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Inhibitory Effects of Biochar on N_2O Emissions through Soil Denitrification in Huanghuaihai Plain of China and Estimation of Influence Time

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Abstract: Biochar application is considered an effective method for reducing nitrous oxide (N_2O) emissions from soil. However, the mechanisms underlying the influence of various biochar dosages on soil N_2O emissions and the duration of one-time biochar application remain unclear. The effects of different biochar application rates and a one-time application on soil N_2O emissions in the Huanghuaihai Plain of China were investigated through a field experiment from 2020 to 2022. In the wheat and maize rotation system, six treatments were administered: no biochar (C0); 2 (C1), 4 (C2), 8 (C3), and 12 t/hm^2 biochar (C4) applied annually; and a one-time application of 12 t/hm^2 biochar (CS) in 2018. Our results indicate that, compared with C0, biochar significantly inhibited soil N_2O emissions, particularly in the C3 and C4 treatments, with reductions of 31.36–56.21% and 36.92–52.45%, respectively. However, CS did not significantly affect soil N_2O emissions during the study period. These findings suggest that the biochar's inhibitory effect on soil N_2O emissions is contingent upon the dosage and frequency of application. A structural equation model revealed that biochar decreases soil N_2O emissions by enhancing the reduction in N_2O during denitrification. Under the conditions of this experiment, based on a logistic ecological model, a one-time application of 12 t/hm^2 biochar was projected to significantly reduce soil N_2O emissions for approximately 1.77 years. On the whole, biochar reduces soil N_2O emissions mainly by regulating N_2O production through denitrification, and the duration of this inhibition of N_2O emissions mainly depends on the application amount and frequency of biochar application.

Keywords: one-time application; functional gene; denitrification; different application amounts; biochar; N_2O emissions

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1. Introduction

Nitrogen fertilizer is a vital component of agricultural production. It significantly contributes to the release of nitrous oxide (N_2O) after being applied to the soil, which is a principal factor increasing N_2O emissions in farmlands [1,2]. Consequently, mitigating N_2O emissions from farmlands is a critical strategy for China to achieve its "carbon peaking and carbon neutrality" objectives [3,4]. Concurrently, crop straw, a major biochar source, not only alleviates the environmental burden of agricultural waste but also diminishes soil greenhouse gas emissions. This dual functionality positions biochar as an agronomic measure well aligned with China's current and forthcoming national circumstances [5].

Numerous studies have demonstrated that biochar can significantly reduce soil N_2O emissions, enhance soil quality, and improve nitrogen use efficiency. These benefits are attributed to biochar's physical adsorption of exogenous nitrogen, alteration of the soil carbon and nitrogen cycles, and impact on related microbial activity [6–8]. For instance, a

recent meta-analysis conducted by Zhang et al. [9] found that biochar application decreased N_2O emissions by 7.09%. Additionally, while the incorporation of a higher amount of biochar into Amazon forest soil markedly reduced N_2O emissions, the application of a lesser amount of biochar did not significantly affect them [10]. The above result indicated that greater biochar addition could increase the activity of N_2O reductase and decrease soil N_2O emissions [11,12]. Concurrently, this process can suppress the enzymatic activity converting NO_3^- and NO_2^- into N_2O, thereby decreasing N_2O production during denitrification [13,14]. However, some studies indicate that biochar can either promote or have no effect on N_2O emissions. For example, Wu et al. [15] reported that adding 40 t/hm^2 of biochar increased soil N_2O emissions by 32.0% and 46.2% over two years, primarily because substantial biochar applications introduce more NO_3^- and NH_4^+, enhancing their transformation [16]. Furthermore, applying very high amounts of biochar drastically reduced the quantity of soil nitrogen available from microbial processes that generate N_2O, indicating that biochar application can modify the soil C:N ratio and, consequently, affect soil N_2O emissions [6,17]. These findings indicate that the impact of biochar on soil N_2O emissions is intricately linked to the quantity of biochar applied.

An increasing number of studies have observed that the aging of biochar in the natural environment may lead to a decline in its efficacy to suppress soil N_2O emissions over time. For instance, Liu et al. [18] reported that a one-time application of 13.5 t/hm^2 biochar significantly reduced soil N_2O emissions in the first two years, yet no considerable difference was observed in the third year. Biochar's influence on the N_2O emissions weakened over time, which may be related to the occupancy of biochar pores and the decomposition of biochar [19–21]. Although a large number of studies have explored the response of soil N_2O emissions to aging biochar, there is currently no way to predict the exact duration of biochar's emission-reducing impact. Therefore, this study aims to estimate the duration of biochar's inhibitory effect on soil N_2O emissions using ecological models, providing crucial insights for future applications regarding the dosage and frequency of biochar use in agriculture.

To develop a reasonable ecological model, it is essential to consider the influence of biochar on soil physicochemical properties and microbial abundance. Nitrification and denitrification are the main ways that biochar affects soil N_2O emissions [22–24]. Nitrification is defined as $NH_4^+ \rightarrow NO_2^- \rightarrow NO_3^-$, and N_2O is produced as a by-product. Denitrification is defined as $NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$, and N_2O is produced as an intermediate product. Previous research has indicated that the porous structure of biochar provides a suitable habitat for nitrifying bacteria, which is beneficial for increasing the abundance of ammonia-oxidizing bacteria (AOB) and ammonia-oxidizing archaea (AOA), and this means that biochar increases the occurrence of nitrification in soil and thus increases the by-product (N_2O) emissions during nitrification [25,26]. Conversely, during denitrification, biochar reduced the nitrite reductase K (nirK) and nitrite reductase S (nirS) gene copy numbers and also increased the nitric oxide reductase (nosZ) gene copy number, which means that biochar can inhibit nitrite reduction and promote N_2O reduction during denitrification and thus reduce N_2O emissions during denitrification [27–29]. Additionally, biochar's ability to adsorb and stabilize soil nitrate nitrogen reduces the substrates available for denitrification, further curtailing N_2O emissions [30–32]. Thus, biochar's role in modulating ammonia oxidation and denitrification processes is evident in its impact on N_2O emissions. Prior studies have identified AOA, AOB, nirK, nirS, and nosZ as crucial functional genes influencing soil N_2O emissions. Soil microorganisms, constrained by environmental resources, respond rapidly to changes in these resources [33,34]. The introduction of biochar imposes a limit on the growth of soil-associated microorganisms, aligning with the logistic ecological model's growth dynamics, which have been substantiated in numerous studies [35–37]. In this study, key functional genes were identified using structural equation models, and ecological models based on logistic differential equations were constructed to predict the temporal effects of biochar on soil N_2O emissions.

