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# Coupling Between and Among Ammonia Oxidizers and Nitrite Oxidizers in Grassland Mesocosms Submitted to Elevated $CO_2$ and Nitrogen Supply

**Marie Simonin[1,2] · Xavier Le Roux[2] · Franck Poly[2] · Catherine Lerondelle[2] · Bruce A. Hungate[3] · Naoise Nunan[1] · Audrey Niboyet[1]**

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**Abstract** Many studies have assessed the responses of soil microbial functional groups to increases in atmospheric $CO_2$ or N deposition alone and more rarely in combination. However, the effects of elevated $CO_2$ and N on the (de)coupling between different microbial functional groups (e.g., different groups of nitrifiers) have been barely studied, despite potential consequences for ecosystem functioning. Here, we investigated the short-term combined effects of elevated $CO_2$ and N supply on the abundances of the four main microbial groups involved in soil nitrification: ammonia-oxidizing archaea (AOA), ammonia-oxidizing bacteria (AOB), and nitrite-oxidizing bacteria (belonging to the genera *Nitrobacter* and *Nitrospira*) in grassland mesocosms. AOB and AOA abundances responded differently to the treatments: N addition increased AOB abundance, but did not alter AOA abundance. *Nitrobacter* and *Nitrospira* abundances also showed contrasted responses to the treatments: N addition increased *Nitrobacter* abundance, but decreased *Nitrospira* abundance. Our results support the idea of a niche differentiation between AOB and AOA, and between *Nitrobacter* and *Nitrospira*. AOB and *Nitrobacter* were both promoted at high N and C conditions (and low soil water content for *Nitrobacter*), while AOA and *Nitrospira* were favored at low N and C conditions (and high soil water content for *Nitrospira*). In addition, *Nitrobacter* abundance was positively correlated to AOB abundance and *Nitrospira* abundance to AOA abundance. Our results suggest that the couplings between ammonia and nitrite oxidizers are influenced by soil N availability. Multiple environmental changes may thus elicit rapid and contrasted responses between and among the soil ammonia and nitrite oxidizers due to their different ecological requirements.

**Keywords** Global change · Grasslands · Nitrification · Ammonia oxidizers · Nitrite oxidizers · Niche differentiation

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✉ Audrey Niboyet
audrey.niboyet@grignon.inra.fr

[1] Institute of Ecology and Environmental Sciences - Paris, UMR 7618 Université Pierre et Marie Curie/CNRS/AgroParisTech, 78850 Thiverval Grignon, France

[2] Microbial Ecology Center, Université de Lyon/Université Lyon 1/CNRS/INRA, UMR CNRS 5557, USC INRA 1364, 69622 Villeurbanne, France

[3] Center for Ecosystem Science and Society and Department of Biological Sciences, Northern Arizona University, Flagstaff, AZ 86011, USA

## Introduction

Environmental changes, such as increases in atmospheric carbon dioxide ($CO_2$) and in nitrogen (N) deposition, are expected to alter the functioning of terrestrial ecosystems, partly through modifications of soil microbial community abundance, structure, and activity [1–5]. Previous studies have focused on the effects of increases in atmospheric $CO_2$ or N deposition in isolation, even though atmospheric $CO_2$ and N deposition are increasing simultaneously [6, 7]. As a result, little information is available about the combined effects of elevated $CO_2$ and N addition on soil microbial communities and the ecosystem functions they perform.

Some microbial processes, such as organic matter decomposition, are carried out by organisms that span a broad range of phylogenies [8], likely with high functional redundancy and a degree of resistance to environmental changes [9, 10]. Other processes, such as nitrification and some trace gas

transformations, are performed by organisms with restricted phylogenies [8] and might, therefore, be more sensitive to environmental changes. For instance, several steps of the N cycle are mediated by only a few microbial groups. Among these, nitrification influences the availability of ammonium ($NH_4^+$) and nitrate ($NO_3^-$) in soils, the two major N sources for plants and microbes [11, 12]. Because the end product of nitrification, $NO_3^-$, is mobile in soils and is the substrate for denitrification, nitrification also contributes to N losses from ecosystems [13]. Nitrification is a two-step process: the first step (the oxidation of $NH_4^+$ to nitrite, $NO_2^-$) is mediated by ammonia-oxidizing bacteria (AOB) and ammonia-oxidizing archaea (AOA) [14], while the second step (the oxidation of $NO_2^-$ to $NO_3^-$) is mediated by nitrite-oxidizing bacteria (NOB), with two main genera of NOB present in soils: *Nitrobacter* and *Nitrospira* [15].

