, A.; Carnol, M. Response of Ammonia-Oxidizing Bacteria and Archaea Abundance and Activity to Land Use Changes in Agricultural Systems of the Central Andes. *Eur. J. Soil Biol.* **2021**, *102*, 103263. [CrossRef]
16. Koch, H.; van Kessel, M.A.H.J.; Lücker, S. Complete Nitrification: Insights into the Ecophysiology of Comammox Nitrospira. *Appl. Microbiol. Biotechnol.* **2019**, *103*, 177–189. [CrossRef] [PubMed]
17. Ginawi, A.; Wang, L.; Wang, H.; Yu, B.; Yunjun, Y. Effects of Environmental Variables on Abundance of Ammonia-Oxidizing Communities in Sediments of Luotian River, China. *PeerJ* **2020**, *8*, e8256. [CrossRef]
18. Zhang, L.-M.; Duff, A.M.; Smith, C.J. Community and Functional Shifts in Ammonia Oxidizers across Terrestrial and Marine (Soil/Sediment) Boundaries in Two Coastal Bay Ecosystems. *Environ. Microbiol.* **2018**, *20*, 2834–2853. [CrossRef] [PubMed]
19. Huang, K.; Xia, H.; Cui, G.; Li, F. Effects of Earthworms on Nitrification and Ammonia Oxidizers in Vermicomposting Systems for Recycling of Fruit and Vegetable Wastes. *Sci. Total Environ.* **2017**, *578*, 337–345. [CrossRef] [PubMed]
20. Deng, L.; Zhao, Y.; Zhang, J.; Bello, A.; Sun, Y.; Han, Y.; Wang, B.; Egbeagu, U.U.; Li, D.; Jong, C.; et al. Insight to Nitrification during Cattle Manure-Maize Straw and Biochar Composting in Terms of Multi-Variable Interaction. *Bioresour. Technol.* **2021**, *323*, 124572. [CrossRef] [PubMed]
21. Wang, L.; Pang, Q.; Peng, F.; Zhang, A.; Zhou, Y.; Lian, J.; Zhang, Y.; Yang, F.; Zhu, Y.; Ding, C.; et al. Response Characteristics of Nitrifying Bacteria and Archaea Community Involved in Nitrogen Removal and Bioelectricity Generation in Integrated Tidal Flow Constructed Wetland-Microbial Fuel Cell. *Front. Microbiol.* **2020**, *11*, 1385. [CrossRef]
22. Karmegam, N.; Jayakumar, M.; Govarthanan, M.; Kumar, P.; Ravindran, B.; Biruntha, M. Precomposting and Green Manure Amendment for Effective Vermitransformation of Hazardous Coir Industrial Waste into Enriched Vermicompost. *Bioresour. Technol.* **2021**, *319*, 124136. [CrossRef] [PubMed]

23. *CJ/T 221-2005*; Determination Method for Municipal Sludge in Wastewater Treatment Plant. Chinese Standard: Beijing, China, 2005.
24. Huang, K.; Li, F.; Wei, Y.; Chen, X.; Fu, X. Changes of Bacterial and Fungal Community Compositions during Vermicomposting of Vegetable Wastes by Eisenia Foetida. *Bioresour. Technol.* **2013**, *150*, 235–241. [CrossRef] [PubMed]
25. Golestan, H.A.; Razavi, H.; Golestan, N.; Fotouhi, F.; Khosravi, A. Development of a Robust TaqMan Probe-Based One-Step Multiplex RT-QPCR for Simultaneous Detection of SARS-CoV-2 and Inuenza A/B Viruses. *BMC Microbiol.* **2023**, *23*, 335.
26. Kasak, K.; Truu, J.; Ostonen, I.; Sarjas, J.; Oopkaup, K.; Paiste, P.; Kõiv-Vainik, M.; Mander, Ü.; Truu, M. Biochar enhances plant growth and nutrient removal in horizontal subsurface flow constructed wetlands. *Sci. Total Environ.* **2018**, *639*, 67–74. [CrossRef]
27. Zhang, J.; Liu, B.; Zhou, X.; Chu, J.; Li, Y.; Wang, M. Effects of Emergent Aquatic Plants on Abundance and Community Structure of Ammonia-Oxidising Microorganisms. *Ecol. Eng.* **2015**, *81*, 504–513. [CrossRef]
28. *GB 3838-2002*; Environmental Quality Standards for Surface Water. Chinese Standard: Beijing, China, 2002.