The Huanghuaihai Plain, an important crop producing area, also records one of the highest nitrogen fertilizer application rates in China, exceeding 600 kg N/hm^2 annually. This figure notably surpasses that of similar farmlands in Europe [38]. Thus, through investigating the effects of biochar at different dosages (2, 4, 8, and 12 t/hm^2) over consecutive years, along with a one-time application of 12 t/hm^2, the objectives of this study were to (1) determine the effect of biochar on soil N_2O emissions in the Huanghuaihai Plain and its mechanism; (2) identify the key functional genes of biochar affecting soil N_2O emissions; and (3) use ecological models to quantify the duration for which biochar inhibits soil N_2O emissions.

2. Experimental Section

2.1. Study Site

The study was conducted at the Jining Experimental Station, Shandong Academy of Agricultural Sciences (116°58′13″ E, 35°40′7″ N), characterized by a warm temperate semi-humid monsoon climate, with an average annual temperature of 13.6 °C. The annual precipitation averages 693 mm, primarily occurring from June to August. The experimental field employs a wheat–maize rotation system, having a light loam soil texture. Prior to 2018, the soil's basic properties in the 0–20 cm layer were as follows: pH 7.82, organic matter 14.25 g/kg, alkali hydrolyzed nitrogen 23.11 mg/kg, available phosphorus 29.89 mg/kg, available potassium 89 mg/kg, and an electrical conductivity of soil 1.72 mS/cm.

2.2. Experimental Design

Field samples for this study were collected from October 2020 to October 2022. The experiment comprised six treatments, each randomly assigned with three replicates, totaling eighteen experimental plots of 20 m^2 each: 0 (C0), 2 (C1), 4 (C2), 8 (C3), 12 t/hm^2 biochar (C4), and one-time application of 12 t/hm^2 biochar in 2018 (CS). Biochar was applied annually before winter wheat sowing for C1, C2, C3, and C4, whereas CS received a single application in 2018. Wheat (JiMai 22) was sown on 5 October 2020 and 4 October 2021 and harvested on 3 June 2021 and 1 June 2022, respectively. Maize (LuDan 510) was sown on 14 June 2021 and 4 June 2022 and harvested on 1 October 2021 and 4 October 2022. JiMai 22 and LuDan 510, selected for the experiment, were acquired from the local market. In the winter wheat season, nitrogen and phosphorus fertilizers were applied at doses of 300 kg N/hm^2 and 150 kg P_2O_5/hm^2, respectively. In the maize season, the applications were 280 kg N/hm^2, 120 kg P_2O_5/hm^2, and 60 kg K_2O/hm^2. The nitrogen fertilizer was evenly divided between base application and topdressing (1:1 ratio) on specified dates, while the phosphorus was applied entirely as a base fertilizer. For maize, all fertilizers were applied in a single session on 14 June 2021 and 4 June 2022. Rotary tillage, sowing, and irrigation were performed concurrently after base fertilizer application in each season, with other management practices being consistent with local standards.

The biochar tested was produced from rice straw via incomplete combustion at 800 °C. Its fundamental properties were as follows: total nitrogen content of 5.12 g/kg, total potassium content of 13.37 g/kg, total phosphorus content of 0.96 g/kg, density of 0.332 g/cm^3, pH of 8.50, and carbon content of 70%. Biochar and fertilizers were mixed and incorporated into the soil to a depth of 20 cm using rotary tillage.

2.3. Sample Collection and Determination

Soil moisture and temperature were measured using a handheld TDR instrument (Spectrum, Tianjin, China). Soil samples, once collected, were cleared of plant roots and gravel and divided into two parts: one for measuring soil inorganic nitrogen, and the other for assessing the copy number of functional genes. NH_4^+ and NO_3^- in the soil were extracted with 0.01 mol/L $CaCl_2$ solution, and the extract was examined using an AA3 flow analyzer (Braun and Lubbe, Norderstedt, Germany). Sample collection occurred at 15–20-day intervals during the wheat season and 7–10-day intervals in the maize season, with increased frequency during fertilization, irrigation, and precipitation events.

Soil DNA was extracted from 0.5 g of soil using the FastDNA Spin Kit for Soils (MP Biomedicals, Solon, OH, USA), dissolved in 100 µL sterile water, and stored at −80 °C. The q-PCR was conducted using a CFX96 Real-Time PCR System (Bio-Rad, Hercules, CA, USA) with a reaction volume of 20 µL, comprising 10 µL SYBR Green (TaKaRa, Kusatsu, Japan), 0.2 µL Rox DYEII, 1 µL DNA template, 0.4 µL of each primer (10 µmol/L), and 8.0 µL sterilized water. The amplification primers and reaction conditions of each gene are shown in Table 1 [39]. Each experiment included strict negative controls.

Table 1. Amplification primers and reaction conditions of qPCR.

Gene	Name	Sequence (5′–3′)	Response Procedures	Reference	Remark
AOB	amoA-1F amoA-2R	GGG GTT TCT ACT GGT GGT CCC CTC KGS AAA GCC TTC TTC	5 min-94 °C, 30 s-94 °C, 30 s-55 °C annealing, 60 s-72 °C, 30 cycles	[40]	S = C or G
AOA	Arch-amoAF Arch-amoAR	STA ATG GTC TGG CTT AGA CG GCG GCC ATC CAT CTG TAT GT		[41]	S = C or G
nirK	nirKF1aCu nirKR3Cu	ATC ATG GTS CTG CCG CG GCC TCG ATC AGR TTG TGG TT	5 min-72 °C; Program 2: 5 min-94 °C, 30 s-94 °C, 5 s-55 °C annealing, 30 s-72 °C, 35 cycles; 1 min-72 °C	[42]	S = C or G R = A or G
nirS	nirSCd3Af nirSR3cd	GTS AAC GTS AAG GAS ACS GC GAS TTC GGR TGS GTC TTG A		[43]	S = C or G R = A or G; S = C or G
nosZ	nosZ-F nosZ-R	AGA ACG ACC AGC TGA TCG ACA TCC ATG GTG ACG CCG TGG TTC		[44]	

Gas samples were collected at the same frequency as the soil samples. N_2O emission fluxes were measured using static box gas chromatography involving a stainless-steel sampling box (50 cm × 50 cm × 50 cm) with a 1.5 mm thickness and a base (50 cm × 50 cm × 15 cm) with a 2 mm thickness [18]. Gas sampling occurred from 8:00 am to 11:00 am. In the gas collection of each box, a 200 mL disposable syringe was used to collect gas samples at 0, 10, 20, and 30 min, respectively. At each gas collection, the temperature in the chamber was recorded. Gas chromatography (Agilent 7890, Santa Clara, CA, USA) was used for sample analysis, with our N_2O flux calculation following:

$$F = \rho \times \frac{V}{A} \times \frac{\Delta C}{\Delta t} \times \frac{273}{273 + T} \quad (1)$$

where F is the N_2O flux (µg/m^2/h); ρ is the density of N_2O under standard conditions (g/m^3); V is the cavity volume (m^3); A is the covered area of the chamber (m^2); $\Delta C/\Delta t$ is the concentration rate of N_2O in the box; and T is the air temperature in the box.

