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Original Research

Soil moisture-driven changes in the balance of ammonia and nitrite oxidation

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Abstract

Soil moisture profoundly regulates nitrification by influencing oxygen (O_2) diffusion, substrate mobility, and microbial activity. However, the mechanisms underlying moisture-driven shifts in the coupling between ammonia (NH_3) and nitrite (NO_2^-) oxidation remain poorly understood. In this study, a laboratory incubation experiment was conducted using agricultural soils under four water-holding capacities (WHC: 40%, 60%, 90%, and 120%) and three ammonium (NH_4^+) levels (50, 100, and 200 mg NH_4^+–N kg^{-1}). Under low nitrogen (N) input, soil moisture exerted minimal influence on NH_3 oxidation. At higher NH_4^+ concentrations, however, oxidation increased substantially at moderate moisture (60%–90% WHC) and dropped under waterlogged conditions (120% WHC) due to O_2 limitation. Quantitative PCR and cDNA analyses indicated clear divergences in the responses of ammonia-oxidizing archaea (AOA), ammonia-oxidizing bacteria (AOB), and nitrite-oxidizing bacteria (NOB) to soil moisture. AOB activity was highest under conditions of high N availability and moderate soil moisture. In contrast, *Nitrobacter* activity declined sharply when soils became saturated, whereas *Nitrospira* showed a much broader tolerance to moisture extremes. Correlation analyses revealed that NO_2^- accumulation was driven by an imbalance between NH_3 and NO_2^- oxidation: a situation particularly pronounced under high N and high moisture conditions, where rapid NH_3 oxidation by AOB outpaced the oxidation capacity of *Nitrobacter*. Together, the results highlight that soil moisture and N availability co-determine the coupling of NH_3 and NO_2^- oxidation by driving microbial community changes and functional gene responses, yielding new insights into moisture-driven regulation of soil N cycling.

Keywords: Soil moisture, Ammonia oxidation, Nitrite oxidation, Ammonia-oxidizing microorganisms, Nitrogen cycling

Highlights

- Interactions between soil moisture and N availability governed the coupling of ammonia and nitrite oxidation in agricultural soils.
- Moderate moisture (60%–90% WHC) enhanced nitrification, whereas waterlogging (120% WHC) suppressed both AOB and NOB activities due to O_2 limitation.
- Imbalanced activities of AOB and *Nitrobacter* under high N input led to NO_2^- accumulation, revealing microbial mechanisms driving moisture-dependent nitrification.

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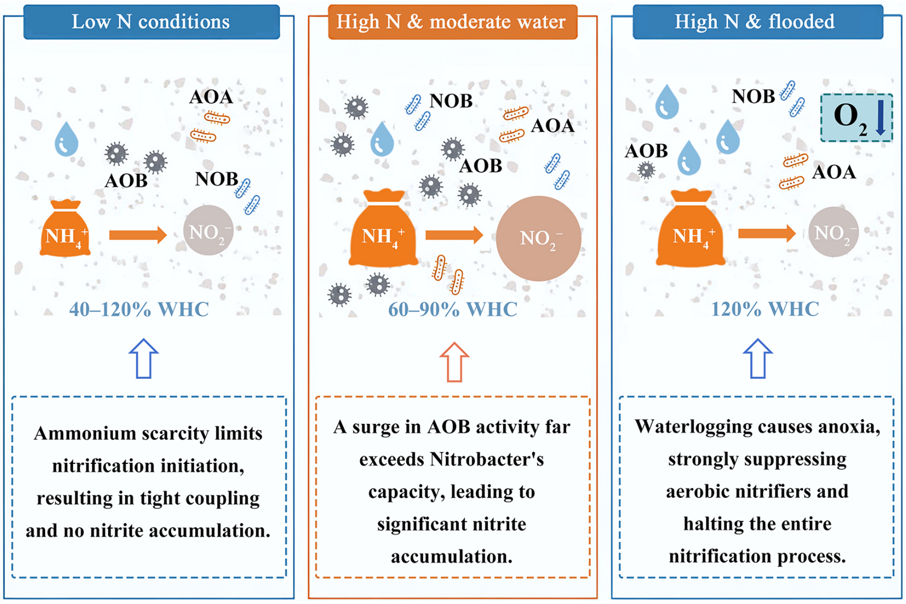
Graphical abstract



Ammonium scarcity limits nitrification initiation, resulting in tight coupling and no nitrite accumulation.

A surge in AOB activity far exceeds Nitrobacter's capacity, leading to significant nitrite accumulation.

Waterlogging causes anoxia, strongly suppressing aerobic nitrifiers and halting the entire nitrification process.

Introduction

Soil nitrogen (N) cycling represents one of the most fundamental biogeochemical processes in terrestrial ecosystems. As a key component of this cycle, nitrification plays a crucial role in regulating N availability, mitigating greenhouse gas (GHG) emissions, and maintaining overall environmental quality. Nitrification is an aerobic, two-step process in which ammonia-oxidizing microorganisms (AOM), including ammonia-oxidizing archaea (AOA) and ammonia-oxidizing bacteria (AOB), oxidize ammonia (NH_3) to nitrite (NO_2^-). This is subsequently followed by the oxidation of NO_2^- to nitrate (NO_3^-) carried out by nitrite-oxidizing bacteria (NOB)[1]. Traditionally, these two steps were thought to be performed by distinct functional microbial groups, forming the classical 'two-step nitrification' model. However, the recent discovery of complete ammonia oxidizers (comammox), which are capable of converting NH_3 to NO_3^- within a single organism, has challenged this long-standing paradigm and introduced additional complexity to our understanding of the nitrification process[2,3].

Under normal conditions, the two steps of nitrification are tightly coupled, resulting in minimal accumulation of NO_2^-. However, environmental disturbances can disrupt this coupling between NH_3 and NO_2^- oxidation, leading to substantial NO_2^- accumulation[4]. Nitrite acts as a key intermediate and precursor for several harmful nitrogenous compounds, including NO_2^-, nitric oxide (NO), and nitrous acid[5]. Therefore, elucidating the factors and mechanisms governing the coupling between NH_3 and NO_2^- oxidation is essential for advancing our understanding of N transformation processes and mitigating their environmental consequences.

