Réponse des groupes microbiens impliques dans la dynamique de l'azote du sol aux facteurs du changement global et aux incendies

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Abstract

Global environmental changes are main drivers of nutrient cycling in ecosystem. However, present studies mainly focus on evaluating the effects of single -and less often a few - global change factors on soil N cycling processes in grasslands. In addition, these studies have not recognized the importance of the response of grassland soil N cycling to co-occurring multiple global change factors and disturbance like fire. It remains unclear how N cycling response to fire could differ under different global change scenarios. This strongly restricts our ability to understand and predict global change effect on grasslands. In this work, two experiments were conducted: (i) a mesocosm experiment to assess the combined effects of increased N deposition and changes in both the precipitation amount and frequency on soil N cycling in a semi-arid Monsoon grassland; and (ii) an *in situ* experiment to assess the main and combined effects of elevated CO_2, warming, increased precipitation, N deposition and fire on soil N cycling in a Mediterranean grassland. This allows studying the -possibly interactive- effects of several global change factors on the abundances of soil N-cycling microbial communities. The microbial groups studied were ammonia oxidizing bacteria (AOB) and archaea (AOA), *nirK*- and *nirS*-nitrite reducers, *nosZI*- and *nosZII*-N_2O reducers, plus *Nitrobacter* and *Nitrospira* for the Mediterranean grassland. The main results are as follows:

1) The responses of different groups of soil (de)nitrifiers to global change scenarios differed strongly in both grasslands. In the Monsoon grassland, AOB abundance mostly responded to nitrogen, whereas AOA were more sensitive to soil water dynamics than nitrogen. The main effects of decreased precipitation amount and altered precipitation frequency differed between the four denitrifier groups studied. The n*irK*- and *nirS*-harboring nitrite reducers and *nosZI*-harboring N_2O reducers were more sensitive to N deposition than *nosZII*-harboring N_2O reducers, and *nirK*- and *nirS*-bacteria responded to reduced precipitation in an opposite direction. This highlights niche differentiation between these groups and indicates that the balance between them may be altered in the future. In addition, the study showed that N_2O emission was related to soil denitrification instead of nitrification in the Monsoon grassland. 2) In the Mediterranean grassland, nitrogen deposition increased the abundance of AOB and to a lesser extent AOA and *Nitrobacter* (+12% to 182%), but not *Nitrospira*. Meanwhile, N deposition increased the abundance of *nirK* and *nosZI* and to a

lesser extent *nirS* (+ 10% to 46%), but not *nosZII*. Instead, Precipitation had a negative main effect on *Nitrobacter* (up to -61%) and no significant effect on the three other nitrifier groups. Further, precipitation increased the abundance of *nirS* (+ 3% to 26%), whereas no significant effect was observed on the three other denitrifier groups. Further, burning had a negative main effect on AOB (- 20% to 56%) and did not affect the abundances of soil denitrifiers immediately after fire, but it decreased the abundance of *nirK* (-6% to 37%) and *nosZI* (-5% to 36%) almost three years after the initial disturbance. No main effect of elevated CO_2 and heating was observed. *Nitrobacter* abundance was mostly affected by global change factors through their effects on AOB abundance, whereas *Nitrospira* abundance was more related to changes of AOA. The effects of multiple global changes and fire on soil (de)nitrifying microbial communities abundance were not additive and thus cannot be predicted by studies on single global change factor.

These results demonstrate that for both grasslands studied, the effects of multiple global change factors and disturbances on soil N cycling could not be predicted simply by studying the effects of one or two factors. The observed interactive effects were explained by environmental variables like soil moisture, mineral N availability, pH and growth of plant belowground parts. This calls for more comprehensive studies in the global change biology domain. Modelling and evaluating the generality of these complex interaction effects is thus a high priority for research to predict the responses of soil N cycling processes to global change and feedbacks on climate in the future.

Key words：Elevated CO_2; Warming; Precipitation Regime; N Deposition; Fire Disturbance; Denitrification and Denitrifiers; Nitrification and Nitrifiers

摘 要

全球环境变化是驱动生态系统营养物质循环过程的重要因素。然而，当前的研究多集中于单因子对生态系统氮周转的影响，很少关注多因子的影响。此外，这些研究没有关注降雨格局变化伴随的氮素湿沉降造成的影响，并且关于全球变化因子与火烧协同作用对氮周转过程的主要微生物群落的影响的相关研究尚不明确，这极大的制约了我们理解和预测全球变化对草地生态系统的影响。因此，本论文通过：（1）围隔实验(mesocosm)研究氮沉降增加、降雨量及频率变化对半干旱季风气候地区土壤氮周转相关微生物群落丰富度的影响；（2）原位实验研究 CO_2 浓度升高、增温、降水增加、氮沉降升高和火灾干扰主效应及交互作用对地中海气候地区草地土壤氮周转相关微生物群落丰富度的影响，拟阐明全球变化多因素主效应及其协同作用对土壤氮周转的影响机制。具体微生物群落包括氨氧化细菌（AOB）、氨氧化古菌（AOA）、*nirK*-和 *nirS*-基因编码的亚硝酸盐还原类微生物、*nosZI*-和 *nosZII*-基因编码的 N_2O 还原类微生物以及亚硝酸盐氧化类微生物 *Nitrobacter* 和 *Nitrospira*。研究结果发现：

（1）在两种气候地区草地生态系统中，各类土壤硝化/反硝化类微生物对全球变化因子的响应均表现出很大差异。在季风性气候草地生态系统，AOB 丰度对全球变化因子的响应主要表现在对氮素的响应。相比之下，AOA 丰度对草地土壤水分动态的敏感性高于氮素。*nirK*-、*nirS*-及 *nosZI*-基因丰度比 *nosZII*-基因丰度对氮沉降的敏感性高，并且 *nirK*-和 *nirS*-基因丰度对降水减少处理响应方向相反。这暗示着不同硝化类/反硝化微生物群落间具有不同的生态位，未来多因素变化条件下氮素周转关键微生物之间的比例关系会发生变化。此外，研究发现，土壤氧化亚氮的排放主要与反硝化作用相关。（2）在地中海气候地区草地生态系统中，氮沉降显著增加 AOB、AOA 和 *Nitrobacter* 丰度，效应值为 12%~182%；并且对 AOB 的影响大于对 AOA 和 *Nitrobacter* 的影响，但氮沉降对 *Nitrobacter* 的丰度没有显著影响；同时，氮沉降增加土壤 *nirK*-、*nirS*-*和* *nosZI*-基因丰度，效应值为 10%~46%；并且对 *nirK*-和 *nosZI*-基因丰度的影响大于对 *nirS*-基因丰度的影响，但氮沉降对 *nosZII*-基因丰度没有显著影响。相比之下，降雨增加对 *Nitrobacter* 有显著的降低效应（高达-61%），但是对另外三种土壤硝化类微生物没有显著影响；降雨增加升高了土壤 *nirS*-基因丰度，效应值为 3%~26%；但对另外三种土壤反硝化类微生物没有显著影响。火烧干扰显著降低了土壤 AOB 的丰度（降低 20% ~ 56%）。在火烧干扰后的短时间内土壤反硝化类微生物丰度没有显著影响，但在火烧干扰处理三年后，火烧处理显著降低土壤的 *nirK*-（降低 6%~37%）和 *nosZI*-（降低 5%~36%）基因丰度。CO_2 升高和增温处理的主效应对土壤硝化/反硝化类微生

物均没有显著影响。全球变化因子通过对 AOB 丰度的影响间接改变 *Nitrobacter* 丰度；然而 *Nitrospira* 丰度对全球环境变化因子的响应则与 AOA 丰度的变化密切相关。全球环境变化因子和火烧干扰处理对土壤硝化/反硝化类微生物群落丰度的影响并非叠加性，因此不能通过研究多个单一因子对其影响预测全球变化多因子的共同效应。

综上所述，两种气候地区的草地生态系统的研究均表明，土壤氮周转过程对全球环境变化因子的响应无法仅通过模拟某单一因子（或两因子）进行预测。全球环境变化多因子间的交互效应通过影响土壤环境因子、土壤氮周转关键微生物数量以及植物生长状况，进而改变氮周转过程。因此，在全球变化研究领域进行更全面的研究势在必行，其中评估这些复杂交互效应的大小并建构普适性模型是预测土壤氮素周转过程对全球变化响应的重中之重。

关键词：CO_2 浓度升高；增温；降雨格局；氮沉降；火烧干扰；草地；硝化/反硝化作用；硝化/反硝化类微生物

Résumé

Les changements globaux sont les principaux moteurs du cycle des nutriments dans les écosystèmes. Cependant, les études actuelles se concentrent principalement sur l’évaluation des effets d’un facteur du changement global et moins souvent de plusieurs facteurs sur les processus du cycle de l’azote dans le sol. De plus, ces études n'ont pas pris en compte le fait que la modification du régime de précipitation avait également une influence sur le régime de dépôt humide d'azote. En outre, la réponse du cycle de l'azote dans les sols des prairies à de multiples facteurs du changement global agissant ensemble -et parfois en même temps que des perturbations telles que les incendies- doit encore être étudiée. Cela limite fortement notre capacité à comprendre et à prévoir les effets du changement global sur les prairies. Dans ce travail de doctorat, deux expériences ont été menées: (i) une expérience en mésocosmes pour évaluer les effets combinés d'une augmentation des dépôts d'azote et de changements dans la quantité et de la fréquence des précipitations sur le cycle de l'azote édaphique dans une prairie semi-aride; et (ii) une expérience in situ pour évaluer les effets combinés de l'augmentation de la concentration en CO_2, du réchauffement, d’ une modification des précipitations, du dépôt d'azote et d’un feu sur le cycle de l'azote du sol dans une prairie méditerranéenne. Cela permet d'étudier les effets de la combinaison de plusieurs facteurs de changement global (et d’ une perturbation, le feu) sur l'abondance des communautés microbiennes du cycle de l'azote. Les groupes microbiens étudiés étaient les bactéries et les archées oxydant l'ammoniac (AOB et AOA, respectivement), les bactéries réductrices de nitrite porteuses des gènes *nirK* ou *nirS*, et les réducteurs de N_2O porteurs des gènes *nosZI*- et *nosZII* pour les deux sites, plus les bactéries oxydant le nitrite du genre *Nitrobacter* et *Nitrospira* pour la prairie méditerranéenne. Les principaux résultats et conclusions sont les suivants :

1) Les réactions des différents groupes de (dé)nitrifiants aux scénarios de changement global différaient fortement quel que soit le type de prairie. Les AOB dépendaient principalement de la disponibilité en azote En revanche, dans les deux prairies, les AOA étaient sensibles à la dynamique de l'eau du sol. Les principaux effets de la diminution des précipitations et de la fréquence des précipitations diffèraient entre les quatre groupes de dénitrifiants étudiés. Les réducteurs de nitrite hébergeant les gènes *nirK* et *nirS* et des réducteurs de N_2O porteurs du gène *nosZI* étaient plus sensibles au dépôt d'azote que les réducteurs de N_2O porteur du gène *nosZII*. Les bactéries de type *nirK* et *nirS* réagissaient à

une réduction des précipitations de façon opposée. Cela met en évidence la différenciation de niche qui existe entre eux et indique que l'équilibre entre ces deux groupes pourrait être modifié à l'avenir. En outre, l'étude a montré que les émissions de N_2O étaient liées à la dénitrification plutôt qu'à la nitrification dans la prairie semi-aride. 2) Dans la prairie méditerranéenne, les dépôts d'azote ont accru l'abondance des AOB (- 20% à 56%) et, dans une moindre mesure, des AOA et *Nitrobacter*, mais pas *Nitrospira*. En parallèle, les dépôts d'azote ont augmenté l'abondance de *nirK* et de *nosZI* et, dans une moindre mesure, de *nirS* (+ 10% à 46%), mais pas de *nosZII*. Les précipitations ont eu un effet principal négatif sur *Nitrobacter* (jusqu'à -61%) et aucun effet significatif sur les trois autres groupes nitrifiants. De plus, les précipitations ont augmenté l'abondance de *nirS* (+ 3% à 26%) sans avoir un effet significatif sur les trois autres groupes de dénitrifiants. De plus, le feu a eu un effet principal négatif sur les AOB (- 20% à 56%) et n'a pas affecté l'abondance des dénitrifiants immédiatement après feu mais a diminué l'abondance de *nirK* (- 6% à 37%) et *nosZI* (-5% à 36%) près de trois ans après le feu. Aucun effet principal de l' élévation du CO_2 ni du réchauffement n'a été observé. L'abondance de *Nitrobacter* était principalement affectée par les facteurs de changement globaux via leurs effets sur l'abondance des AOB, tandis que l'abondance des *Nitrospira* était davantage liée aux changements d'abondance des AOA. Les effets de multiples changements globaux et du feu sur l'abondance des (dé)nitrifiantes du sol n' étaient pas additifs et ne peuvent donc pas être prédits par des études étudiant les facteurs du changement global de façon isolée.

Ces résultats démontrent que pour les deux prairies étudiées, les effets des facteurs du changement global et du feu sur le cycle de l'azote du sol ne pouvaient être prédits simplement en étudiant les effets d'un ou de deux facteurs. Les effets interactifs observés ont été expliqués par quelques variables environnementales telles que l'humidité du sol, la disponibilité de l'azote minéral, le pH et la croissance des parties souterraines de la plante. Nos résultats appellent des études plus complètes dans le domaine de la biologie et l' écologie du changement global. La modélisation et l'évaluation de la généralité de ces effets d'interaction complexes entre différents facteurs constituent donc une priorité majeure pour les chercheurs qui veulent prédire les réactions des processus du cycle de l'azote des sols au changement global ainsi que les rétroactions sur le climat dans la période à venir.

Mots-Clés: CO_2 élevé; Réchauffement climatique; Régime de précipitation; Dépôt d'azote; Perturbation par le feu; Dénitrification; Nitrification

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1 Introduction

1.1 Context

1.1.1 Soil N Cycling Processes

Although nitrogen (N) is one of the most widely distributed elements in nature, it limits or regulates primary production in most terrestrial ecosystems [1, 2]. A mechanistic understanding of the soil N cycling processes is therefore critical when analyzing the functioning of terrestrial ecosystems and their responses to natural and anthropogenic changes and disturbances [3]. Nitrification and denitrification are two major processes of the N cycle that play key roles for the production and transformation of N forms important for plant nutrition, namely nitrate (NO_3^-) and ammonium (NH_4^+) (Fig. 11). They also partly determine N losses, through the production of NO_3^- subjected to leaching to ground water and of N-containing gases from the soil system to the atmosphere, including nitrous oxide (N_2O), a potent greenhouse gas. Hence de(nitrifiers) are not only important to the local soil environment, but also to the global environment. It is thus important for researchers to work on the environmental determinants of these processes and their responses in face of the changes and disturbances that characterize the Anthropocene [4].

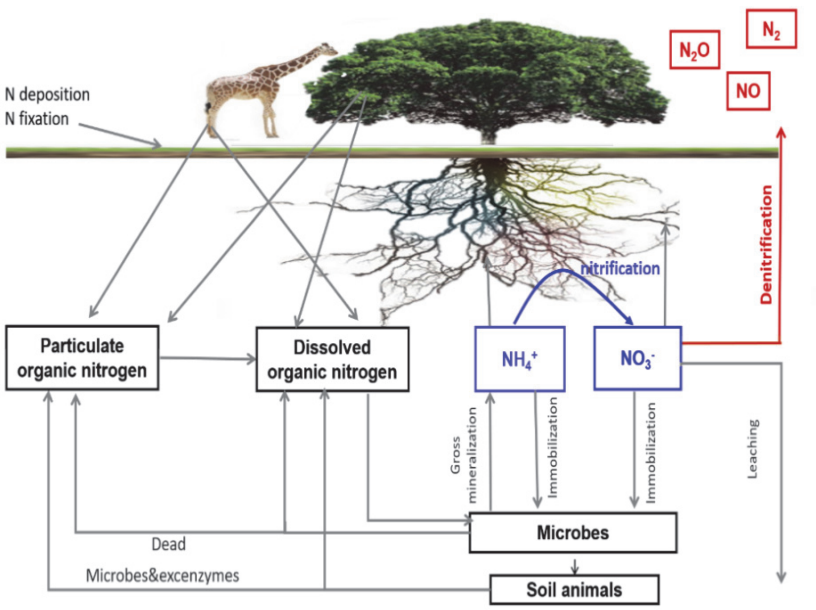

Fig. 11 Overview of the terrestrial nitrogen cycle [4]. Nitrification and denitrification are highlighted in blue and green, respectively

1.1.2 Nitrifiers

Nitrification is a stepwise process consisting of the oxidation of ammonia (NH_3) to nitrite (NO_2^-) and subsequently to NO_3^- (Fig. 12) [4-6] . Nitrification in soil is generally divided into autotrophic and heterotrophic nitrification. It is widely accepted that autotrophic nitrification is the prevalent nitrification process in most soils [7]. In soil, nitrification is mainly carried out by chemoautotrophic ammonia-oxidizing bacteria (AOB) and archaea (AOA) [8] and nitrite-oxidizing bacteria (NOB, mainly represented by *Nitrobacter* and *Nitrospira* genera) [9-10].

For a long time, AOB were believed to be the only soil microorganisms responsible for the first step of nitrification (ammonia oxidation), which is often assumed to be the limiting step as NO_2 generally does not accumulate in the environment. It was thus thought that only AOB possess the gene encoding the key enzyme ammonia monooxygenase, AMO [5]. In the 2000, it has been found that some archaea can also perform ammonia oxidation [11]. This has been supported by strain isolation and physiological studies [12-15], metagenomic studies [16] and nitrification inhibition coupled with molecular analysis [17-18]. The *amoA* gene, which codes for a subunit of the AMO enzyme, has been used extensively as a molecular marker gene for cultivation-independent studies of AOB and AOA in soil systems [11, 19, 20]. In the majority of terrestrial ecosystems, soil AOA were often found outnumber AOB [11, 21-25] and a few studies reported relationships between nitrification rates in soil and AOA abundance [20, 26].Whereas soil AOB are largely monophyletic on the basis of 16S rRNA and their diversity is relative low as compared with other functional groups [27]. The obligate chemoautotrophic lifestyle of AOB leads to the large probability of constrains of both the diversity and abundance of AOB [28]. Nitrite oxidizing bacteria (NOB) are distributed more widely, among *Alpha*, *Beta* and *Gamma* classes of proteobacteria and the *Nitrospira* phylum, for *Nitrobacter*, *Nitrotoga*, *Nitrococcus*, *Nitrospina* and *Nitrospira* respectively [29, 30], but *Nitrobacter* and *Nitrospira* are the most important NOB in soils [9, 10]. *Nitrospira* were often viewed as canonical nitrite-oxidizing bacteria (NOB) [31-34]. However, some studies discovered the first complete ammonia oxidizers (“comammox”) in the bacterial genus *Nitrospira* [35, 36]. In addition, it was reported that nitrifier denitrification is another pathway of nitrification where ammonia is oxidized to nitrite followed by a reduction step of nitrite to nitric oxide, N_2O and N_2 [37].

1.1.2.1 Ammonia-Oxidizing Bacteria and Ammmonia-Oxidizing Achea

It is widely accepted that a variety of environmental factors control the abundances of ammonia - and nitrite-oxidizers and nitrification rates in soil [8, 38-40]. These factors include substrate (i.e. NH_4^+ and NO_3^- for AOB/AOA and NOB, respectively) concentration, temperature, pH [41] and moisture/oxygen availability [42-43].

AOB are generally chemolitho-autotrophs and are obligate aerobes [5]. Pratscher et al. (2011) showed that AOB were often favored by high concentration of NH_4^+ [24]. In contrast, AOA are autotrophic or mixotrophic microbes [24] and their abundance often does not respond to soil NH_4^+ level [41, 44, 45]. For instance, Simonin et al. (2015) found an increase in the abundance of AOB with N addition while no response was found in AOA abundance [46]. There are also some results found positive responses of AOA to N addition when ammonia concentrations are low [15, 24, 47-48]. Several studies documented that high level of fertilization inhibits AOA [12, 49-51]. In addition, AOA are favored by acidic conditions [52-53]. It is generally accepted that NH_3 rather than NH_4^+ is used as substrate for ammonia oxidization [5, 54]. Actually, as soil pH decreases, the availability of ammonia is decreased by increasing ionization to NH_4^+ [55], and this can favor the growth of AOA, because the half-saturation constant of AOA for ammonia is significantly lower than AOB (i.e. higher affinity, allowing AOA to perform better than AOB under low NH_3 concentration [12]). In addition, AOA was often detected under environmental surveys at the oxic-anoxic interface [56-59], which indicated an adaptation to low oxygen conditions. Further, the low K_m for O_2 found for *N. maritimus* as well as other AOA [12, 60] supported a hypothesis that AOA are very likely better adapted to low O_2 than AOB, while AOB are adapted to more aerobic conditions [61].

The latest study found that the addition of organic substances could facilitate the growth of AOA strains PS0 and HCA1, showing their characteristics of mixotrophic growth [62]. In contrast, some studies reported that the presence of organic substances had inhibitory effect on the growth of some certain AOA strains such as *Nitrosopumilus maritimus* SCM1 and *Nitrosocaldus yellowstonii* [13-14].

1.1.2.2 Nitrite oxidizers

Recent studies indicated that nitrite oxidation, the second step of nitrification (Fig. 12), can limit the rate of nitrification in particular conditions, such as in disturbed soil systems [63-64] so that more work is need on nitrite oxidizers (microbial ecology studies have studied mostly ammonia oxidizers and information on the ecology of nitrite oxidizers in soil remains scarce). Previous studies found two major genera of NOB in soils, i.e., *Nitrobacter* and *Nitrospira* [9, 10, 65, 66]. *Nitrospira* are assumed to be generally characterized by low half-saturation constants for NO_2^- and O_2, thus being favored by low availability of NO_2^- and O_2; whereas the half-saturation constants of *Nitrobacter* are higher so that *Nitrobacter* outcompete *Nitrospira* when the concentrations of NO_2^- and O_2 are high [67-70]. This has been supported by studies on NOB dynamics in soils [10, 26, 71] and chemostats [72]. Further, some *Nitrobacter* [73, 74] and *Nitrospira* [75] [76] can grow heterotrophically or mixotrophically. Recently, it has been shown that mixotrophic *Nitrobacter* can dominate the

Nitrobacter community in some soils [26]. Currently, it is increasingly accepted that the main ecological difference between these two groups is related to N availability: *Nitrobacter* are favored by high N availability and *Nitrospira* by low N availability [76], whereas the mixotrophic capacity of some *Nitrobacter* can be important to some extent [26, 77].

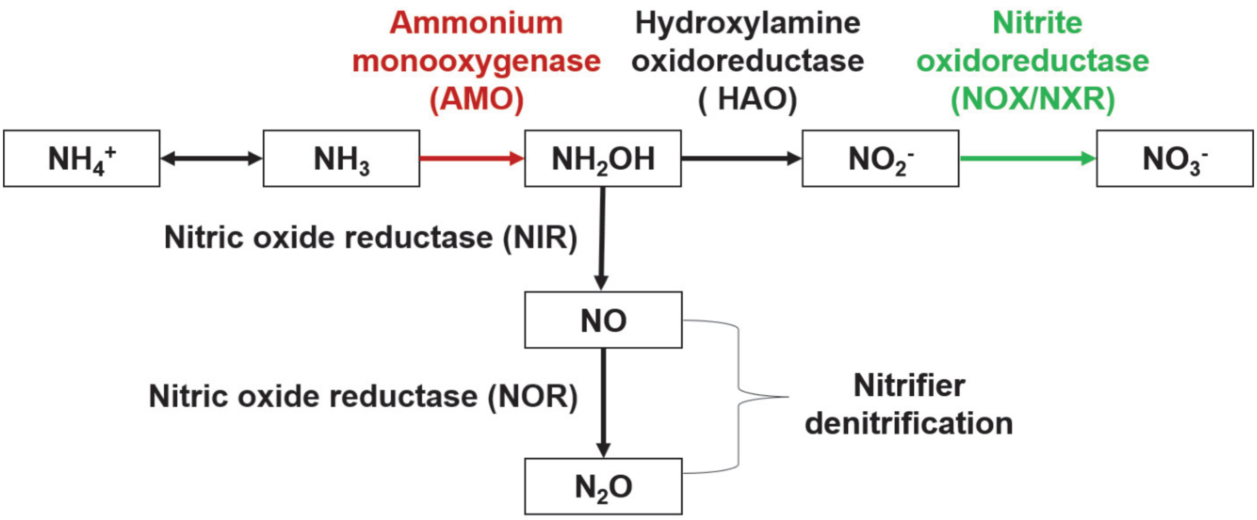

Fig. 12 Nitrification pathway diagram [6]. The main processes I have worked on during my Ph.D., i.e. ammonia oxidation (red) and nitrite oxidation (green)

1.1.3 Denitrifiers

Denitrification is a stepwise process reducing NO_3^- and NO_2^- to gaseous nitrogen (N) compounds, with nitric oxide (NO), N_2O or N_2 as end products [78] (Fig. 13). However, many nitrate reducing bacteria do not participate to further steps of denitrification and are thus not denitrifiers.

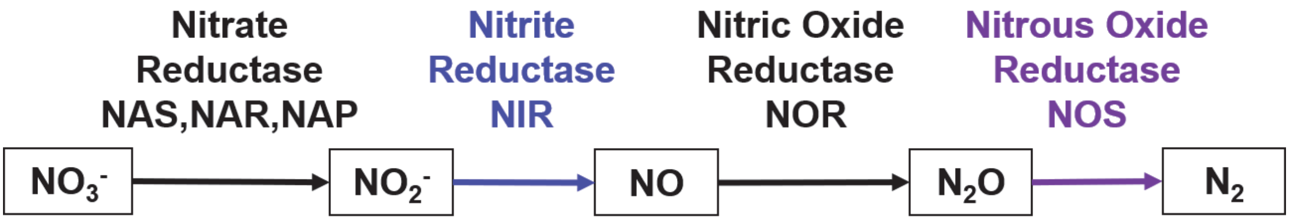

Fig. 13 Denitrification pathway diagram [6]. The main processes I have worked on during my Ph.D., i.e. nitrite reduction (blue) and N_2O reduction (purple)

The ability to perform one step of the denitrification process is widespread among several phylogenetic groups [79]. This makes a functional approach preferable for studying this process, and targeting functional genes specific of one step (Fig. 13) allows the characterization and quantification of a group of functionally similar organisms.