The cumulative soil N_2O emissions were calculated as follows:

$$E = \sum \frac{(f_i + f_{i+1})}{2} \times (t_{i+1} - t_i) \times 24 \times 10^{-5} \quad (2)$$

where E is cumulative soil N_2O emissions (kg/ha); f is the N_2O flux (µg/m^2/h); i is the ith measurement; $(t_{i+1} - t_i)$ is the number of days between two adjacent measurements; and $\times 10^{-5}$ is the unit conversion of N_2O.

2.4. Construction of an Ecological Model to Predict Biochar's Duration of Effectiveness

Based on the results of experimental analyses, key marker functional genes were identified, facilitating the subsequent construction of the model. The Logistic model posits an environmental constraint, known as the environmental capacity (K), which limits the number of microbial populations carrying these functional genes. The instantaneous growth rate, denoted as r', was assumed to be a linear function of the population number (N). As N approaches K, r' decreases, reaching zero when the population number equals the environmental capacity ($N = K$), indicating no further growth. Conversely, when N

is zero, r' is at its maximum, equivalent to the intrinsic growth rate of the population (r). Therefore, it can be assumed that

$$r' = r\left(1 - \frac{N}{K}\right) \tag{3}$$

According to the Logistic differential equation model,

$$\frac{dN}{dt} = rN\left(1 - \frac{N}{K}\right) \tag{4}$$

By solving this equation, we can obtain the following:

$$\frac{KdN}{[N(K-N)]} = rdt \tag{5}$$

The following given condition is considered:

$$\frac{K}{[N(K-N)]} = \frac{1}{N} + \frac{1}{K-N} \tag{6}$$

After integration, we can obtain the following:

$$\int \left[\frac{1}{N} + \frac{1}{K-N}\right] dN = \int rdt + C \tag{7}$$

The solution is formalized in

$$\log N - \log(K-N) = rt + C \tag{8}$$

Further sorting produce equations are

$$N = \frac{K}{1 + e^{a-rt}} \tag{9}$$

$$\frac{K-N}{N} = e^{a-rt} \tag{10}$$

and

$$y = \log\left(\frac{K-N}{N}\right) \tag{11}$$

By performing unary regression, we can obtain

$$y = a - rt \tag{12}$$

We can then obtain parameters a and r.

These mathematical findings enable the calculation of the effective duration for a one-time application of 12 t/hm^2 biochar in mitigating soil N_2O emissions, informed by the result of an increased copy number value for key marker functional genes from the biochar treatment.

2.5. Statistical Analysis

A one-way ANOVA was conducted using SPSS22 software. The Pearson correlation coefficient was employed to examine the relationship between gas emission fluxes and influencing factors, with the significance threshold being set at $p < 0.05$. Graphs were generated using Origin8.5 software, and all data were presented as mean ± standard deviation. The comprehensive impact of soil physicochemical properties and microorganisms on soil N_2O emissions was assessed through structural equation modeling (SEM) using Amos17.0

software. Excel 2021 was utilized to estimate the duration of soil N_2O emission reduction by biochar based on the Logistic model.

3. Results

3.1. Effects of Biochar Doses and Application Frequency on Soil N_2O Emissions

Throughout the experimental period, annual biochar additions consistently reduced cumulative soil N_2O emissions, with treatments C3 and C4 exhibiting the most pronounced inhibitory effects in each growing season (Figure 1). Relative to C0, cumulative N_2O emissions in the wheat seasons decreased by 44.69% and 56.21% for C3 and by 49.39% and 52.45% for C4. In the maize seasons, these reductions were 34.65% and 31.36% for C3 and 38.62% and 36.92% for C4. An inverse quadratic linear relationship was observed between the biochar doses and cumulative N_2O emissions, indicating a decrease in emissions with increasing biochar application. The one-time application of 12 t/hm^2 biochar (CS) showed a reduction in cumulative N_2O emissions compared to C0, but this result was not significant, indicating its ineffectiveness in substantially reducing soil N_2O emissions three years post-application. The study highlighted that biochar's inhibition of soil N_2O emissions was predominantly observed at emission peaks (Figure 2). During the wheat season, peak N_2O fluxes in treated soils occurred following base and topdressing fertilizer applications, remaining low throughout the rest of the season. In the maize season, the first emission peak occurred post-fertilization, followed by a declining trend, with a second peak emerging from late July to mid-August annually. Although the maize season soil exhibited higher N_2O flux intensity than in the wheat season, biochar's emission mitigation was more effective during the wheat season.

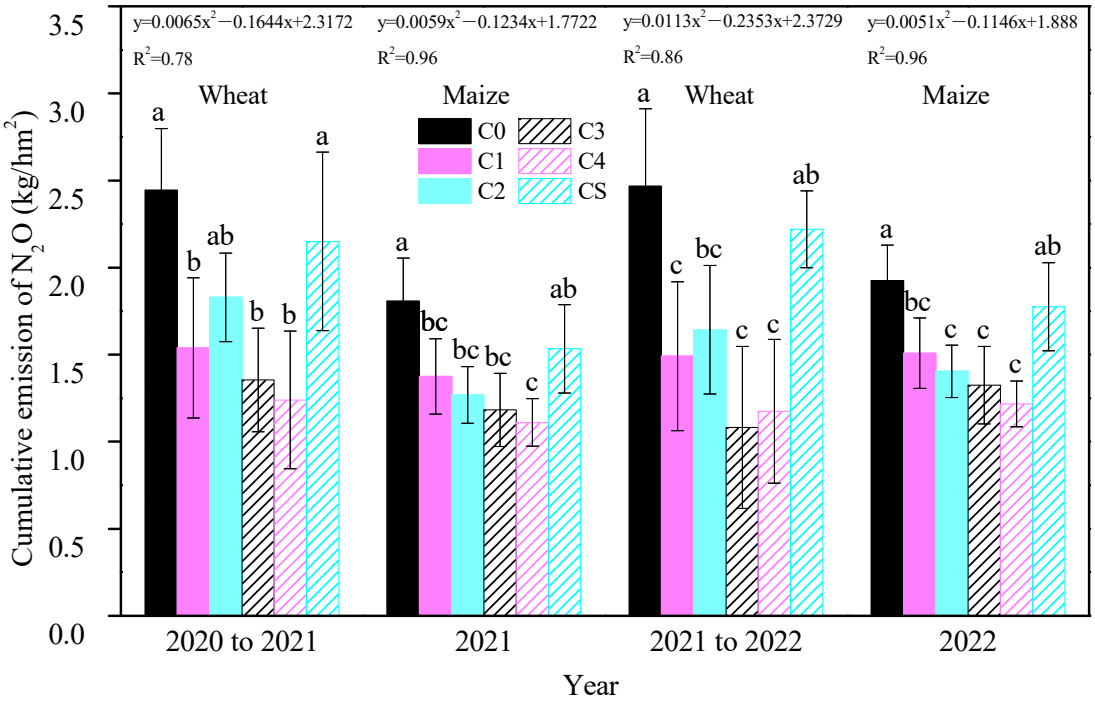


Figure 1. Cumulative soil N_2O emissions in each season and treatment. Bars are shown as the means with standard errors. Different small letters for the same season indicate a significant difference between the groups ($p < 0.05$). The equation represents the linear relationship between different biochar inputs and soil cumulative N_2O emissions in each season.