Soil moisture, as a key environmental factor influencing microbial activity and nutrient diffusion, exerts a profound effect on all stages of the nitrification process. On one hand, soil moisture directly controls oxygen (O_2) availability, thereby affecting the growth and activity of aerobic ammonia-oxidizing and nitrite-oxidizing microorganisms. Previous studies have demonstrated that higher water content in inter-aggregate pores can impede O_2 diffusion, creating a microenvironment that favors denitrification over nitrification[6,7]. AOB and NOB differ in their affinity for O_2; for instance, *Nitrobacter winogradskyi* (an NOB) is less competitive than *Nitrosomonas europaea* (an AOB) under low-O_2 conditions, placing *Nitrobacter winogradskyi* at a disadvantage in hypoxic soils[8]. Guisasola et al.[9] further refined the method developed by Wiesmann to simultaneously estimate the O_2 affinity constants for AOB and NOB (the O_2 affinity constant for AOB (KOA) and O_2 affinity constant for NOB (KON), respectively). Their results showed that KOA was 0.74 ± 0.02 mg O_2 dm^{-3}, whereas KON was 1.75 ± 0.01 mg O_2 dm^{-3}, indicating that NO_2^- oxidation is more sensitive to fluctuations in dissolved O_2 concentrations than NH_3 oxidation[9]. Beyond O_2 availability, soil moisture also influences nitrification by regulating the diffusion rates of nutrients and substrates. Under either excessively low or high soil moisture conditions, substrate diffusion can be constrained or microbial activity suppressed, thereby reducing nitrification rates[10]. Such constraints may decouple the oxidation of NH_3 and NO_2^-, leading to the accumulation of NO_2^-.

Moreover, different ammonia-oxidizing and nitrite-oxidizing microorganisms may exhibit distinct responses to soil moisture, leading to shifts in the dominant microbial communities under varying moisture conditions. These community shifts can, in turn, influence both the rate and pathway of nitrification. For example, some studies have suggested that AOA may have a competitive advantage over AOB under low-O_2 conditions, whereas AOB exhibit significantly higher maximum NH_3 oxidation rates (V_{max}) compared to both AOA and comammox[11]. However, a study by Liu et al.[12] reported that the gene abundances of both AOA and AOB increased with rising soil moisture levels, ranging from 50% to 85% water-filled pore space (WFPS). The effects of soil moisture on AOB abundance remain inconsistent across studies. Hastings et al.[13] observed that increasing soil moisture may enhance AOB abundance by alleviating water stress, whereas Nguyen et al.[14] suggested that excessive soil moisture could restrict O_2 diffusion, thereby reducing the abundance of nitrifying bacteria. Additionally, the presence and activity of comammox organisms may also be regulated by soil moisture, potentially altering the dominant nitrification pathways in soils[15,16].

Despite the recognized importance of soil moisture in regulating nitrification, studies explicitly addressing its influence remain limited, and systematic investigations into the coupling between NH_3 and NO_2^- oxidation under varying moisture conditions are still scarce. Most existing research on O_2-mediated coupling has been conducted in wastewater treatment systems rather than in natural soils[17]. Although nitrification has been extensively investigated in wastewater treatment systems, the mechanistic understanding derived from aquatic media cannot be directly transferred to soils. Unlike wastewater, soil is a heterogeneous porous matrix in which water content simultaneously affects O_2 diffusion, substrate transport, redox gradients, and microbial niche partitioning at the microscale. As a result, the coupling between NH_3 oxidation and NO_2^- oxidation in soils is expected to be more spatially variable and more sensitive to environmental thresholds than in relatively homogeneous aquatic systems. Furthermore, NH_4^+ availability plays a crucial role in regulating soil nitrification. Higher NH_4^+ concentrations typically stimulate ammonia oxidation by increasing substrate availability, especially for AOB[18]. Nevertheless, the combined effects of soil moisture and NH_4^+ availability are still poorly understood.

Therefore, this study aims to quantify the coupling between NH_3 and NO_2^- oxidation under controlled soil moisture and NH_4^+ conditions. Specifically, we investigate how variations in soil moisture and NH_4^+ conditions affect the abundance and activity of ammonia-oxidizing and nitrite-oxidizing microorganisms, and how these microbial responses collectively regulate the rate and pathway of soil nitrification. The findings are expected to provide new mechanistic insights into the regulation of N cycling in terrestrial ecosystems.

Materials and methods

Soil sampling

Soil samples were collected from the topsoil layer (0–20 cm) of a peach orchard in Yuncheng City, Shanxi Province, China. The sampling site was an unfertilized agricultural field. To obtain representative samples, a composite sampling strategy was employed by collecting soils from multiple locations within the field. The collected soils were thoroughly mixed *in situ*, and subsamples were obtained using the quartering method. Samples were transported to the laboratory of Sun Yat-sen University under cold-chain conditions, air-dried at room temperature in the shade, gently ground, and passed through a 2 mm sieve for homogenization. A portion of the processed soil was stored at 4 °C for incubation experiments, while the remaining samples were kept at −20 °C for physicochemical analyses. The basic physicochemical properties of the soil are as follows: total carbon (TC) 10.9 g kg^{-1}, total nitrogen (TN) 1.08 g kg^{-1}, NH_4^+–N 23.20 mg kg^{-1}, NO_2^-–N 0.46 mg kg^{-1}, NO_3^-–N 21.43 mg kg^{-1}, and pH 8.0.

Experimental design

Prior to the experiment, soils were adjusted to 30% of water-holding capacity (WHC) with double-distilled water (ddH_2O) and pre-incubated at 25 °C in the dark for 7 d to restore microbial activity. Soil moisture was maintained by periodic rewetting during pre-incubation. Afterward, 50 g of fresh soil was placed into 100 mL glass bottles covered with perforated aluminum foil to ensure aerobic conditions, and the incubation temperature was kept at 25 °C for all treatments. Soil moisture was adjusted to four levels of WHC: 40% (W_1), 60% (W_2), 90% (W_3), and 120% (W_4). Simultaneously, ammonium chloride (NH_4Cl) solutions were added to achieve three NH_4^+–N levels: 50 mg N kg^{-1} (A1), 100 mg N kg^{-1} (A2), and 200 mg N kg^{-1} (A3). The volume of NH_4Cl solution required for each treatment was calculated according to the target N concentration and moisture level, and evenly applied to the soil surface using a micropipette. Each treatment was replicated three times, and soil samples were collected on days 0, 1, 3, 4, 5, 6, 7, and 9 of incubation for subsequent analyses.

Chemical analyses

The TC and TN contents of the soils were determined using an elemental analyzer (FlashSmart 11206125, Thermo Scientific, USA). Ammonium and NO_3^- were simultaneously extracted with 0.01 M $CaCl_2$ at a soil-to-solution ratio of 1:10 (w/v). The soil slurries were shaken horizontally at 200 rpm for 1 h, followed by centrifugation at 690 × g for 15 min, and subsequently filtered through 0.45 μm polypropylene membrane syringe filters (25 mm, VWR Europe). Nitrite was extracted separately using ddH_2O at a 1:5 (w/v) ratio, following the same shaking and centrifugation procedure. Nitrite concentrations were determined colorimetrically using the Griess reagent[19]. Nitrate concentrations were quantified indirectly via the vanadium (III) reduction method[20], in which NO_3^- is reduced to NO_2^-, allowing the determination of total $NO_2^- + NO_3^-$; the NO_3^- concentration was then obtained by subtracting the measured NO_2^- concentration. Ammonium concentrations were determined using the indophenol blue colorimetric method[21]. Soil pH was measured in a 1:2.5 soil-to-water suspension using a pH meter (FE28-Standard, Mettler-Toledo, Switzerland).