The effects of inorganic or organic N additions on soil ammonia oxidizers in grassland ecosystems are well documented; in contrast, very few studies have investigated the effects of elevated $CO_2$ (alone or combined with N additions) [16, 17] (see Table 1). Most of these studies have been conducted in long-term experiments, i.e., for 2 to 10 years under field conditions (Table 1). It is, thus, difficult to know whether the reported effects on soil ammonia oxidizers are progressively induced after several years of treatment or are generated quickly after treatment application. Furthermore, no studies have reported the effects of elevated $CO_2$ and N addition on soil nitrite oxidizers in grassland ecosystems. Most studies have focused solely on the first step of nitrification, as it is considered to be the rate-limiting step [28]. However, $NO_2^-$ can accumulate in disturbed soil systems [29–31], which indicates that nitrite oxidation may sometimes be limiting for nitrification. Moreover, it has been demonstrated that the two steps of nitrification can respond differently to environmental changes [32]: in a grassland ecosystem exposed to simulated global changes, N additions, and elevated precipitation affected ammonia and nitrite oxidation differently. Elevated $CO_2$ and N additions are expected to have both direct and indirect effects on soil ammonia and nitrite oxidizers. For instance, N additions could have a direct effect on soil $NH_4^+$ availability, the substrate for nitrification, and thus, on the soil nitrifiers. But elevated $CO_2$ and N addition could also have indirect effects on soil nitrifiers by increasing shoot and root biomass and associated root exudates, with impacts on soil moisture and soil carbon (C) and oxygen contents, variables to which soil nitrifiers are sensitive [1]. Elevated $CO_2$ and N addition may induce different responses between the soil ammonia and nitrite oxidizers, given the different ecological requirements of these two functional groups, as ammonia oxidizers are predominately autotrophs [33], while different nitrite oxidizers can also be mixotrophs [34]. Furthermore, among soil ammonia oxidizers, AOA and AOB are thought to occupy different ecological niches, e.g., in terms of soil pH, oxygen concentration, and nutrient conditions [35–37]. Similarly, among soil nitrite oxidizers, *Nitrobacter*- and *Nitrospira*-like NOB have different ecological requirements, *Nitrobacter* being adapted to high-nitrite and high-oxygen environments, while *Nitrospira* are adapted to low-nitrite and low-oxygen environments [38]. Hence, the responses of the soil-nitrifying microbial communities to the combined effects of elevated $CO_2$ and N addition are difficult to predict given the multi-factor parameters in play and could differ depending on the microbial group considered.

Here, we investigated the short-term and combined effects of elevated $CO_2$ and inorganic N supply on nitrifier abundances in grassland mesocosms planted with *Dactylis glomerata*. We measured the abundances of the four main soil microbial groups involved in nitrification (AOA, AOB, *Nitrobacter*, and *Nitrospira*) using quantitative PCR and analyzed their responses to elevated $CO_2$ and/or N additions. We then explored the relationships between these abundances and key plant/soil variables to identify the main drivers of the observed responses to the treatments. We also examined the relationships between gross and potential nitrification rates and soil ammonia- and nitrite-oxidizer abundances to determine whether changes in nitrifier abundances could explain changes in nitrification rates. Finally, we investigated the links between and among ammonia- and nitrite-oxidizer abundances to decipher the coupling of the different microbial groups involved in nitrification under the combined effects of elevated $CO_2$ and inorganic N supply.

## Materials and Methods

### Experimental Design, Treatments, and Soil Sampling

Experimental pots (15×20×50 cm) were filled with a sandy-loam soil and assigned to one of 12 growth chambers, set up inside a large greenhouse at the Université Paris Sud (Orsay, France;see [39] for full description). The soil had a total N content of 0.23 g $kg^{-1}$, a total C content of 2.46 g $kg^{-1}$, an organic matter content of 4.26 g $kg^{-1}$, a cation exchange capacity of 1.81 cmol $kg^{-1}$, and a pH of 8.5 (INRA, Arras, France). The experimental pots were sown with *D. glomerata*, a fast-growing perennial grass common in a wide variety of habitats worldwide [40], at a density of 2,000 seeds per square meter. Maximum daily photosynthetically active radiation (PAR) values recorded during the study ranged between 150 and 1,680 μmoL $s^{-1}$ $m^{-2}$. One month later, when the seedlings had fully emerged, two $CO_2$ treatments (ambient vs. elevated) crossed with two N treatments (no N addition vs. N addition) were applied. Six chambers were ventilated with ambient air taken from outside the greenhouse ($CO_2$=381±6 μmol $mol^{-1}$) and the six others with ambient air enriched with $CO_2$ ($CO_2$= 645±9 μmol $mol^{-1}$). The elevated $CO_2$ concentration chosen

**Table 1** A review of the effects of elevated $CO_2$, organic or inorganic N supply, and of the combined effects of elevated $CO_2$ and N supply on the abundance of the soil ammonia oxidizers (AOA and AOB) in grassland ecosystems