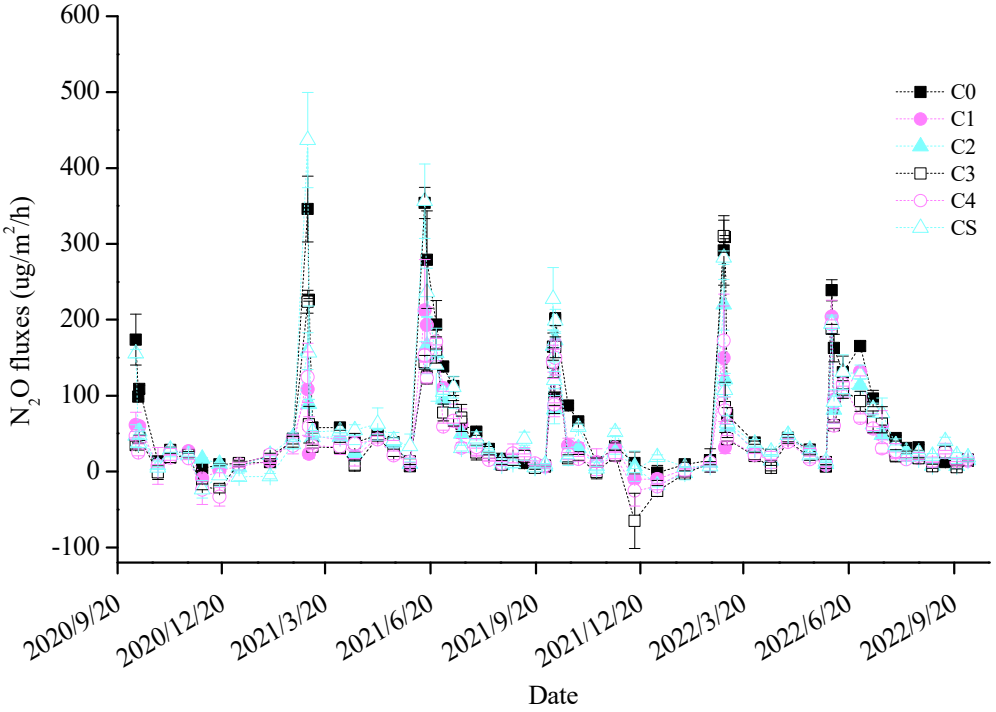


Figure 2. Changes in soil N_2O fluxes in each treatment. Broken lines are shown as means with standard errors. The same applies to the figures below.

3.2. Effects of Biochar Dosage and Frequency on Soil Inorganic Nitrogen, Temperature, and Moisture

The soil NH_4^+ content trends were similar across all treatments, peaking swiftly post-fertilization before decreasing rapidly (Figure 3a). Notably, in the first maize season, the C2 and C4 treatments decreased average soil NH_4^+ content by 10.2% and 20.1%, respectively. In the maize season of the second year, only C2 treatment significantly reduced the average soil NH_4^+ by 20.1%. The soil NO_3^- trends followed a consistent pattern across treatments (Figure 3b), increasing after fertilization and then gradually declining. In the first wheat season, the C3, C4, and CS treatments significantly raised the average soil NO_3^- by 12.35–25.69%. During the first maize season, the biochar treatments elevated the average soil NO_3^- content by 21.18–59.02%. In the second wheat season, all treatments except C1 increased the average soil NO_3^- content by 13.26–25.29%, and in the second maize season, all except C2 enhanced the soil NO_3^- content by 13.52–18.80%. The soil temperature and moisture dynamics are depicted in Figure S1. The soil temperature changes were gradual over time across the treatments, while the soil moisture exhibited more significant fluctuations due to irrigation and precipitation. The biochar treatments, particularly C3, C4, and CS, modestly increased the average soil temperature by 1.22–4.30%, 1.90–5.61%, and 1.62–4.40%, respectively (Table S1). Only the CS treatment significantly reduced soil moisture in the first wheat season by 16.14%, whereas the other biochar treatments did not notably affect soil moisture during the whole test period.

3.3. Effects of Biochar Dosage and Frequency on Functional Genes

Seasonal variations were observed in the copy numbers of the nirK, nirS, and nosZ genes in the soil across the different treatments, with significantly higher values being noted during the maize season than in the wheat season (Figure 4). Excluding the CS treatment, the biochar applications resulted in significant reductions in soil nirK gene copy numbers of 17.90–29.55%, 10.70–24.12%, 16.30–23.55%, and 10.99–21.97% across the four growing seasons, respectively (Table 2). The CS treatment led to a notable decrease of 8.50% in the nirK gene copy number in the first wheat season only. During the second maize season, all biochar treatments except CS significantly reduced the nirS gene copy number by 9.72–12.99%. In other seasons, the impact of biochar on the soil nirS gene copy

number was not significant. All biochar treatments except CS enhanced the nosZ gene copy number to various extents, particularly in the C3 and C4 treatments, with increases of 9.11% and 8.92%, 7.49% and 8.94%, 6.39% and 5.65%, and 9.00% and 13.95% in the respective seasons. Additionally, slight seasonal shifts were noted in the AOB gene copy number across the treatments, marked by a substantial rise in the middle of the second maize season (Figure S2a). The biochar treatments led to significant increases in the soil AOB gene copy number, with increments of 56.18–197.44%, 28.23–83.93%, 68.85–284.63%, and 70.68–151.61% in the respective growing seasons. The AOA gene copy number in the soil exhibited no significant seasonal variation (Figure S2b). All biochar treatments except for CS notably augmented the soil AOA gene copy number by 28.26–69.09%, 18.84–70.96%, 28.53–90.57%, and 21.00–25.80% across the four seasons, respectively.