DNA extraction and quantitative PCR (qPCR) of functional genes

Fresh soil samples (0.5 g) were incubated for 3 d before DNA extraction. DNA was extracted using the DNeasy PowerSoil Kit (Tiangen Biotech, Beijing, China), with a blank control included to monitor potential contamination. DNA quality and concentration were assessed using a NanoDrop spectrophotometer (ND-1000, Thermo Scientific, USA). The abundances of ammonia- and nitrite-oxidizing bacteria were quantified by targeting the *amoA* and *nxrB* genes, respectively, while those of denitrifiers were determined using the *nirS* and *nirK* genes. Quantitative real-time PCR (qPCR) was performed in triplicate on an ABI Prism 7900 system (Applied Biosystems, USA). Each 20 μL qPCR reaction contained 10 μL of 2× RealStar Green Fast Mixture (GenStar, Cat. No. A301), 0.5 μL each of forward and reverse primers (10 μM), 2 μL of DNA template, and 7 μL of nuclease-free water. The thermal cycling conditions consisted of an initial denaturation at 95 °C for 2 min, followed by 40 cycles of denaturation at 95 °C for 15 s, annealing at 60 °C for 20 s, and extension at 72 °C for 30 s. Standard curves were generated from serial dilutions of plasmid DNA containing known copy numbers of target genes (*amoA*, *nxrB*, *nirS,* and *nirK*), and gene copy numbers were calculated by comparing the sample Ct values with those of the calibration curves[22]. All primers used in this study are listed in Table 1.

Soil RNA extraction and reverse transcription

Total RNA was extracted from soil samples incubated for 3 d using the RNeasy PowerSoil Total RNA Kit (Qiagen, Hilden, Germany). RNA concentration and purity were immediately assessed using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, USA). The amount of RNA used for reverse transcription was calculated based on its final concentration[23]. Complementary DNA (cDNA) was synthesized with the HiScript III 1st Strand cDNA Synthesis Kit (+gDNA

Table 1 Primers used for quantitative real-time PCR (qPCR) analysis

Gene	Primer	Sequence (5'-3')	Amplicon size [bp]	Ref.
Archaeal *amoA*	Amo19F CrenamoA6	STAATGGTCTGGCTTAGACG GCGGCCATCTGTATGT	635	Leininger et al. [45]; Schauss et al.[46]
Bacterial *amoA*	amo41F amoA2R	GGGGTTTCTACTGGTGGT CCCCTCGGGAAGGCTTTTC	491	Rotthauwe et al.[47]
Nitrobacter-like *nxrB*	nxrB-1F nxrB-1R	ACGTGGAGACCAAGCCGGG CCGTGCTGTTGAYCTGTTGA	411	Vanparys et al.[48]
Nitrospira-like *nxrB*	nxrB169f nxrB638r	TACATGGTGTGGAACA CGGTTCTTGGTCRATCA	485	Pester et al.[49]
Comammox *amoA*	ComamoAF ComamoAR	AGGNGAYTGGGAYYTTCGG CGGACAWABRTGAABCCCAT	436	Wang et al.[50]
nirK	copper583F copper909R	TCATGGTGCTGCGCGKGACGG GAACTTGCCGGTKGCCCAGAC	326	Yan et al.[51]
nirS	heme832F heme1606R	TCACACCCCGAGCCGCGCGT AGKCGTTGAACTTKCCGGTCGG	774	Yan et al.[51]

wiper) (Vazyme Biotech, Nanjing, China) according to the manufacturer's instructions. Each 20 μL reaction contained 1 μg of total RNA and 10× random hexamer/oligo(dT) primers. The thermal program consisted of incubation at 25 °C for 10 min, reverse transcription at 45 °C for 1 h, and enzyme inactivation at 94 °C for 5 min, followed by holding at 4 °C. Negative controls lacking either reverse transcriptase or RNA template were included to confirm the absence of DNA or reagent contamination. The synthesized cDNA was stored at −80 °C until further analysis.

Statistical analysis

The effects of soil moisture content on the abundances of the AOA-*amoA*, AOB-*amoA*, *Nitrobacter-nxrB*, *Nitrospira-nxrB*, *nirK*, and *nirS* genes, as well as AOR and NOR activities, were assessed using one-way analysis of variance (ANOVA). Prior to ANOVA analysis, the normality and homogeneity of variances were evaluated using Levene's test. Differences among groups were determined by Duncan's post hoc test, with statistical significance set at $p < 0.05$. Pearson correlation analysis was performed to explore the relationships among net nitrification rate, copy numbers of AOA-*amoA*, AOB-*amoA*, *Nitrobacter-nxrB*, and *Nitrospira-nxrB* genes, and soil NO_2^-–N concentrations. All statistical analyses were performed in R programming (version 4.1.3) using the 'stats' and 'ggpubr' packages, and GraphPad Prism (version 10.1.2), and visualizations were created with the assistance of Adobe Illustrator 2018.

Results

Dynamics of soil NH_4^+–N, NO_2^-–N, and NO_3^-–N concentrations

Ammonia acts as the substrate for NH_3 oxidation, producing NO_2^- as an intermediate. This NO_2^- then serves as the substrate for the subsequent NO_2^- oxidation process, which produces NO_3^-. When the NH_4^+ concentration was 50 or 100 mg N kg^{-1}, there were no significant differences in the NH_4^+ decrease rates among the different soil moisture treatments (Fig. 1a, b). However, at a concentration of 200 mg N kg^{-1}, the NH_4^+ decrease rates in the W_2 and W_3 treatments were significantly higher than that in W_1 (Fig. 1c). In addition, under the same N application level, the NH_4^+ decrease rate was the slowest in the W_4 treatment (Fig. 1a–c). In the W_2 and W_3 treatments, a pronounced accumulation of NO_2^- was observed on the third day of incubation (Fig. 1e, f), with the highest NO_2^- concentration detected in soils receiving 200 mg N kg^{-1}. Significant differences ($p < 0.05$, $n = 3$) in NO_2^- concentration were found among different moisture treatments. At the same N application level, NO_3^- concentrations did not differ significantly among the soil moisture treatments, except for W_4 (Fig. 1g–i).

Variation of AOM and NOB abundance affected by N dose and WHC

The gene abundances of AOA-*amoA*, AOB-*amoA*, Nitrobacter-*nxrB*, Nitrospira-*nxrB*, *nirK*, and *nirS* were quantified using quantitative PCR (qPCR) on the third day of incubation (Fig. 2). Although AOA-*amoA* abundance increased at W_1 and W_4 WHC, its response to N addition lacked a consistent trend across treatments (Fig. 2a). The gene abundance of AOB-amoA was regulated by both N dose and soil moisture, showing a positive synergistic effect. Specifically, higher soil moisture (W_3/W_4) promoted gene abundance to a greater extent under high N addition levels than under low levels, highlighting their significant interaction (Fig. 2b). Moreover, the abundance of comammox-*amoA* shared a similar trend with that of AOA-*amoA*, and it was low across all soil samples (Fig. 2c).