| Treatment | References | Grassland type | Nitrifier community | Duration of treatment | Treatment level | Effects reported |
|---|---|---|---|---|---|---|
| $CO_2$ | [17] | Temperate grassland (Germany) | AOA and AOB | 10 years | 440 ppm (in dry, medium, and wet plots) | Increase in the AOA abundance in the dry plots and slight decreases in the medium and wet plots; no effect on the AOB abundance |
| N | [18] | Upland grassland (China) | AOA and AOB | 6 years | 10 g N $m^{-2}$ $year^{-1}$ (as urea and $NH_4NO_3$) | Decrease in the AOA abundance at one of the two sampling dates; increase in the AOB abundance |
| N | [19] | Icelandic grassland (Iceland) | AOA and AOB | 4 years | 10 g N $m^{-2}$ $year^{-1}$ (as urea) | Increase in the AOA abundance; AOB were undetectable. |
| N | [20] | Temperate grassland (New Zealand) | AOA and AOB | 83 years | 50 and 100 kg N $ha^{-1}$ $year^{-1}$ (as urea) or 300 and 600 kg N $ha^{-1}$ $year^{-1}$ (as animal urine) | No effect on the AOA abundance; increase in the AOB abundance only with the highest concentration of animal urine |
| N | [21] | Temperate grassland (New Zealand) | AOA and AOB | 97 years | 1,000 kg N $ha^{-1}$ $year^{-1}$ (as animal urine) | Decrease in the AOA abundance and increase in the AOB abundance |
| N | [22] | Mediterranean grassland (USA) | AOB | 6 years | g N $m^{-2}$ $year^{-1}$ (as $Ca(NO_3)_2$) | Increase in the AOB abundance |
| N | [23] | Upland grassland (Switzerland) | AOA and AOB | 2 years | 30 g N $m^{-2}$ (as $NH_4NO_3$) | Increases in the AOA and AOB abundances |
| N | [24] | Temperate grassland (New Zealand) | AOA and AOB | 56 years | 600 kg N $ha^{-1}$ $year^{-1}$ (as bovine urine) | No effect on the AOA abundance; increase in the AOB abundance |
| N | [25] | Semiarid temperate grassland (China) | AOA and AOB | 6 years | 0, 1, 2, 4, 8, 16, 32, and 64 g N $m^{-2}$ $year^{-1}$ (as urea) | No effect on the AOA abundance, except a decrease with the highest N loading; increase in the AOB abundance with increasing N concentrations |
| N | [26] | Upland grassland (China) | AOA and AOB | 6 years | 1.5 and 7.5 g N $m^{-2}$ $year^{-1}$ (as $NH_4$, $NO_3$, or $NH_4NO_3$) | Increases in the AOA and AOB abundances in the high N treatments whatever the N form |
| N | [27] | Upland grassland (China) | AOA and AOB | 5 years | 10 g N $m^{-2}$ $year^{-1}$ (as $NH_4NO_3$) | No effect on the AOA abundance; increase in the AOB abundance |
| $CO_2$ × N | [16] | Mediterranean grassland (USA) | AOB | 2 years | 680 ppm 7 g N $m^{-2}$ $year^{-1}$ (as $Ca(NO_3)_2$) | Decrease in the AOB abundance under elevated $CO_2$; no effect of N addition on the AOB abundance; no interactive effect of the $CO_2$ and N treatments |

No published data were found on the soil nitrite oxidizers

was close to the middle of the range of the IPCC scenarios for the second half of the twenty-first century [7]. Within each chamber, two N treatments were applied—one pot received 10 g N-$NH_4NO_3$ $m^{-2}$ through six applications of an $NH_4NO_3$ solution at 2-week intervals, while the other pot received the equivalent amount of distilled water alone. The total amount of N added was equivalent to 100 kg N $ha^{-1}$, which is in the range of common fertilization regimes in grasslands [41] and comparable to very high N deposition rates that already occur in a few industrialized regions of the world [6, 42]. Each of the four treatments (Ctrl, +$CO_2$, +N, and +$CO_2$+N) was replicated six times, resulting in a total of 24 experimental mesocosms (2 $CO_2$ treatments × 2 N treatments × 6 replicates). After 10 weeks of plant growth under treatments, when roots had fully colonized, soil and shoots were well developed (about 50 cm high), plants were harvested and separated into below- and above-ground material (see [40] for further details).

Soil from the top 10 cm was collected in each pot, homogenized, and sieved (2 mm). Part of the freshly sieved soil was immediately used for measurements of microbial activities and soil variables, while the rest of the soil was stored at −20 °C before DNA extraction and measurements of microbial abundances.

### Soil DNA Extraction and Quantification of the Abundance of Soil Ammonia Oxidizers

DNA was extracted from 0.7 g of frozen soil using the Power Soil™ DNA Isolation Kit (MO BIO laboratories, Carlsbad, CA, USA), following the manufacturer's instructions.

The abundances of AOA and AOB were measured by quantitative PCR targeting the *amoA* functional gene encoding for ammonia monooxygenase which is specific of these groups. Amplification was performed using gene primers CrenamoA23f and CrenamoA616r for the AOA [43] and amoA_1F and amoA_2R for the AOB [44]. The final reaction volume was 20 μL and contained (final concentrations) 0.75 μM of CrenamoA616r and 1 μM of CrenamoA23f for the archaeal *amoA* or 0.5 μM of each primer for the bacterial *amoA*, 2 % bovine serum albumin (BSA), 1× of QuantiTect SybrGreen PCR Master Mix (Qiagen, Courtaboeuf, France), and 10 ng of soil DNA extract or $10^7$–$10^2$ gene copies number per microliters of an in-house plasmid containing cloned archaeal (54d9 fosmide fragment [45]) and bacterial (*Nitrosomonas europaea*, GenBank accession number:L08050) *amoA* genes. The samples were run in duplicate ($n$=6 in all treatments) on a Lightcycler 480 (Roche Diagnostics, Meylan, France) as follows: for archaeal *amoA*, 15 min at 95 °C, 45 amplification cycles (45 s at 94 °C, 45 s at 55 °C, and 45 s at 72 °C) and 10 s at 40 °C; for bacterial *amoA*, 15 min at 95 °C, 45 amplification cycles (30 s at 95 °C, 45 s at 54 °C, 45 s at 72 °C, and 15 s at 80 °C), and 30 s at 40 °C.