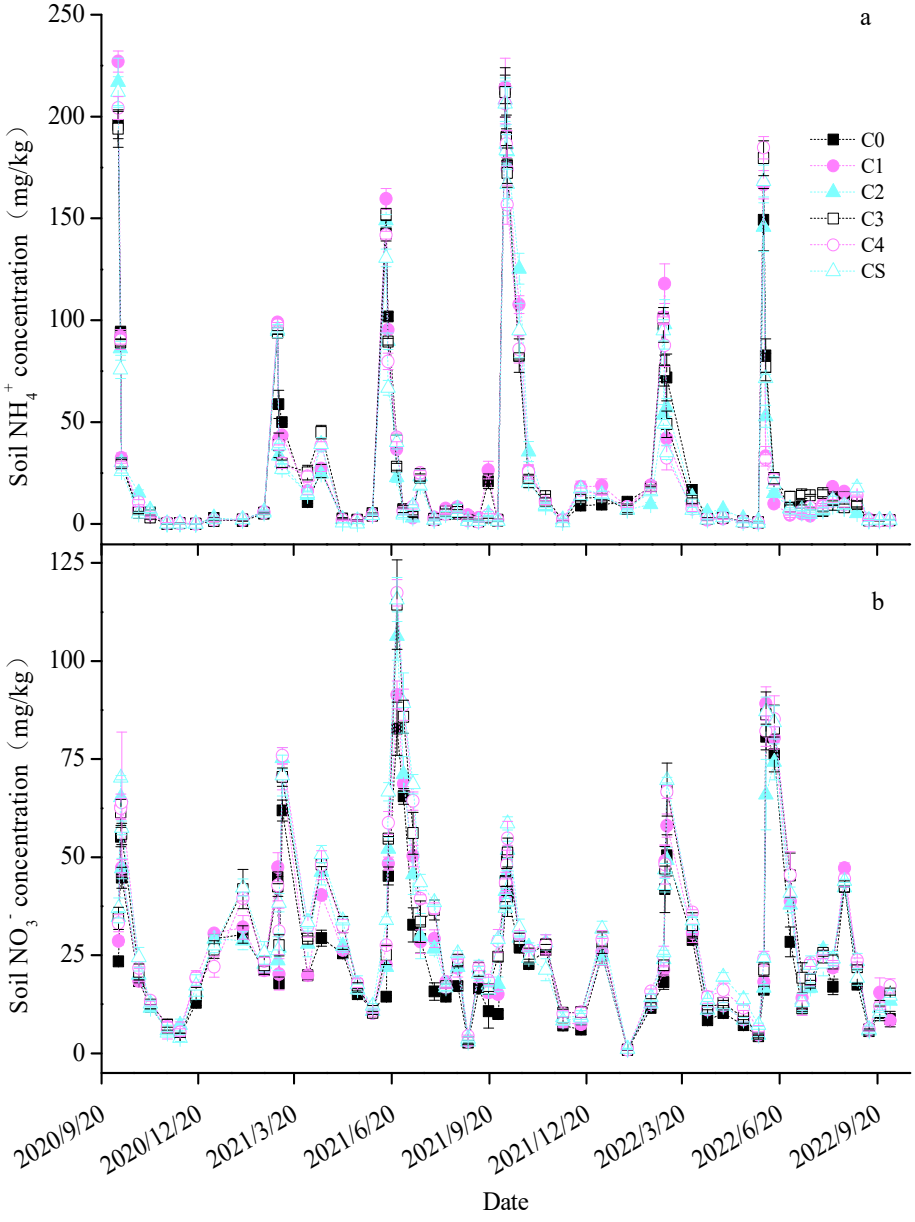


Figure 3. Changes in the soil NH_4^+ (**a**) and NO_3^- (**b**) content in each treatment.

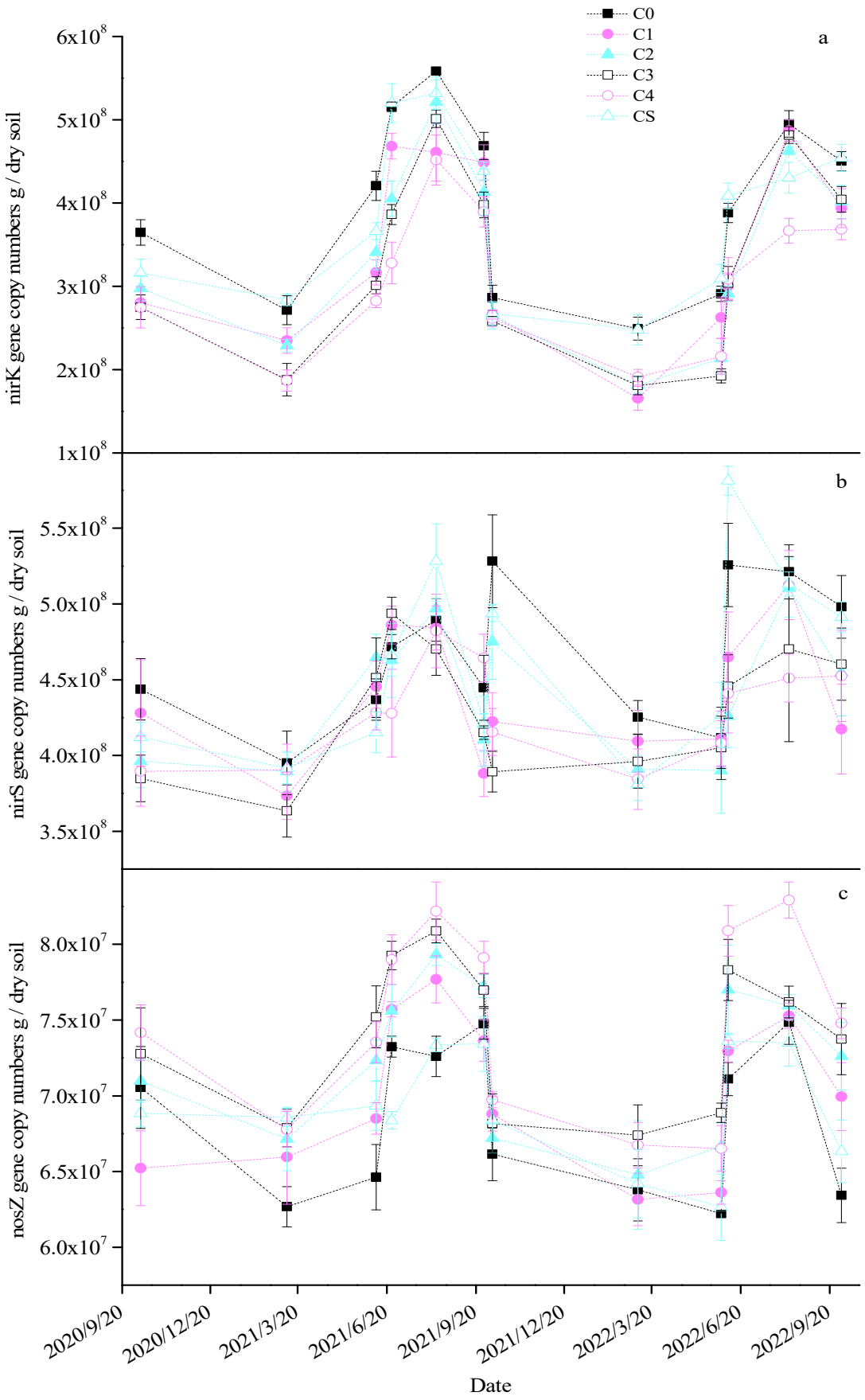


Figure 4. Changes in the nirK (**a**), nirS (**b**), and nosZ (**c**) gene copy numbers in each treatment.