Based on DNA abundance, *Nitrospira* was the predominant NOB across all soil samples (Fig. 2e). At the 50 mg NH_4^+–N addition level, gene abundance of *Nitrospira-nxrB* showed no significant differences among soils with varying moisture contents ($p < 0.05$). At the 100 mg NH_4^+–N level, the gene abundance of *Nitrospira-nxrB* was higher in soils at W_1 and W_4 WHC. The gene abundance of *Nitrobacter* remained consistently low across all treatments, with the lowest values observed under saturated conditions (W_4) at the same N level (Fig. 2d). The gene abundance of the *nirS* increased with increasing soil moisture across all N treatments, whereas the gene abundance of *nirK* did not show a clear trend, indicating a greater sensitivity of *nirS*-containing denitrifier populations to soil moisture variation (Fig. 2f, g).

Active nitrifying microorganism abundance affected by N dose and WHC

The expression levels of functional genes involved in NH_3 and NO_2^- oxidation were assessed using cDNA-based quantification (Fig. 3). The cDNA abundance patterns of AOB-*amoA* and AOA-*amoA* differed from their respective DNA-level profiles. Specifically, at the 50 mg NH_4^+–N addition level, AOB-*amoA* cDNA abundance was significantly higher under W_4 WHC compared to the other moisture treatments ($p < 0.05$), while no significant differences were observed under the other two N levels. For AOA-*amoA*, higher cDNA abundance was observed only

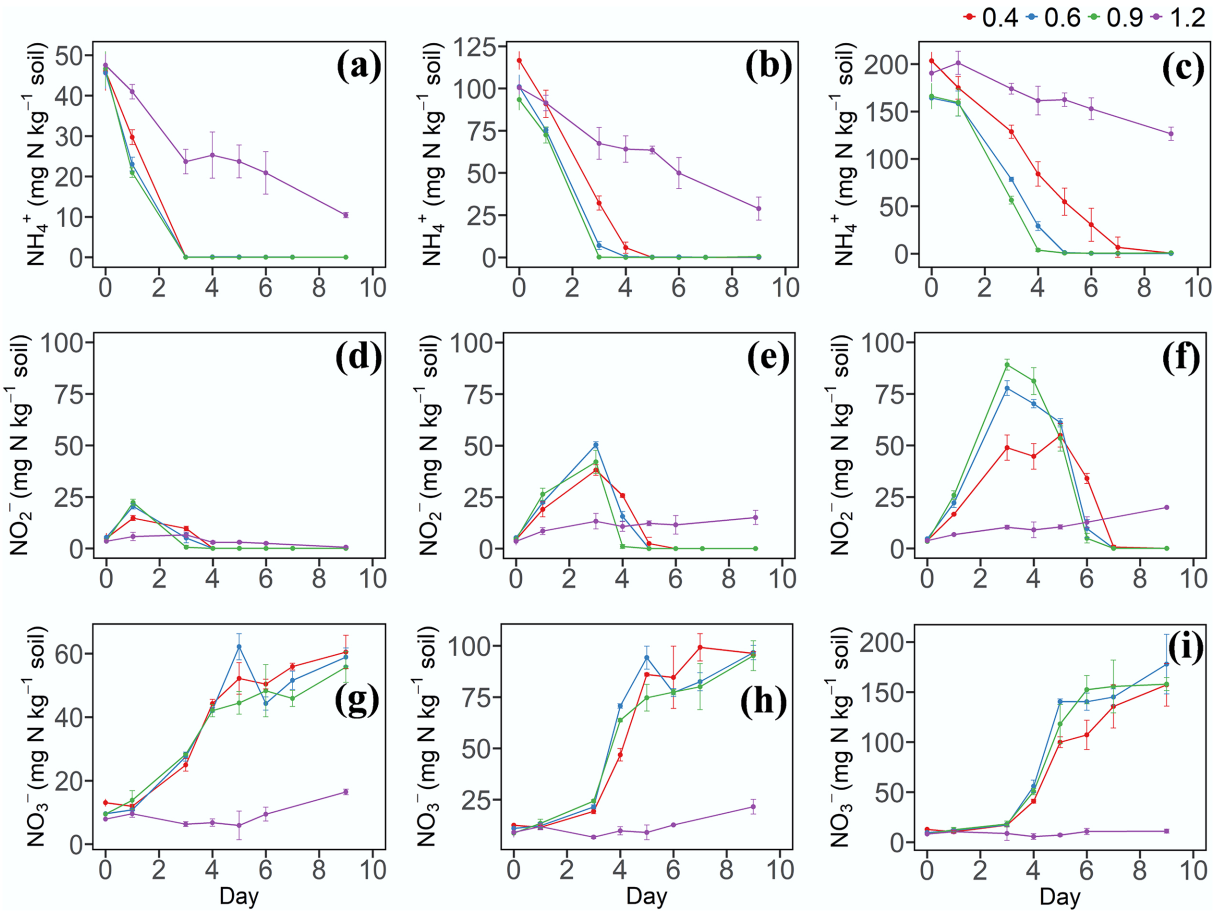


Fig. 1 (**a**)–(**c**) Dynamic changes of NH_4^+–N, (**d**)–(**f**) NO_2^-–N, and (**g**)–(**i**) NO_3^-–N in soil under different soil water contents and N levels. 0.4, 0.6, 0.9, and 1.2 represent W_1, W_2, W_3, and W_4 WHC, respectively. (**a**), (**d**), and (**g**) represent soil with a N content of 50 mg N kg^{-1}. (**b**), (**e**), and (**h**) represent soil with a N content of 100 mg N kg^{-1}. (**c**), (**f**), and (**i**) represent soil with a N content of 200 mg N kg^{-1}. Error bars indicate standard deviations (n = 3). Abbreviations: NH_4^+: ammonium, NO_2^-: nitrite, NO_3^-: nitrate, WHC: water holding capacity.

under W_2 and W_4 at the high N addition level. Notably, the AOB-*amoA* cDNA abundance was markedly higher than that of AOA-*amoA* under W_4 conditions. Compared to the DNA-based results, the cDNA/DNA ratio of *Nitrobacter-nxrB* was higher than that of *Nitrospira-nxrB*, indicating that although *Nitrospira* exhibited higher relative abundance at the DNA level, only a small fraction of the population was transcriptionally active.