Melting curve analysis confirmed the specificity of amplification for both AOA and AOB. Amplification efficiencies of 91 and 94 % were obtained for AOA and AOB quantification, respectively. Dilution series were performed to control for possible PCR inhibition by co-extracted compounds, and no inhibition was observed. The ratio of the abundance of AOA to AOB was calculated. This ratio enables the comparison of the relative numbers of AOA and AOB *amoA* gene copies between treatments, but does not reflect the exact cell proportion of AOA to AOB in soils as the mean *amoA* gene copy numbers per cell may vary between these two groups.

### Quantification of the Abundance of Soil Nitrite Oxidizers

The abundance of *Nitrobacter*-like NOB was measured by quantitative PCR targeting the functional gene *nxrA* [38], which encodes for the nitrite oxidoreductase. The amplification was performed using the gene primers F1norA [46] and R2norA [47]. The final reaction volume was 20 μL and contained (final concentrations) 0.5 μM of each primer, 1× of QuantiTect SybrGreen PCR Master Mix (Qiagen, Courtaboeuf, France), and 40 ng of soil DNA extract or $10^7$–$10^2$ copies using a linearized plasmid containing cloned *nxrA* gene of *Nitrobacter hamburgensis* X14 (DSMZ 10229). The samples ($n$=6 in all treatments, except in the high N treatment where $n$=5) were run twice on a Lightcycler 480 (Roche Diagnostics, Meylan, France) as follows: 15 min at 95 °C and 45 amplification cycles (30 s at 95 °C, 45 s at 55 °C, and 45 s at 72 °C).

The abundance of *Nitrospira* was measured by quantitative PCR targeting *16S* rRNA gene sequences specific for this group [38]. The amplification was performed using the gene primers Ns675f and Ns746r [48]. The final reaction volume was 25 μL and contained (final concentrations) 0.4 μM of each primer, 1× of QuantiTect SybrGreen PCR Master Mix (Qiagen, Courtaboeuf, France), and 10 ng of soil DNA extract or $10^7$–$10^2$ *Nitrospira* copies of linearized plasmid DNA (GenBank accession number: FJ529918). The samples ($n$=6 in all treatments) were run twice on a Lightcycler 480 (Roche Diagnostics, Meylan, France) as follows: 15 min at 95 °C and 45 amplification cycles (30 s at 95 °C, 30 s at 66 °C, and 1 min at 72 °C).

For both genes, melting curves confirmed the specificity of the amplification and amplification efficiencies were of 95–96 %, with no PCR inhibition observed.

The ratio of the abundance of *Nitrospira* to *Nitrobacter*-like NOB was calculated. This ratio allows the comparison of the numbers of *Nitrospira 16S* gene copies and *Nitrobacter*-like *nxrA* gene copies between treatments, but does not reflect the exact cell proportion of *Nitrospira* to *Nitrobacter*-like NOB in soils, as the mean *nxrA* or *16S* gene copy numbers per cell may vary between these two groups.

### Plant/Soil Variables and Nitrification Rates

Previously measured plant/soil variables [39] were used to determine whether changes in the abundances of the soil ammonia and nitrite oxidizers in response to the treatments were related to changes in plant biomass (shoot and root biomass) or soil variables (soil respiration, gross N mineralization and soil water content). Soil respiration was used as an indicator of soil heterotrophic activity and oxygen status, gross N mineralization rates as a proxy of soil N availability, and soil water content as an indicator of soil oxygen status [39]. In addition to the soil variables that were previously measured, we conducted measurements of Olsen phosphorus (P) concentration (at the INRA, Arras, France) and used this variable as a proxy of soil P availability.

Potential nitrification rates (determined using nitrifying enzyme activity assays) and gross nitrification rates (determined using $^{15}N$ pool dilution technique; for further details, see [39]) were used to determine whether changes in the abundances of each of the four microbial groups involved in nitrification were related to changes in potential and gross nitrification rates.

### Statistical Analysis

We assessed the interactive effects of elevated $CO_2$ and inorganic N supply on the abundances of soil ammonia oxidizers (AOA and AOB), soil nitrite oxidizers (*Nitrobacter*-like NOB and *Nitrospira*), and on the AOA/AOB and *Nitrospira*/*Nitrobacter*-like NOB abundance ratios. Statistical analyses were carried out using PROC MIXED in SAS 9.3 (SAS Institute, Cary, NC). The experiment was analyzed as a full factorial split-plot design, with two factors ($CO_2$ and N) at two levels (ambient and elevated) and their interaction. The $CO_2$ treatment was included as a whole-plot factor, and the N treatment as a split-plot factor. Where necessary, data were log-transformed prior to the analysis to ensure conformity with the assumptions of normality and homogeneity of variances. Effects with $P<0.05$ are referred to as significant and effects with $0.05 \leq P < 0.10$ as marginally significant. The effects of each treatment (elevated $CO_2$, N addition, and elevated $CO_2$ combined with N addition) were calculated as: % effect=[treatment−control]/ [control]×100 ($n=6$ in the control and in the treatment).