29. Xue, R.; Wang, C.; Liu, X.; Liu, M. Earthworm Regulation of Nitrogen Pools and Dynamics and Marker Genes of Nitrogen Cycling: A Meta-Analysis. *Pedosphere* **2022**, *32*, 131–139. [CrossRef]
30. Zhou, X.; Wang, X.; Zhang, H.; Wu, H. Enhanced Nitrogen Removal of Low C/N Domestic Wastewater Using a Biochar-Amended Aerated Vertical Flow Constructed Wetland. *Bioresour. Technol.* **2017**, *241*, 269–275. [CrossRef]
31. Verhamme, D.T.; Prosser, J.I.; Nicol, G.W. Ammonia Concentration Determines Differential Growth of Ammonia-Oxidising Archaea and Bacteria in Soil Microcosms. *ISME J.* **2011**, *5*, 1067–1071. [CrossRef]
32. Fu, Q.; Yan, J.; Li, H.; Li, T.; Hou, R.; Liu, D.; Ji, Y. Effects of Biochar Amendment on Nitrogen Mineralization in Black Soil with Different Moisture Contents under Freeze-Thaw Cycles. *Geoderma* **2019**, *353*, 459–467. [CrossRef]
33. Li, J.; Fan, J.; Zhang, J.; Hu, Z.; Liang, S. Preparation and Evaluation of Wetland Plant-Based Biochar for Nitrogen Removal Enhancement in Surface Flow Constructed Wetlands. *Environ. Sci. Pollut. Res.* **2018**, *25*, 13929–13937. [CrossRef]
34. Zhao, L.; Wang, Y.; Yang, J.; Xing, M.; Li, X.; Yi, D.; Deng, D. Earthworm-Microorganism Interactions: A Strategy to Stabilize Domestic Wastewater Sludge. *Water Res.* **2010**, *44*, 2572–2582. [CrossRef] [PubMed]
35. Liu, W.; Huo, R.; Xu, J.; Liang, S.; Li, J.; Zhao, T.; Wang, S. Effects of Biochar on Nitrogen Transformation and Heavy Metals in Sludge Composting. *Bioresour. Technol.* **2017**, *235*, 43–49. [CrossRef]
36. Zhu, Y.; Li, H.; Wu, Y.; Yin, X.A.; Zhang, G. Effects of Surface-Modified Biochars and Activated Carbon on the Transformation of Soil Inorganic Nitrogen and Growth of Maize under Chromium Stress. *Chemosphere* **2019**, *227*, 124–132. [CrossRef] [PubMed]
37. Knowles, O.A.; Robinson, B.H.; Contangelo, A.; Clucas, L. Biochar for the Mitigation of Nitrate Leaching from Soil Amended with Biosolids. *Sci. Total Environ.* **2011**, *409*, 3206–3210. [CrossRef]
38. Cardelli, R.; Becagli, M.; Marchini, F.; Saviozzi, A. Effect of Biochar, Green Compost, and Vermicompost on the Quality of a Calcareous Soil: A 1-Year Laboratory Experiment. *Soil Sci.* **2017**, *182*, 248–255. [CrossRef]
39. Liu, X.; Ren, J.; Zhang, Q.; Liu, C. Long-Term Effects of Biochar Addition and Straw Return on N_2O Fluxes and the Related Functional Gene Abundances under Wheat-Maize Rotation System in the North China Plain. *Appl. Soil Ecol.* **2019**, *135*, 44–55. [CrossRef]
40. Li, J.; Wang, S.; Luo, J.; Zhang, L.; Wu, Z.; Lindsey, S. Effects of Biochar and 3,4-Dimethylpyrazole Phosphate (DMPP) on Soil Ammonia-Oxidizing Bacteria and NosZ-N_2O Reducers in the Mitigation of N_2O Emissions from Paddy Soils. *J. Soils Sediments* **2021**, *21*, 1089–1098. [CrossRef]
41. Yang, Y.; Lu, Z.; Azari, M.; Kartal, B.; Du, H.; Cai, M.; Herbold, C.W.; Ding, X.; Denecke, M.; Li, X.; et al. Discovery of a New Genus of Anaerobic Ammonium Oxidizing Bacteria with a Mechanism for Oxygen Tolerance. *Water Res.* **2022**, *226*, 119165. [CrossRef]
42. Li, M.; Zhang, J.; Yang, X.; Zhou, Y.; Zhang, L.; Yang, Y.; Luo, L.; Yan, Q. Responses of Ammonia-Oxidizing Microorganisms to Biochar and Compost Amendments of Heavy Metals-Polluted Soil. *J. Environ. Sci.* **2021**, *102*, 263–272. [CrossRef]
43. Kumar, S.; Pratap, B.; Dubey, D.; Dutta, V. Microbial Communities in Constructed Wetland Microcosms and Their Role in Treatment of Domestic Wastewater. In *Emerging Eco-Friendly Green Technologies for Wastewater Treatment*; Springer: Berlin/Heidelberg, Germany, 2020; pp. 311–327.
44. Levy-Booth, D.J.; Prescott, C.E.; Grayston, S.J. Microbial Functional Genes Involved in Nitrogen Fixation, Nitrification and Denitrification in Forest Ecosystems. *Soil Biol. Biochem.* **2014**, *75*, 11–25. [CrossRef]
45. Sun, Y.; Zhu, L.; Xu, X.; Meng, Q.; Men, M.; Xu, B.; Deng, L. Correlation between Ammonia-Oxidizing Microorganisms and Environmental Factors during Cattle Manure Composting. *Rev. Argent. Microbiol.* **2019**, *51*, 371–380. [CrossRef] [PubMed]
46. Pester, M.; Schleper, C.; Wagner, M. The Thaumarchaeota: An Emerging View of Their Phylogeny and Ecophysiology. *Curr. Opin. Microbiol.* **2011**, *14*, 300–306. [CrossRef] [PubMed]