Correlations among nitrite concentration and nitrification processes

Soil moisture significantly affected the coupling between NH_3 and NO_2^- oxidation (Fig. 4). NAOR (net NH_3 oxidation rate from day 0 to 3) was positively correlated with the AOB : AOA gene abundance ratio (R^2 = 0.54, $p < 0.05$), reflecting differences in the contributions of bacterial and archaeal NH_3 oxidizers. NNOR1 (net NO_2^- oxidation rate from day 0 to 3) also showed a positive correlation with the *Nitrobacter* : *Nitrospira* ratio (R^2 = 0.24, $p < 0.05$), suggesting shifts in nitrite-oxidizing community structure. Moreover, NO_2^- concentration was positively correlated with both the NAOR : NNOR1 ratio (R^2 = 0.40, $p < 0.05$) and the AOB : *Nitrobacter* gene abundance ratio (R^2 = 0.56, $p < 0.05$), indicating that imbalances between functional groups may contribute to NO_2^- accumulation.

Discussion

The effect of soil moisture content on the oxidation processes of ammonia and nitrite

Net NH_3 oxidation rates did not differ significantly among the W_1, W_2, and W_3 WHC treatments in soils amended with 50 mg of N, indicating that under relatively low N input, soil moisture had little effect on NH_3 oxidation. However, when the N input was increased to 100 mg NH_4^+–N, net NH_3 oxidation rates under W_2 and W_3 WHC were significantly higher than those under W_1 WHC. This shift suggests that under high NH_4^+ concentrations, NH_4^+ is no longer the limiting factor for NH_3 oxidation, and soil moisture becomes the dominant factor regulating the oxidation rate. This finding is consistent with previous studies; for instance, Cheng et al.[24] observed that the total nitrification rate was significantly higher at 90% WHC compared to 70% WHC in forest soils. In fact, numerous studies have reported that mineralization and nitrification rates tend to increase with rising soil moisture across arable land, grasslands, and forest soils[25,26]. This enhancement may be attributed to two complementary mechanisms. First, increasing soil moisture can alleviate water limitation and improve the mobility of dissolved substrates such as NH_4^+, thereby increasing substrate availability for ammonia oxidizers. Second, moderate soil moisture may still maintain sufficient O_2 supply for nitrification. However, this effect should be interpreted cautiously, because further increases in soil

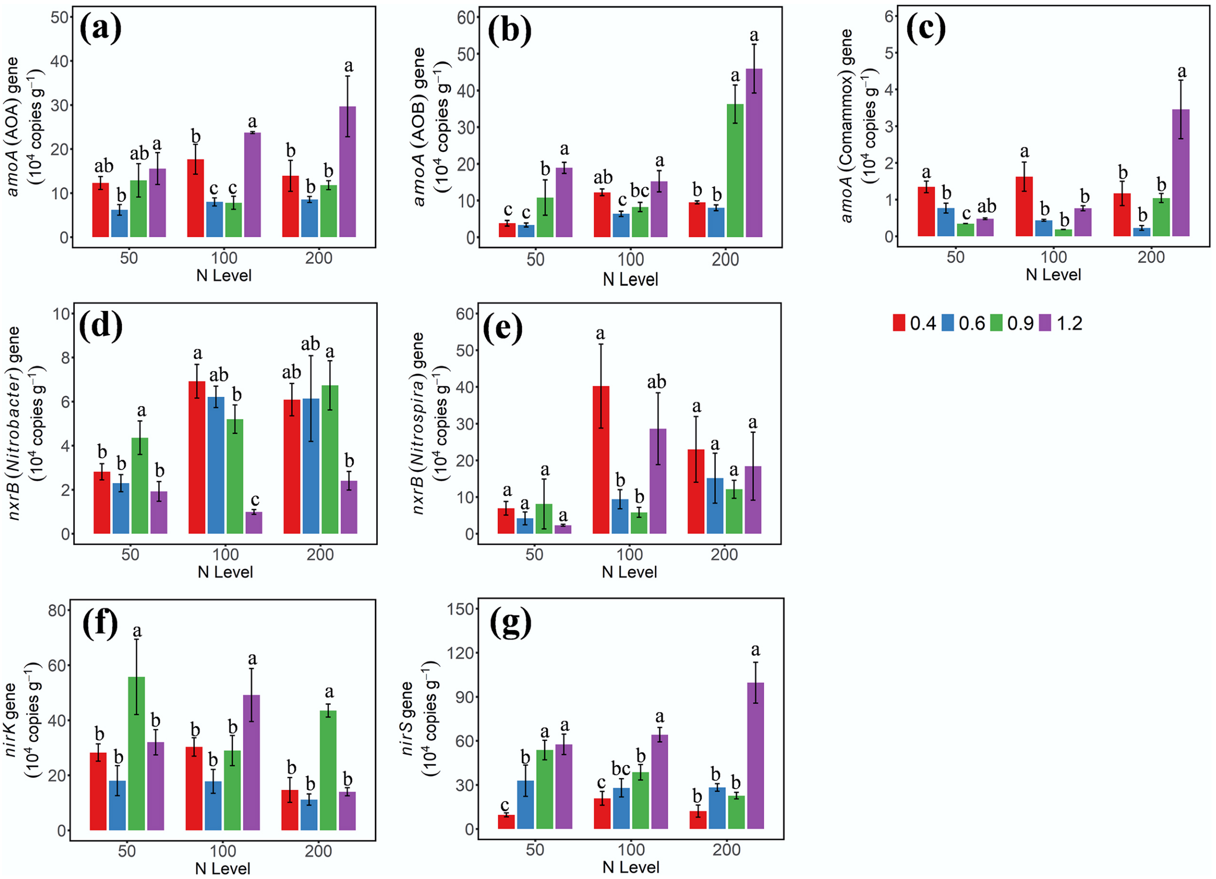


Fig. 2 Gene abundances of (**a**) AOA-*amoA*, (**b**) AOB-*amoA*, (**c**) *amoA*-comammox, (**d**) *nxrB-Nitrobacter*, (**e**) *nxrB-Nitrospira*, (**f**) *nirK*, and (**g**) *nirS* on day 3 under different soil moisture and N treatments. 0.4, 0.6, 0.9, and 1.2 represent W_1, W_2, W_3, and W_4 WHC, respectively. Error bars indicate standard deviations ($n = 3$). Different lowercase letters indicate significant differences ($p < 0.05$) between moisture content treatments within the same N application level.

moisture generally reduce soil aeration and restrict O_2 diffusion, which can ultimately suppress nitrification[27]. In contrast to this trend, net NH_3 oxidation rates were consistently lowest under W_4 WHC across all N addition treatments. The decline in nitrification under saturated conditions was most likely associated with O_2 limitation caused by reduced soil aeration. These observations are consistent with the findings of Wang et al.[27], who reported that soil NO_3^-–N concentrations accumulated more rapidly when WFPS was ≤ 80% than when WFPS ≥ 90%.