Correlations were carried out using PROC CORR in SAS to investigate relationships between and among ammonia-oxidizer (AOA and AOB) and nitrite-oxidizer (*Nitrobacter*-like NOB and *Nitrospira*) abundances. In addition, correlations were performed to investigate relationships between the abundances of the soil ammonia and nitrite oxidizers and plant/soil variables and nitrification rates.

## Results

### Ammonia-Oxidizer Abundance

The average abundance of AOA across all treatments was $2.58\times10^6$ *amoA* copies per gram of dry soil. The abundance of AOA was not affected by any of the treatments (Fig. 1a; $CO_2$, $P=0.96$; N, $P=0.31$; $CO_2 \times N$, $P=0.80$). The average abundance of AOB across all treatments was $1.10\times10^6$ *amoA* copies per gram of dry soil. Inorganic N addition increased the abundance of AOB (Fig. 1a; N, $P=0.002$). Elevated $CO_2$ had no effect on AOB abundance and the interaction between elevated $CO_2$ and N addition was not significant (Fig. 1a; $CO_2$, $P=0.41$; $CO_2 \times N$, $P=0.94$).

Inorganic N addition decreased the average of AOA to AOB abundance ratio from 5.9 to 1.5 (Fig. 1b; N, $P<0.001$). Elevated $CO_2$ and the $CO_2 \times N$ interaction did not significantly affect the AOA/AOB ratio (Fig. 1b; $CO_2$, $P=0.54$; $CO_2 \times N$, $P=0.49$).

### Nitrite-Oxidizer Abundance

The average abundance of *Nitrobacter*-like NOB across all treatments was $2.18\times10^3$ *nxrA* copies per gram of dry soil. Inorganic N addition increased the abundance of *Nitrobacter*-like NOB (Fig. 1a; N, $P=0.004$). Elevated $CO_2$ had no effect on the abundance of *Nitrobacter*-like NOB and the interaction between elevated $CO_2$ and N addition was not significant ($CO_2$, $P=0.35$; $CO_2 \times N$, $P=0.12$). The average abundance of *Nitrospira* across all treatments was $8.83\times10^5$ *16S* copies per gram of dry soil. Inorganic N addition decreased the abundance of *Nitrospira* (Fig. 1a; N, $P=0.01$). The effects of elevated $CO_2$ and of the interaction between elevated $CO_2$ and N addition were not significant (Fig. 1a; $CO_2$, $P=0.55$; $CO_2 \times N$, $P=0.33$).

Inorganic N supply significantly decreased the ratio of *Nitrospira* to *Nitrobacter*-like NOB abundances (Fig. 1c; N, $P<0.001$). This decrease was less pronounced under elevated $CO_2$ (Fig. 1c; $CO_2 \times N$, $P=0.05$). Elevated $CO_2$ alone had no effect on this ratio (Fig. 1c; $CO_2$, $P=0.17$).

### Relationships Between and Among Ammonia-Oxidizer and Nitrite-Oxidizer Abundances

When all the treatments were taken into account, the abundance of *Nitrobacter*-like NOB was significantly and positively correlated to the abundance of AOB (Fig. 2a), while the abundance of *Nitrospira* was significantly and positively correlated (logarithmic relationship) to that of AOA (Fig. 2b). The abundance of AOB was positively correlated to the abundance of AOA in the treatments including N addition, but not in the treatments with no N addition (Fig. 2c). The abundance