Overall, denitrifiers oxidize organic carbon for energy by using NO_3^- or NO_2^- as an electron acceptor when O_2 concentration is low. Most denitrifiers are facultative anaerobes and use O_2 rather than NO_3^- if O_2 is available [42].

1.1.3.1 Nitrite reducers

A key step of denitrification is NO_2^- reduction, being performed by bacteria harboring NIR genes (Fig. 13), i.e. the *nirK* or *nirS* gene [80]. The abundance of *nirK*-harboring bacteria generally increases with soil organic carbon concentration [44, 81-88]. The abundance of *nirS*-harboring bacteria have been reported to be often positively related to soil NO_3^- concentration [81, 89, 90]. In addition, previous studies also reported that *nirK* abundance had a positive correlation with temperature [91]. The *nirS* gene abundance had strong correlations with NH_4^+ and NO_3^-, and pH [91]. It was reported that the *nirK*-harboring denitrifiers appear to be more sensitive to soil nutrient changes than *nirS*-harboring denitrifiers[84, 92].

1.1.3.2 N_2O reducers

Another key step of denitrification is N_2O reduction, which is performed by bacteria harboring NOS genes (Fig. 13), i.e. the *nosZI* or *nosZII* gene [93, 94]. The *nosZI* groups were reported consisting exclusively of *Alpha-, Beta-, and Gamma*-proteo bacteria. Because about eighty percentage of genomes with *nosZI* also possess *nirK* or *nirS*, organisms possessing *nosZI* genes are likely to be complete denitrifiers [95, 96]. In contrast, nearly half of organisms with *nosZII* appear to be non-denitrifying N_2O reducers [96]. Previous studies suggested that *nosZII* was more abundant or equally as compared with *nosZI* clade I in a range of environments [93]. Many studies reported the dominance of *nosZII* over *nosZI*, with ratios ranging from 1.5 to 10 with quantitative PCR method [93, 97, 98]. Based on abundance analysis, Hallin et al. (2018) summarized that *nosZI* could have key role in N_2O reduction in aquatic systems, whereas the *nosZII* reducers are generally more relevant in soils Many studies indicated that the abundance of *nosZI*-N_2O reducers was positively correlated with the C:N ratio [99, 100]. Other studies found that the abundance of *nosZI*-harboring bacteria were mainly driven by soil NO_3^- concentration [81] or negatively correlated to soil pH [44].The abundance of *nosZII*-harboring bacteria has been reported to be affected mainly by soil organic carbon content [81], and to be modified by soil pH, soil moisture, soil total N and Ca concentration [99].

1.1.4 Coupling Between Different Groups of Nitrifiers and Denitrifiers

Overall, organisms performing one step of nitrification or denitrification are expected to depend to some degree on the organisms performing the previous step(s). However, niche differentiation exists between different groups of nitrifiers or denitrifiers performing a same N process as indicated above. Consequently, some degree of coupling has been observed between different groups of (de)nitrifiers sharing similar ecological requirements. For instance, analyzing the responses of many N-cycling microbial groups in soil along fertilization gradients, Ma et al. (2016) reported a strong coupling between the abundances of soil *Nitrobacter* and AOB, and a coupling between the abundances of AOA, *narG*-nitrate reducers and *nirK*-nitrite reducers [44]. Similarly, Assemien et al. (2019) found that soil NO_3^- was the main driver of the abundances of both *nirS*- and *nosZI*-harboring bacteria, whereas soil organic carbon was an important driver for *nirK*-harboring bacteria and also (though to a lesser extent) for *nosZII*-harboring bacteria [81].

This suggests that some microbial groups largely share similar environmental determinants (e.g. AOB and *Nitrobacter*) whereas a high niche differentiation exists between other groups, such as *Nitrobacter* and *Nitrospira* [46]. However, this view oversimplifies the complexity of N-related microbial communities of course, as functional diversity exists within one group. A first example in this context is that soil *Nitrobacter* include two contrasted groups of bacteria (chemolithotrophs and mixotrophs) with quite different niches regarding N, organic C and O_2 availabilities [26]. Another example is that Xie et al. (2014) detailed how different groups of *nirK*-bacteria or different groups of *nirS*-bacteria have contrasted responses along a grazing gradient in relation to different soil environmental drivers [101]. This should be kept in mind when interpreting results of changes in the abundances of a particular N-cycling group targeted by a specific functional gene.

1.1.5 Global Change: Multiple Factors at Stake

From the industrial revolution period to the past century, due to the increase of the Earth's population, the increased exploitation of the earth's resources, and the rapid development of science and technology, humans are currently affecting and changing the global ecosystem at an unprecedented speed and scale [102]. Along with the surge in population and the ever-increasing demand for resource use, global problems caused by natural and anthropogenic impacts of global ecosystems have emerged. Global processes and the processes of change under their interactions are defined as global change. In essence, global change refers to the remarkable change in the human-environment relationship that has occurred over the last few centuries [103]. This has even led to the idea that we now live in a new era which can be called ‘the Anthropocene’ [104].

Global change not only includes climate change, but also changes in population, economy, energy use, land use, biodiversity, nutrient cycling, the linkages and interactions between these different changes [103]. Among them, the impact of global environmental changes caused by human beings on the diversity and functioning of terrestrial ecosystems has received increasing attention [105-107]. Global environmental changes characterized in particular by elevated atmospheric CO_2

concentrations, warming, changes in precipitation patterns, and increased nitrogen deposition [108, 109] have been exacerbated by anthropogenic disturbances like fertilization.

1.1.5.1 Rising Atmospheric CO_2

Increased human activity has led to an increase in greenhouse gas emissions. For example, industrial activities and combustion of fossil fuels emit large amounts of greenhouse gases such as CO_2, methane (CH_4) and nitrous oxide (N_2O). From 1750 to 2011, the global atmospheric CO_2 concentration has increased from ~280 ppm to ~391 ppm (Fig. 14) [110]. The average value today this year is 408 ppm (https://www.co2.earth/daily-co2). This rise strongly results from the increment of the global consumption of fossil fuels [111]. Atmospheric CO_2 concentration is expected to exceed 700 ppm by the end of this century [112].

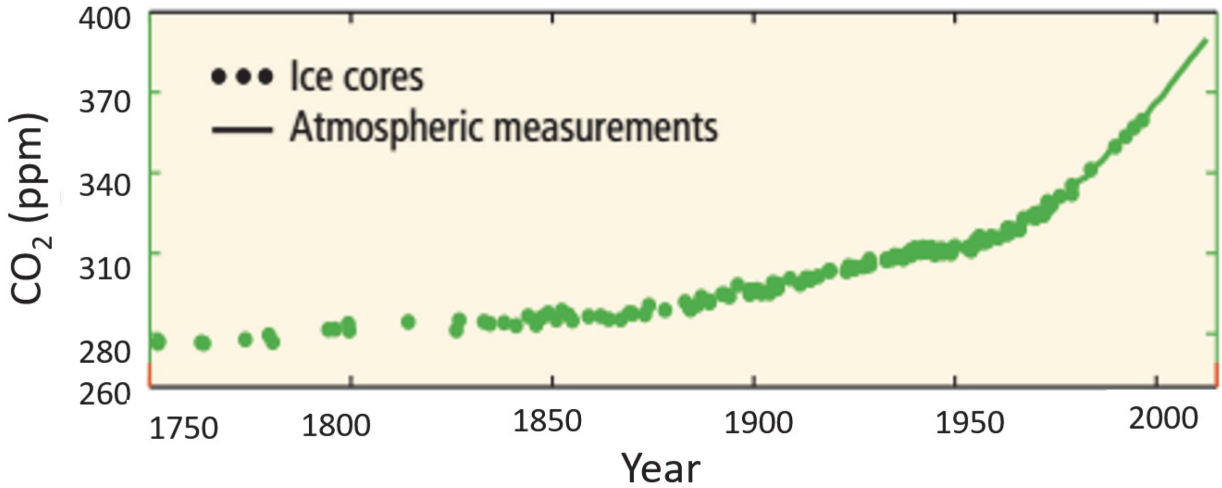

Fig. 14 Atmospheric carbon dioxide (CO_2) concentration from 1750 [110]

1.1.5.2 Warming

As reported by the WMO (2005), the global average surface temperature has increased by 0.6-0.7°C since pre-industrial periods (1850-1900) [113]. However, the temperature increased sharply at +0.18°C per decade since 1976. The 10 warmest years for the earth’ s surface temperature occurred since 1990 [114] and the period from 1983 to 2012 was very likely the warmest period of the last 800 years in the Northern Hemisphere. It is increasingly accepted that the acceleration of the warming during the last four decades mainly resulted from the increasing atmospheric concentrations of greenhouse gases due to human activities [115-117].

At this rate, global temperatures would increase by 1.5°C around 2040 (Fig. 15) [118]. Warming would be varied spatially or seasonally [119]. In many locations, particularly in regions of Northern Hemisphere mid-latitude winter (December-February), the temperature of the regional warming would be more than double as compared with global average [118]. Previous predicted that there would be substantially warmer and wetter winters and marginally warmer summers in the next 100 years California [120, 121].

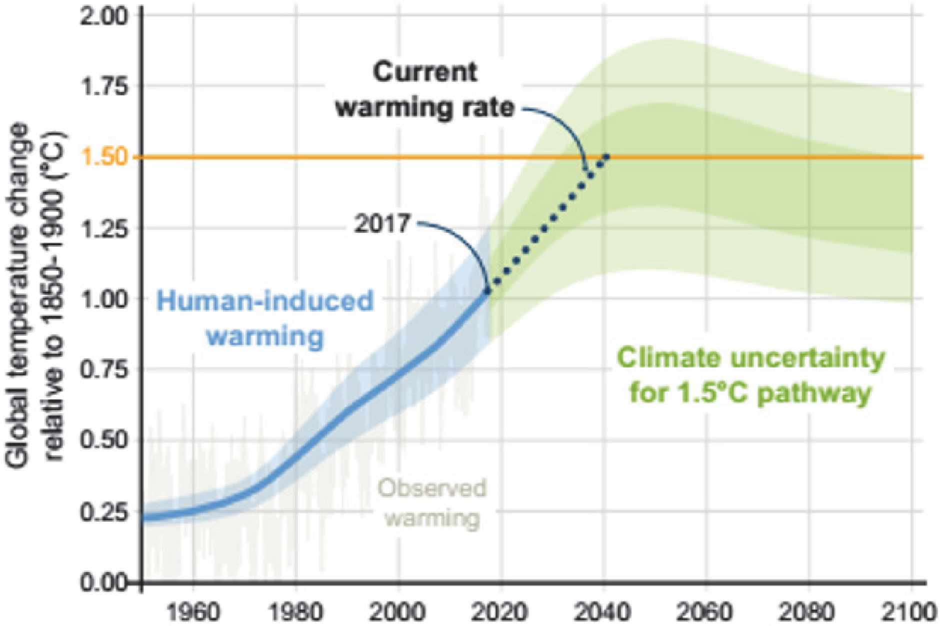

Fig. 15 Human-induced warming as compared to pre-industrial period, which has already reached about 1°C above pre-industrial levels in 2017. Prediction based on the present warming rate, the temperature would be above 1.5°C around 2010 [118]

1.1.5.3 Changed precipitation regime

Precipitation is distributed unevenly over the globe. The average distribution of precipitation is controlled mainly by the availability of moisture and the atmospheric circulation patterns, being influenced by temperature [122]. Therefore, human-induced increase of temperature and the consequent +7% increase of the water holding capacity of the atmosphere per centigrade warming [123, 124] will very likely alter the atmospheric water vapor concentrations and overall evaporation [125, 126]. The latter will change the formation of cloud [124, 127], contributing to the alteration of precipitation patterns, including the amount, frequency, type and intensity of precipitation [128]. The annual mean precipitation is likely to increase in the regions of the equatorial Pacific and high latitudes and in many mid-latitude wet regions (Fig. 16). In contrast, mean precipitation will likely decrease in most mid-latitude and subtropical dry regions. Based on observations for the 1900-2005 period, Trenberth et al. (2017) reported that precipitation has already increased in northern and central Asia, northern Europe, and eastern North and South America [124], but decreased in the Mediterranean, Sahel, southern Asia, and southern Africa. In addition to the precipitation amount, the intensity, frequency and distribution of are also considered as crucial [129]. Trenberth et al. (2007)

documented that atmospheric water vapor concentrations increased by 5% over the 20th century, leading to increased intensity of precipitation events [124]. Changes in extreme events have also been observed since about 1950. An increase in the number of extreme precipitation events, such as extreme droughts, heavy rains, and floods [129, 130] [131], can have a significant impact on ecosystems.

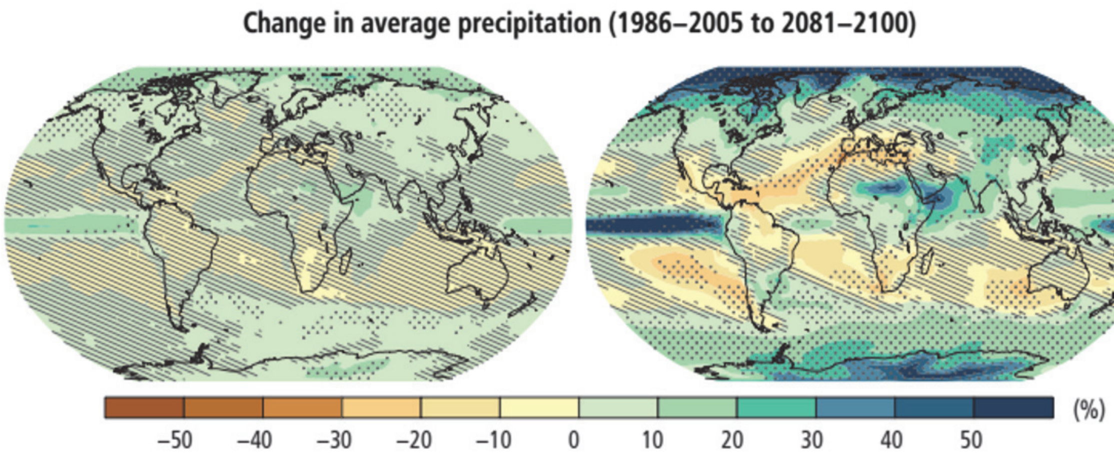

Fig. 16 Average precipitation changes expected for the 2081 – 2100 period as compared to 1986 – 2005 under the RCP2.6 (left) and RCP8.5 (right) scenarios. Predictions are based on multi-model mean projections [132]

1.1.5.4 Increased N deposition

In addition to climate change factors, nitrogen (N) deposition is another important factor of global environmental change. Since the industrial revolution of the 19th century, the phenomena of fossil fuels burning, the application of chemically synthesized N fertilizers and development of livestock production surged all over the world. These processes have released large amounts of NH_x (including NH_3, RNH_2) and NO_x -which are active N compounds- into the atmosphere [133-135]. It is estimated that human induced inputs of activated N has enhanced from 15Tg N yr^{-1} to 165Tg N yr^{-1} from 1860 to 2002, from 55% to 60% of the activated N being released as NH_x and NO_x [136]. This is leading to a steep increase in atmospheric N deposition (Fig. 17) [108]. For instance, Goulding et al. (1998) found that 70-80% of the N emitted into the atmosphere will be returned to terrestrial and aquatic ecosystems in the form of wet or dry N deposition [137], and *ca*. 38% of the deposited N settles in terrestrial ecosystems [136]. N deposition mainly occurs as wet N deposition.

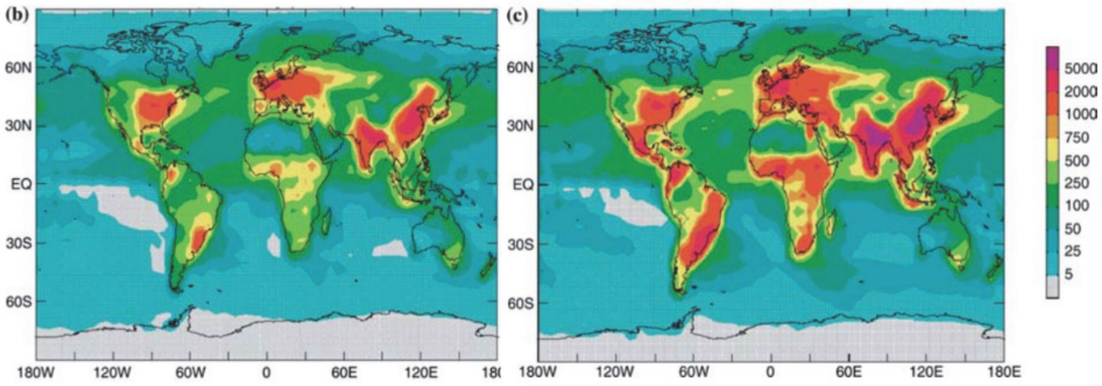

Fig. 17 Total inorganic nitrogen deposition (mg N m^{-2} yr^{-1}) spatial patterns in early 1990s (a), and 2050 (b) [108]

1.1.5.5 Modified Fire Regime

Fires have been a common natural disturbance from the late Devonian Period [138]. Fire is recognized as a global phenomenon [139, 140] that affects more land area than any other natural disturbance [141]. It has been estimated that more than 30% of the land surface is subjected to a significant frequency of fires [142]. Owing to ongoing global changes, it is expected that fire regimes will immediately respond to climate change [143] in terms of frequency/recurrence, size, seasonality, and fire intensity.

Flannigan et al. (2009) estimated that historical (1960 – 2000) global annual area burned by wildland fires range from 273 to 567 Mha, with an average of 383 Mha [144]. Approximately 80 – 86% of the global area burned occurs in grassland and savannas, primarily in Africa and Australia, but also in South Asia and South America, while the remainder occurs in forested regions [145, 146] (Fig. 18).

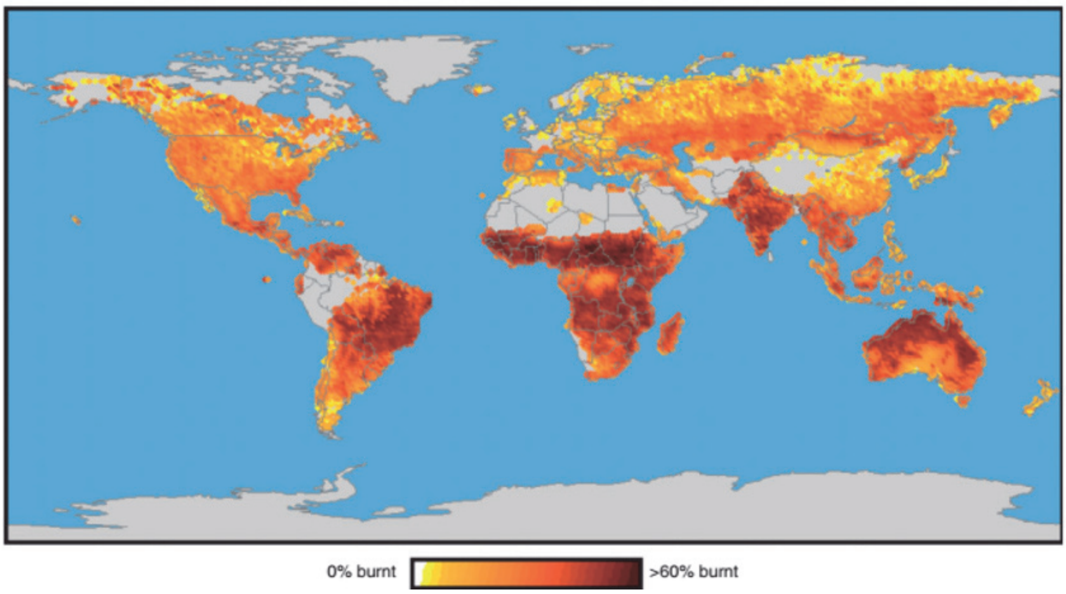

Fig. 18 Global map of average annual area burned (percentage of cell burned) for the 1960-2000 period [144]

Historically, ground fires are thought to have occurred every 15 to 20 years in red fir forests, 4 to 20 years in mixed conifer forests, 6 to 8 years in coastal redwood forests, and 5 to 10 years in grasslands and woodlands [147-148]. Numerous important factors determine fire frequencies in grasslands, including climate, vegetation type, and extreme weather events such as drought or heavy winds [149]. Reconstructed fire history shows that fire frequency is highly dependent on climate [150]. Consequently, models suggest a likely increase in both ignition rates and fire spread with the warmer temperatures, lower humidity, higher winds, and drier fuels that are expected under future climate scenarios [151, 152]. If precipitation remains high during some years, fire risk during dry years is likely to increase as a result of increased plant productivity -and thus increased fuel amount for fire- in previous years. Torn et al. (1998) projected that climate change will greatly increase the number of wildfires [151]. Moritz et al. (2012) examined the pixel-wise agreement among the 16 global climate models (GCMs) in increase and decrease of different climate variables to assess general trajectories from recent historical to the 2010 – 2039 and 2070–2099 periods [153].

More specifically, in the Mediterranean region, over the last 60 years, human activity has already influenced the pattern, frequency and intensity of fires [154-156]. Fires are frequent [157-158] and are likely to be altered further by human beings in the coming century by climate change, especially in the western US [152, 159-160].

As common processes in many ecosystems, especially in grassland ecosystems, fires are one of the major drivers of the structure and functioning of ecosystems due to their pervasive effects on carbon and nutrient cycling [161] and microclimates [162].

1.1.6 Grassland Ecosystems: Global Coverage and Classification of Grassland

Grassland, which is the largest of the four major natural biomes [163], is a terrestrial ecosystem dependent on disturbance [164]. Grassland is dominated by herbaceous vegetation and with significant seasonality of productivity [165], being maintained by factors such as drought, grazing, fire, and/or freezing temperatures [166]. However, grasslands not only include non-woody systems but also shrublands, woodlands, savannas, and tundra [167]. The area of the grassland (excluding Antarctica and Greenland) was estimated ranging from ~31 to 43 % of land area or from ~42 to 56 million km^2 [168, 169]. FAO (2019) estimates that 70% of the world agricultural area and 26% of the world land area are covered by grasslands [170]. Grasslands not only act as the production base for animal husbandry, providing environmental protection, but also stores organic carbon in terrestrial ecosystems, providing net primary productivity. However, due to the rapid increase in population accompanying with the effects of climate change, the grassland ecosystem process and function has seriously been affected, particularly soil nitrogen cycling process.

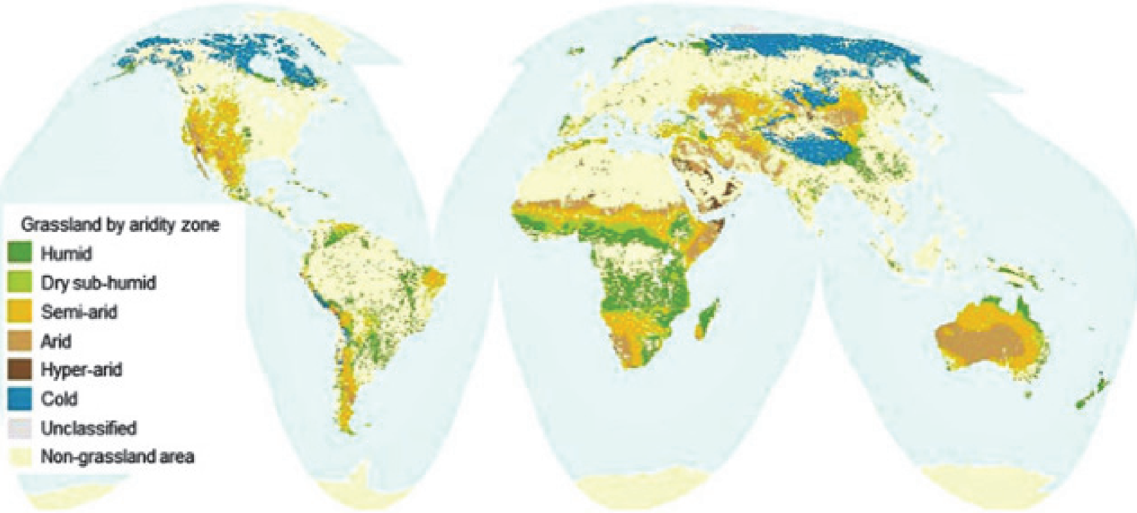

Fig. 19 Grassland and climate zones. From Unger and Jongen et al. (2014) [171]

1.2 Research Status about Impacts of Global Change Factors on Soil N Cycling

Extensive studies have reported that global change factors, such as elevated atmospheric carbon dioxide (CO_2), warming, changes in precipitation regime and elevated N deposition, but also fire disturbances like fire, can alter the function of soil including N cycling. In particular, global change effects have been reported on microbial nitrification and denitrification processes in soil [172, 173]. As detailed below, each of the global change factors listed above can alter the activity, the abundance and the structure of soil nitrifying and denitrifying microbial communities. Indeed, each of these factors can modify soil environmental variables to which nitrification and denitrification processes are sensitive. For instance, nitrification process is favored by well-aerated soils with high concentration of NH_4^+, and moderate temperatures but is unfavored by acidic conditions [174, 175]. Denitrification is favored by anaerobic conditions with high NO_3 availability, moderate temperatures and high labile C availability, and also could be affected by changes in soil pH [78, 175-178]. Hereafter, I briefly review how each of these global environmental change factors can influence the activity and abundances of soil nitrifiers and denitrifiers.

1.2.1 CO_2 Effect on Nitrifiers and Denitrifiers

Elevated concentration of atmospheric CO_2 often stimulates plant photosynthesis [179], increases plant carbon inputs to the soils [180-182] and enhances plant water use efficiency [183] and nutrient use efficiency [184]. This alters soil C and N availabilities, soil moisture and oxygen level, which might in turn affect nitrifiers and denitrifiers.