The effect of soil moisture on net NH_3 oxidation varied with the level of NH_4^+ addition. At low NH_4^+ concentrations, changes in moisture had little impact on net NH_3 oxidation. However, as NH_4^+ availability increased, the effect of moisture became more pronounced, showing a significant positive correlation at higher NH_4^+ levels. Notably, excessive moisture (120% WHC) consistently suppressed net NH_3 oxidation across all NH_4^+ treatments.

Consistent with the net NH_3 oxidation rates, no significant differences were observed in soil NO_2^- concentrations among the 40%, 60%, and 90% WHC treatments under low NH_4^+ input (50 and 100 mg N kg^{-1}). However, when NH_4^+ was elevated to 200 mg N kg^{-1}, soils at 60% and 90% WHC exhibited significantly higher NO_2^- accumulation compared to the 40% WHC treatment, whereas at 120% WHC, NO_2^- levels remained comparatively low for all NH_4^+ levels. Further, soil NO_3^- concentrations remained relatively low under the highest soil moisture treatment (W_4) for all NH_4^+ levels.

This interpretation is consistent with previous studies. For example, a modeling and incubation study demonstrated that as soil saturation increases beyond an optimal threshold, nitrification rates drop sharply while denitrification becomes the dominant pathway, with the greatest N loss observed under near-saturated conditions[28]. Similarly, a global synthesis found that denitrification rates are strongly positively correlated with increases in soil water-filled pore space and soil NO_3^- content, and negatively correlated with soil O_2 availability—supporting the view that in high-moisture soils, O_2 limitation shifts the balance from nitrification toward denitrification (and gaseous N losses)[29]. This pattern suggests that under saturated or near-saturated moisture conditions, nitrification is inhibited—likely due to limited O_2 diffusion—and concurrently, enhanced denitrification or other anaerobic N-transformation processes may consume NO_2^-/NO_3^-, preventing their accumulation.

Responses of AOB and AOA to soil moisture content

AOB and AOA play critical roles in soil nitrification processes; however, their responses to soil moisture content exhibit distinct patterns. In this study, under the 50 mg NH_4^+–N treatment, AOB-*amoA* cDNA abundance was significantly higher at the W_4 WHC than at other moisture conditions ($p < 0.05$), whereas no significant differences were observed under the other two NH_4^+ levels. This response may be closely related to the residual NH_4^+ concentrations in the soil. In

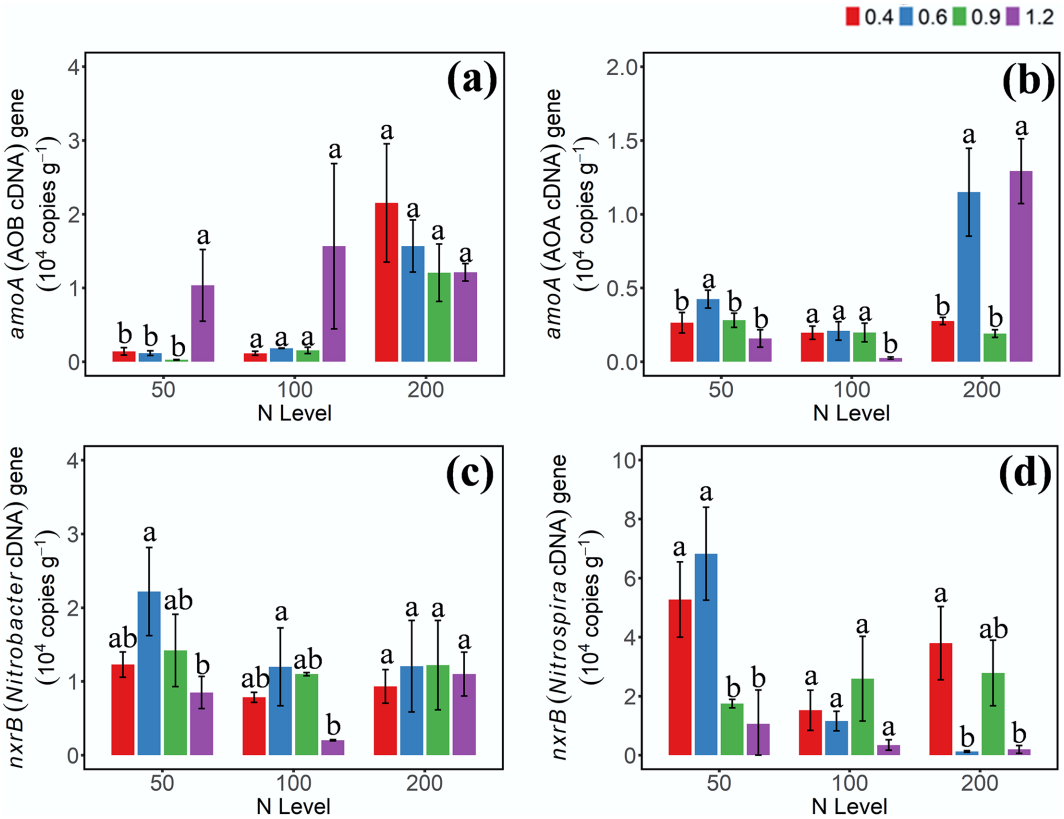


Fig. 3 Gene abundances of (**a**) AOB-*amoA*, (**b**) AOA-*amoA*, (**c**) *Nitrobacter-nxrB*, and (**d**) *Nitrospira-nxrB* cDNA on day 3 under different moisture and N addition treatments. 0.4, 0.6, 0.9, 1.2 represent W_1, W_2, W_3, and W_4 WHC, respectively. Error bars indicate standard deviations (*n* = 3). Different lowercase letters indicate significant differences ($p < 0.05$) between moisture content treatments within the same N application level.