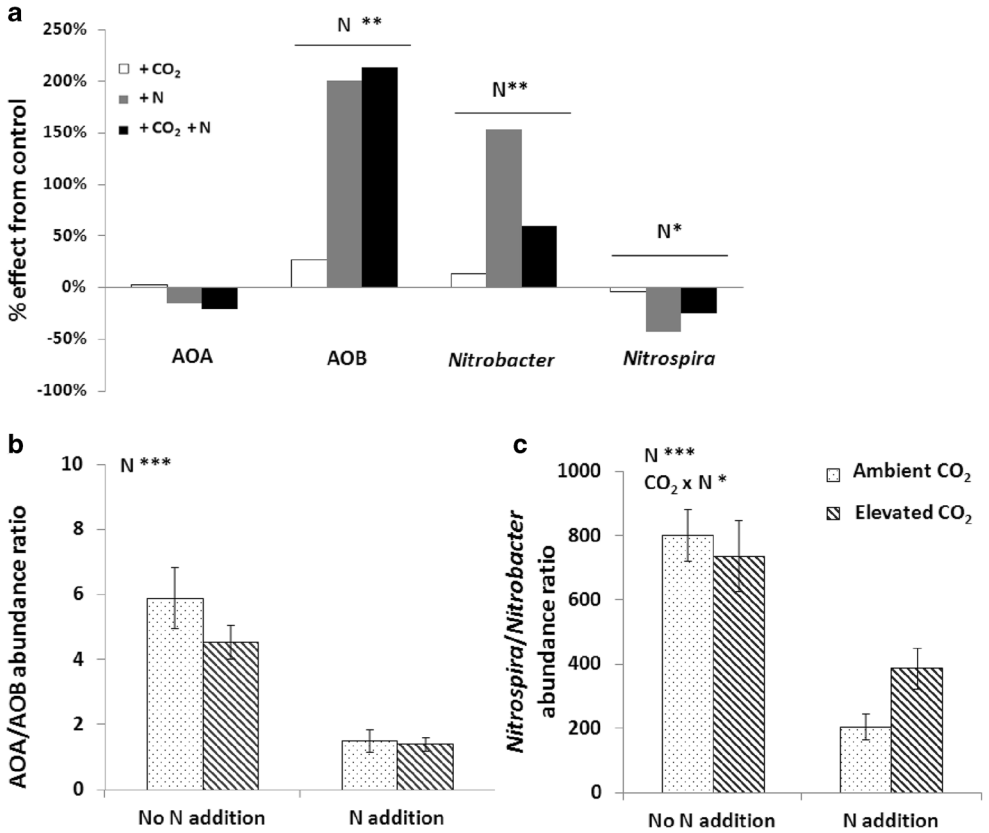


**Fig. 1** **a** Changes in the abundance of soil ammonia-oxidizing archaea (*AOA*), ammonia-oxidizing bacteria (*AOB*), and nitrite-oxidizing bacteria (*Nitrobacter* and *Nitrospira*) in the different treatments relative to the control. Treatments are elevated $CO_2$ without N addition (+$CO_2$, *white bars*), N addition without $CO_2$ elevation (+N, *gray bars*), and elevated $CO_2$ combined with N addition (+$CO_2$+N, *black bars*). Data are expressed as the percentage change from the control treatment (where $CO_2$ and N are both at ambient levels). **b** Ratios between the abundances of AOA to AOB and **c** the abundances of *Nitrospira* to *Nitrobacter*-like NOB in the four treatments (no N addition vs. N addition; *dotted bars* ambient $CO_2$, *hashed bars* elevated $CO_2$). Means and standard errors are presented ($n$=6 except for the N treatment where $n$=5 for the *Nitrospira*/*Nitrobacter* abundance ratio). Significant effects are indicated (*$P$<0.05; **$P$<0.01; ***$P$<0.001)

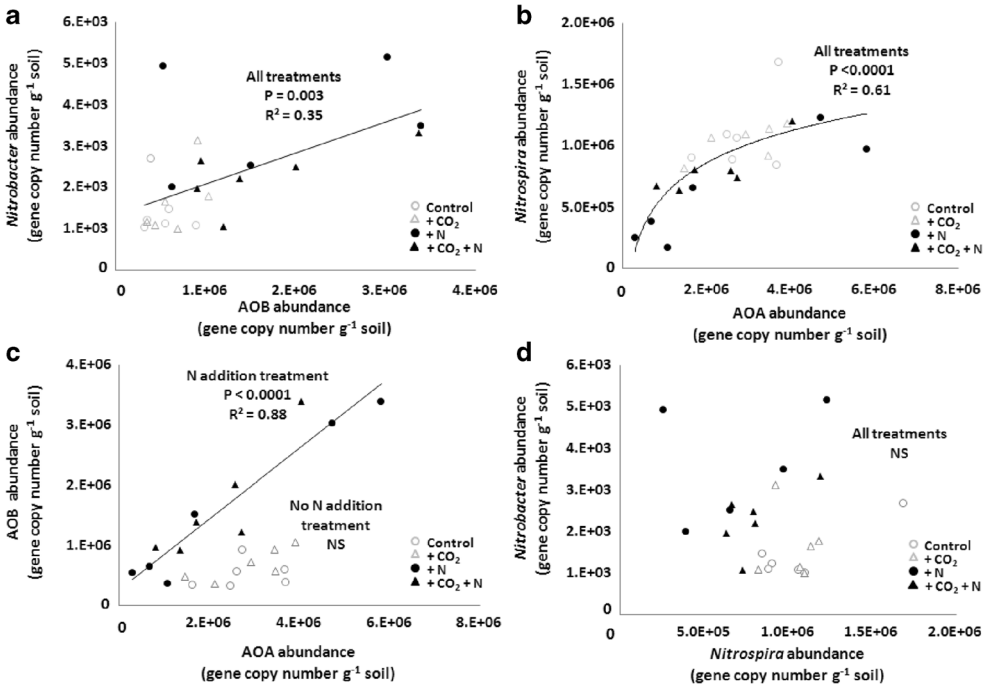


**Fig. 2** Correlations between and among ammonia-oxidizer and nitrite-oxidizer abundances. When significant, the linear or logarithmic regression is drawn, and the associated $P$ and $R^2$ values are indicated

of *Nitrobacter*-like NOB was not correlated to the abundance of *Nitrospira* (Fig. 2d).