Table 2. The mean values for AOA, AOB, nirK, nirS, and nosZ gene copy numbers for each treatment. Different small letters of the same season and same gene indicate a significant difference between the groups ($p < 0.05$).

Growing Season	Treatment	AOB	AOA	nirK	nirS	nosZ
2020–2021 Winter wheat	C0	$(6.89 \pm 2.60) \times 10^6$ d	$(3.23 \pm 0.14) \times 10^7$ e	$(3.52 \pm 0.17) \times 10^8$ a	$(4.25 \pm 0.18) \times 10^8$ a	$(6.60 \pm 0.21) \times 10^7$ b
	C1	$(1.56 \pm 0.15) \times 10^7$ b	$(3.75 \pm 0.18) \times 10^7$ d	$(2.77 \pm 0.17) \times 10^8$ cd	$(4.16 \pm 0.23) \times 10^8$ a	$(6.66 \pm 0.22) \times 10^7$ b
	C2	$(1.87 \pm 0.14) \times 10^7$ a	$(5.61 \pm 0.17) \times 10^7$ b	$(2.89 \pm 0.15) \times 10^8$ c	$(4.17 \pm 0.15) \times 10^8$ a	$(7.02 \pm 0.16) \times 10^7$ a
	C3	$(2.05 \pm 0.16) \times 10^7$ a	$(6.34 \pm 0.17) \times 10^7$ a	$(2.55 \pm 0.15) \times 10^8$ de	$(4.00 \pm 0.20) \times 10^8$ a	$(7.20 \pm 0.21) \times 10^7$ a
	C4	$(1.82 \pm 0.11) \times 10^7$ ab	$(4.81 \pm 0.12) \times 10^7$ c	$(2.48 \pm 0.15) \times 10^8$ e	$(4.03 \pm 0.17) \times 10^8$ a	$(7.18 \pm 0.15) \times 10^7$ a
	CS	$(1.08 \pm 0.12) \times 10^7$ c	$(3.87 \pm 0.14) \times 10^7$ d	$(3.22 \pm 0.11) \times 10^8$ b	$(4.06 \pm 0.11) \times 10^8$ a	$(6.89 \pm 0.10) \times 10^7$ ab
2021 Summer maize	C0	$(1.08 \pm 0.09) \times 10^7$ d	$(3.46 \pm 0.22) \times 10^7$ f	$(5.14 \pm 0.09) \times 10^8$ a	$(4.69 \pm 0.15) \times 10^8$ a	$(7.35 \pm 0.10) \times 10^7$ d
	C1	$(1.47 \pm 0.12) \times 10^7$ c	$(4.54 \pm 0.19) \times 10^7$ d	$(4.59 \pm 0.24) \times 10^8$ b	$(4.53 \pm 0.14) \times 10^8$ a	$(7.57 \pm 0.11) \times 10^7$ c
	C2	$(1.54 \pm 0.17) \times 10^7$ bc	$(5.52 \pm 0.16) \times 10^7$ c	$(4.46 \pm 0.25) \times 10^8$ b	$(4.57 \pm 0.16) \times 10^8$ a	$(7.74 \pm 0.11) \times 10^7$ bc
	C3	$(1.99 \pm 0.16) \times 10^7$ a	$(7.75 \pm 0.19) \times 10^7$ a	$(4.28 \pm 0.13) \times 10^8$ b	$(4.60 \pm 0.11) \times 10^8$ a	$(7.90 \pm 0.09) \times 10^7$ ab
	C4	$(1.76 \pm 0.16) \times 10^7$ ab	$(6.31 \pm 0.21) \times 10^7$ b	$(3.90 \pm 0.24) \times 10^8$ c	$(4.58 \pm 0.23) \times 10^8$ a	$(8.01 \pm 0.16) \times 10^7$ a
	CS	$(1.39 \pm 0.17) \times 10^7$ c	$(4.00 \pm 0.18) \times 10^7$ e	$(4.97 \pm 0.18) \times 10^8$ a	$(4.72 \pm 0.19) \times 10^8$ a	$(7.17 \pm 0.09) \times 10^7$ d
2021–2022 Winter wheat	C0	$(5.09 \pm 0.22) \times 10^6$ d	$(3.20 \pm 0.12) \times 10^7$ e	$(2.76 \pm 0.13) \times 10^8$ a	$(4.55 \pm 0.21) \times 10^8$ a	$(6.41 \pm 0.13) \times 10^7$ c
	C1	$(1.70 \pm 0.15) \times 10^7$ ab	$(4.65 \pm 0.18) \times 10^7$ d	$(2.31 \pm 0.15) \times 10^8$ b	$(4.14 \pm 0.19) \times 10^8$ bc	$(6.52 \pm 0.12) \times 10^7$ bc
	C2	$(1.43 \pm 0.08) \times 10^7$ b	$(7.33 \pm 0.21) \times 10^7$ b	$(2.20 \pm 0.15) \times 10^8$ b	$(4.19 \pm 0.21) \times 10^8$ bc	$(6.62 \pm 0.20) \times 10^7$ abc
	C3	$(1.96 \pm 0.24) \times 10^7$ a	$(8.86 \pm 0.39) \times 10^7$ a	$(2.11 \pm 0.08) \times 10^8$ b	$(3.97 \pm 0.17) \times 10^8$ c	$(6.81 \pm 0.15) \times 10^7$ a
	C4	$(1.94 \pm 0.21) \times 10^7$ a	$(6.39 \pm 0.21) \times 10^7$ c	$(2.23 \pm 0.14) \times 10^8$ b	$(4.03 \pm 0.17) \times 10^8$ bc	$(6.77 \pm 0.12) \times 10^7$ ab
	CS	$(8.60 \pm 0.39) \times 10^6$ c	$(3.32 \pm 0.11) \times 10^7$ e	$(2.75 \pm 0.16) \times 10^8$ a	$(4.34 \pm 0.13) \times 10^8$ ab	$(6.51 \pm 0.19) \times 10^7$ bc
2022 Summer maize	C0	$(9.26 \pm 1.04) \times 10^6$ c	$(3.75 \pm 0.17) \times 10^7$ c	$(4.46 \pm 0.13) \times 10^8$ a	$(5.15 \pm 0.22) \times 10^8$ a	$(6.98 \pm 0.15) \times 10^7$ e
	C1	$(1.59 \pm 0.16) \times 10^7$ b	$(5.00 \pm 0.10) \times 10^7$ b	$(3.94 \pm 0.17) \times 10^8$ b	$(4.65 \pm 0.27) \times 10^8$ b	$(7.27 \pm 0.13) \times 10^7$ cd
	C2	$(1.87 \pm 0.12) \times 10^7$ b	$(6.05 \pm 0.14) \times 10^7$ a	$(3.85 \pm 0.11) \times 10^8$ b	$(4.64 \pm 0.23) \times 10^8$ b	$(7.52 \pm 0.20) \times 10^7$ bc
	C3	$(2.21 \pm 0.23) \times 10^7$ a	$(6.29 \pm 0.15) \times 10^7$ a	$(3.97 \pm 0.15) \times 10^8$ b	$(4.59 \pm 0.35) \times 10^8$ b	$(7.61 \pm 0.18) \times 10^7$ b
	C4	$(2.33 \pm 0.21) \times 10^7$ a	$(6.18 \pm 0.21) \times 10^7$ a	$(3.48 \pm 0.18) \times 10^8$ c	$(4.48 \pm 0.24) \times 10^8$ b	$(7.95 \pm 0.13) \times 10^7$ a
	CS	$(1.58 \pm 0.15) \times 10^7$ b	$(3.86 \pm 0.18) \times 10^7$ c	$(4.31 \pm 0.17) \times 10^8$ a	$(5.29 \pm 0.09) \times 10^8$ a	$(7.11 \pm 0.14) \times 10^7$ de