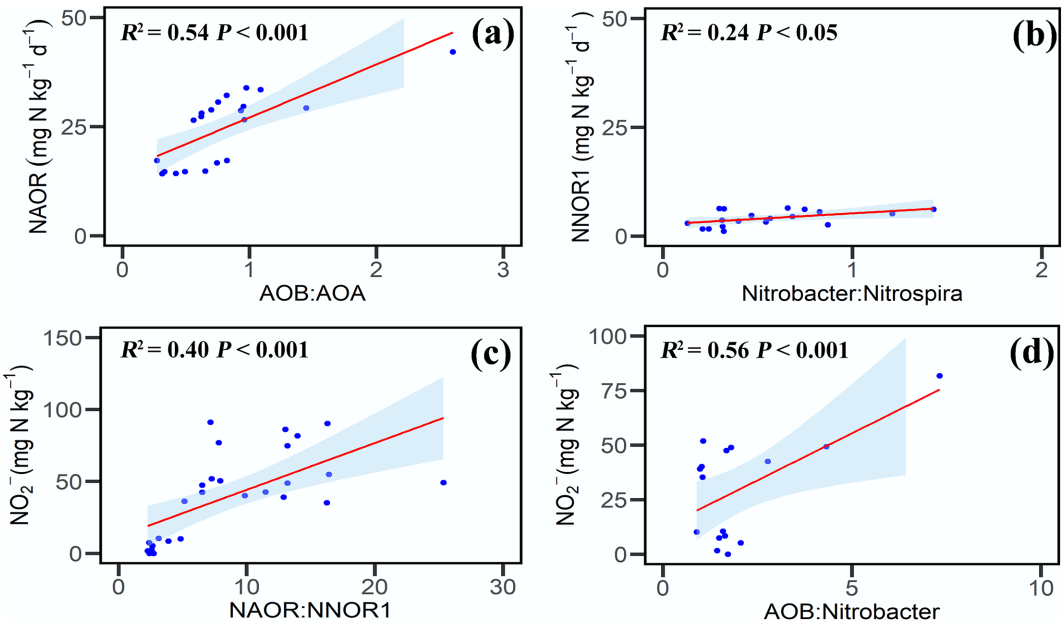


Fig. 4 Relationships between net nitrification rate, nitrite concentration, and gene abundance. (**a**) NAOR and the AOB : AOA gene abundance ratio. (**b**) NNOR1 and the Nitrobacter : Nitrospira ratio. (**c**) NO_2^- concentration and the NAOR:NNOR1 ratio. (**d**) NO_2^- concentration and the AOB : Nitrobacter ratio. Symbols represent individual soil samples; lines denote significant correlations; and asterisks indicate statistically significant correlations ($p < 0.05$). Abbreviations: NAOR is the net ammonia oxidation rate from day 0 to 3; NNOR1 is the NO_2^- oxidation rate from day 0 to 3.

contrast, AOA-*amoA* cDNA abundance declined under higher moisture levels, except in soils treated with 200 mg NH_4^+–N. Interestingly, although AOA-*amoA* DNA abundance was relatively high at both W_1 and W_4 WHC, the corresponding AOA-*amoA* cDNA abundance remained low, suggesting that transcriptional activity did not parallel population size. This observation does not necessarily contradict previous studies reporting a competitive advantage of AOA under high-moisture or low-O_2 conditions, but instead suggests that the response of AOA activity to moisture may be nonlinear and strongly dependent on NH_4^+ availability and soil-specific environmental conditions[30,31].

Ample research suggests that AOB have a competitive advantage under high NH_4^+ conditions due to their efficient ammonia-oxidizing capacity. This finding aligns with our observations, as the elevated NH_4^+ concentration at W_4 WHC likely created favorable conditions for AOB growth and activity[32]. In our experimental system, substrate availability, alongside O_2 availability, played a key role in driving the nitrification process. This was evidenced by significant differences in NAOR and NO_2^- accumulation in soils amended with 200 mg NH_4^+–N ($p < 0.05$), whereas such differences were not observed under the 50 and 100 mg NH_4^+–N treatments. These findings suggest that sufficient NH_4^+ supply is an important condition for enhanced AOB-driven nitrification, particularly under high moisture levels.

Differential responses of *Nitrobacter* and *Nitrospira* to soil moisture conditions

Nitrospira and *Nitrobacter* represent the two major lineages of NOB, yet their ecological niches and physiological strategies differ markedly, resulting in contrasting responses to N input and soil moisture conditions. In our study, *Nitrospira-nxrB* gene abundance was substantially higher than *Nitrobacter-nxrB* across all treatments, and increased consistently with rising NH_4^+ levels. Moreover, *Nitrospira* abundance peaked under both low (40% WHC) and excessively high moisture (120% WHC), suggesting that this lineage possesses a broader tolerance to substrate and O_2 fluctuations. These findings align with previous reports describing *Nitrospira* as an oligotrophic, K-selected NOB with high affinity for NO_2^- and superior ability to persist under nutrient limitation or microaerobic conditions[33,34].

In contrast, *Nitrobacter-nxrB* abundance increased from 50 to 100–200 mg N kg^{-1} NH_4^+ addition but did not rise further at higher N levels. Moreover, unlike *Nitrospira*, *Nitrobacter* abundance decreased sharply under 120% WHC, especially at high NH_4^+ inputs. This pattern is consistent with its classification as a fast-growing, r-strategist NOB that prefers well-aerated, nitrite-rich environments[35]. The suppression of *Nitrobacter* under saturated soil likely reflects O_2 limitation, because this lineage relies strictly on O_2 as the terminal electron acceptor and exhibits poor tolerance to anoxia or reduced redox potential[36]. In addition, micro-scale acidification under high water saturation, which is known to inhibit *Nitrobacter* more strongly than *Nitrospira*, may further constrain its growth and activity[37].

The transcriptional responses revealed even more pronounced lineage-specific differences. *Nitrospira-nxrB* cDNA abundance was highest under low N addition and under 40%–60% WHC, indicating that although *Nitrospira* is numerically dominant at the DNA level, its active fraction declines when substrate concentrations become excessive. This supports the view that many *Nitrospira* cells in soils may remain in a dormant or low-activity state, activating only under conditions of moderate NO_2^- supply that match their oligotrophic lifestyle. In contrast, *Nitrobacter-nxrB* cDNA showed limited sensitivity to N or moisture treatments, except for a notable decline under 100 mg N kg^{-1} at 120% WHC, reinforcing its vulnerability to O_2 constraints and redox instability. The generally higher cDNA/DNA ratios observed in *Nitrobacter* than in *Nitrospira* suggest that, although less abundant, *Nitrobacter* maintains a larger proportion of metabolically active cells, consistent with its copiotroph strategy and rapid-response physiology[38].

These findings highlight that soil moisture and N availability regulate NO_2^- oxidation not only through their effects on O_2 diffusion and substrate supply, but also by shifting the competitive balance between K-selected *Nitrospira* and r-selected *Nitrobacter*. Moderate soil moisture promotes the activity of *Nitrobacter*, whereas *Nitrospira* gains a competitive advantage under both drier and more saturated conditions, reflecting its greater ecological plasticity and ability to tolerate fluctuating microaerobic environments. These lineage-specific responses ultimately shape NO_2^- oxidation dynamics and may influence nitrification bottlenecks under variable hydrological regimes.

Mechanistic drivers of soil nitrite accumulation

The strong positive correlations between peak NO_2^- concentrations and both the net NH_3 oxidation rate (NAOR) and the net NO_2^- oxidation rate (NNOR) highlight the central role of process imbalances in regulating NO_2^- accumulation. Such imbalances arise when the capacity of NO_2^- oxidizers cannot match the production of NO_2^- from NH_3 oxidation, a phenomenon widely reported as a key driver of NO_2^- accumulation in soils[39]. Consistent with this mechanism, NO_2^- accumulation in our soils was positively associated with the relative abundance ratio of AOB to *Nitrobacter*, suggesting that a dominance of fast-growing AOB over *Nitrobacter* can lead to transient NO_2^- buildup under conditions where NO_2^- oxidation becomes rate-limiting. Previous work has also shown that NO_2^- oxidation is particularly sensitive to environmental stressors such as high NH_4^+ levels and reduced O_2 availability, both of which can disproportionately suppress *Nitrobacter* activity and thereby promote NO_2^- accumulation[40,41].