### Treatment Effects on Plant/Soil Variables and Relationships with Ammonia- and Nitrite-Oxidizer Abundances

Shoot biomass, root biomass, and soil respiration were increased under elevated $CO_2$ and N supply, and the largest increases occurred when both treatments were elevated (Supplementary Fig. 1a). AOA abundance was not correlated to these variables, while AOB abundance was positively correlated with shoot biomass, root biomass, and soil respiration (Table 2). *Nitrobacter*-like NOB was positively correlated to shoot and root biomass, while *Nitrospira* abundance was negatively correlated to these two variables (Table 2).

Elevated $CO_2$ increased gross N mineralization in the high N treatment (Supplementary Fig. 1a), but no significant correlations were found between the nitrifier abundances and gross N mineralization rates (Table 2). Elevated $CO_2$ and inorganic N supply decreased Olsen P concentration (Supplementary Fig. 1a), but no significant correlations were found between the nitrifier abundances and Olsen P concentrations (Table 2).

Finally, soil water content decreased in the N addition treatment, but this negative effect was negated by elevated $CO_2$ (Supplementary Fig. 1a). AOA and AOB abundances were not correlated to soil water content. In contrast, *Nitrobacter*-like NOB was negatively correlated to soil water content, while *Nitrospira* abundance was positively correlated to this variable (Table 2).

### Treatment Effects on Nitrification Rates and Relationships with Ammonia- and Nitrite-Oxidizer Abundances

The potential nitrification rates increased in response to N addition and were further stimulated by elevated $CO_2$ at high N, while gross nitrification rates were not altered by any of the treatments (Supplementary Fig. 1b). Changes in potential and gross nitrification rates were not correlated to changes in the abundances of the nitrifier groups studied (Table 3).

## Discussion

### Combined Effects of Elevated $CO_2$ and Inorganic N Supply on Soil Ammonia Oxidizers

In this experiment, the short-term responses of AOA and AOB to elevated $CO_2$ and inorganic N supply differed. The abundance of AOB increased in response to N addition, while the abundance of AOA was not affected by this treatment, supporting the idea of a degree of niche differentiation between these two groups in soils [36, 49, 50]. The increase of AOB abundance in the high N treatment could be explained by higher ammonium availability, resulting from the direct addition of ammonium to the soil, and greater gross N mineralization in this treatment. These results are consistent with the literature for grassland ecosystems; all available studies have indeed reported increases in the abundance of AOB in response to $NH_4^+$ or urea addition (see Table 1). In addition, the positive correlations between AOB abundance and root biomass or soil respiration both suggest that beyond possible side effect on oxygen level, higher soil C inputs from roots in the high N treatment could have contributed to the stimulation of mineralization and hence AOB growth.

In contrast, AOA abundance was not altered by the high N treatment and was not correlated to plant/soil variables. This result indicates that AOA and AOB do not respond similarly to changes in soil environmental parameters, and in particular, to changes in soil N availability. It has already been shown that AOA may be favored under low N conditions [49, 51, 52], and a number of studies have demonstrated that the abundance of AOA remains unaffected or even decreases in response to N addition in grasslands ([18, 20, 21, 24, 25, 27]; see Table 1), although this is not always the case ([23, 26]; see Table 1) and possibly not for all AOA populations [37, 53].

**Table 2** Correlations between the abundances of the soil nitrifiers and variables of the plant/soil system ($n$=24)

| | AOA | | AOB | | *Nitrobacter* | | *Nitrospira* | |
|---|---|---|---|---|---|---|---|---|
| | $r$ | $P$ value | $r$ | $P$ value | $r$ | $P$ value | $r$ | $P$ value |
| Shoot biomass | | 0.21 | **0.50** | **0.01** | **0.49** | **0.02** | **−0.56** | **0.005** |
| Root biomass | | 0.46 | **0.54** | **0.006** | *0.41* | *0.053* | **−0.43** | **0.04** |
| Soil respiration | | 0.56 | **0.45** | **0.03** | | 0.20 | | 0.41 |
| Gross N mineralization | | 0.62 | | 0.21 | | 0.38 | | 0.88 |
| Olsen P | | 0.61 | | 0.16 | | 0.65 | | 0.25 |
| Soil water content | | 0.17 | | 0.56 | **−0.41** | **0.05** | **0.67** | **<0.001** |

When the correlation is significant (in bold) or marginally significant (in italics), the Spearman's correlation coefficient ($r$) is given

**Table 3** Correlations between the abundances of the soil nitrifiers and nitrification rates ($n$=24)

| | AOA | | AOB | | *Nitrobacter* | | *Nitrospira* | |
|---|---|---|---|---|---|---|---|---|
| | $r$ | $P$ value | $r$ | $P$ value | $r$ | $P$ value | $r$ | $P$ value |
| Potential nitrification | 0.75 | | 0.13 | | 0.71 | | 0.45 | |
| Gross nitrification | 0.31 | | 0.32 | | 0.99 | | 0.51 | |

No significant correlation was found

Given the current global change context, our results suggest that the abundance of soil AOB may increase in ecosystem experiencing rising atmospheric N deposition, while the abundance of soil AOA may remained unaffected, leading to changes in the soil ammonia-oxidizing community.