3.4. SEM of Biochar's Effects on Soil N_2O Emissions

The results from the structural equation modeling (SEM) indicated that the nosZ gene significantly reduced soil N_2O emissions, evidenced by a path coefficient of −0.08 (Figure 5). The soil NO_3^- content, an important factor, influenced N_2O emissions both directly and indirectly through the nosZ gene, yielding a path coefficient of 0.70 + 0.17 × (−0.08) = 0.69. Soil moisture and temperature were found to significantly impact the nosZ gene, with soil temperature also affecting NO_3^- content. Hence, in our study, soil temperature and moisture indirectly influenced soil N_2O emissions. The nirK gene had no direct effect on soil N_2O emissions, but the SEM revealed an association with the nosZ gene, potentially affecting its copy number. Under drought conditions, the denitrification process in soil was less pronounced. However, soil denitrification was aggravated after precipitation and irrigation. Biochar application could significantly reduce soil N_2O emissions by reducing denitrification N_2O emissions.

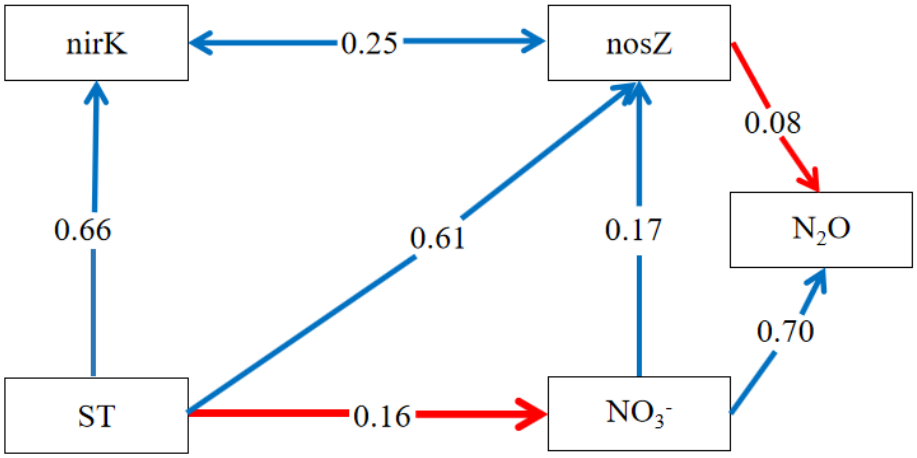


Figure 5. SEM of factors influencing soil N_2O emissions. The blue line indicates a positive correlation, and the red line indicates a negative correlation. A→B represents the action of A on B, and A↔B represents the interaction between A and B.

3.5. Prediction of the Inhibition Time of Biochar on Soil N_2O Emissions Based on the Logistic Ecological Model

It is evident that the nosZ gene emerged as the key functional gene in the biochar-mediated inhibition of soil N_2O emissions. From the C4 treatment data in Table S2, the peak increase in the nosZ gene copy number was approximately 1×10^7, denoted as the K value in 1.5. For evaluating the nosZ gene copy number increase for the C5 treatment in Table S2, data from 7 October 2021, 6 March 2022, and 31 May 2022 were chosen. Parameter a was determined to be 0.2907, and r was considered as −0.0041, following the methodology in Section 2.4, yielding $y = 0.2907 - (-0.0041t)$. In correlating these findings with the N_2O emission fluxes on corresponding dates (Figure 2), it was shown that an increase in the nosZ gene copy number to 1×10^6 notably suppressed soil N_2O emissions. Consequently, the CS processing duration was calculated from the maximum (1×10^7) to the minimum (1×10^6) value, which was approximately 1.77 years. Therefore, based on the logistic ecological model, a one-time application of 12 t/hm^2 of biochar would cause a rapid surge leading to a peak in nitrous oxide-reducing bacteria, followed by a decline to the base level, effectively restraining soil N_2O emissions for an estimated duration of 1.77 years.

4. Discussion

4.1. Effects of Biochar on Soil Environment and Inorganic Nitrogen

In our study, an increase in average soil temperature was observed following biochar application, aligning with previous findings that attribute this to biochar's black color and greater heat absorption capacity [45,46]. However, biochar had no significant effect

on soil moisture in our study. The soil moisture levels were primarily influenced by precipitation and irrigation, with the biochar treatment demonstrating no significant water retention effect. Our results differ from those of many past studies [47–49] and may be linked to the characteristics or preparation conditions of the biochar used. It has been reported that straw biochar produced at lower temperatures exhibits considerably higher water retention capabilities compared to that prepared at higher temperatures [50,51]. As temperature increases, biochar's degree of aromatization intensifies, its hydrophobic properties are enhanced, and the quantity of oxygen- and nitrogen-containing functional groups diminishes, leading to a reduction in biochar's water-holding capacity [52,53].