Table 11 A review of the effects of elevated CO_2 on plant-soil ecosystems variables, including: environmental variables, plant growths, activity and abundance of (de)nitrifiers, and soil N_2O emissions. ↑, positive effect; ↓, negative effect;

ns, no significant effect; -, no study available; gray arrows indicate responses that have been observed only in a limited number of studies as compared to more generally observed

CO_2	Effects	References
Direct effects	**á** plant photosynthesis and growth	[179, 184]
	á plant water use efficiency WUE	[183]
	á nutrient use efficiency	[184]
Indirect effects	**á** soil water content (through WUE)	[185-191]
	á organic C inputs to soil (through higher root growth)	[181, 182]
DEA	**á â ns**	**á**: [192-198] **â**: [173, 195, 199-203] **ns**: [173, 204-210]
NEA	**á â ns**	**á**: [46, 194, 202] **â**: [173, 201, 203, 204, 211, 212] **ns**: [199, 205, 207, 210, 213]
AOB	â **ns**	â: [214, 215] **ns**: [46, 203, 216-221]
AOA	á **ns**	á: [217, 220] **ns:** [46, 203, 215-217, 220]
Nitrobacter	**Ns**	**ns**: [26, 46]
Nitrospira	**Ns**	**ns**: [26, 46]
nirK	**á â ns**	**á**: [222] **â**: [215] **ns**: [217, 221, 223]
nirS	**á â ns**	**á**: [222, 224] **â**: [217] **ns**: [220, 223]
nosZI	**Ns**	**ns**: [221-223, 225]
nosZII	**-**	
N_2O	**á** â **ns**	**á**: [173, 226-230] â: [231] **ns**: [206, 232, 233]

Previous results about elevated CO_2 effects on soil N cycling are rather inconsistent [173, 234] (Table 11). Denitrifying enzyme activity (DEA) was often found to be significantly increased by elevated CO_2. For instance, Ineson et al. (1998) found higher denitrification rates in *Lolium perenne* mesocosms growing under elevated CO_2 and high N inputs [235]. Similarly, Carnol et al. (2002) reported a positive response of DEA to elevated CO_2 in a 4 years mesocosm experiment [196]. Higher denitrification under elevated CO_2 can be attributed to the stimulation of denitrifiers by higher fine root amount, higher exudation of labile C compounds and anerobic conditions resulting from increased soil water content (due to higher WUE) and increased soil respiration that also reduce oxygen level in the soil atmosphere [236, 237]. However, there are also several studies that have reported that DEA was decreased at elevated CO_2 or remained unchanged at elevated CO_2 (Table 11). Such responses of DEA to elevated CO_2 have been attributed to an insignificant effect of elevated

CO_2 on soil water content, an insufficient response of soil O_2 availability induced by elevated CO_2, and/or been attributed to soil NO_3^- limitation [199].
Similarly, nitrifying enzyme activity (NEA) also showed contrasted responses to elevated CO_2 and was found either to be increased, decreased or not changed by elevated CO_2 (see Table 11).

The abundances of soil nitrifiers and denitrifiers have also been reported to be modified by elevated CO_2 (Table 11). Generally, little effect of elevated CO_2 on the abundance of soil nitrifiers and denitrifiers has been found. For instance, the abundance of soil AOA and AOB showed no response to elevated CO_2 in a long-term FACE grassland experiment conducted in Minnesota [221]. Other studies have reported no response of the abundance of soil AOA and soil AOB to elevated CO_2, either in the field [203, 216, 217, 221, 223] and in greenhouse experiments [46]. However, positive response of the abundance of soil AOA to elevated CO_2 was reported by Regan et al. (2001) [217] in a dry block which was furthest from water table, and negative response of the abundance of AOB was reported by Horz et al. (2004) [214] in a Californian annual grassland. Nitrite-oxidizers have mostly been reported to be insensitive to elevated CO_2. Simonin et al. (2015) reported no effect of elevated CO_2 on the abundance of *Nitrobacter* bacteria and *Nitrospira* bacteria in grassland mesocosms [46]. This lack of response was consistent with results from a study conducted in a Californian annual grassland showing no significant response of the abundance of *Nitrobacter* and *Nitrospira* to elevated CO_2 [26].

The responses of the abundances of soil denitrifiers, such as *nirK*-, *nirS*- and *nosZ*-harboring bacteria to elevated CO_2 are also variable (as shown in Table 11). For instance, Regan et al. (2001) [217] observed that the responses of the abundance of soil *nirK*-, *nirS*- and *nosZI*-harboring bacteria to elevated CO_2 depended on soil depth and the soil water content, with negative effect on *nirK*-harboring bacteria at MED block (intermediate to water table) whereas only slight negative trend at DRY (furthest to water table) and WET (nearest to water table) block between soil depth of 15-22.5 cm. Similarly, the *nirS*-harboring bacteria was decreased by elevated CO_2 in the MED and WET blocks in soil depth of 15 -30 cm. Tu et al. (2017) [223] and He et al. (2010) [221] found no significant effect of elevated CO_2 on these denitrifying functional genes. However, in the SoyFACE experimental site established on a farmland, He et al. (2014) [222]observed significant positive responses of *nirK*- and *nirS*- harboring bacteria to elevated CO_2. Such differences could mainly result from the soil nutrient state, with often lower N in grassland ecosystems, and higher N in the soybean agro-ecosystem. In addition, a negative response of soil denitrifier abundances to elevated CO_2 was reported in some field experiments. Finally, elevated CO_2 often has positive effect on soil N_2O emission as reviewed by Barnard et al. (2005), although some studies found no significant effect (such as Carter et al. 2011; Hungate et al. 1997) [186, 233] or even negative effect under low N conditions [231].

1.2.2 Warming Effect on Nitrifiers and Denitrifiers

Table 12 A review of the effects of warming on plant-soil ecosystems variables, including: environmental variables, plant growths, activity and abundance of (de)nitrifiers, and soil N_2O emissions. ↑, positive effect; ↓, negative effect; ns, no significant effect; -, no study available; gray arrows indicate responses that have been observed only in a limited number of studies as compared to more generally observed

Warming	Effects	References
Direct effects	á soil temperature	[238]
Indirect effects	â soil moisture	[239-241]
	Altered plant species composition	[242]
	á photosynthesis	[243]
	á productivity	[243, 244]
	á soil labile C	[244]
DEA	á ns	á: [173, 174, 205, 233, 244-248] ns: [173, 247, 249]
NEA	á â ns	á: [205, 207, 250-252] â: [174, 194, 218] ns: [173, 249, 252, 253]
AOB	á â ns	á: [205, 241] â: [250, 254] [254]ns: [218, 252, 255-259]
AOA	á â ns	á: [252, 260] â: [241, 254, 255, 257, 261] ns: [241, 255, 256, 259, 260, 262, 263]
Nitrobacter	á	á: [264, 265]
Nitrospira	â	â: [241, 250]
nirK	á â ns	á: [261, 266, 267] â: [241, 268] ns: [205, 259]
nirS	á â ns	á: [266] â: [241] ns: [259, 261, 267, 269]
nosZI	á â ns	á: [205] â: [241, 267] ns: [259, 266]
nosZII	-	
N_2O	á â ns	á: [230, 245, 270-272] â: [273] ns: [233]

Warming increases soil temperature and often reduces soil moisture [239] (Table 12). It can also have indirect effects on soil microbial activities through changes in the length of the growing season [274] and in plant species composition [242]. Previous studies have shown that warming can significantly increase DEA in the field [205, 233, 246], in mesocosms [244] and in incubation experiments [245, 247, 248]. The positive response of soil DEA to warming was mainly attributed to increased soil labile C [244]. The response of soil microbial activities to warming could however be offset by a decrease of soil moisture [240, 275]. Several studies reported no effect of warming on DEA, as in a long-term field experiment in tundra plant communities in Northern Sweden [249] and an incubation experiment with 6 different soil texture types [247]. A meta-analysis actually reported no overall effect of warming on DEA (see the review by Barnard and Leadley 2005) [173] (Table 12).Positive responses of NEA to warming have also been reported by several studies (see Table 12). In contrast, negative responses of soil NEA have also been reported, likely due to increased soil respiration which lead to decreased soil O_2 concentration[174]. Besides, several studies reported no effect of warming on NEA, e.g. in a long-term field experiment in Northern Sweden [249] and in an artificial grassland ecosystem established in Belgium [253]. Overall, a meta-analysis reported no effect of warming on NEA (see the review by Barnard and Leadley 2005) [173] (Table 12).

Numerous studies have reported the responses of the abundances of soil nitrifying and denitrifying functional genes to warming (see Table 12). For instance, Long et al. (2012) [263] showed that warming did not significantly change the abundance of AOA, being consistent with the result from an elevated temperature experiment in a pristine forest soil [254]. In contrast, a study showed a decrease of AOA diversity under soil warming in the rhizosphere of a boreal forest tree [276]. According to some authors, the ammonia oxidizers, especially the AOB, may be in an inferior position to compete with plants as temperature increases [277, 278].

Previous studies have shown that the response of the abundances of soil denitrifying functional genes to warming was variable. For example. Zhang et al. (2013) [259] reported no significant effect of warming on *nirK, nirS and nosZI*-harboring bacteria in a grassland experiment. Similarly, Jung et al. (2011) [261]found no response of the abundance of *nirS* to warming in an Antarctic soil, and Cantarel et al. (2012) [205] observed no effect of warming on the abundance of *nirK* in an upland grassland ecosystem. However, Jung et al. (2011) [261] found a positive response of the abundance of *nirK* to warming. Warming generally increases soil N_2O emission, likely via increasing soil microbial activity [245] or soil labile carbon content [244].

1.2.3 Precipitation Effect on Nitrifiers and Denitrifiers

Previous studies on the effects of changes in precipitation regime on soil N cycling have mostly focused on changes in the amount of precipitation [205, 279] [280], whereas fewer studies have considered changes in the duration of the rainy season [232, 233] and in the distribution of precipitation [281]. Changes in precipitation regime obviously lead to changes in soil water content and soil oxygen content which can affect soil nitrifiers and denitrifiers. In particular, increased precipitation increases soil water content and soil heterotrophic respiration thus decreasing soil O_2 availability [240], which would benefit the growth of denitrifiers but would be unfavorable for nitrifiers [78, 174] [247] (Table 13).

Table 13 A review of the effects of elevated precipitation amount on plant-soil ecosystems variables, including: environmental variables, plant growths, activity and abundance of (de)nitrifiers, and soil N_2O emissions. ↑, positive effect; ↓, negative effect; ns, no significant effect; -, no study available; gray arrows indicate responses that have been observed only in a limited number of studies as compared to more generally observed

Elevated Precipitation Amount	**Effects**	**References**
Direct effects	**á** soil water content	
Indirect effects	**á** plant productivity	[243, 282, 283]
DEA	**á** â **ns**	**á**: [194, 213, 247, 284] **â**: [285] **ns**: [285]
NEA	**â ns**	**â**: [285, 286] **ns:** [194]
AOB	**á ns**	**á**: [250, 287, 288] **ns**: [255, 259, 289]
AOA	**á ns**	**á**: [287, 288] **ns**: [255, 259, 289]
Nitrobacter	**â**	**â:** [26]
Nitrospira	**Ns**	**ns :** [26]
nirK	**á ns**	**á**: [287] **ns**: [259, 289-291]
nirS	á **ns**	á: [287] **ns**: [259, 289-292]
nosZI	á **ns**	á: [287, 292] **ns**: [259, 289-291]
nosZII	-	
N_2O	**á** â	**á**: [205, 232, 290, 292-295] â: [290]

An increased amount of precipitation has been reported to increase DEA [194, 213, 284] (Table 13) mainly through decreasing soil O_2 availability. Increased precipitation has been found to either reduce NEA (Chen et al. 2013, Niboyet et al. 2011 for potential nitrite-oxidation) [213, 286] due to decreased soil O_2 availability, or to increase NEA likely due to higher N mineralization rates and associated substrates for nitrifiers (Niboyet et al. 2011 for potential ammonia-oxidation) [213]. High frequency precipitation (more dry-rewetting cycles) increased net N mineralization [296, 297].

The effects of altered precipitation on the abundances of soil nitrifiers and denitrifiers are rather inconsistent (Table 13). Increased precipitation has no significant effect ;[198, 255, 259] or positive effect [287, 288] on the abundance of soil ammonia-oxidizers (Table 13). Consistently, studies investigating decreased precipitation effects have reported either no significant effect on soil ammonia-oxidizers or decreases in the abundance of soil AOB [198, 262] and AOA [262, 290, 298]. Very few studies have investigated the response of soil nitrite-oxidizers to altered precipitation. Le Roux (2016) [26] reported negative response of *Nitrobacter* bacteria to elevated precipitation but no response of soil *Nitrospira* to this treatment for a Californian annual grassland, the abundance of *Nitrobacter* being strongly and positively correlated to soil potential nitrite oxidation [26].

Numerous studies have documented the effect of altered precipitation on the abundance of soil denitrifiers, except on soil *nosZII*-harboring bacteria (Table 13; Table 14). An increase in precipitation amount has been reported to either increase or have no significant effect on the abundances of soil *nirK*-, *nirS*-, and *nosZI*-harboring bacteria (Table 13). Concurrently, soil N_2O emissions have been often been reported to increase with elevated precipitation, while soil N_2O emissions are mostly reduced by decreased precipitation amount (Table 14).

Table 14 A review of the effects of decreased precipitation on plant-soil ecosystems variables, including: environmental variables, plant growths, activity and abundance of (de)nitrifiers, and soil N_2O emissions. ↑, positive effect; ↓, negative effect; ns, no significant effect; -, no study available; gray arrows indicate responses that have been observed only in a limited number of studies as compared to more generally observed

Decreased Precipitation Amount	**Effects**	**References**
Direct effects	**â** soil water content	[246]
Indirect effects	**â** plant productivity	[243]
DEA	á **ns**	**á**: [299] **ns**:[205]
NEA	**á ns**	**á**:[299] **ns**:[205, 300]
AOB	**á ns**	**á**: [198, 262] **ns**: [254, 255, 298, 301]
AOA	**á ns**	**á**: [262, 290, 298] **ns**: [198, 254, 255, 290, 298, 300-302]
Nitrobacter	-	
Nitrospira	-	
nirK	**á â ns**	**á**: [290, 303] **â**: [262, 302] **ns**: [290, 300, 301])[198]
nirS	**á â ns**	**á**: [290, 303] **â**: [262, 301, 304] **ns**: [198, 290, 301, 304]
nosZI	**á â ns**	**á**: [290, 303] **â**: [262] **ns**: [198, 246, 290, 300, 301]
nosZII	**â**	**â**: [262]
N_2O	**á â ns**	**á**: [225, 254] **â**: [280, 290, 305]; **ns**: [233]

1.2.4 Nitrogen Deposition Effect on Nitrifiers and Denitrifiers

Nitrogen deposition generally increases net plant primary productivity (NPP) [306] and increases the soil nitrogen content and more particularly mineral N availability [307]. But N deposition also alters the rate of nitrogen turnover in soil [308, 309], reduces soil pH, alters litter quality, consequently changing soil conditions for soil (de)nitrifiers [307].

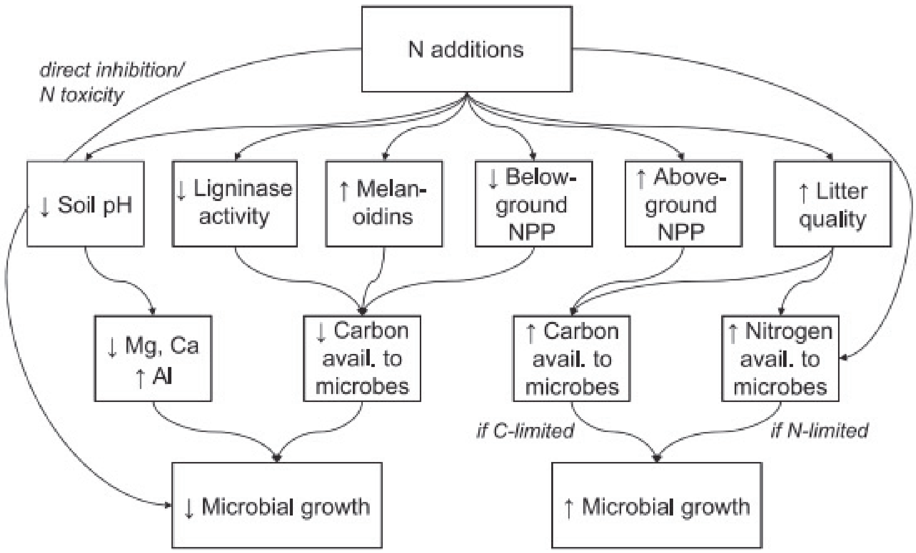

Fig. 110 Diagram of potential mechanisms for N addition effects on soil microbes, most of these mechanisms being relevant to understand the possible responses of nitrifiers and denitrifiers to N deposition [307]

Numerous studies have reported positive effects of N addition on soil N cycling processes including nitrification and denitrification (see the review by Barnard et al. 2005) [173] (Table 15). N addition increases ammonium and nitrate availability for nitrifiers and denitrifiers, resulting in increased NEA and DEA [20, 173, 208, 310]. However, some studies found that the DEA showed no significant response to N addition [311], which could be attributed to the nutrient status of the soil [312]. Indeed, in soil systems with lower fertility, nitrogen addition increased DEA, but in high soil nutrient condition, negative effect of N addition on DEA was observed [312].

Table 15 A review of the effects of Nitrogen addition on plant-soil ecosystems variables, including: environmental variables, plant growths, activity and abundance of (de)nitrifiers, and soil N_2O emissions. ↑, positive effect; ↓, negative effect; ns, no significant effect; -, no study available; gray arrows indicate responses that have been observed only in a limited number of studies as compared to more generally observed

N addition	Effects	References
Direct effect	á N availability	[307]
Indirect effect	á ANPP	[306]
	á litter quality	[307]
	â BNPP	[307]
	â soil pH	[307]
DEA	á ns	á: [173, 208, 300, 310, 313-316] ns: [233, 300]
NEA	á â ns	á: [4, 46, 198, 208, 300, 311, 312, 317-319] â: [312] ns: [319]
AOB	á â ns	á: [4, 46, 47, 49, 263, 298, 300, 317, 319-330] â: [22, 263, 328, 331] ns: [214, 263, 288, 289, 300, 332-335]
AOA	á â ns	á: [47, 300, 312, 323, 325, 328, 329, 336] â: [289, 320, 321, 325, 330, 337] ns: [44-46, 49, 198, 222, 259, 263, 288, 300, 317, 319, 327, 329, 332, 333, 338-340]
Nitrobacter	á	[26, 44, 46]
Nitrospira	á â ns	á: [331] â: [46, 341] ns: [26, 44] [342]
nirK	á â ns	á: [92, 261, 321, 323, 325, 343, 344] â: [259, 325, 332, 338, 345] ns: [44, 45, 198, 289, 303, 312, 329, 346-348]
nirS	á â ns	á: [92, 261, 312, 323, 325, 343, 349, 350] â: [259, 321, 325, 332, 345] ns: [44, 45, 198, 289, 303, 312, 346]
nosZI	á â ns	á: [312, 321, 325, 332, 343, 349] â: [259, 325] ns: [44, 45, 198, 261, 289, 303, 312, 346]
nosZII	â ns	â: [351] ns: [173, 351-353]
N_2O	á ns	á: [173, 186, 240, 293, 294, 321, 349, 354-359] ns: [290]

N addition has been found to elicit inconsistent responses in the abundances of soil nitrifiers and denitrifiers (Table 15). Responses of AOB vary with fertilizer type, ecosystem, study type, and soil pH, and are generally favored in soils fertilized with inorganic N sources[47]. Carey et al. (2016) suggested that elevated N supply enhances soil nitrification potential by increasing AOB populations [47]. Current studies indeed frequently reported a positive response of AOB which dominate N-rich environments [20] [360], to N addition (see Table 15). The abundance of AOA, which are favored by lower NH_3 and lower pH conditions, often showed no significant response to N addition (see Table 15). However, some studies showed that AOA abundance was decreased by N addition [307] [337] or increased by N addition [25, 300, 307, 361]. Actually, N deposition can alter the ratio of AOA to AOB. For example, N addition could induce the decrement of soil pH [25], which favored AOA rather than AOB [52].

Nitrobacter are generally increased by N addition [26, 44, 46] because of their higher half-saturation constants and higher growth rates as compared to *Nitrospira* which favor *Nitrobacter* under conditions of high nitrite NO_2^- availability. In contrast, *Nitrospira* which have low half-saturation constants for NO_2^- and lower growth rates [67-70] showed inconsistent response to N addition (Table 15).

The responses of the abundance of soil denitrifiers to N addition are also rather inconsistent (Table 15), depending on soil nutrient availability, labile C content, soil depth, fertilizer type, ecosystem, and soil pH.

1.2.5 Fire Effect on Nitrifiers and Denitrifiers

Burning can have a direct effect on soil (de)nitrifiers, as it induces direct temperature elevation to soils and may lead to heat-induced mortality of soil microbes [362] [363]. However, Delmas et al. (1995) reported that low intensity fires (e.g. flames with 2-5 m/min and 1-2 m height) induced heat wave not penetrating very deep (soil temperature changed only within 3 cm) [364]. Consequently, microbes are probably not impacted directly by burning due to heating. Besides direct effect, burning most often indirectly affects the abundance of soil microbes via altering soil physical and chemical properties such as soil water content, soil temperature, and the availability of soil nutrients and labile carbon exudated by roots (Fig. 111) [365]. There are many reports including numerous reviews on the effects of fire on soil properties [366] [143]. This includes effects on soil temperature, soil water content, but also availability of soil organic matter and nutrient (Table 16). In addition, burning also can affect plant growth [367, 368].

Burning has been reported to increase [369, 370], decrease [369] or to have no effect [4] on NEA, sometimes depending on soil sampling timing after burning [369-371]. Similarly, the responses of DEA to burning are also inconsistent (Table 16).

In general, burning would increase the abundance of AOB due to increased soil pH [372-373] or increased NH_3 concentration [20, 23, 25, 50,373]. However, a grassland study has reported no burning effect on the abundance of AOB [4]. The abundance of AOA has been shown to respond negatively to burning, which may result from increased soil pH [372], because AOA tended to be favored by lower pH as compared to AOB. The effects of fire on soil nitrite-oxidizers have been rarely investigated. In a study conducted in Tallgrass Prairie, no response of the relative abundance of *Nitrospira* bacteria has been found [342].

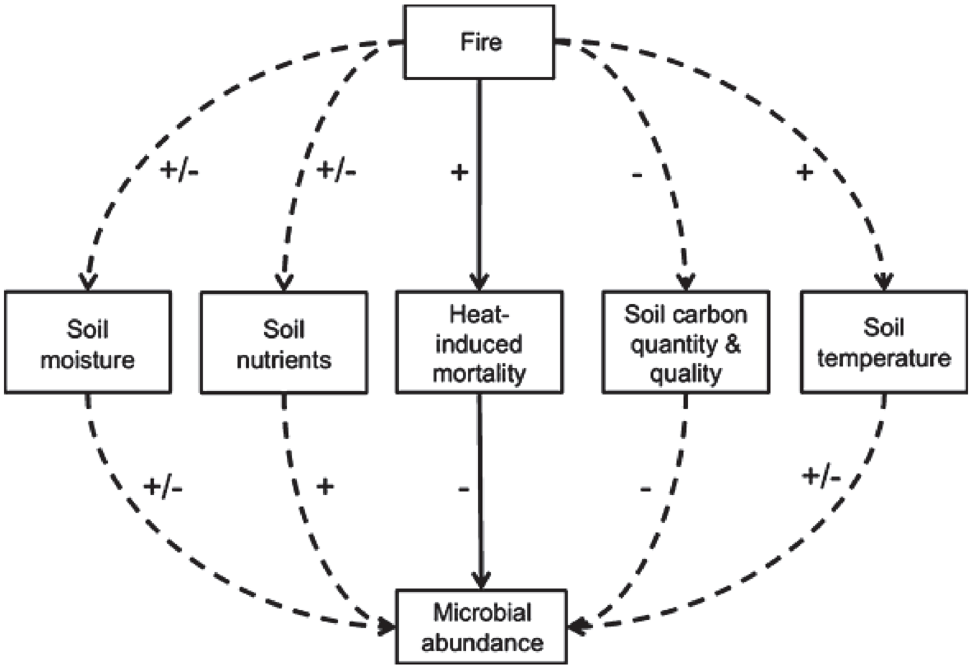

Fig. 111 Diagram of potential direct (solid arrows) and indirect (dashed arrows) mechanisms through which fire disturbance positive (+) or negative (-) effects on microbial abundance [365]

Fire increased the abundance of soil *nirK*- and *nirS*-harboring bacteria likely due to an increase of soil pH [370]. No study to date has been reported the response of *nosZII*-harboring bacteria to fire disturbance. The response of soil N_2O emission to burning was often expected to be positive because of burning induced changes of soil environmental variables such as soil water content and availability of soil NO_3^- and C, or altered soil (de)nitrifier activity and abundance (Table 16). For instance, Niboyet et al. (2011) reported that fire increased soil N_2O emission after 2 and 3 years of fire disturbance which was attributed to the changes of soil water content and labile carbon [284]. Other studies also found positive response of fire disturbance but was mainly ascribed to the fire induced increase of soil NH_4^+ concentration [374-375].