## Combined Effects of Elevated $CO_2$ and Inorganic N Supply on Soil Nitrite Oxidizers

The two bacterial groups of nitrite oxidizers responded differently to the N treatment, which highlights their niche differentiation in soil. Indeed, N addition increased *Nitrobacter*-like NOB abundance, while it decreased *Nitrospira* abundance. These results suggest that *Nitrobacter* growth is favored at high nutrient conditions—and in particular at high nitrite availability—while *Nitrospira* is favored at low nutrient conditions, which is consistent with previous report for an agricultural soil [38]. In addition, the direction of the correlations with plant/soil variables was reversed between *Nitrobacter* and *Nitrospira*. On the one hand, *Nitrobacter*-like NOB abundance was negatively correlated to soil water content and positively to shoot and root biomass. These correlations suggest that *Nitrobacter* growth is stimulated at high oxygen content associated to low soil water content and also at high soil C availability associated to increased root biomass in the high N treatment. In particular, increases in soil C availability could have benefited to mixotrophic *Nitrobacter* [34]. On the other hand, *Nitrospira* abundance was positively correlated with soil water content and negatively to root biomass, indicating a preference for low oxygen conditions and low C availability. Our results are consistent with the literature, since *Nitrobacter* are known to have lower N substrate and oxygen affinities and thus to be adapted to high-nitrite and oxygen environments, whereas *Nitrospira* bacteria are known to have higher N substrate and oxygen affinities and to be adapted to low-nitrite and oxygen environments [38, 54–56]. The different responses of nitrite oxidizers to the N treatment resulted in a decrease of the *Nitrospira*/*Nitrobacter* abundance ratio in presence of N addition. This decrease was however mitigated when elevated $CO_2$ and N addition were combined, because the increase of *Nitrobacter*-like NOB abundance and the decrease of *Nitrospira* were less pronounced in this treatment than in the N treatment alone. This interactive effect of $CO_2$ and N on the *Nitrospira*/*Nitrobacter* abundance ratio may be explained by higher soil water content in this treatment compared to the N treatment that may have limited the increase of *Nitrobacter*-like NOB and the decrease of *Nitrospira*.

*Nitrobacter* and *Nitrospira*, thus, differ in their ecological requirements and do not respond similarly to changes in soil N and C availability and soil water content. This niche differentiation may have important ecological implications in the context of global change, in particular, rising atmospheric N deposition and changes in precipitation regimes might have consequences on the relative abundance of the two groups of nitrite oxidizers in soil.

## Coupling Between and Among Ammonia- and Nitrite-Oxidizer Abundances

In our experiment, we found that there was a coupling between AOB and *Nitrobacter* and between AOA and *Nitrospira*. AOB and *Nitrobacter* abundances were favored at high N and were positively correlated, whereas AOA and *Nitrospira* abundances were not altered or even decreased at high N and were also positively correlated. These positive correlations between these microbial groups could thus result from their similar ecological requirements. Though, and as correlations between nitrification rates and nitrifiers abundances were not significant in our soil, we cannot state that the AOB-*Nitrobacter* couple drives nitrification at high N conditions, and we cannot identify which nitrifier couple (AOA-*Nitrospira* or AOB-*Nitrobacter)* drives nitrification at ambient N. Studying the dynamics of nitrifiers at the RNA level [43, 57] would be interesting to determine which nitrifier groups drive the nitrification process according to soil N availability.

The coupling among ammonia oxidizers or nitrite oxidizers has been also investigated and we found that AOB abundance was positively correlated to AOA abundance. This positive correlation was observed only in the high N treatment, i.e., when AOA and AOB populations adapted to high N availability were selected [37, 53]. However, we could not identify the common environmental driver of AOA and AOB abundances in this treatment, explaining this positive relationship.

Conversely, *Nitrobacter*-like NOB abundance was not correlated to *Nitrospira* abundance whatever the N treatment, suggesting that the niche separation is greater between nitrite oxidizers than ammonia oxidizers for N requirements. This assumption is supported by the absence of effect of N addition on AOA abundance, while *Nitrospira* abundance was significantly decreased.

After exposure to treatments of only 10 weeks in grassland mesoscosms, N addition significantly altered soil ammonia- and nitrite-oxidizer abundances, and elevated $CO_2$ when combined with N supply affected the *Nitrospira* to *Nitrobacter*

abundance ratio. Our results demonstrate that the responses of ammonia and nitrite oxidizers to elevated $CO_2$ and inorganic N addition are contrasted between and among these functional groups. AOB and *Nitrobacter* abundances are coupled and are both stimulated at high N availability, while AOA and *Nitrospira* abundances are coupled and are likely promoted at low N availability. In our soil, changes in ammonia-oxidizer abundances are essentially controlled by changes in soil N and C availability, while changes in nitrite-oxidizer abundances are driven by soil N and C availability, and also soil oxygen content (associated to soil water content). Our results further suggest that the niche separation for N requirements seems to be more pronounced among nitrite oxidizers than ammonia oxidizers.

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