The observation that NAOR was strongly correlated with the AOB : AOA *amoA* ratio further confirms that AOB were the principal drivers of NH_3 oxidation in this system, aligning with the broader consensus that AOB generally dominate under elevated NH_4^+ concentrations and in moderately moist soils[31]. Likewise, NNOR was positively correlated with the *Nitrobacter* : *Nitrospira* ratio, indicating that *Nitrobacter* played a leading role in NO_2^- oxidation despite the well-known oligotrophic lifestyle and high substrate affinity of *Nitrospira*[42]. This pattern may reflect the higher growth rate and substrate tolerance of *Nitrobacter*, enabling it to respond more rapidly to increasing NO_2^- levels under moderate moisture conditions.

However, under high soil moisture (120% WHC), the regulatory framework governing NO_2^- accumulation shifted markedly. In these nearly saturated soils, NO_2^- and NO_3^- concentrations remained low despite active NH_3 oxidation, indicating that denitrification masked nitrification signals by consuming NO_2^- and NO_3^- as electron acceptors. Hence, the pathway responsible for NO_2^- dynamics appeared to vary with soil moisture. At 40%–90% WHC, NO_2^- accumulation was more consistent with nitrification-derived buildup caused by incomplete coupling between NH_3 oxidation and NO_2^- oxidation. At 120% WHC, however, enhanced denitrification likely altered or consumed NO_2^- intermediates, thereby suppressing their net accumulation. The sharp increases in *nirS* gene abundance with rising moisture content, together with nitrogen-dependent responses of *nirK* communities, support the activation of oxygen-sensitive denitrifiers under high moisture. This observation is consistent with the established ecological differentiation of these functional groups: *nirS*-type denitrifiers typically dominate under low-oxygen or intermittently anoxic conditions, whereas *nirK*-bearing microorganisms include taxa associated with both denitrification and nitrification pathways[43]. The enhanced *nirS* abundance under saturated conditions, therefore, aligns with classical models of moisture-driven denitrification[44], explaining the observed suppression of inorganic N pools. The pathway responsible for NO_2^- dynamics appeared to vary with soil moisture. At 40%–90% WHC, NO_2^- accumulation was more consistent with nitrification-derived buildup caused by incomplete coupling between NH_3 oxidation and NO_2^- oxidation. At 120% WHC, however, enhanced denitrification likely altered or consumed NO_2^- intermediates, thereby suppressing their net accumulation.

Environmental implications

This study provides new mechanistic insight into how soil moisture and NH_4^+ availability jointly regulate the coupling between NH_3 oxidation and NO_2^- oxidation, thereby shaping the fate of reactive N in terrestrial ecosystems. By integrating process-based measurements with functional gene and transcript analyses, our results demonstrate that the balance between AOB- and AOA-driven NH_3 oxidation and the differential contributions of *Nitrobacter* and *Nitrospira* to NO_2^- oxidation are key determinants of whether NO_2^- accumulates or is efficiently oxidized to NO_3^-. The finding that NO_2^- accumulation is strongly associated with microbial community composition—particularly high AOB : *Nitrobacter* ratios—highlights the importance of microbial ecological interactions in governing transient N intermediates that influence nitrification efficiency and downstream N losses.

Limitations

Despite providing important insights into the moisture-N regulation of nitrification pathways, this study has several limitations that should be acknowledged. First, the study did not employ selective inhibitors such as acetylene, 1-octyne, or chlorate to differentiate the contributions of AOA, AOB, and specific NOB groups. As a result, causal links between gene abundance/expression and process rates remain inferential rather than experimentally verified. In addition, the incubation experiment was conducted under controlled laboratory conditions, which inevitably simplifies the physical and biological complexity of natural soils. Factors such as heterogeneous pore structure, fluctuating redox conditions, plant-microbe interactions, and microscale O_2 dynamics were not fully represented, potentially limiting the extrapolation of our results to field environments. Moreover, denitrification was inferred from nirS/nirK abundances and N-balance patterns; direct measurements of denitrification rates, N_2O/N_2 production, or NO_3^- reduction were not conducted. Future studies combining selective inhibition, isotope tracing, and direct gaseous N flux measurements will be necessary to quantitatively separate the contributions of nitrification and denitrification pathways under varying moisture and N regimes.

Conclusions

This study demonstrates that soil moisture and N availability jointly regulate the coupling efficiency between NH_3 oxidation and NO_2^- oxidation by reshaping nitrifier community structure, functional gene expression, and microbial activity. Increasing N inputs and moderate moisture levels (60%–90% WHC) enhanced AOB-dominated NH_3 oxidation, often exceeding the nitrite-oxidizing capacity of *Nitrobacter*, thereby promoting NO_2^- accumulation. The strong positive correlations between NO_2^- accumulation and both the NAOR : NNOR ratio and the AOB : Nitrobacter abundance ratio further confirm that imbalances between NH_3 oxidation and NO_2^- oxidation are a primary driver of NO_2^- buildup. Under waterlogged conditions (120% WHC), the apparent accumulation of NO_2^- and NO_3^- was substantially reduced despite active NH_3 oxidation. This pattern reflects the increased importance of denitrification under oxygen-limiting, high-moisture conditions, as indicated by the sharp rise in *nirS* abundance. These findings highlight that at high soil moisture, NO_2^- consumption through denitrification masks the accumulation that would otherwise result from decoupled NH_3 and NO_2^- oxidation, demonstrating a shift from a nitrification-dominated system toward a more tightly integrated nitrification-denitrification regime. However, this study did not employ selective nitrification inhibitors (e.g., acetylene, 1-octyne, chlorate), limiting our ability to definitively attribute measured NH_3 and NO_2^- oxidation rates to specific functional groups. Further work should incorporate inhibition assays and isotope tracing approaches to directly quantify the contributions and interactions of AOA, AOB, NOB, and denitrifiers under dynamic moisture-nitrogen regimes.

Author contributions

The authors confirm their contributions to the paper as follows: All authors contributed to the study conception and design. Zhijie Li: writing – original draft. Jiani Ma: writing – original draft. Miao Chen: writing – original draft. Weiqi Kuang: conceptualization. Gaochao Cai: material preparation, data collection, and formal analysis. Yubin Wang: material preparation, data collection, and formal analysis. Yunyun Cao: material preparation, data collection, and formal analysis. Shurong Liu: funding acquisition and writing – review and editing. All authors reviewed the results and approved the final version of the manuscript.

Data availability

The datasets used or analyzed during the current study are available from the corresponding author on reasonable request.

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Declarations

Competing interests

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.

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