Table 16 A review of the effects of burning on plant-soil ecosystems variables, including: environmental variables, plant growths, activity and abundance of (de)nitrifiers, and soil N_2O emissions. ↑, positive effect; ↓, negative effect; ns, no significant effect; -, no study available; gray arrows indicate responses that have been observed only in a limited number of studies as compared to more generally observed

Burning	Effects	References
Direct effects	**á** soil temperature	[362, 363, 368, 376, 377]
	á soil C quantity and quality	[365]
Indirect effects	**á** BNPP	[367, 368]
	á soil water content	[368, 378]
	â soil water content	[135]
	â soil NH_4^+	[370]
	á soil pH	[371, 372, 379-382]
	â soil pH	[300]
	ns pH	[383]
DEA	**á â** ns	**á**: [314, 370, 384] **â**: [369, 370] **ns**: [369]
NEA	**á** â **ns**	**á**: [369, 370, 385] **â**: [369] **ns**: [4]
Microbial enzyme activity	**á â**	**á**: [386] **â**: [387, 388]
AOB	**á ns**	**á**: [370, 372, 373, 385] **ns**: [4]
AOA	**á â**	**á**: [370] **â**: [372]
Nitrobacter	**-**	
Nitrospira	**ns**	**ns**: [342]
nirK	**á ns**	**á**: [370] **ns**: [135, 370, 389]
nirS	**á ns**	**á**: [370] **ns**: [135, 370, 389]
nosZI	**á ns**	**á**: [389] **ns**: [135, 370, 389]
nosZII	**-**	
N_2O	**á â ns**	**á**: [284] **â**: [135] **ns**: [284]

1.2.6 Research Knowledge Gaps Regarding Effects of Global Change Factors on nitrifiers and denitrifiers

An increasing number of studies have assessed the interactive effects of multiple global change factors on the activity and/or abundance of soil nitrifiers and denitrifiers [4, 26, 46, 194, 198, 205, 207, 208, 213, 214, 232, 258-260, 284, 300, 313, 390, 391]. Those studies have highlighted that the response of soil nitrification and denitrification to multiple global change factors was not necessarily additive, the effects of multiple global change factors amplifying or dampening each other. These studies have mostly focused on two global change factors, while fewer studies have reported the effects of more than three co-occurring global change factors on soil nitrifiers and denitrifiers [4, 26, 198, 205, 208, 213, 214, 259, 284]. In these studies, changed precipitation amount and frequency have not been unraveled and the coupling between changed precipitation regime and changed regime of wet N deposition has not been considered. Furthermore, the interactive effects of global change factors and disturbances such as fire on nitrification and denitrification have been poorly investigated to date (apart from few exceptions [4, 232, 284]). Only one study to date has investigated the response of the abundance of the soil nitrifiers to the combined effect of fire and multiple global environmental changes: Docherty et al. (2012) have studied the response of AOB following a wildfire event part of a global change experiment [4], who only reported one date after fire (9 months after wild-fire) and with only 2 blocks for wild-fire treatment. In addition, no study has investigated the response of other soil nitrifying groups (AOA, *Nitrobacter*, *Nitrospira*) and of soil denitrifying groups to the interactive effects of multiple global change factors and fire disturbance. In addition, the temporal responses of the soil nitrifiers and denitrifiers according to time after fire and under different global change scenarios have never been evaluated so far.

1.3 Objectives of My Ph.D. Work

The main objective of my Ph.D. work was to study the -possibly interactive- effects of several global change factors (namely increased N deposition; altered rainfall regime; warming; and/or CO_2 elevation) and fire disturbance on microbial communities involved in soil N dynamics in grasslands, focusing on different groups of nitrifiers and denitrifiers. To address this, I have worked on two experiments:

(i) a mesocosm experiment assessing the combined effects of increased N deposition and changes in both the amount and the frequency of rainfall in semi-arid Monsoon grassland (Songnen grassland, see chapters 2 and 3). In this experiment, a first novelty was to decouple the modification of precipitation amount and the modification of precipitation frequency; and a second novelty was to mimic chronic wet N deposition by coupling N input to precipitation events; and

(ii) an *in situ* experiment assessing the combined effects of increased N deposition, enhanced precipitation, warming, elevated CO_2 and fire in a Mediterranean grassland (Californian grassland, see chapters 3 and 4). The novelty was to assess the effect of these 5 global change factors and all their combinations (32 treatments, replicated) on 8 microbial groups involved in soil N cycling (4 groups of nitrifiers and 4 groups of denitrifiers).

1.4 Experimental Designs Used in My Ph.D.

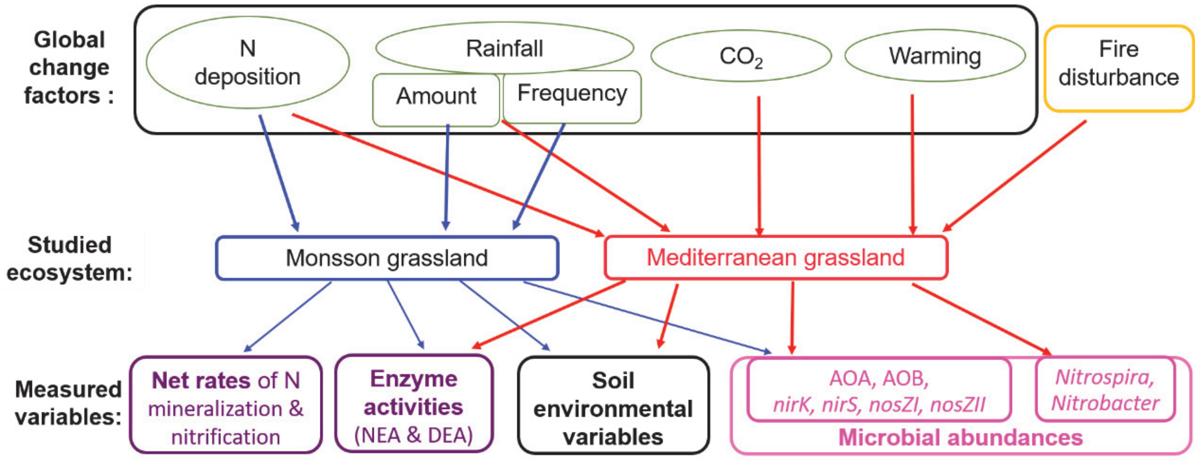

Fig. 112 Diagram of Experimental Designs Used in My Ph.D. (In the experiment conducted in Monsoon grassland, global change factors include N deposition, - rainfall amount and +/- rainfall frequency; In the experiment conducted in Mediterranean grassland, global change factors include N deposition, + rainfall amount plus extending 3-week rainfall period, N deposition, elevated CO_2, warming and fire disturbance)

1.4.1 Experimental Design in Semi-Arid Monsoon Grassland

An experiment based on 36 mesocosms was set up in a greenhouse at the experimental facility of Northeast Normal University, China (43° 51´N, 125°19´E, 236 m a.s.l.). Each mesocosm was a 38-liter cylinder (34 cm in diameter and 42 cm in depth) containing *ca.* 50 kg of grassland soil collected in the Grassland Ecosystem Field Station of the Northeast Normal University (123°44´E, 44º40´N, 167 m a.s.l.), part of the Songnen Grassland area of China. Soil was air dried and sieved (2 cm) to roughly mix the soil and remove stones and large plant fragments before filling the mesocosms with soil. The mesocosms were buried in the ground to buffer temperature changes of the belowground part during the day, with the top edge of each mesocosm being placed 3 cm above ground level to avoid surface runoff. Each mesocosm was planted with 30 individuals of *Leymus chinensis* (Trin.) Tzvel, the dominant grass species in Songnen Grassland [392]. Plants were pre-cultivated in soil until 3-4 leaves developed. Planting was made in April 2013, one year prior to treatment inception, under ambient conditions and without any fertilization. Mesocosms were considered deep enough because in the field most of the root system of *L. chinesis* is found in the 5-10 cm soil layer. The mesocosms were mown once a year in late August to simulate farmers' practice in Songnen grassland. Mesocosms were also regularly weeded to remove unplanted species.

The experiment consists of a three factors factorial design with randomized block. The three factors were N deposition (with two levels: 0 and +10 g N-NH_4NO_3 m^{-2} yr^{-1}); precipitation amount (with two levels: ambient precipitation amount, 370 mm and -30% ambient amount); and precipitation frequency (with three levels: ambient frequency, +50% and -50% ambient precipitation frequency; the total precipitation amount remaining the same). Three replicates were used for each of 12 treatments, and the 36 mesocosms were arranged according to three blocks, each including the 12 treatments. Based on a 60-years record of daily precipitations (from 1953 to 2013; data from the Climatic Station of Changling County, Jilin Province), the semi-arid climate in this area is characterized by a low annual precipitation amount (*ca.* 430 mm) with a short rainy season typically from May to September (average total precipitation amount during this period is 370 mm) and a long dry period from October to April (average total precipitation amount for this period is 50 mm). From October to April, all mesocosms were exposed to natural precipitation events without any treatments applied. During the growing season from May to September, both in 2014 and in 2015, the treatments (precipitation amount × precipitation frequency × N deposition) were simulated. Light transmissive material was used as shelter to avoid interference of natural N deposition and precipitation. N deposition in the North China Plain is estimated to be as high as 8.3 g-N m^{-2} yr^{-1} and could become higher in the future [393]. Further, Wang et al. (2008) observed that wet deposition in the China Northern Loess area accounts for over 90% of the total atmospheric N deposition [394], and Xing & Zhu (2002) reported a N-NH_4^+: N-NO_3^- ratio of 3:1 to 4:1 for wet inorganic N deposition [395]. Here we used 10 g-N m^{-2} per rainy season for simulating wet N deposition, applied as ammonium-nitrate with a N-NH_4^+-:N-NO_3^- ratio of 3.4:1. To simulate chronic N deposition as experienced in the field, the N input was coupled with simulated precipitation by dissolving N in water used for each prescribed precipitation, so that the prescribed total precipitation amount during the growing season (370 mm) corresponded to a total wet deposition of 10 g-N m^{-2}. The current precipitation frequency for the study area was defined according to a recent 10 years record of precipitation frequency during growing season (from 2000 to 2010; data from the Climatic Station of Changling County, Jilin Province). Precipitation frequency changes as compared to the 10-year average were chosen according to the minimum and maximum observed frequencies, leading to treatments with -50% and +50% days of precipitation events. Whatever the precipitation frequency treatments, all the mesocosms received the same total amount of precipitation per month (and same total precipitation over the growing season). The largest simulated precipitation amount in a single day was 24 mm. Detail experimental design see

Table 17. Mesocosms were slowly hand watered (with a watering sprayer) to minimize surface runoff. The experimental treatments lasted for two years. In the first year, the experimental treatments were carried out, without sampling; plants and soil were sampled in the second experimental year.

Table 17 Monthly values of precipitation amount (ambient precipitation amount, A_{ctrl}; reduced precipitation amount, $A_{-30\%}$) and frequency (reduced by 50%, $F_{-50\%}$; normal frequency, F_{ctrl}; increased by 50%, $F_{+50\%}$)F) used for the experiment in the semi-arid Monsoon grassland

	Precipitation amount (mm)		$F_{-50\%}$	F_{ctrl}	$F_{+50\%}$
	A_{ctrl}	$A_{-30\%}$	(days)	(days)	(days)
May	23	7	5	9	14
June	66	20	6	11	17
July	144	43	6	12	18
August	97	29	5	10	15
September	41	12	4	7	11

1.4.2 Experimental Design in Mediterranean Grassland

A field experiment was set up at the Jasper Ridge Global Change Experiment (JRGCE) in the Jasper Ridge Biological Preserve (Fig. 113) located in the eastern foothills of the Santa Cruz Mountains in northern California 37°24'N, 122°14'. The JRGCE began in the fall of 1998, and provides global change scenarios with four global-change factors, each at two levels (ambient and elevated): atmospheric CO_2 concentration (ambient and +275 ppm) manipulated in a free-air CO_2 enrichment (FACE) system, temperature (ambient and soil surface warming of 0.8-1℃ for 12 years and then 1.5 – 2℃) by using overhead infrared heat lamps, precipitation (ambient and +50% above ambient and 3 weeks elongation of the growing season) manipulated first with drip irrigation (1998 – 2000) and then with sprayed sprinklers irrigation system (2001 – 2004) system, and N supply (ambient and +7 g $Ca(NO_3)_2$-N m^{-2} yr^{-1}) twice per year. The first simulated N deposition event was 2 g-N m^{-2} addition in solution early in the growing season (November) to mimic the accumulated dry N deposition flushed into the system with the first rains. The second simulated N deposition event was manipulated later in the season (January – February), with 5g N m^{-2} addition as slow-release pellets (Nutricote 12 – 0 – 0, Agrivert, Riverside, California, United States) [396, 397].

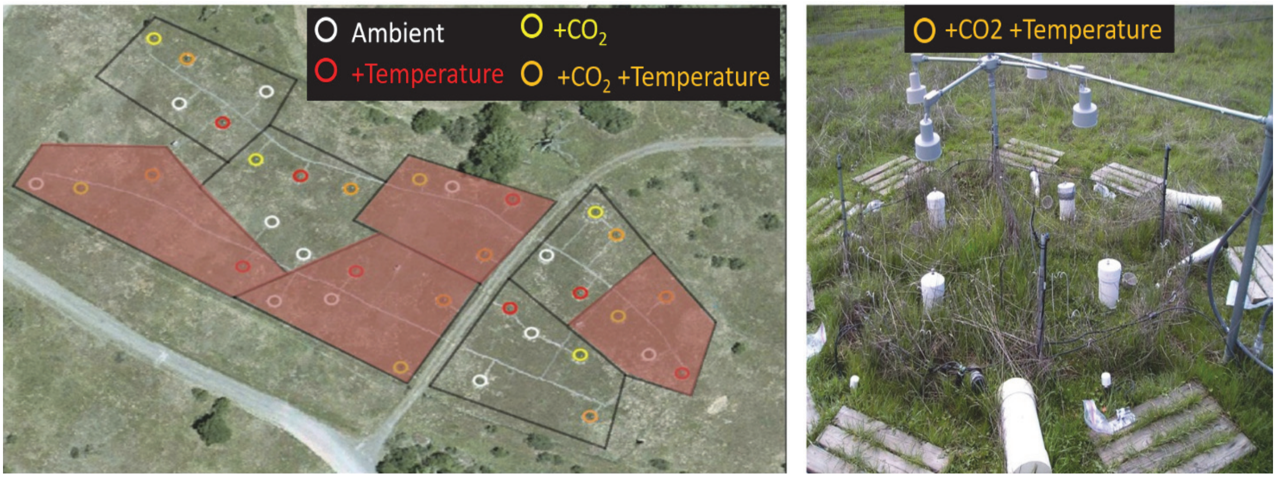

Fig. 113 Map of Jasper Ridge Global Change Experiment area at the Jasper Ridge Biological Preserve. Circles

represent 2 - m - diameter global change treatment plots (n = 36 total). Orange circles indicate plots that receive both $+CO_2$ and heat treatments. Red circles indicate plots that receive heat treatment, but ambient CO_2. Yellow circles indicate plots that receive $+CO_2$ treatment, but ambient temperature. White circles indicate plots at ambient CO_2 and temperature. In blocks with two white circles, one is an infrastructure - free control. For each circular plot, one quadrant receives +N treatment, one quadrant receives elevated precipitation treatment, one quadrant receives both +N and elevated precipitation treatment, and one quadrant is held at ambient precipitation and N. Red shading indicates the areas which were subjected to a low - intensity experimental burn in July 2011 [109, 368]

The elevated levels of the global change factors studied were selected according to scenarios predicted to occur at the end of the 21st century for central California [398]. Thirty-two circular plots (2-m diameter) were established at the JRGCE. Each plot was divided into four subplots (each with *ca.* 0.78 m^2) using fiberglass barriers (0.5 in depth) to separate soil between neighboring quadrants and soil between plot and surrounding grassland. The experiment was organized as a randomized block split-plot design, with CO_2 and temperature treatments manipulated at the plot level, and precipitation and N treatments manipulated at the subplot level. There were eight replicates (i.e. blocks) for all 16 treatments. In addition, an accidental and low-intensity fire occured in two of the eight blocks of the experiment in July 2003 [284, 377, 388]. Then, a controlled burned was conducted by the California Department of Forestry and Fire Protection in July 2011 in half of the blocks, resulting in a new experiment with all combinations between five factors, 32 treatments, with four replicates for each. This prescribed fire was conducted based on a plan to achieve typical grassland fire intensities while also maintaining high confidence that the burns remained within predetermined areas. Air temperatures at the time of burning were 23-25°C and relative humidity was 45-50%. The prescribed fire consumed 100% of the aboveground vegetation, with maximum flame lengths of 0.6 to 1.3 m and rates of spread of 5.6 to 15 m/minute.

For the paper on global change scenarios effect on soil nitrifiers and denitrifiers (see section 1.2), the following keywords were used and search in Web of Science:
((AOB or AOA or "ammonia and oxidi" or nitrifier* or nitrifying or Nitrobacter or Nitrospira or "nitrite oxidi*") and ((fire or fires or burning) or "elevat* CO2" or "elevat* carbon dioxide" or "increased CO2" or "increased carbon dioxide" or "change* CO2" or "change* carbon dioxide" or "elevat* temperature" or "increased temperature" or warming or "nitrogen deposition" or "nitrogen addition " or "N deposition" or "N addition" or "elevated precipitation" or elevated rainfall*" or "decreased precipitation" or decreased rainfall*" or "altered precipitation" or "altered rainfall*")) and soil* for searching papers published on nitrifiers and *((nirK or nirS or nosZ or denitrifier* or denitrifying or "nitrite oxidi*" or " nitrous oxide*' or N2O) and ((fire or fires or burning) or "elevat* CO2" or "elevat* carbon*

dioxide" or "increased CO2” or “increased carbon dioxide" or “change CO2” or “change* carbon dioxide" or "elevat* temperature” or "increased temperature” or warming or "nitrogen deposition” or “nitrogen addition " or "N deposition” or “N addition" or “elevated precipitation” or elevated rainfall*” or “decreased precipitation” or decreased rainfall*” or “altered precipitation” or “altered rainfall*”)) and soil* for searching published papers for denitrifiers and soil N_2O emission.

2 Responses of Soil Nitrifiers to Chronic N Deposition and Changes in Precipitation Amount and Frequency in a Semi-Arid Monsoon Grassland

2.1 Introduction

Human-induced global changes have significantly altered most terrestrial ecosystem processes, especially nitrogen (N) cycling processes [194, 256]. Rising atmospheric N deposition along with alteration of precipitation regime are two important aspects of these global changes [134]. Some areas on the globe are particularly exposed to high N deposition loads, due to intensive agricultural and fossil fuel combustion [133, 134, 136]. In particular, N deposition in the North China Plain is estimated to be as high as 8.3 g-N m^{-2} yr^{-1} and could become higher in the future [393]. Regarding the nature of N deposition in this area, Wang et al. (2008) observed that wet deposition in the China Northern Loess area accounts for over 90% of the total atmospheric N deposition [394], and Xing & Zhu (2002) reported a NH_4^+-N: NO_3^--N ratio of 3:1 to 4:1 for wet inorganic N deposition [395]. Many studies have shown that enhanced N deposition deeply influences soil N cycling processes such as N mineralization, nitrification and denitrification [399].

Together with increased N deposition, global change is also characterized by changes in precipitation patterns. According to the report of IPCC (2013), changes in precipitation patterns will not be uniform. In many mid-latitude and subtropical dry regions, mean precipitation will likely decrease [132], and Liu et al. (2005) found that precipitation has already decreased over the 1960-2000 period in the North China Plain and north central China [400]. However, for terrestrial areas, climate models predict that besides changes in precipitation amount, reduced or increased precipitation frequency – with or without modifications in total precipitation amount – will occur [124, 129, 401, 402]. Changes in precipitation frequency can lead to significant changes in ecological processes [403, 404] [405] and are expected to have large impacts on N cycling particularly in arid and semi-arid ecosystems [297, 402]. Indeed, changes in precipitation frequency can alter the whole water balance in the soil-plant continuum, affecting the growth of root systems and changing root N uptake. For example, reduced rainfall frequency without altering total rainfall amount induces larger rainfall pulses, hence providing more water to deep roots, whereas increased rainfall frequency induces shallower root systems with higher fine root biomass of wheat *(Triticum aestivum* L. cv.

SST33) [406]. Previous studies have shown that rainfall frequency (or drying-rewetting frequency) influences soil N cycling in different terrestrial ecosystems [407-410]. Indeed, modified rainfall frequency induces changes in soil moisture regime with a direct effect on soil N transformation [411]. Meanwhile, it also induces changes in vegetation growth [412] and root exploration of the soil with cascading effects on soil microbial community as well as roots-microbes interactions [413]. However, only few studies have conducted experiments with changes in precipitation frequency decoupled from possible changes in total annual precipitation amount, and all of these studies have been conducted in mesic grasslands [171, 414].

Moreover, wet N deposition is tightly coupled to precipitation events [415, 416] so that altering precipitation frequency also modifies the temporal distribution of wet N deposits. But the response of N cycling to simulated N deposition is often studied based on a single or a few N addition event(s) during the year [193, 417-420]. The interactive effects of modified rainfall regime and of co-occurring wet N deposition on soil N cycling have never been investigated so far.

Leymus chinensis is a typical species of perennial grassy pastures widely distributed in the eastern part of the Eurasian grassland, including the outer Baikal region of Russia, the northern eastern part of the Mongolian People's Republic, as in China's Northeast Plain, North China Plain and the Loess Plateau. In the grassland area of Northeast China (so called Songnen grassland), *Leymus chinensis* is the dominant species. This semi-arid grassland is highly limited by both water and N [421, 422]. At the same time, it is located in areas prone to high N deposition loads [393] and where precipitation regime is expected to be modified over the next decades according to the precipitation history [400] so that important consequences of these global change factors can be expected.

In this chapter, a grassland mesocosm experiment was conducted to investigate the responses of soil N-cycling microbial processes (net N mineralization, Net-N-min; and nitrifying enzyme activity, NEA) and abundances of nitrifiers (Ammonia-Oxidizing-Archaea, AOA; and Ammonia-Oxidizing-Bacteria, AOB) along with soil environmental variables (soil water content and mineral N availabilities) to changes in precipitation amount (-30%), precipitation frequency (±50% in term of events per month during the growing season as compared to current precipitation regime according to history of the local climate projections) and co-occurring wet N deposition. We hypothesized that (1) soil Net-N-min and NEA would be increased by reduced precipitation amount because of more aerobic conditions induced by reduced precipitation; simultaneously, reduced precipitation amount may increase plant belowground growth, which would result in either increased soil organic matter for microbes or increased competition between plant and soil microbes; (2) increased rainfall frequency for a given total rainfall amount (i.e. more rainfall events with low intensity) would increase soil Net-N-min, NEA and nitrifying abundance, which could be due to more aerobic conditions with frequent dry-rewetting cycles. This positive effect could be increased by the co-occurring chronic N deposition, which could provide more N nutrient in this N limited grassland; (3) as compared to AOB abundance, AOA abundance would be less sensitive to treatments because of the strong adaptive property of AOA (Table 14; Table 15).

2.2 Study site

Soil was collected in the Grassland Ecosystem Field Station of the Northeast Normal University (123°44´E, 44º40´N, 167 m a.s.l.), part of the Songnen Grassland area of China. This area is characterized by a typical temperate semi-arid Monsoon climate. Mean annual precipitation ranges from 250 to 490 mm, with more than 70% falls from June to August. The annual mean air temperature is 6.4°C, ranging from minimum *ca.* -27°C in January to maximum *ca*. 30°C in July, respectively. The frost-free period is about 140 days (generally from early May to late September). Soil texture for the 0-10cm layer is 17.9% clay, 8.1% silt and 74.0% sand, corresponding to a sandy loam soil according to International Society of Soil Science Standard (ISSS). Soil pH, EC and bulk density are 7.85, 2.17 dS m^{-1} and 1.85 g cm^{-3}, respectively (0-10 cm layer). Soil total carbon, dissolved carbon and total nitrogen concentrations are 7.22 ± 0.05, 0.20 ± 0.01 and 0.74 ± 0.01 mg g^{-1} respectively. Soil ammonium and nitrate concentrations are 0.92 ± 0.08 and 0.07 ± 0.01 µg-N g^{-1}. The vegetation type in the study area is a meadow steppe, mainly composed of perennial grasses, and the dominant species is *Leymus Chinensis* [392]. *Leymus chinensis* is widely distributed in the arid and semi-arid grasslands of northern China, Russia, and eastern Mongolia. *Leymus chinensis* is very resistant to cold, drought and salt and alkali. Other species in the area include the grass species *Calamagrostis epigejos*, *Stipa baicalensis* and *Phragmites australis*; the forbs *Artemisia scoparia*, *Artemisia mongolica*, *Kalimeris integrifolia*, *Hemarthria sibirica* and *Carex diriuscula*; and the legumes *Apocynum venetum, Melissitus rutenica*, and *Lathyrus quinquenervius* [392].

2.3 Materials and Methods

2.3.1 Experimental Design

See section 1.4.1

2.3.2 Soil Sampling and Measurements

2.3.2.1 Soil temperature and soil water content

Soil temperature and volumetric water content (SWC) (%) were determined for the 0-10 cm layer with a TRIME Pico 64 field moisture TDR-sensor [423] every two weeks from early June to late September in 2015.

2.3.2.2 Soil environmental variables

On 11th August 2015, three 10 cm-deep soil cores were collected in each mesocosm, using an auger (3 cm in diameter). The remaining holes were filled with fine quartz sand to limit alteration of gaseous diffusion from soil. After sieving (2-mm) the sampled soil, fresh soil sub-samples were stored at -80°C before DNA extraction and quantitative PCR (qPCR) analyses. Other fresh soil sub-samples were stored at 4°C before determination of the concentrations of soil ammonium, NH_4^+ (µg -N g^{-1} dry soil), and total dissolved organic carbon, DOC (mg-C g^{-1} dry soil). Fresh soil sub-samples were also used to quantify net N mineralization rate, Net N-min (µg-N g^{-1} dry soil day^{-1}), and nitrifying enzyme activity, NEA (µg-N g^{-1} h^{-1}). Other soil sub-samples were air-dried before analysis of soil total carbon (TOC) and total nitrogen concentration (mg g^{-1} dry soil).

TOC was analyzed with an elemental analyzer (Vario TOC; Elementar, Hanau, Germany) after acidizing the air-dried soil samples with 1 M HCl. Total soil N concentration was analyzed with an elemental analyzer (Pyrocube, Elementar, Hanau, Germany) after grounding with a Wiley ball mill (MM400, Retsch, Hanau, Germany). NH_4^+ concentrations were determined by extraction with KCl. Briefly, 10 g (equivalent dry weight) fresh soil sub-samples were extracted with 50 ml KCl (2 M) solution, extracted in a shaker (180 rpm) for 1h, and the supernatants were filtered (0.45 µm) before analysis. Concentrations of inorganic N were then analyzed using a continuous flow analyzer (Alliance Flow Analyzer, Futura, Frépillon, France). To determine the soil DOC concentration, 10 g fresh soil were extracted using 50 ml K_2SO_4 (0.5 M) solution, and the extracts were analyzed with a Vario TOC element analyzer (Elementar, Hanau, Germany).