Soil NH_4^+ and NO_3^- play critical roles as substrates for the nitrification and denitrification processes in soil microorganisms [54,55]. In our study, soil NO_3^- was identified as a primary factor influencing soil N_2O emissions, and it was observed that biochar application increased soil NO_3^- levels, with particularly notable effects in the C3, C4, and CS treatments. Owing to its large specific surface area and efficient adsorption capabilities, biochar minimizes leaching, thereby retaining NO_3^- within the soil [56,57]. Furthermore, a reduction in soil NH_4^+ content was noted, implying that the rise in soil NO_3^- was partly due to enhanced nitrification. This inference is supported by the observed increases in AOB and AOA gene copy numbers in soils treated with biochar. Additionally, an increment in biochar application was found to suppress the amplification of nirK and nirS gene copy numbers, suggesting that biochar inhibits NO_3^- reduction, thereby augmenting soil NO_3^- concentration [58]. A positive correlation was established between the quantity of biochar added and the nosZ gene copy number in the soil. Notably, the nosZ gene copy number exhibited positive associations with both soil temperature and NO_3^- levels. The elevated soil temperatures enhance the activity of N_2O-reducing bacteria, leading to an increased nosZ gene copy number [59–61]. Additionally, a higher concentration of NO_3^- in the soil favors a rise in nosZ gene copy number [62].

4.2. Influence of Biochar on Soil N_2O Emissions and Its Temporal Effects

Biochar application has been found to affect N_2O production from nitrification and denitrification, effectively reducing soil N_2O emissions [24]. Our research indicated a significant increase in AOB and AOA gene frequencies and NO_3^- concentration in the soil post-biochar treatment, suggesting that biochar enhances nitrification, which may indirectly affect soil N_2O emissions [63]. Nonetheless, the overall impact of biochar was a reduction in soil N_2O emissions, underscoring its efficacy in inhibiting N_2O generation during denitrification. The SEM of relevant factors revealed that biochar's primary mechanism for reducing soil N_2O emissions was through increasing the nosZ gene copy number, thereby facilitating the N_2O reduction [64,65]. It was also observed that biochar could reduce N_2O emissions by increasing the abundance of N_2O-reducing bacteria in a field experiment in North China [25]. Hence, denitrification is identified as the crucial process through which biochar mitigates soil N_2O emissions in this experimental context. Additionally, the rise in soil NO_3^- levels appeared to stimulate microbial activity associated with N_2O reduction [66]. This inhibition mechanism was also found in our results, especially in the treatment with a high amount biochar application, and this may be because the addition of biochar creates a more favorable environment for nitrous oxide-reducing bacteria, which, in turn, enhances the N_2O reduction process [67]. Furthermore, higher amounts of biochar showed superior efficacy in adsorbing NH_4^+, thus diminishing the nitrification substrate availability and further reducing N_2O emissions [68,69].

Our findings indicate that the annual application of 8 t/hm^2 biochar was most effective in inhibiting N_2O emissions in the wheat season, whereas it was 12 t/hm^2 in the maize season, which is consistent with the results of many previous studies [70–72]. Biochar's emission reduction is attributed to alterations in soil physicochemical properties and microbial activities, revealing a temporal dependency in its inhibitory effect. In 2018, a one-time application of 12 t/hm^2 biochar (CS treatment) was implemented; however, no significant N_2O emission reduction was exhibited throughout the study period. Given

the pre-planting application of biochar in the wheat season and its time-sensitive effect, biochar was found to be more effective in reducing N_2O emissions during the wheat season than in the maize season. Over time, the beneficial properties of biochar, such as its large specific surface area and strong adsorption, tend to diminish, affecting soil structure and microbial activity, thereby impacting its N_2O emission reduction capacity [73–76]. Furthermore, biological processes, including the invasive growth of saprophytic mycelia and extracellular enzymes, can alter biochar's structure within the soil, potentially leading to its fragmentation and influencing its functionality [77].

According to the above results, to accurately estimate the duration of biochar's inhibitory effect on soil N_2O emissions, we adopted a logistic ecological model. The findings suggest that the inhibitory impact of 12 t/hm^2 biochar on soil N_2O emissions persists for approximately 1.77 years under the conditions of this experiment. Therefore, reapplying 12 t/hm^2 biochar at intervals of roughly 1.77 years can effectively and sustainably reduce soil N_2O emissions. This aligns with the work of Liu et al. [18], who noted that a single application of 13.5 t/hm^2 biochar significantly reduced soil N_2O emissions in the first two years, with no marked difference observed at the onset of the third year. A clear correlation exists between biochar's duration and application rate. Based on our experiments and the practical aspects of farmland management, in a wheat–maize rotation system, the initial biochar application should occur before wheat sowing in the first cycle, followed by a second application before maize sowing in the second cycle (around 1.5 years after the previous application) and a third application before maize sowing in the fourth cycle (approximately 2 years after the last application), continuing with alternating intervals of 1.5 and 2 years. While prior research has generally advocated for annual biochar applications, considering economic and labor costs, extending the interval between biochar applications has proven to be an efficient strategy [78,79]. For instance, using a 12 t/hm^2 biochar application, as recommended in this study, could reduce the average annual cost of biochar by 37.5%, significantly reduce labor requirements, and optimize biochar's value.

The primary cause of increased soil N_2O emissions has been identified as fertilizer application, with reductions in fertilization to lower these emissions potentially impacting crop yield negatively [80,81]. However, integrating biochar with fertilizers has been shown to decrease soil N_2O emissions without compromising crop yield. Experimental results indicate that biochar application effectively diminishes soil N_2O emissions via denitrification. Within this study's parameters, the optimal biochar application rate for the targeted area, under standard nitrogen fertilization conditions, was determined to be 12 t/hm^2. Furthermore, it was recommended that biochar be applied at alternating intervals of 1.5 and 2 years to sustain its efficacy.

5. Conclusions

In this study, we examined the effect of biochar on soil N_2O emissions and its time-limited effect. Biochar significantly reduced soil N_2O emissions compared to the control (C0), particularly during fertilization, irrigation, and precipitation events. SEM analysis revealed that biochar diminished N_2O emissions by enhancing N_2O reabsorption, with this effect being notably pronounced in treatments with larger biochar quantities (C4). However, the suppression of N_2O emissions by large, one-time applications of biochar was not sustained. Utilizing a logistic ecological model, it was determined that the biochar's inhibitory influence on soil N_2O emissions gradually declines, persisting for approximately 1.77 years. Thus, when evaluating the overall cost and benefits of biochar usage, the quantity of biochar applied and the frequency of its application emerge as critical considerations.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/su16135813/s1. Table S1: Mean table of soil properties under the six experimental treatments. Data are the mean with standard errors. Lowercase letter in the same column and in the same season represents significant differences among experimental treatments at the level of 0.05; Table S2: Compared with C0, the increased value of nosZ gene copy numbers under the six experimental treatments. Figure S1: Variation of the soil temperature (a) and soil moisture (b)

under the six experimental treatments. Data are shown as the means with standard errors; Figure S2: Variation of the AOB (a) and AOA (b) gene copy numbers under the six experimental treatments. Data are shown as the means with standard errors.

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Abbreviations

N_2O	Nitrous oxide
AOB	Ammonia-oxidizing bacteria
AOA	Ammonia-oxidizing archaea
nirK	Nitrite reductase K
nirS	Nitrite reductase S
nosZ	Nitric oxide reductase

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