2.3.2.3 Soil net N mineralization and nitrifying enzyme activity

Soil net N mineralization (Net N-min) rate was measured during aerobic incubation according to Hart et al. (1994) [424]. 10 g (equivalent dry mass) fresh soil were placed in a 100 ml glass flask which was then covered with a thin perforated parafilm to allow gas exchange but minimize water loss. The soil was incubated for 28 days at 25°C in the dark. Ammonium and nitrate concentrations (µg-N g^{-1} dry soil) were determined before and after incubation as described above. Net N-min was calculated as the net accumulation rates of total mineral N (sum of NH_4^+ and NO_3^-) during the incubation[424]. NEA was determined according to the shaken-slurry method [424]. For each sample, 15 g soil were mixed with 100 ml of 1.5 mM ammonium-sulfate and incubated at 25°C. Then, 10 ml slurry samples were sampled after 2, 4, 17.5, 22 and 24 h and centrifuged. Supernatants were filtered (0.45 µm) and immediately analyzed as described above for NO_3^-. NEA was calculated from the linear increase of NO_3^- during the incubation.

2.3.2.4 Soil DNA extraction and quantification of the abundances of ammonia-oxidizing bacteria and archaea

Soil DNA was extracted from *ca.* 0.5 g of soil using PowerSoil DNA isolation kit (MO BIO laboratories, Carlsbad, CA, USA) according to the manufacturer's instructions. The concentration of the extracted genomic DNA was determined on a Nano Drop 2000 device (Fisher Scientific, Schwerte, Germany). The abundances of ammonia-oxidizing archaea (AOA) and ammonia-oxidizing bacteria (AOB) were quantified with a Lightcycler 480 (Roche Diagnostics, Meylan, France), targeting the *amoA* gene. The sets of primers used were CrenamoA23f/CrenamoA616r for AOA and AmoA-1F/AmoA-2R for AOB. Further details about the primers are provided in Table 21. Possible PCR inhibition by co-extracted compounds was evaluated by serial dilution and no inhibition was observed. Amplification efficiencies were 90~98% and the R^2 value of the standard curves were always higher than 0.99.

Table 21 Sets of primers used for amplification of the *amoA* gene of AOA and AOB by real-time PCR

Target genes	Primers	Sequences (5`-3`)
***amoA*-AOA** [425]	CrenamoA23F	ATGGTCTGGCTWAGACG
	CrenamoA616R	GCCATCCATCTGTATGTCCA
***amoA*-AOB** [426]	AmoA-1F	GGGGTTTCTACTGGTGGT
	AmoA-2R	CCCCTCKGSAAAGCCTTCTTC

2.3.3 Data Analysis and Calculation

Data were analyzed with the SPSS 22.0 software (SPSS Inc., Chicago, IL, USA). The effects of wet N deposition, precipitation amount, precipitation frequency and their interactions on soil abiotic and biotic variables were tested with three-way ANOVA, with block as a random factor and N deposition, precipitation amount and precipitation frequency as fixed factors. Data were log-transformed if needed to meet the assumptions of ANOVA (normality was tested with Shapiro-Wilk test, and equal variance was tested using Levene's test). When treatment effects were significant, mean comparisons were performed with *Tukey's* post hoc tests. Main effects of N deposition, reduced precipitation amount, reduced precipitation frequency and increased precipitation frequency as compared to control (no N deposition; mean history precipitation amount and mean history precipitation frequency) were tested using *T-test*. Differences were considered statistically significant for $P < 0.05$. The correlations between abundances and soil environmental variables were examined using Spearman's rank correlation coefficient.

In addition, path analysis was performed using Amos25® (Amos Development Corporation, Crawfordville, FL, USA) to explore the possible causal links between the abundance of nitrifiers, the nitrifying enzyme activity and soil environmental variables. The complete model with all possible causal relationships considered is presented in Fig. 21. A χ^2 test was used to evaluate the model, *i.e.* whether the covariance structures implied by the model adequately fitted the actual covariance structures of the data (a non-significant χ^2 test with $P > 0.05$ indicates an adequate fit by the model). Within a model, the coefficients of paths indicate by how many standard deviations the effect variable would change if the causal variable was changed by one standard deviation.

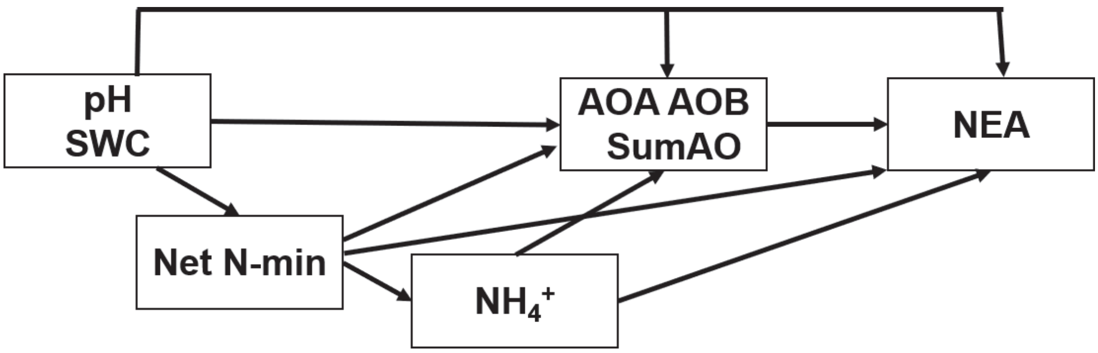

Fig. 21 Complete model used for structural equation modeling aimed at identifying the main drivers of changes in the abundance and enzyme activity of soil nitrifiers in response to N deposition and changed precipitation amount and frequency

Treatment effect size was quantified as response ratio (R) which was calculated as: R=Ln(T/C), where T is the value under the treatment considered, and C is the value under control conditions. For instance, a response ratio with positive value indicates positive treatment effect, and negative value indicate negative treatment effect.

2.4 Results

2.4.1 Soil Environmental Variables

Soil temperature was not influenced by any treatment during the experiment (Fig. 22). The main effects of nitrogen deposition (N), precipitation amount (A) and precipitation frequency (F) on SWC at sampling date were all significant (Table 22). N deposition (N_{10}) and reduced precipitation amount ($A_{-30\%}$) tended to decrease soil water content (SWC) (Table 22). The N_{10} effect on SWC was significant on June 3rd, August 1st and August 11th, and the $A_{-30\%}$ effect was significant on July 14th, August 1st and August 11th. Precipitation frequency (F) effect on soil water content was more variable, a significant difference being observed on 17th July. SWC increased under increased precipitation frequency ($F_{+50\%}$), which was significant at 3rd June 1st August and 11th August, whereas no significant effect of decreased precipitation frequency ($F_{-50\%}$) was observed. A significant N×A× F interaction effect was observed on SWC (Table 22).

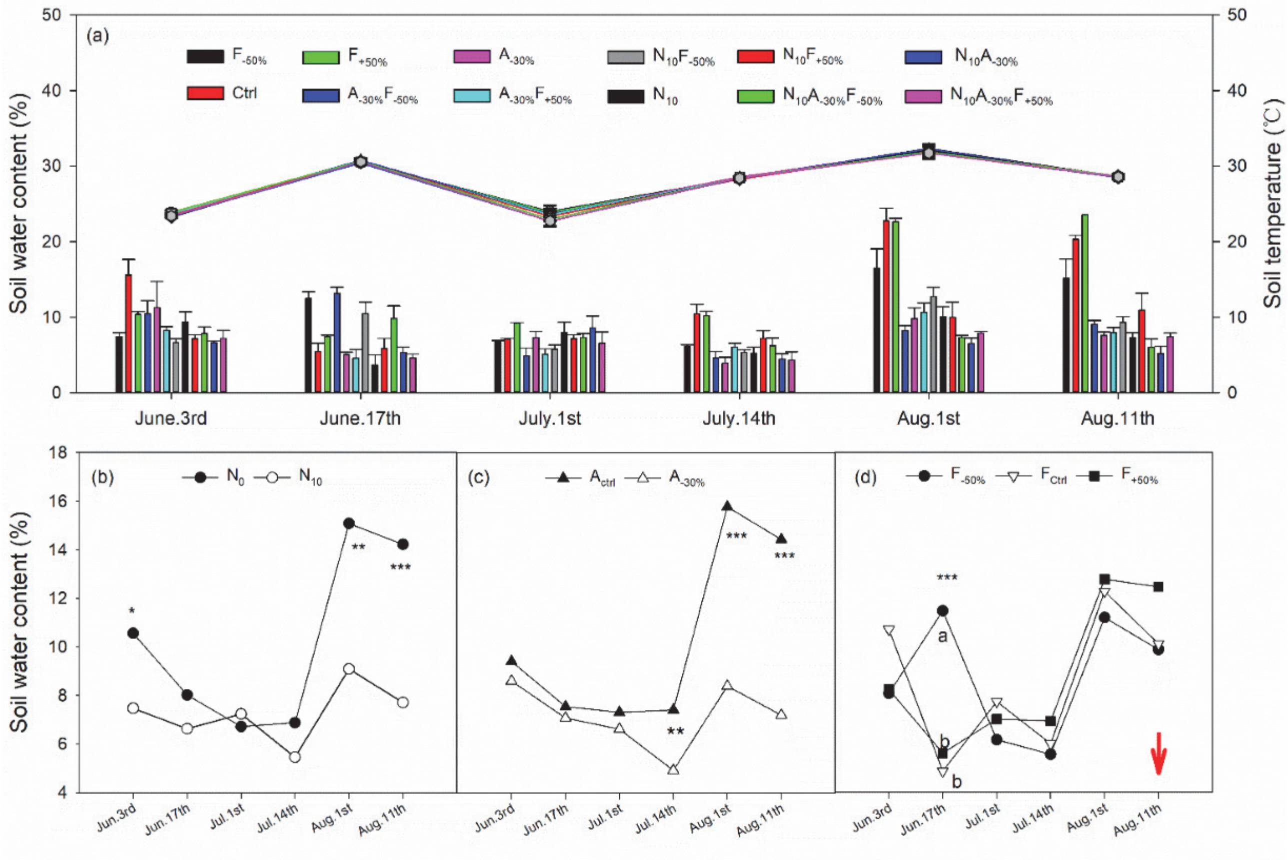

Fig. 22 (Top) Values of water content of the 0-10 cm soil layer (bars) and soil temperature at 10 cm depth (dots) at the 6 sampling dates during the growing season, for the 12 treatments. (Bottom) Soil water content according to (b) nitrogen deposition (N), (c) precipitation amount (A), and precipitation frequency (F) at the 6 sampling dates. N_0, no N deposition; N_{10}, N deposition with 10 g NH_4NO_3 m^{-2} yr^{-1}; A_{ctrl}, normal precipitation amount (370mm); $A_{-30\%}$, precipitation amount reduced by 30%; $F_{-50\%}$, rainfall frequency reduced by 50%; F_{ctrl}, normal precipitation frequency; $F_{+50\%}$, precipitation frequency increased by 50%. Arrow indicates soil sampling date. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$

The main effects of nitrogen deposition (N), precipitation amount (A) and precipitation frequency (F) on soil ammonium concentration (NH_4^+) were significant (Table 22). Overall, NH_4^+-N increased from 1.45μg-N g^{-1} for N_0 to 1.83μg-N g^{-1} for N_{10}, and $A_{-30\%}$ decreased NH_4^+-N from 1.74μg-N g^{-1} to 1.54μg-N g^{-1} for A_{ctrl}. Soil NH_4^+ was significantly influenced by the N × F interaction (Table 22).

Table 22 ANOVA results summarizing the effects of nitrogen (N) deposition, precipitation amount (A) and precipitation frequency (F), along with all their possible interactions, on soil water content (SWC) at sampling date, ammonium content (NH_4^+), net N mineralization rate (Net N-min), nitrifying enzyme activity (NEA), and the abundances of ammonia-oxidizing bacteria (AOB) and ammonia-oxidizing archaea (AOA). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$

Variables	Nitrogen deposition (N)	Precipitation amount (A)	Precipitation frequency (F)	N x F	A x F	N x A	N x A x F
SWC	***	***	**	ns	*	***	*
NH_4^+	***	*	***	*	ns	ns	ns
Net N-min	ns	**	***	*	ns	ns	**
NEA	***	ns	*	ns	ns	ns	ns
AOB	***	ns	*	ns	ns	ns	ns
AOA	ns	ns	ns	ns	***	**	ns

2.4.2 Net Nitrogen Mineralization and Nitrifying Enzyme Activity

The main effects of precipitation amount (A) and frequency (F) on soil net nitrogen mineralization (Net N-min) were significant, whereas no main effect of nitrogen deposition (N) was observed on Net N-min (Table 22).

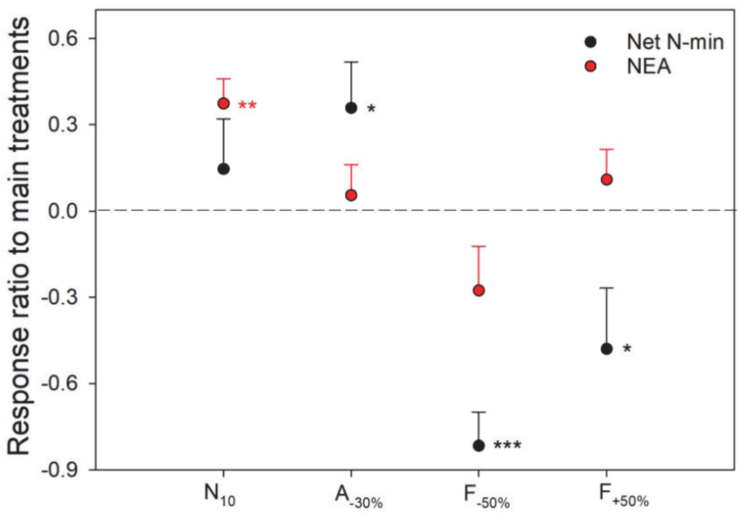

Fig. 23 Response ratio of soil net N mineralization (net N-min) and nitrifying enzyme activity (NEA) to the following main treatments: simulated N deposition (N_{10}), reduced precipitation amount ($A_{-30\%}$) and precipitation frequency changes (either reduced by 50%, $F_{-50\%}$; or increased by 50%, $F_{+50\%}$). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$

Net N-min significantly increased from 45.50μg-N g^{-1} day^{-1} for A_{ctrl} to 65.30μg-N g^{-1} day^{-1} for $A_{-30\%}$ (response ratio of 0.15), whereas both $F_{-50\%}$ and $F_{+50\%}$ significantly decreased Net N-min (Fig. 23) with a response ratios of -0.81 and -0.48, respectively. The main effects of nitrogen deposition (N) and precipitation frequency (F) on NEA were significant, whereas no main effect of precipitation amount (A) and interaction between treatments was observed on NEA (Table 22).

Overall, NEA significantly increased from 82.86μg-N g^{-1} h^{-1} for N_0 to 120.92μg-N g^{-1} h^{-1} for N_{10} (response ratio of 0.38).

2.4.3 Abundance of Ammonia Oxidizing Bacteria and Their Drivers

The abundance of ammonia oxidizing bacteria (AOB) was significantly affected by the main treatment of N and F without any significant interaction effect between treatments (Table 22). AOB abundance significantly increased from 3.11×10^5 copies g^{-1} soil for N_0 to 3.94×10^5 copies g^{-1} soil for N_{10} (response ratio of 0.13). $F_{-50\%}$ decreased AOB abundance to 3.09×10^5 copies g^{-1} soil as compared to 3.58×10^5 copies g^{-1} soil for F_{ctrl}, whereas no significant difference was found for $F_{+50\%}$ as compared to F_{ctrl} (Fig. 24).

Overall, the abundance of AOB was negatively related to soil water content (SWC), and positively – though weakly – related to soil net nitrogen mineralization (net N-min). The changes in SWC and net N-min explained 13% of the variance of the AOB abundance. Further, NEA was positively correlated with the abundance of AOB (Fig. 24-Right).

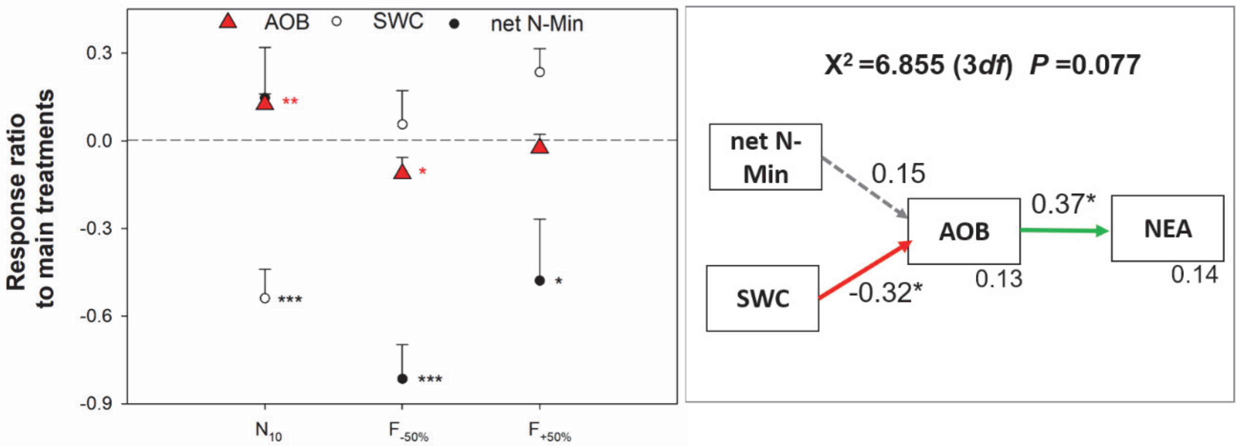

Fig. 24 (Left) Response ratio of the abundance of soil ammonia-oxidizing bacteria (AOB) to N deposition (N_{10}) and precipitation frequency changes (either reduced by 50%, $F_{-50\%}$; or increased by 50%, $F_{+50\%}$). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; the response ratios of soil water content (SWC) and net N mineralization (net N-Min) are also presented. (Right) Structural equation model result relating changes in the abundance of AOB and soil nitrifying enzyme activity (NEA) to net N-min and SWC. Values near the arrows are path coefficients. A green arrow indicates a positive correlation, a red arrow indicates a negative correlation, and a grey dash arrow indicates a marginally significant correlation. The percentage of variance explained by the model for each explained variable are indicated at the bottom-right of each corresponding box. *, $P < 0.05$

Thus, the main positive effect of N deposition on the abundance of AOB and NEA was (at least partly) mediated by decreased SWC and to a lesser extent increased N-min. In contrast, the main negative effect of altered precipitation frequency on the AOB abundance and NEA was partly mediated by decreased net N-min with a weaker role of SWC (Fig 2-4).

2.4.4 Abundance of Ammonia Oxidizing Archaea and Their Drivers

The abundance of AOA was significantly affected by the A × F and N × A interactions, without any main treatment effect (Table 22). Under ambient precipitation amount (A_{ctrl}), N_{10} significantly increased the abundance of AOA to 8.11×10^7 copies g^{-1} soil as compared to 6.47×10^7 copies g^{-1} soil for N_0 (response ratio of 0.20). However, the positive response to N deposition was not observed under reduced precipitation amount ($A_{-30\%}$) (Fig. 25-Top-Left). In addition, the abundance of AOA was positively affected by $A_{-30\%}$ under N_0, whereas this effect was dampened under N_{10} (Fig. 25-Top -Right).

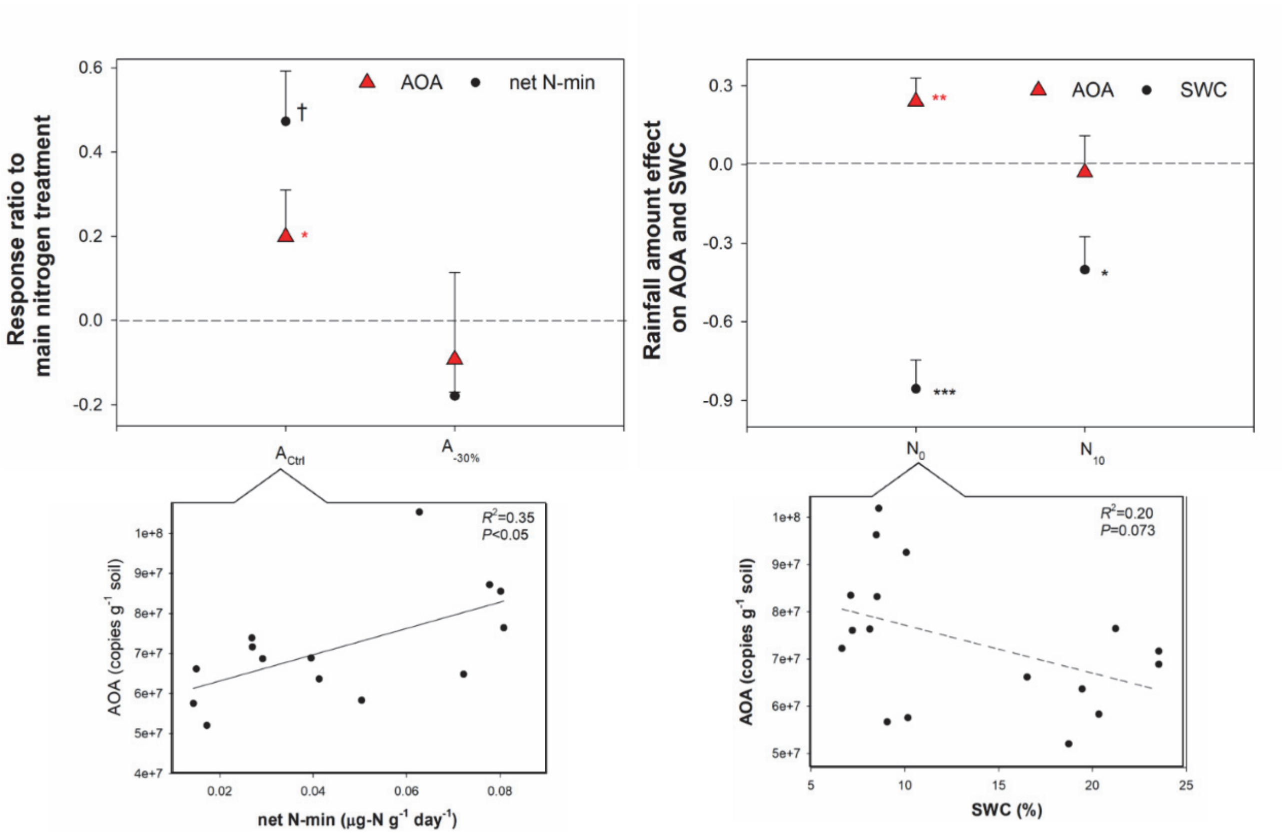

Fig. 25 (Top-Left) Response ratios of the abundance of soil ammonia-oxidizing archaea (AOA) and of net N mineralization (net N-Min) to N deposition according to precipitation amount (control, A_{Ctrl}, or reduced by 30%, $A_{-30\%}$); and (Bottom-Left) correlation between AOA and net N-Min under A_{Ctrl}. (Top-Right) Response ratios of AOA and soil water content (SWC) according to N deposition (no N deposition, N_0, or N deposition, N_{10}); and (Bottom-Right) correlation between AOA and SWC under N_0. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$

The abundance of AOA was significantly and positively related to net N-min under A_{ctrl} whatever the precipitation frequency and nitrogen deposition treatments (R^2=0.35, $P < 0.05$). AOA tended to be negatively related to SWC under N_0 across all precipitation amount and frequency treatments (R^2=0.20, P=0.073).

2.5 Discussion

Although global change biology has emerged as a research field over the last decades [427], with a strong focus on climate change effects on ecosystems, researches distinguishing the responses of soil N processes to changed precipitation frequency and changed total precipitation amount are still limited. Further, many studies have analyzed the effect of increased atmospheric N deposition on soil N cycling but generally by prescribing one or a few N addition event(s) per year rather than manipulating chronic N deposition [428, 429]. Mimicking the coupling between changed precipitation frequency and wet N deposition occurring through precipitation events has not been done so far in global change studies. The present study is thus novel because it evaluates the possibly interactive effects of changed precipitation amount, altered rainfall frequency and chronic N deposition co-occurring with simulated precipitation events on soil nitrifiers and N processes. AOA were dominant over AOB in the study soil, which is consistent with previous studies in many grasslands [45, 327, 430].

For NEA and AOB, the only significant effects observed were the main effects of N deposition and of precipitation frequency, whereas no interaction effect between global change factors was found. The path analysis and response ratio computation showed that N deposition (N_{10}) acted by decreasing soil water content (SWC), likely through increase plant production leading to increased transpiring foliar surfaces, as observed for rice (*Oryza sativa*) cultivars [431]. The decrease in SWC provided more aerobic conditions favorable for AOB, as AOB are favored by sufficiently high oxygen availability [40, 432]. The positive correlation between NEA and AOB abundance suggests a dominant role of AOB for nitrification in this semiarid grassland. This is consistent with previous reports in Tibetan alpine meadows [44], although AOA have been reported also have an important role in some grasslands [26]. Furthermore, N deposition tended to increase soil net nitrogen mineralization, which contributed the increase of the abundance of AOB. Indeed, AOB are known to be favored by higher N availability [433] and many previous studies have reported a positive effects of N inputs on AOB and NEA [46, 173], N_{10} increased soil NEA. However, no relationship was observed between the abundance of AOB and soil ammonium concentration. This may be due to quick uptake of ammonium by plants and/or quick immobilization by soil microorganisms. More generally, soil ammonium concentrations result from various N processes decreasing or increasing the ammonium pool, and snapshot concentration measurements thus reflect quasi-steady state concentrations rather than turnover. It is then possible that soil ammonium concentration is not a good proxy of actual ammonium availability.

The positive response of Net N-min to decreased precipitation amount found here is surprising, N mineralization is generally positively correlated with SWC [288, 434, 435] and SWC was decreased under the $A_{-30\%}$ treatment. Our results contrast with previous studies reporting no significant effect, such as for a North American grassland (along a gradient of increasing precipitation; Barrett et al. 2002). In our study, the positive response of net N-min to $A_{-30\%}$ maybe ascribed to increased belowground biomass production (data not shown) and possibly higher, labile C inputs by roots through exudation. Because the study site is characterized with low soil organic carbon content (6.58 mg g^{-1}), increased belowground biomass could alleviate energy limitation for soil microbial mineralizers.

In addition, N mineralization was strongly decreased by changed precipitation frequency. N mineralization is influenced by dry-rewetting cycles [411]. It is possible that for $F_{+50\%}$, smaller and more frequent rainfall events only wet the surface soil layers where mineralization substrate and nitrifiers are not concentrated. By contrast, for $F_{-50\%}$, large rainfall events penetrate deeper into soil layers, where microbial activity and organic nitrogen are lower, and wet the soil layers less frequently.

In contrast to results for AOB, no significant main effect of treatments was found on the abundance of AOA, including the main N effect. Similar results have also been reported by Shen et al. (2008) [25], Ma et al. (2016) [44] and Assemien et al. (2017) [433] who found that long-term N fertilization increased AOB abundance without detectable effect on AOA abundance. This is due to the fact that AOA and AOB tend to differ in their N substrate affinity and energy use efficiency [260]. AOA was reported to have better low-substrate tolerance and have greater potential for mixotrophic growth as compared to AOB [390, 436]. The abundance of AOA was significantly strongly affected by the A × F interaction, and to a lesser extent by the N × A interaction. In particular, the abundance of soil AOA was significantly increased by N deposition (N_{10}) under ambient precipitation amount (A_{ctrl}), which can be explained by the increment of soil net N-min by N_{10} (Fig. 25), resulting more substrate for AOA. In contrast, no significant response of net N-min or AOA to N_{10} was found under $A_{-30\%}$. This indicated that, under $A_{-30\%}$, the abundance of AOA and net N-min was limited by soil water content rather than N nutrient. Further, under no N deposition treatment (N_0), $A_{-30\%}$ significantly decreased SWC, contributing to the significant increment of the abundance of AOA. This is can be ascribed to the aerobic condition favored the growth of AOA. In contrast, under N_{10} treatment, the decrease of SWC was much lower than under N_0 treatment, contributing the lower increment of AOA under N_{10} treatment as compared to under N_0 (Fig. 25).

2.6 **Summary**

It is essential to study interactive effects of global change factors such as chronic N deposition, altered precipitation amount and frequency on soil N dynamics. Based on a mesocosm experiment for the Songnen grassland, interactive effects between factors are very important. For instance, P amount and frequency for AOA (most important effect) and N × F for N-min. Conclusions can be made in this study that the responses of AOB and AOA to the global change scenarios differed strongly. AOB mostly responded to N: either directly with N deposition treatment, or indirectly with P amount or frequency changes by altering N cycling. In contrast, AOA were particularly sensitive to soil water dynamics than N dynamics at this study site. This shows that the manipulating P frequency independently of P amount in global change experimental studies is fundamental, particularly in ecosystems where AOA are expected to play an important role for soil N dynamics.

3 Response of Soil Denitrifiers and N_2O emissions to Chronic N Deposition and Precipitation Amount and Frequency in a semi-arid Grassland

3.1 Introduction: Possible Feedbacks Between Global Change Factors and Soil N_2O Emissions

Many studies have shown that enhanced N deposition deeply influences soil N cycling processes and generally results in increased emissions of nitrous oxide, N_2O, by soil [173, 358]. This can feedback on climate change because N_2O has a warming potential per molecule 300 times higher than carbon dioxide and is also a major stratospheric ozone-destructing compound [437-439]. In addition, numerous studies have documented soil N_2O emission can be altered by precipitation regime [205, 232, 280, 286, 410]. In terrestrial ecosystems, N_2O emitted from soil is mainly the product of nitrification and denitrification [440, 441]. The first step of nitrification (the oxidation of ammonium, NH_4^+, to nitrite, NO_2^-) is carried out by ammonia-oxidizing bacteria (AOB) and ammonia oxidizing archaea (AOA) and can generate N_2O as a byproduct [442, 443]. Nitrification is generally favored by aerobic conditions [5, 444]. In contrast, denitrification is a stepwise process reducing nitrate, NO_3^-, and NO_2^- to gaseous nitrogen (N) compounds, with NO, N_2O or N_2 as end products [78], which is favored by anaerobic conditions [445]. A key step of denitrification is nitrite reduction, being performed by bacteria harboring the *nirK* or *nirS* gene that have a major role for N_2O production [80]. Another key step is N_2O reduction, which is performed by bacteria harboring the *nosZI* or *nosZII* gene [93, 94]. N_2O emissions from soil depend on the balance between its production and consumption, and hence on the responses of nitrifiers, NO_2^- reducers and N_2O reducers to changes in soil environmental conditions [446].

Different studies [173, 256, 354] have analyzed the effects of N deposition on N_2O fluxes and underlying microbial processes [173, 321, 354]. For instance, in the review paper of Barnard et al. (2005) [173], it was reported that soil N_2O emission was stimulated by N addition either in the field with the effect sizes of 128%, or in the laboratory with 328%. But these studies generally used one or a few N addition events, which does not mimic the actual chronic N inputs associated to precipitation events. Numerous studies analyzed the effect of modified precipitation regime on N_2O emissions by soils [205, 232]. For instance, Brown et al. (2012) reported that altered precipitation (elevated precipitation amount and extend rainfall season) stimulated soil N_2O emission in JRGCE, an annual grassland [232]. But the treatment applied included both an increased precipitation amount and number of rainfall events [232]. Wet N deposition is often tightly coupled to rainfall events [415, 416] so that altering precipitation frequency also modifies the temporal distribution of chronic wet N deposition. But to what extent N deposition, changed precipitation frequency and modified precipitation amount can have interactive effects on N_2O emissions remains to be studied.

As dominant and typical species of perennial grass, *Leymus chinensis* is widely distributed in the eastern part of the Eurasian grassland and is the dominant species in Songnen, a semi-arid grassland. Songnen grassland is highly limited by both water and N [421] and the location area is prone to high N deposition loads [393]. The precipitation regime in this area is expected to be modified over the next decades according to the precipitation history [389]. Consequently, the effects of chronic wet N deposition, reduced precipitation amount (-30%) and altered precipitation frequency (either +50% or -50%) on the abundances of 4 major soil denitrifier groups, denitrifying enzyme activity and soil N_2O emissions in this semi-arid grassland were studied.

We hypothesized that (1) reduced precipitation amount would induce decreased soil moisture which would be unfavorable to soil denitrifiers; in contrast, reduced precipitation amount would hinder plant growth and hence decrease the competition for nitrate between plants and denitrifiers; (2) decreased rainfall frequency for a given total rainfall amount (i.e. fewer rainfall events of higher intensity) would increase average soil N_2O emission by generating periods of higher soil moisture generating more anoxia, which would be favorable to denitrifying enzyme activity and abundance. This effect would be amplified by wet N deposition that would increase nitrate availability in soil and increase plant growth and likely root exudation, which are both favorable to denitrifiers; (3) conversely, increased rainfall frequency would decrease soil N_2O emission because this would limit periods of high soil moisture, hence constraining more soil microorganisms through drought and limiting anoxic periods in this semi-arid ecosystem, which would be particularly unfavorable for denitrifiers. We also used structural equation modelling (SEM) to identify and hierarchize the environmental drivers underlying the response of denitrifiers to N deposition, changed precipitation frequency and modified precipitation amount. In addition, I evaluated by a literature search what extent the results obtained for this semi-arid grassland are consistent with results obtained for other grassland ecosystems. I discuss the implications of our results for a better understanding of the effect of multiple global change factors acting in concert on soil N dynamics and N_2O emissions.

3.2 Study site

See Chapter 2.2

3.3 Materials and Methods

3.3.1 Experimental Design

See Chapter 1.4.1

3.3.2 Sampling and Measurements

3.3.2.1 Soil environmental variables

Soil temperature, water content, and total and dissolved organic carbon (TOC and DOC, respectively) were measured as described in section 2.3.2.2. Nitrate (NO_3^-) concentrations were determined by extraction with KCl. Briefly, 10 g (equivalent dry weight) fresh soil sub-samples were extracted with 50 ml KCl (2 M) solution, extracted in a shaker (180 rpm) for 1h, and the supernatants were filtered (0.45μm) before analysis. Concentrations of NO_3^--N were then analyzed using a continuous flow analyzer (Alliance Flow Analyzer, Futura, Frépillon, France).

3.3.2.2 Soil denitrifying enzyme activity (DEA)

Soil denitrifying enzyme activity (DEA) was measured according to a protocol modified from Enwall et al. (2005) [447] and Patra et al. (2005) [448]. Triplicate soil samples (25 g) were placed in 250 ml flasks containing 25 ml of substrate with 1 mM glucose and 1 mM KNO_3. The flasks were capped with gas tight stopper and anaerobic conditions in the flasks were generated by replacing the flask atmosphere with N_2. Acetylene (10%) was added to the flask headspaces to prevent N_2O reduction. The soil slurries were incubated at 25°C on a rotary shaker for 3 h, and gas samples were collected every 30 min. N_2O was analyzed on a gas chromatograph (7890A, Agilent, Santa Clara, USA). DEA was calculated from the increase of N_2O concentration in the flask headspace, which was always linear during the incubation.

3.3.2.3 Measurements of N_2O emission flux from soil

N_2O emissions were measured every three days from May 1st to September 30th in 2015 (i.e. second year of treatment) using the closed static chamber technique [449]. The chamber was composed of a steel collar with a gutter (about 3cm deep) at the top end and a PVC lid (35 cm in height and 30 cm in diameter), with two small fans to mix the gases inside the chamber. The steel collar was inserted into the soil (10 cm deep) immediately after transplanting the plants to the mesocosms, and the collars were maintained in the mesocosms for the whole experimental period. During gas measurements, the collar was covered by a PVC lid, which was shaded on the top with polystyrene foam and covered with aluminum foil to minimize temperature and pressure fluctuations for the enclosed gases, whereas the sides were left partially uncovered to allow for light entering the chamber. Before setting the chamber, the gutter on the top edge of the collar was filled with water for airtight seal. A 2.5 mm diameter hole in each lid was tightly fitted with a gas-check valve connected to a flexible Teflon pipe (2-3 mm diameter) with 15 cm stretched into the chamber and 5 cm outside on the top to facilitate gas sampling. This design produced a 44 L chamber volume that fits criteria defined in guidelines for N_2O chamber methodologies [450]. During a pre-experiment, gas samples were collected 0, 15, 30, 45, 60, 90 and 120 min after chamber closure. The results showed that N_2O concentration in the chamber increased linearly with time during the first 45-60 min ($R^2 > 0.9$). Therefore, during the experiment, two gas samples at 0 and *ca.* 50 min were collected. N_2O measurements were made between 10:00 and 12:30 am and emission rates were assumed to be closed to the average gas flux for that day. Air sampling was carried out using a 100 ml polyurethane syringe, flushing 100 ml of air back 3 times through the connected Teflon pipe (modified after de Klein et al. 2003) [451]. The air samples were drawn into pre-evacuated Tedlar® air-sampling bags (Delin Company, Dalian, China, 300 ml). N_2O concentrations of the air samples were quantified within a few days by gas chromatography (7890A, Agilent).

3.3.2.4 Soil DNA extraction and quantification of denitrifier abundances

Table 31 Sets of primers used for amplification of soil denitrifier groups by real-time PCR

Target genes	Primers	Sequences (5-3)
nirK [452]	*nirK*1F	GGMATGGTKCCSTGGCA
	*nirK*5R	GCCTCGATCAGRTTRTGG
nirS [453]	cd3AF	GTSAACGTSAAGGARACSGG
	R3cd	GASTTCGGRTGSGTCTTGA
nosZI [454]	nosZ-1181F	CGCTGTTCITCGACAGYCAG
	nosZ-1880R	ATGTGCAKIGCRTGGCAGAA
nosZII [93]	*nosZII*-F	CTI GGI CCI YTK CAY AC
	nosZII-R2	I GAR CAR AAI TCB GTR

Soil DNA extraction method is as described in Chapter 2.3.2.4. The abundances of nitrite-reducers (harboring the *nirK* or *nirS* gene) and N_2O-reducers (harboring the *nosZI* or *nosZII* gene) were quantified with a Lightcycler 480 (Roche Diagnostics, Meylan, France). The sets of primers were *nirK*1F/*nirK*5R for *nirK*-harboring bacteria, Cd3aF/R3cd for *nirS*-harboring bacteria, NOSZ-1181F/NOSZ-1880R for *nosZI*-harboring bacteria and nosZ-II-F/nosZ-II-R2 for *nosZII*-harboring bacteria. Further details about the primers are provided in Table 31. Possible PCR inhibition by co-extracted compounds was evaluated by serial dilution and no inhibition was observed. Amplification efficiencies were 80~93% and the R^2 value of the standard curves were always higher than 99%.

3.3.3 Data Analysis and Calculation

The ANOVA and mean comparison analyses were performed as described in Section 2.3.3. The complete structural equation model, with all possible causal relationships considered, is presented in Fig. 31.

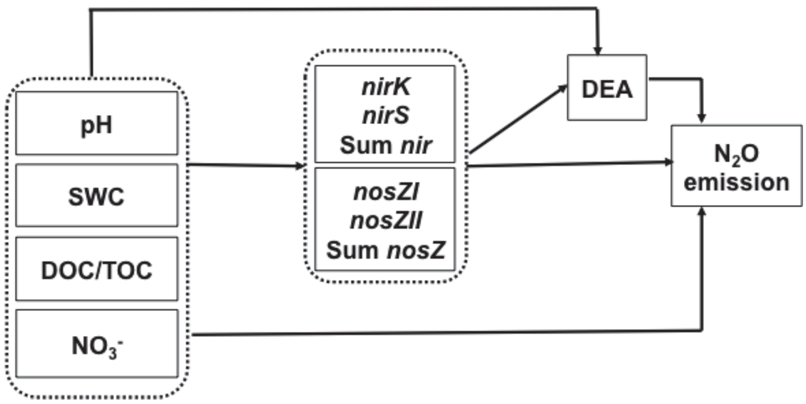

Fig. 31 Complete model used for structural equation modeling aimed at identifying the main drivers of changes in the abundances of soil denitrifiers, denitrifying enzyme activity (DEA), and N_2O emission from soil in response to N deposition, changed precipitation amount and changed precipitation frequency. SWC, DOC, TOC, NO_3^- and DEA refer to soil water content, dissolved organic carbon, total organic carbon, nitrate concentration and denitrifying enzyme activity

For the literature survey on N addition effect on soil N dynamics and N_2O emission rates, the percentage change of a given variable induced by the N treatment as compared to the control was calculated as: % N effect = (value under N treatment － value under control) / value under control × 100%. I selected studies from the literature according to the following criteria: (1) only grassland and steppe ecosystems were considered; (2) because addition levels can influence N effect on N_2O emission [173], N rates as close as possible to 10g-N m^{-1} yr^{-1} were selected (i.e. if several N rates were applied in a study, the rate closest to 10g-N m^{-1} yr^{-1} was considered); (3) when a study reported N effects for different sites or vegetation covers, results were considered as independent replications. When N effects were measured several times in the same year, I selected data collected at the date closest to the peak biomass period. Results obtained for a given ecosystem over several years were averaged. A total of 29 references were selected. N effect values were collected either from text, from tables or from figures using GetData Graph Digitizer 2.24 (http://getdata-graph-digitizer.com).

Considering all dataset with both nitrification in Chapter 2 and denitrification, soil N_2O emission was correlated with denitrification rather than nitrification, therefore, soil N_2O emission was further analyzed together with denitrification in this Chapter.

3.4 Results

3.4.1 Soil Environmental Variables

The main effects of nitrogen deposition (N), precipitation amount (A), precipitation frequency (F) along with the N×A× F interaction effect on SWC were significant (Table 32). The response of SWC to the treatments is detailed in Chapter 2.4.1. The main effects of N and A on soil nitrate concentration (NO_3^-) were both significant, without any N×A interaction (Table 32). Overall, increased N deposition increased soil NO_3^- from 1.05 μg g^{-1} for N_0 1.41 μg g^{-1} for N_{10}, while $A_{-30\%}$ increased NO_3^- from 1.06 μg g^{-1} for A_{ctrl} to 1.41 μg g^{-1} under $A_{-30\%}$. No main or interactive effects of nitrogen deposition, precipitation amount and precipitation frequency were observed for both soil total organic carbon (TOC) and dissolved organic carbon (DOC) (Table 32).

Table 32 ANOVA results summarizing the effects of nitrogen deposition (N), precipitation amount (A) and precipitation frequency (F), along with their possible interactions, on soil water content (SWC), nitrate content (NO_3^-), total organic carbon (TOC) and dissolved carbon content (DOC). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$

Variables	Nitrogen depositio n (N)	Precipitatio n amount (A)	Precipitatio n frequency (F)	N x F	A x F	N x A	N x A x F
SWC	***	***	**	ns	*	***	*
NO_3^-	**	**	ns	*	ns	ns	ns
TOC	ns	ns	ns	ns	ns	ns	ns
DOC	ns	ns	ns	ns	ns	ns	ns

3.4.2 Abundances of Nitrite Reducers and N_2O Reducers

The abundances of soil *nirK*- and *nirS*-harboring nitrite-reducers were significantly affected by main effect of N, A, and F. In addition, a significant N×F interaction effect was observed on *nirK* abundance while a A×F interaction effect was found for *nirS* abundance. In contrast, the main effect of N on the abundance of *nosZI*-harboring N_2O reducers was significant, as was the main effect of A on the abundance of *nosZII* N_2O reducers. A N × A × F interactive effect was observed on the abundance of both *nosZI*- and *nosZII*-harboring bacteria (Fig. 32).

Table 33 ANOVA results summarizing the effects of nitrogen deposition(N) , precipitation amount (A) and precipitation frequency (F), along with all their possible interactions, on the abundances of *nirK-, nirS-, nosZI-* and *nosZII*-harboring bacteria, denitrifying enzyme activity (DEA), and soil N_2O fluxes. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$

Variables	Nitrogen deposition (N)	Precipitation amount (A)	Precipitation frequency (F)	N x F	A x F	N x A	N x A x F
nirK	**	**	**	**	ns	ns	ns
nirS	**	***	**	ns	**	ns	ns
nosZI	***	ns	ns	ns	***	ns	*
nosZII	ns	*	ns	ns	ns	ns	**
DEA	ns	ns	**	**	*	ns	*
N_2O	**	***	ns	*	ns	**	ns

Overall, N_{10} significantly decreased the abundances of *nirK*, *nirS* and *nosZI* by 20%, 27% and 47% respectively (i.e. response ratios of -0.25, -0.44, and -0.55, respectively; see Fig. 32 and Fig.33). Decreased total precipitation amount decreased the abundance of *nirS* from 7.96×10^6 copies g^{-1} soil for A_{ctrl} to 4.72×10^6 copies g^{-1} soil for $A_{-30\%}$, but increased the abundances of *nirK* and *nosZII* by 28% and 30% respectively (response ratios of 0.25 and 0.12 respectively; see Fig. 32). The $F_{-50\%}$ and $F_{+50\%}$ treatments significantly decreased the abundance of *nirS*-harboring nitrite reducers by 54% and 37% respectively (response ratios of -0.39 and -0.27 respectively; Fig.33). In addition, $F_{-50\%}$ decreased the abundance of *nirK* by 37% whereas no significant effect of $F_{+50\%}$ on the abundance of *nirK* was found.

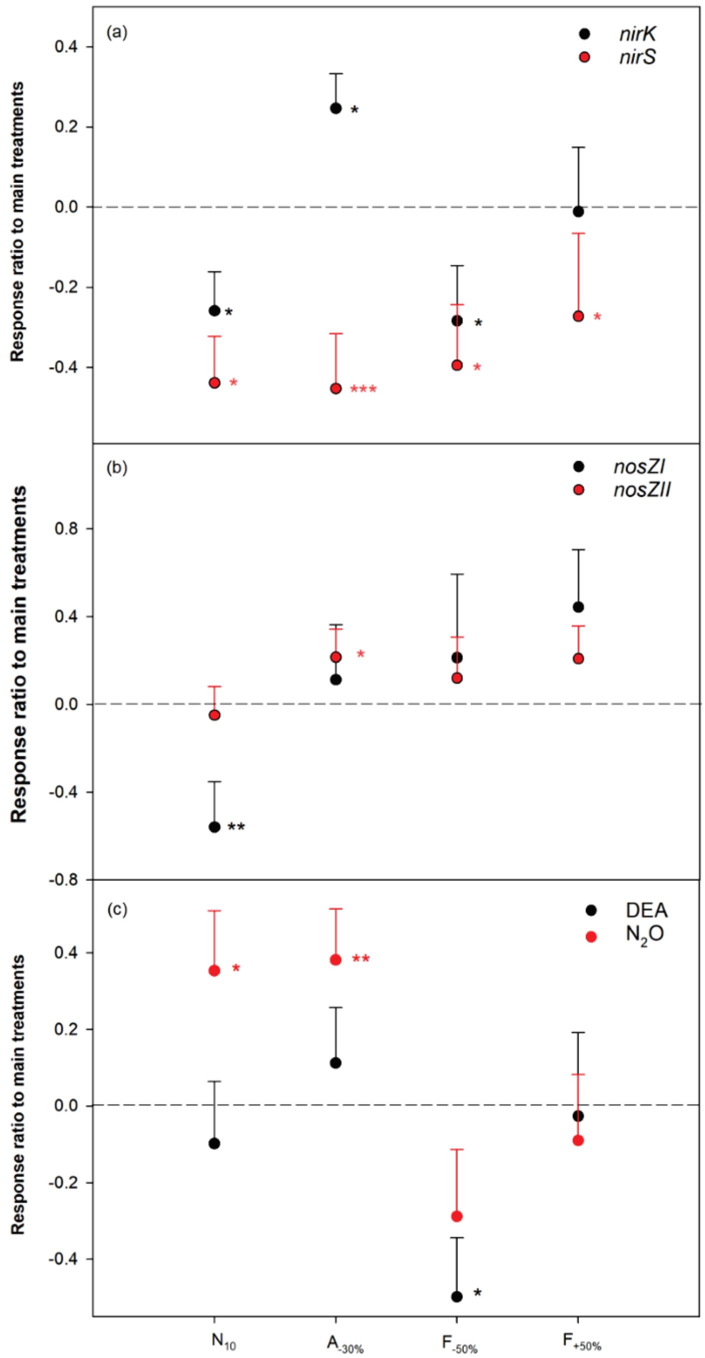

Fig. 32 Response ratio of (a) denitrifying enzyme activity (DEA) and nitrous oxide emission (N_2O); (b) the abundances of *nirK-, nirS*-nitrite reducers; and (c) the abundances of *nosZI-* and *nosZII*-N_2O reducers to the following main treatments: N deposition (N_{10}), reduced rainfall amount ($A_{-30\%}$) and rainfall frequency changes (either reduced by 50%, $F_{-50\%}$; or increased by 50%, $F_{+50\%}$). For each main treatment: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$

Under $F_{ctrl,}$ N deposition significantly decreased the abundance of *nirK* from 4.72×10^5 copies g^{-1} soil under N_0 to 2.76×10^5 copies g^{-1} soil (response ratio of -0.59) under N_{10}, whereas this negative effect was dampened under altered precipitation frequency (Fig.33-Left). Under $N_{0,}$ the abundance of *nirK* significantly decreased from 2.76×10^5 copies g^{-1} soil under F_{ctrl} to 2.34×10^5 copies g^{-1} soil under $F_{-50\%}$ (response ratio of -0.14 (Fig.33-Right). In contrast, no significant effect of precipitation frequency was found on the abundance of *nirK* when precipitation events were coupled to wet N deposition (Fig.33-Right).

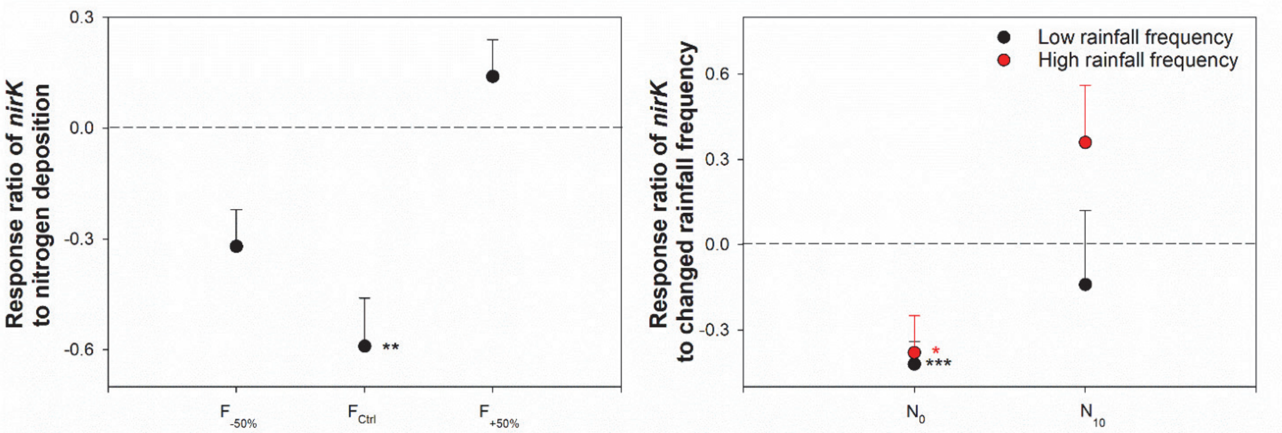

Fig.33 Response ratio of the abundance of *nirK*-harboring bacteria to (Left) N deposition (N_{10}) and (Right) changed rainfall frequency (either reduced by 50%, $F_{-50\%}$; or increased by 50%, $F_{+50\%}$). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$

The N×A×F interaction effect on the abundances of *nosZI*- and *nosZII*- N_2O-reducers was significant (Table 33). Fig. 34 details this 3-way interaction effect for the abundance of *nosZI*-harboring bacteria. Under normal precipitation amount and frequency ($A_{ctrl}F_{ctrl}$), the abundance of *nosZI* did not respond to N deposition, whereas under reduced precipitation frequency ($A_{ctrl}F_{-50\%}$) N_{10} decreased the abundance of *nosZI* by 44.2% (response ratio of -0.95). Similar negative effects of N deposition on the abundance of *nosZI*–harboring N_2O reducers were found under $A_{-30\%}F_{-50\%}$ and $A_{-30\%}F_{+50\%}$ treatments Fig. 34-Top-Left). Reduced precipitation amount significantly increased the abundance of *nosZI* under control conditions (N_0F_{ctrl}) and to a lesser extent under $N_0F_{+50\%}$, but not under reduced precipitation frequency ($N_0F_{-50\%}$). Reduced precipitation amount also increased the abundance of *nosZI*-harboring bacteria under $N_{10}F_{ctrl}$, but decreased *nosZI* abundance when chronic N deposition was combined with reduced precipitation frequency ($N_{10}F_{-50\%}$), whereas no significant effect was observed under the $N_{10}F_{-50\%}$ treatment (Fig. 34-Top-Right). Reduced precipitation frequency ($F_{-50\%}$) significantly increased the abundance of *nosZI* N_2O-reducers under control conditions ($A_{ctrl}N_0$), and this positive effect was dampened under the $A_{-30\%}N_0$ and $A_{ctrl}N_{10}$ treatments (Fig. 34-Bottom-Left). In contrast, reduced precipitation frequency decreased *nosZI* abundance when chronic N deposition was combined with reduced precipitation amount ($N_{10}A_{-30\%}$) condition (Fig. 34-Bottom-Left). Increased precipitation frequency increased the abundance of *nosZI* under A_{ctrl} N_{10} whereas no significant effect of increased precipitation frequency was observed under the other treatments (Fig. 34-Bottom-Right).

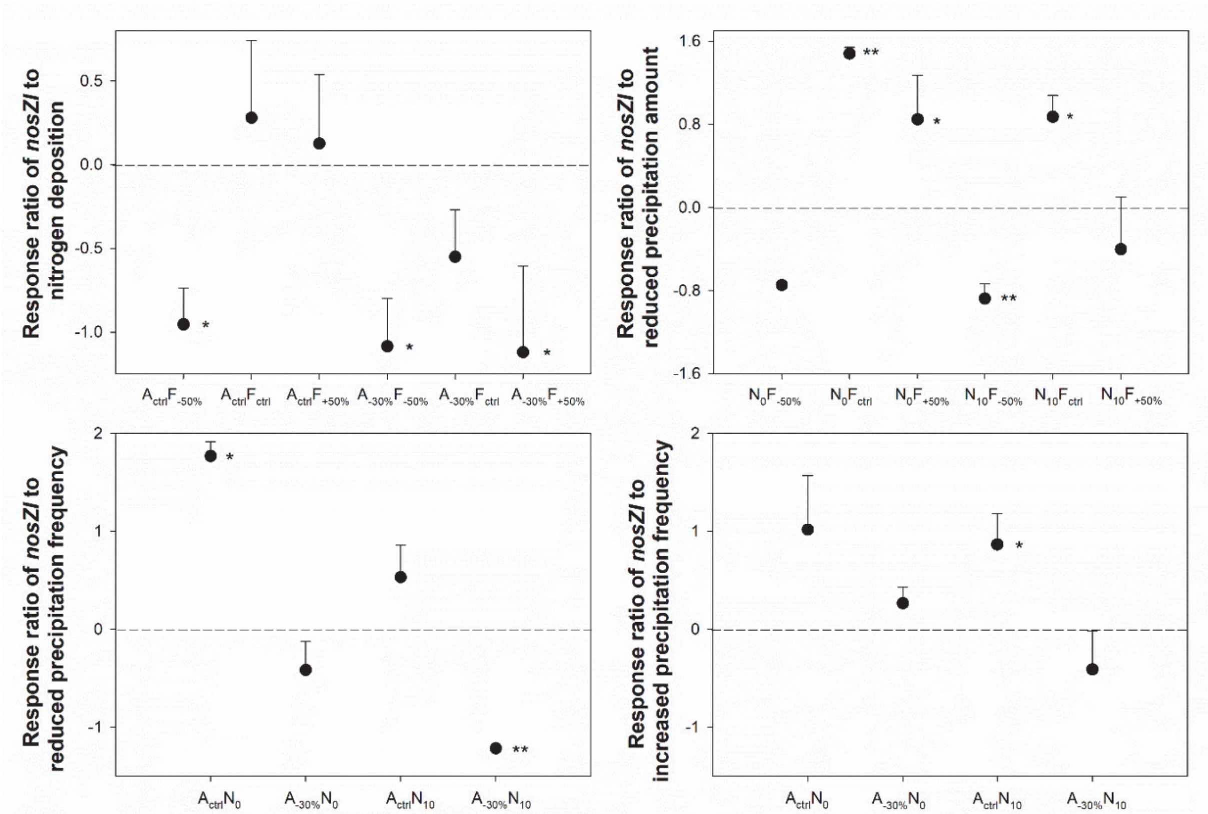

Fig. 34 Response ratio of the abundance of *nosZI*-harboring bacteria induced by (Top-Left) N deposition according to other treatments; (Top-Right) reduced precipitation amount according to other treatments; (Bottom-Left) precipitation frequency reduced by 50% according to other treatments; and (Bottom-Right) precipitation frequency increased by 50% according to other treatments. N_0, no N deposition; N_{10}, N deposition with 10g NH_4NO_3 $m^{-2.}$ yr^{-1}; A_{ctrl}, normal total precipitation amount; $A_{-30\%}$, precipitation amount reduced by 30%; $F_{-50\%}$, precipitation frequency reduced by 50%; F_{ctrl}, normal precipitation frequency; $F_{+50\%}$, precipitation frequency increased by 50%. *, P < 0.05; **, P < 0.01; ***, P < 0.001

3.4.3 Denitrifying Enzyme Activity (DEA) and Soil N_2O Emissions

The main effect of precipitation frequency (F) on DEA was significant as were the N×F, A×F and A×N×F interaction effects (P <0.05) (Table 33). Overall, DEA significantly decreased from 685 ng N_2O-N g^{-1} h^{-1} for F_{ctrl} to 434 ng N_2O-N g^{-1} h^{-1} for $F_{-50\%,}$ but no significant $F_{+50\%}$ effect on DEA was found.

Mean N_2O emission over the growing season (May-September) was highly correlated with the mean N_2O flux measured from two weeks before to two weeks after soil sampling (R^2>0.9 data not shown). Hereafter, we focus on the N_2O flux integrated over the growing season which ranged from 7.8 to 26.2 µg N_2O-N m^{-2} h^{-1} according to treatment. The main effects of N and W and the N×W interaction effect (and to a lesser extent the N×F effect) were significant on N_2O emission (Table 33

).

Considering all the dataset, N_2O emissions were strongly and positively related to *nirK*-harboring nitrite reducers, and negatively related to soil water content (SWC) and to a lesser extent to the abundance of *nosZI*-harboring N_2O reducers (Fig. 35). These drivers explained 48% of the variance in the changes in N_2O emissions. Note that we also tried to account for the possible roles of ammonia oxidizing bacteria and archaea and of nitrifying enzyme activity (see Chapter 2) but these variables were never retained in the SEM for explaining N_2O emissions.

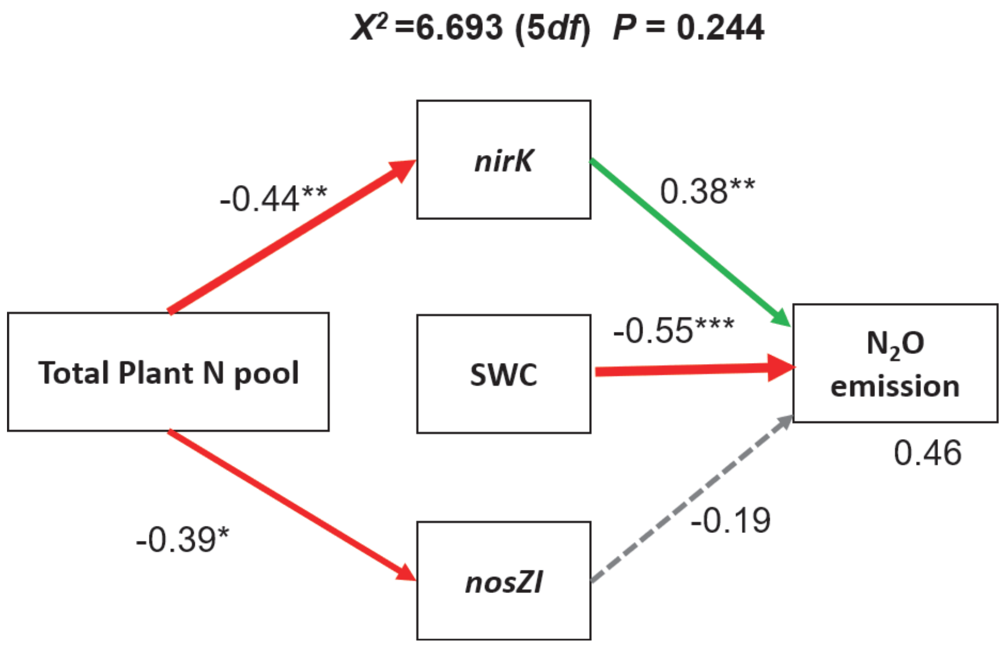

Fig. 35 Best structural equation model linking N_2O emission to microbial, soil environmental variables and plant N pool. Values near the arrows are path coefficients. The green arrow indicates a positive effect and the red arrows indicate negative effects. The percentage of variance in N_2O emission explained by the model is indicated at the bottom-right of the corresponding box. †, $0.05 < P < 0.1$; **, $P < 0.01$; ***, $P < 0.001$

Given that the strong interaction effect on N_2O emissions was the N×W effect, we further analyzed this effect and underlying drivers. Decreased rainfall amount increased N_2O emissions without N deposition (N_0), but not when rainfalls were associated to N deposition (

Fig. 36-Left-Top). In both cases, N_2O emissions were positively related to the abundance of *nirK*-harboring bacteria (and to a lesser extent to soil nitrate concentration under N_0), and negatively related to SWC, with the changes in these drivers explaining 42-43% of the variance in N_2O emissions (

Fig. 36-Left-Bottom). Chronic N deposition increased N_2O emissions under A_{ctrl}, but not under $A_{-30\%}$ (Fig. 3-6-Right-Top). Consistently, N_2O emissions were positively related to soil nitrate concentration only under $A_{-30\%}$, while N_2O emissions were positively related to *nirK* abundance and

negatively related to SWC whatever the precipitation amount. These three drivers explained 48% of the variance in N_2O emissions under both A_{ctrl} and $A_{-30\%}$ conditions (Fig. 36-Right-Bottom). Overall, the A x N interaction effect on N_2O emissions was thus strongly related to the strong A x N interaction effect observed on SWC (Table 2-2).

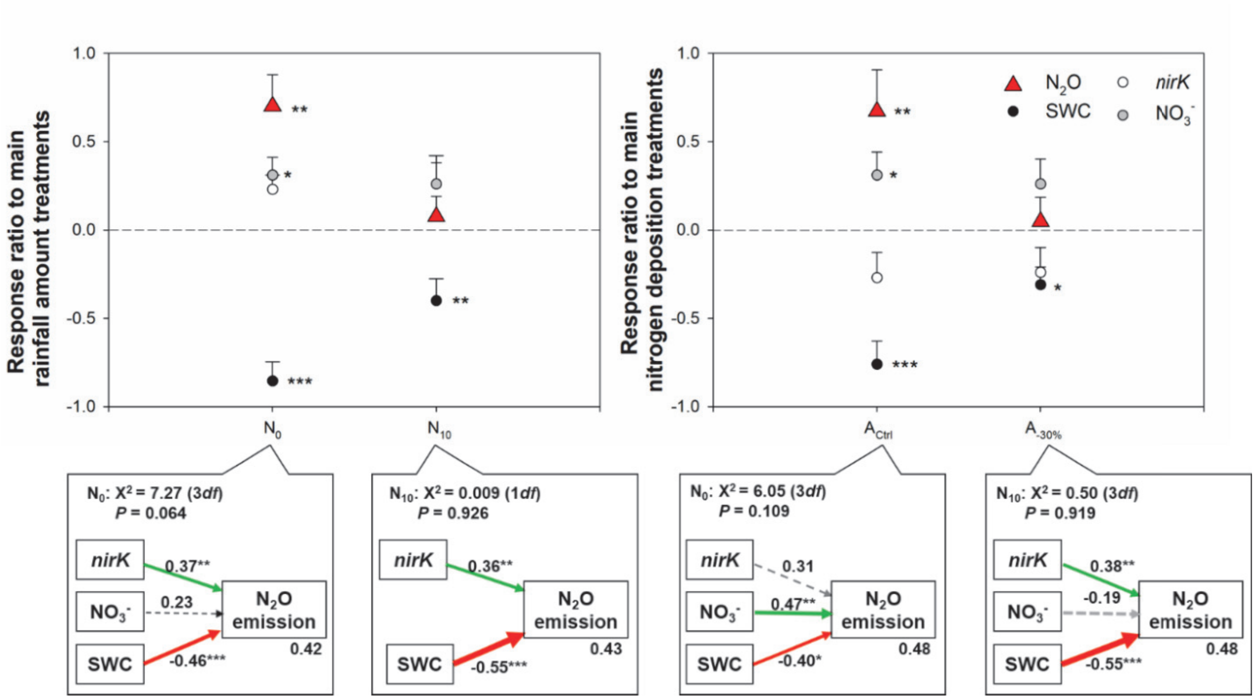

Fig. 36 Response ratio of N_2O to (Top-Left) reduced precipitation amount according to N deposition, and to (Top-Right) N deposition according to precipitation amount. The response ratios of soil water content (SWC) and of the abundance of *nirK*-harboring bacteria (*nirK*) are also presented. (Bottom) Best structural equation models linking N_2O emission to microbial and soil environmental variables under different N deposition or precipitation amount scenarios. The green arrows indicate positive effects and the red arrows indicate negative effects. Values near the arrows are path coefficients. The percentage of variance explained by the model for each explained variable is indicated at the bottom-right of each corresponding box. N_0, no N deposition; N_{10}, N deposition with 10 g NH_4NO_3 $m^{-2\cdot}$ yr^{-1}; A_{ctrl}, normal precipitation amount; $A_{-30\%}$, precipitation amount reduced by 30%. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$

3.5 Discussion

As mentioned in Chapter 2, many studies have been conducted on the response of soil N cycling processes and N_2O emissions to global change factors. However, only few studies have conducted experiments with changes in precipitation frequency without altering total annual precipitation amount [171, 414]. Besides, some global change experiments involved concurrent modifications of both total precipitation amount and number of precipitation events, without decoupling both aspects [232]. Further, many studies have analyzed the effect of increased atmospheric N deposition on soil N cycling but generally using very few N addition event(s) per year rather and mimicking more N fertilization than chronic N deposition [428, 429]. No study of global change effects on soil N processes and microbial groups simulated chronic N deposition co-occurring with precipitation events as far as we know. The novelty of our study is thus that it evaluates the possibly interactive effects of three global change factors: (1) changed precipitation amount, (2) altered rainfall frequency, and (3) chronic N deposition co-occurring with simulated precipitation events, on N_2O emissions from soil and the enzyme activity and abundances of soil denitrifiers.

3.5.1 Response of Soil Denitrifiers and N_2O Emissions to Main Effect of Global Change Factors

3.5.1.1 Lack of stimulation of denitrifiers and weak stimulation of N_2O emissions by N deposition

In our study, N_2O emissions from soil only weakly responded to N deposition, with a 27.6% increase on average despite the high amount of N added. This stimulation is lower than that reported in most previous studies (Fig. 3-7). Concurrently, and contrary to our assumptions, the main effect of N deposition on the abundances of *nirK*- and *nirS*-nitrite reducers, as on *nosZI*-N_2O reducers, was significant but negative, and N deposition also tended to decrease DEA. This is inconsistent with the results of most previous studies reporting increased denitrifier abundances in response to N addition (Fig. 3-7).

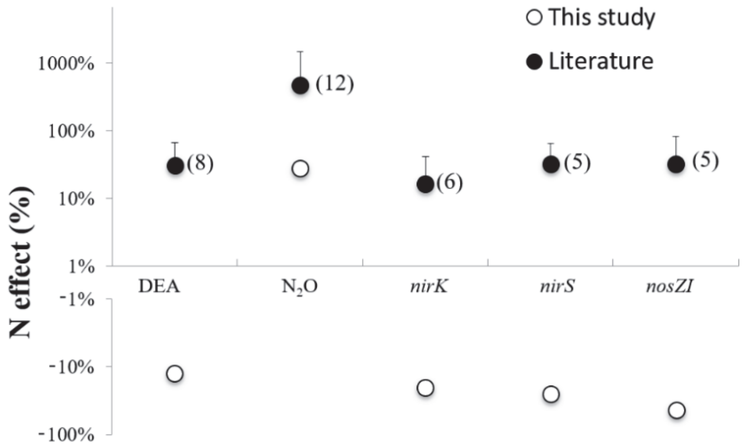

Fig. 37 Comparison of the effects of N addition on N_2O emission from soil and denitrifying enzyme activity and abundances for grassland/steppe ecosystems, as observed in our study (white dots) and reported in the literature (black dots), Denitrifying enzyme activity, DEA; N_2O emission from soil, N_2O; abundances of *nirK*- and *nirS*-harboring nitrite reducers, *nirK* and *nirS*, respectively; and abundance of *nosZI*-harboring N_2O reducers, *nosZI*. For previously published studies, bars show 95% confidence intervals of the mean size effects (number of experiments were indicated between brackets)

For instance, N addition increased DEA by 34% in a Mediterranean grassland [213]. This may be due to the following reasons. First, N addition could have increased soil acidity as fertilization is often associated to decreased pH [307]. However, N deposition did not modify soil pH (7.82 for N_{10} and 7.78 for N_0). Second, it is possible that most of the N added through chronic N deposition benefited plants rather than soil microbes. I verified this by analyzing plant N pools in the mesocosms under all treatments and observed an increase in the total plant N pool (increase representing 4.2 to 13.4 g-N m^{-2}) and a weak increase in the total soil N pool (representing -0.4 to 2.8 g-N m^{-2}) (Fig. 38). This is likely due to the capacity of the dense root system of the perennial grass species *L. chinensis* to uptake efficiently the small amount of N brought to the soil through each rainfall event during the growing season. Third, likely due to the N-promoted plant growth (particularly for aboveground parts) and associated higher plant transpiration, soil water content decreased strongly when N deposition co-occurred with rainfalls, for instance from 20% under $F_{CTRL}N_0$ to 9% $F_{CTRL}N_{10}$. The latter value is particularly low and likely unfavorable to denitrifiers and their activity. These mechanisms, both directly and indirectly linked to the higher capacity of plants than soil microbes to benefit from chronic wet N deposition, explained the overall weak increase of N_2O emission by N deposition. This result, very unusual as compared to results reported for other grassland ecosystems (Fig. 3-7), could be due to the eco-physiology of the dominant plant species of the Songnen grassland and its ability to efficiently capture low N amounts and withstand intense drought [455]. It is also likely due to the more realistic simulation of wet N deposition used here. Indeed, mimicking wet N deposition by chronic N addition associated to each precipitation event during the plant growing season may lead to a different outcome of the plants-microbes competition for N than using one or a few addition events with much higher N amounts. This should be further explored in future researches.

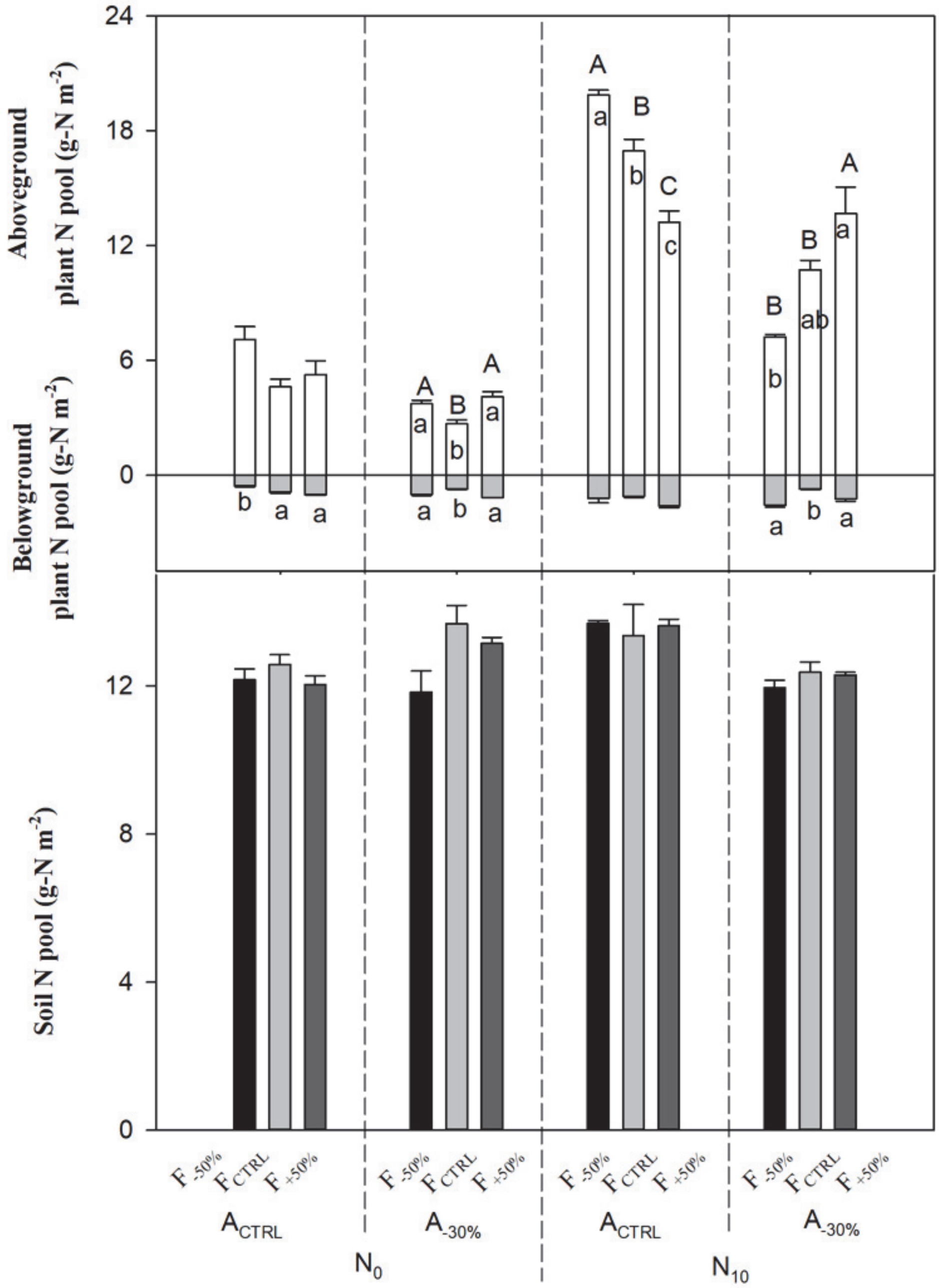

Fig. 38 Treatments effects on plant N pool (above-ground and below-ground)and soil N pool. N_0, no N deposition; N_{10}, N deposition with 10g NH_4NO_3 m^{-2} yr^{-1}; A_{ctrl}, normal total precipitation amount; $A_{-30\%}$, precipitation amount reduced by 30%; $F_{-50\%}$, precipitation frequency reduced by 50%; F_{ctrl}, normal precipitation frequency; $F_{+50\%}$, precipitation frequency increased by 50%. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$

If N deposition on a plant cover dominated by plant species very efficient at retrieving added N induces an increase of the amount of transpiring leaf surfaces, and a cascading negative effect on SWC and denitrification, then this negative effect would be critical in drier environments. But it could be dampened or even reversed in wetter environments. Analyzing the results from our literature search, I did observe that the response of soil N_2O emission to N addition across ecosystems indeed depend on precipitation amount, N addition increasing N_2O emission in environments with high annual precipitation but not in drier ecosystems (Fig. 39).

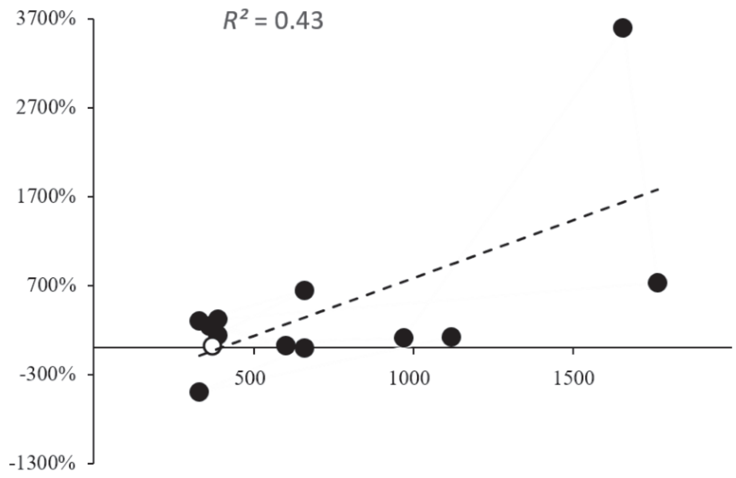

Fig. 39 Relationship between N deposition effect on N_2O emission by soils and precipitation amount in grassland ecosystems. The white dot corresponds to the present study, and black dots to results from the literature

3.5.1.2 Contrasted effects of reduced precipitation amount on denitrifier groups

Reduced precipitation amount ($A_{-30\%}$) induced a strong decrease of the abundance of soil *nirS*-harboring bacteria. This is consistent with our hypothesis and with several previous studies reporting that the abundances of soil denitrifiers are positively correlated with SWC in arid or semi-arid grasslands [303]. However, the abundance of *nirK*- and *nosZII*-harboring bacteria significantly increased in response to decreased precipitation. Because $A_{-30\%}$ tended to decrease plant below-ground growth (data not shown), this could decrease the competition between plant and denitrifiers for nitrate. In addition, no significant response of DEA to decreased precipitation amount was observed in this study, which could be due to the opposite effects of $A_{-30\%}$ on the abundance of *nirK*- and *nirS*-harboring bacteria.

3.5.1.3 Opposite effects of changed precipitation frequency on nitrite reducers and N_2O reducers

Out of expectation, $F_{-50\%}$ significantly decreased the abundance of nitrite reducers (both *nirK*- and *nirS*-harboring bacteria), while the abundances of N_2O reducers (both *nosZI*- and *nosZII*-harboring bacteria) were not affected. Because annual amount is low in these grasslands (280-370 mm during the growing season), soil NO_3^- is not prone to leaching [456]. However, under F_{-50}% individual precipitation events corresponded to a higher water amount on average, which likely enhanced the leaching of soil nitrate below the 0-10 cm soil layer, resulting in decreased N substrate for nitrite reducers. Consistently to the decrease in the abundances of both *nirK*- and *nirS*-harboring bacteria, DEA was significantly decreased by $F_{-50\%}$ as compared to $F_{ctrl,}$. Several previous studies have also found that changes in DEA are positively related to changes in the abundance of nitrite reducers (such as Yin et al. 2014[457]). Overall, N_2O emission tended to decrease with $F_{-50\%}$ likely due to a decreased production of N_2O (decreased abundance of nitrite reducers and DEA whereas the abundance of N_2O reducers was not affected.

Denitrification pulses were reported after water addition in several arid and semiarid ecosystems [458-460]. However, in this study, the abundances of soil nitrite reducers, DEA and N_2O emission showed no significant response to the main treatment of $F_{+50\%}$ (except a decrease in the abundance of *nirS*-harboring bacteria). This is maybe because under $F_{+50\%}$, individual precipitation events were associated to too low water amount (0.5 to 8 mm on average) to really induce anaerobic conditions in the upper soil layer.

Taken together, our results illustrate that the four groups of denitrifiers studied often responded in different ways to the main effects of the 3 global change factors, supporting the view that they have different ecological niches as previously reported by Assemien et al. (2019) [461], who reported that both *nirS*- and *nosZI*- harboring bacteria intensely depended on soil NO_3^- availability whereas *nirK*- and *nosZII*-harboring bacteria were mainly linked with soil pH and soil organic carbon. In addition, other studies, such as Hallin et al. (2009) also documented the niche differentiation between *nosZI*- and *nosZII*–harboring bacteria in a review paper, which is consistent with my results [360].

3.5.2 Importance of Interaction Effects Between Reduced Precipitation Amount and Frequency and N Deposition on Soil Denitrifiers and N_2O Emissions

A significant interaction effect of N×F was observed on the abundance of *nirK*-harboring bacteria. The effect of N deposition on the abundance of *nirK*-harboring bacteria was negative under F_{ctrl}, but altered precipitation frequency dampened the negative effect. This is maybe because altered precipitation frequency changed soil NO_3^- vertical distribution by leaching (see discussion above), modifying the availability of N substrate for *nirK*-harboring bacteria. In addition, under N_0, altered precipitation frequency (either $F_{-50\%}$ or $F_{+50\%}$) significantly decreased the abundance of *nirK*-harboring bacteria. However, when N deposition co-occurred with precipitation, the effect of decreased precipitation frequency tended to be positive. Under N_0, soil *nirK*-harboring bacteria were likely very N limited in this soil, and the $F_{-50\%}$ treatment induced larger water pulses that could move more soil NO_3^- into deeper soil layers (outside the 0-10cm layer sampled). The $F_{+50\%}$ treatment increased plant below-ground growth (data not shown), likely increasing the competition for nitrate between plant and *nirK*-harboring bacteria. When precipitation events were coupled to wet N deposition, the additional input of N into the soil alleviated these two mechanisms, which likely explained the observed interaction effect.

A significant N×A×F interactive effect was found on the abundances of both *nosZI* and *nosZII*-harboring N_2O reducers. For instance, under control conditions for precipitation, $A_{ctrl}F_{+50\%}$, and $A_{-30\%}F_{ctrl}$, N_{10} did not significantly influence *nosZI* abundance. In contrast N_{10} decreased *nosZI* abundance under the $A_{ctrl}F_{+50\%}$, $A_{-30\%}F_{-50\%}$ and $A_{-30\%}F_{+50\%}$ treatments. Under $A_{-30\%}$, N_{10} decreased the abundance of *nosZI*-harboring N_2O reducers. It is difficult to interpret these results, because the ecological drivers of N_2O reducers are less known than those of nitrite reducers, and because the global change factors likely have multiple effects on plant growth and labile C exudation by roots, soil N availability (either directly or through plant-microbes competition), soil water balance and oxygen level in the soil atmosphere (either directly for the nature of water inputs by rainfall events, or indirectly through an influence on leaf area and transpiration). For instance, N addition increased soil N availability, which favor the growth of *nosZI*-harboring bacteria; but decreased P amount may induce higher soil O_2 concentration by decreasing SWC, which would be unfavorable. Further, $F_{+50\%}$, which indicates more dry-rewetting cycles, may further affect soil water and O_2 state and affect plant belowground biomass. This supports previous reports indicating that precipitation frequency can strongly influence soil nutrient cycling [410] and that N deposition can influence soil water balance [307].

Similarly, interaction effects between global change factors (N x A and to a lesser extent N x F) were observed on N_2O emission. Under N_0 condition, $A_{-30\%}$ significantly increased soil N_2O emission, and this could result from a synergy between the positive response of the abundance of *nirK*-harboring bacteria and the increased NO_3^- concentration. In contrast, when N deposition co-occurred with precipitation events, the response of N_2O emission to $A_{-30\%}$ was not significant anymore. This was explained by the lower response ratios of both SWC and NO_3^- concentration. Under $A_{ctrl,}$ soil N_2O emission increased by N deposition via increasing NO_3^- concentration. In contrast, when precipitation amount decreased ($A_{-30\%}$), no stimulation of N_2O emission by N deposition, which maybe resulted from soil water limitation. However, overall and out of expectation, soil N_2O emission was negatively correlated with soil water content across the different treatments.

Taken together, these results show that chronic wet N deposition co-occurring with rainfalls lead to decreased or unchanged N cycling rates and slight increase of N_2O emissions, which is a rather unusual situation as compared to previous studies conducted in other grassland ecosystems. This may be due to the remarkable ability of dominant plants of the Songnen grassland to efficiently capture N associated to precipitation events (thus restricting any positive effect of N deposition on N availability for soil microbes) and grow accordingly (thus increasing soil water uptake and enhancing drought stress for soil microorganisms). In addition, significant interaction effects were observed between altered rainfall frequency and N deposition on N_2O emissions and many soil abiotic and biotic variables, which shows that studies focusing only on altered precipitation regime or only on wet N deposition are not sufficient to infer how global change will affect N dynamics in terrestrial ecosystems.

3.6 Summary

Using a mesocosm experiment for the semiarid Songnen grassland, we analyzed for the first time the effects of chronic N deposition, reduced precipitation amount and altered frequency, along with all their interactions, on the abundances of soil nitrite reducers and N_2O reducers, DEA, and soil N_2O emission. Surprisingly, N deposition tended to decrease denitrifier abundances and DEA in this semiarid grassland. This was explained by the very efficient capture of added N by the dominant grass species and by the increased plant growth leading to increased transpiration of decreased soil moisture with N deposition. The main effects of decreased precipitation amount and altered precipitation frequency differed between the four denitrifier groups studied. For instance, *nirK*- and *nirS*-harboring nitrite reducers and *nosZI*-harboring N_2O reducers were more sensitive to N deposition than *nosZII*-harboring N_2O reducers, and *nirK*- and *nirS*-bacteria had opposite responses to reduced precipitation, which supports the view that these groups have distinct ecological niches. The responses of the abundance of each denitrifier group, denitrifying enzyme activity and soil N_2O emission to N deposition, altered precipitation amount and frequency imply complex interaction effects between these three global change factors. It is thus impossible to predict how denitrifiers and denitrification respond to global change scenarios involving multiple factors only from the knowledge of single factor effects. In particular, interactive effects between N deposition and decreased precipitation amount, and N deposition and altered precipitation frequency were observed on soil N_2O emission, which was linked to changes in the abundance of *nirK*-harboring bacteria, soil water content and soil nitrate content. This illustrates the complex interplay that occurs between the water and nitrogen cycle and depends on the global change scenario considered. Our results demonstrate the need to analyze the rarely studied interactions between precipitation frequency, precipitation amount and wet N deposition to adequately predict how global change will affect soil N dynamics and N_2O emissions by soil in the future.

4 Responses of Soil Nitrifiers to a Fire Disturbance and Multi-factorial Global Change Scenarios in a Mediterranean Grassland

4.1 Introduction

Many Californian ecosystems are threatened by multiple global change factors that will co-occur, including increased atmospheric CO_2 concentration, warming, altered precipitation regime, and enhanced nitrogen (N) deposition. Along with the increase of global consumption of fossil fuels, the atmospheric concentration of CO_2 has been rising from ~280 ppm in 1750 to nearly 400 ppm now [110], and is expected to reach around 605-755 ppm at the 2070 horizon [462, 463]. Surveys over the past 50 years suggest that warming may be operating in California [464], and model simulations indicate that annual temperature is likely to rise by 1.7-2.2°C in the next century in California [121, 464], with a trend toward warmer winter and spring temperatures [464]. Changes in precipitation predicted for California differ between climate models. For example, one model (Parallel Climate Model) indicated that precipitation might increase slightly in Northern California by the end of century, whereas another model (Geophysical Fluid Dynamics Laboratory CM2.1) predicted decreased precipitation [464]. According to Field et al. (1999), winter precipitation will increase and will more often fall as the form of rain than snow [121]. The increase of atmospheric N deposition and its effects on ecosystems in the United States have been recognized for longtime [465], dry deposition of nitrate being particularly high over California [466]. In western United States, N deposition ranges from 1 to 4 kg ha^{-1} yr^{-1}, reaching 30 to 90 kg ha^{-1} yr^{-1} in some urban and agricultural areas [467]. Stickman et al. (2019) estimated the amount of the N deposition in 2013 and 2014 in California to be *ca.* 29 kg ha^{-1} yr^{-1} [468].

An additional aspect of global change impacting Californian ecosystems is fire. California is indeed a fire-prone area where fire activity has greatly increased over the recent years[159]. Westerling et al. (2006) attributed this increase of the wildfires to warmer spring and summer temperatures [159]. This trend will likely be reinforced according to climate change scenarios [469, 470] and predicted increased fuel loads, implying a higher risk of large, damaging fires in parts of California.

Each of these facets of global change can affect key soil microbial functional groups including nitrifiers. Nitrification corresponds to the oxidation of ammonia into nitrite and nitrate, an important process for plant nutrition (role in N availability and ammonium/nitrate balance). Ammonia oxidation, driven by both ammonia oxidizing bacteria and archaea (AOB and AOA, respectively) [12], is often assumed to be the rate-limiting step of nitrification, but studies reported that nitrite oxidation can limit the rate of nitrification in disturbed soils [63, 64]. Dominant nitrite oxidizing bacteria (NOB) in soil, namely *Nitrobacter* and *Nitrospira,* can thus also play an important role in soil N dynamics. Increased atmospheric CO_2 concentration could affect nitrifiers mainly indirectly through modified plant growth, increased water use efficiency and cascading effects on soil water balance and/or altered C and nutrient dynamics [214, 215]. An effect of higher atmospheric CO_2 concentration has been reported on soil AOB and AOA [46, 214, 221, 471] and on NOB [26, 46].Warming has also been shown to influence nitrifiers [260, 391], although this depends on the amplitude of the increase in temperature [245]. Altered precipitation can affect soil nitrifiers, directly through its effect on soil moisture and indirectly through altered plant growth. N deposition has also been reported to influence soil nitrifiers as they critically depend on N supply [44, 472] (see the review in Simonin et al. 2015 [46]. In addition, fire can also influence soil nitrifiers as reported in grassland ecosystems [4]. As the effect of fire on soil temperature is often restricted to the very upper soil layer (e.g. 0-2 cm, Delmas et al. 1995 [364]), fire effect on nitrifiers is likely mainly indirect, through changes of soil carbon and nitrogen pools [473], soil texture [379, 474], aggregate formation [475], bulk density [379], pH [476], water content [368], and plant compartment [377].

The previous studies cited above have mostly focused on the effects of one or two global change factors. Fewer studies have reported the effects of multiple (≥3) interactive global change factors on soil nitrifying groups [26, 205, 214, 259]. Actually, the response of soil nitrifying groups to multiple global change factors and fire disturbance has only been reported by Docherty et al. (2012) [4], but focusing only on AOB. In addition, Docherty et al. (2012) only reported one date after fire (9 months after wild-fire) and with only 2 blocks for wild-fire treatment [4]. The temporal responses of the major soil nitrifier groups to fire under different global change scenarios involving factors like CO_2, warming, changed precipitation regime and N deposition, have never been evaluated so far.

We studied the responses of four major groups of soil nitrifiers (AOB, AOA, *Nitrobacter* and *Nitrospira*) to a prescribed fire disturbance under 16 global change scenarios, based on the Jasper Ridge Global Change experimental site in California [109, 214]. The experimental design involves 5 factors (fire, CO_2, warming, precipitation, N deposition) with 2 levels for each, and all their possible combinations, leading to 32 treatments (16 global change scenarios, each with or without fire) replicated 4 times. The responses of the abundances of the four nitrifier groups (by quantitative PCR) just before fire, just after fire, and 9 and 33 months after the fire were surveyed. The objectives were to:

(1) evaluate the importance of the main effect of each factor and of 2-, 3-, 4- and 5-way

interaction effects on nitrifier group abundances. A neutral assumption was that the number of significant effects detected would be proportional to the number of effects considered (i.e. with such an experimental design, the number of the main effects tested at each date for 4 nitrifier groups is 20 (4 groups x 5 factors), but the number of 4-way interaction effects tested is 20 for instance);

(2) analyze how these effects on nitrifiers change with time following fire. I assumed that the effects of main treatments like N deposition would be strong and visible at all dates and across treatments for AOB and *Nitrobacter*. Because AOB has lower phylogenetic diversity as compared to other N-cycling functional groups [5, 214], which may lead to somewhat high sensitivity in gene abundance [259]; and *Nitrobacter* are favored by high NO_2^- [67, 70]. However, the five global factors manipulated here are known to often act through indirect effects involving modified plant growth, soil properties, soil C and N cycling over time [365]. Thus, the expectation was that complex interaction effects could arise when different global change factors are combined, and that these effects could evolve according to time after fire. In particular, we assumed that fire effect on soil nitrifiers could differ depending on the global change scenarios considered and on the time since the initial disturbance, and will be dampened through time after the initial disturbance; and

(3) identify the main drivers explaining the effect of burning on the four groups of soil nitrifiers under different global change scenarios using measurements of soil environmental variables important for nitrifiers (moisture, ammonium, organic C and pH) that were previously collected. We assumed that the interaction effects observed between fire and other global change factors could be explained by similar interaction effects on key soil environmental variables.

4.2 Study Site

The study was conducted at the Jasper Ridge Global Change Experiment (JRGCE) in the Jasper Ridge Biological Preserve, located in the eastern foothills of the Santa Cruz Mountains in northern California 37°24'N, 122°14'W. The study site is characterized by a Mediterranean-type climate with a cool, wet winter from November to March, and a hot, dry summer from June to October. At the study site, mean annual air temperature is 13.3°C and average of precipitation 787 mm, more than 80% falling between November and March, 1998 – 2006 average [213]. The grassland is dominated by annual grasses (*Avena barbata* and *Bromus hordeaceus*) and annual forbs (*Geranium dissectum* and *Erodium botrys*) [397, 477]. The soil is a fine, mixed, Typic Haploxeralf developed from Franciscan complex alluvium sandstone [284, 388].

Thirty-two circular plots (2 m diameter) were established at the JRGCE in the fall of 1998, allowing four global-change factors manipulations with two levels (ambient and elevated) for each factor: atmospheric CO_2 concentration (ambient and +275 ppm) manipulated with a free-air CO_2

enrichment (FACE) system; temperature (ambient and soil surface warming of +1.5-2.0°C) by using overhead infrared heat lamps; precipitation (ambient and +50% above ambient with 3 weeks elongation of the growing season) manipulated first with drip irrigation (1998 – 2000) and then with sprayed sprinklers irrigation system; and N supply (ambient and +7 g $Ca(NO_3)_2$-N m^{-2} yr^{-1}) twice per year. Each year, the first simulated N deposition event is a 2 g N m^{-2} addition in solution early in the growing season (November) to mimic the accumulated N deposition flushed into the system with the first rains. The second simulated N deposition event occurs later in the season (January – February), with 5 g N m^{-2} addition as slow-release pellets (Nutricote 12 – 0 – 0, Agrivert, Riverside, California, United States) [396, 397]. Each plot was divided into four quadrants (each with *ca.* 0.78 m^2) using fiberglass barriers (0.5 in depth) to separate soil between neighboring quadrants and soil between plot and surrounding grassland. A randomized block split-plot design was used: the CO_2 and temperature treatments were manipulated at the plot level, while the precipitation and N treatments were manipulated at the subplot level The levels of treatments were chosen according to scenarios for central California at the end of the 21st century [109, 284, 396, 397]. Eight replicates (blocks) for these 16 global change treatments (all combinations between the 4 factors with 2 levels: 2^4) were set up initially. An accidental and low-intensity fire occurred in two of the eight blocks of the experiment in July 2003 [284, 377, 388]. A controlled burned was conducted in July 2011 to mimic a fire disturbance in half of the 8 blocks (including the 2 previously burned ones), resulting in five factors and all their combinations (total of 32 treatments) with four replicates for each.

4.3 Materials and Methods

4.3.1 Experimental Design

See chapter 1.4.2.

4.3.2 Sampling and Measurement

4.3.2.1 Plant and Soil Variables

Plant and soil variables were measured as part of a project former to the present Ph.D. and will be used here as putative drivers of the abundances of the nitrifiers groups. Soil cores (5 cm diameter; 7 cm deep) were sampled in each quadrant in July 2011 at two dates (4 days before fire and 2 days after fire), April 2012 (9 months after fire) and April 2014 (33 months after fire). Large roots and rocks were removed by hand and soil sample was thorough mixed. Fresh soil samples were used for measuring the following soil environmental variables: gravimetric soil water content (SWC), pH, dissolved organic carbon content (DOC) and NH_4^+ concentration. Above-ground net plant primary productivity (ANPP), below-ground net plant primary productivity (BNPP) and total net plant primary productivity (TNPP) were assessed according to Dukes et al. (2005) [396]. Soil nitrifying enzyme activity (NEA) data obtained on the same soil samples part of another study (Niboyet, Le Roux et al, pers. com) were used to determine whether abundances of nitrifier groups correlated with NEA.

4.3.2.2 Soil DNA Extraction and Quantification of the Abundance of Nitrifier Groups

We used *ca.* 0.5 g of frozen soil to extract soil DNA using PowerSoil DNA isolation kit (MO BIO laboratories, Carlsbad, CA, USA) according to the manufacturer's instructions. The quantity of extracted DNA was assessed using the Quant-iT™ Picogreen dsDNA Assay Kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

The abundances of ammonia-oxidizing bacteria (AOB) and ammonia-oxidizing archaea (AOA) were measured by quantitative PCR targeting the *amoA* functional gene encoding for ammonia monooxygenase. The sets of primers used were AmoA-1F/AmoA-2R for AOB [426] and CrenamoA23f/CrenamoA616r for AOA [425]. The final reaction volume was 20 µLand contained (final concentrations) 0.5 µMof each primer for the bacterial *amoA* or 1 µM of CrenamoA23f and 0.75 µM of CrenamoA61 for archaeal *amoA* , along with 2 % bovine serum albumin (BSA), 1× of QuantiTect SybrGreen PCR Master Mix (Qiagen, Courtaboeuf, France) and 10^2–10^7 gene copies number per microliters of the target DNA sequences, using a linearized plasmid containing cloned archaeal *amoA* genes of 54d9 fosmide fragment [11] and bacterial *amoA* genes of *Nitrosomonas europaea* DNA (GenBank accession number: L08050) as standards. The samples were run on a Lightcycler 480 (Roche Diagnostics, Meylan, France) as follows: for AOA, first with 15 min at 95° C for initial denaturation step; 50 amplification cycles (45s at 94°C, 45s at 55°C, and 60s at 72°C); one cycle melting step (15s at 95°C, 30s at 60°C and continuous 95°C; and one cycle cooling step with 10s at 40°C ; for AOB, first with 15 min at 95°C for initial denaturation step; 45 amplification cycles (30s at 95°C, 45s at 54°C, 45s at 72°C, and 15s at 80°C); one cycle melting step (1s at 95°C, 20s at 68°C and continuous at 98°C ; and one cycle cooling step with 10 s at 40°C. Melting curve analysis confirmed the specificity of amplification for both AOA and AOB. Amplification efficiencies of 81-87% obtained for AOA and 87-90% AOB quantification, respectively.

The abundance of *Nitrospira* was measured by quantitative PCR targeting the *16S rRNA* gene sequences specific for this group [10]. The sets of primers used were Ns675f and Ns746r [478]. The final reaction volume was 25 µL, containing 1× of QuantiTect SybrGreen PCR Master Mix (Qiagen, Courtaboeuf, France), 0.4 µM of each primer, and 10^2–10^7 *Nitrospira* copies using a linearized plasmid DNA (GenBank accession number: FJ529918). The samples were run on a Lightcycler 480 (Roche Diagnostics, Meylan, France) as follows: first with 15 min at 95°C for initial denaturation step; 45 amplification cycles (30s at 95°C, 30s at 66°C, and 1 min at 72°C); one cycle melting step (1s at 95°C, 20s at 68°C and continuous at 98°C); and one cycle cooling step with 10s at 40°C. Melting curves confirmed the specificity of the amplification. The amplification efficiency was 85% and no PCR inhibition was observed during tests by dilution.

The abundance of *Nitrobacter*-like *NOB* was quantified by targeting the functional gene *nxrA* [10], encoding for the nitrite oxidoreductase. The sets of primers used were F1norA and R2norA [10, 479]. The final reaction volume was 20 μL, containing 1× of QuantiTect SybrGreen PCR Master Mix (Qiagen, Courtaboeuf, France), 0.5 μM of each primer, and 10^2–10^7 gene copies using a linearized plasmid containing cloned *nxrA* gene of *Nitrobacter hamburgensis* X14 (DSMZ 10229). The samples were run on a Lightcycler 480 as follows: first with 15 min at 95°C for initial denaturation step; 45 amplification cycles (30s at 95°C, 45s at 55°C, and 45s at 72°C); one cycle melting step (5s at 95°C, 1 min at 65°C and continuous at 97°C; and one cycle cooling step with 10s at 40°C. The amplification efficiency was 89% and no PCR inhibition was observed during tests by dilution.

4.3.3 Data Analysis

Outliers were detected using Tukey's method prior to the statistical analyses: values greater than the 75th percentile plus 1.5 times the interquartile distance, or less than the 25th percentile minus 1.5 times the interquartile distance were treated as outliers (Shoemaker and North Haven 2008). The block effect was assessed using PROC GLM in SAS 9.4 (SAS Institute, Cary, NC, USA). If significant, data were normalized per block prior to the detection of the outliers (the ratio of the individual value to the average value of each block was calculated to normalize for the block effect). The outlier numbers of AOB are 4, 3, 0 and 1, respectively -0.13, 0.06, 9 and 33 months after fire; the corresponding outlier numbers of AOA are 2, 1, 0 and 2; of *Nitrospira* are 4, 0, 7 and 0; and of *Nitrobacter* are 8, 3, 5, and 0.

T-test was performed on the data obtained before the 2011 fire to assess a possible residual effect of the 2003 burning by comparing blocks with no burning, blocks with only one (2011) burning events, and with two (2003 and 2011) burning events.

First, data were first analyzed using PROC MIXED in SAS 9.4 using a full factorial split-plot analysis of variance in order to assess the overall effects of the burning disturbance and other global environmental changes treatments on the abundance of the four groups of soil nitrifiers (AOB, AOA, *Nitrospira* and *Nitrobacter*). Data were analyzed for each sampling date by including the CO_2, warming and burn treatments as whole-plot factors, and the precipitation and N treatments as split-plot factors. The normality of the residuals and the independence of the residuals related to the predicted values were analyzed, if these criteria were not met, data were transformed with Box-Cox using Minitab® 18.0 (Minitab Inc., PA, USA) software prior to analyses. Effects with $P < 0.05$ are referred to as significant.

Second, based on the results of the mixed-model analysis obtained for the four sampling dates, the most significant effect observed that including burning was selected for each nitrifier group. Accordingly, the effect of burning was compared under the different relevant global change scenarios using T-test. For example, the heat (H) × nitrogen (N) × burning (B) interaction effect was the most significant interaction effect involving burning observed across the sampling dates. Therefore, I focused on the H × N × B interaction effect and the burning effect was compared between the control, H, N and H × N conditions using *T-tests* (Fig. 45), checking data normality and homogeneity of variance after data transformation if needed. For graphical representation, main treatment effect size was calculated as: *Effect* = $T - C$, where the T is the value with treatment, and the C is the value with control. Effects with $P < 0.05$ are referred to as significant.

Third, in order to explore and hierarchize the links between changes in the abundances of soil nitrifiers following fire and environmental variables (SWC, pH, DOC, NH_4^+ and ANPP and BNPP), path analysis was performed using Amos25® (Amos Development Corporation, Crawfordville, FL, USA). The complete model with all possible causal relationships considered is presented in Fig. 41.

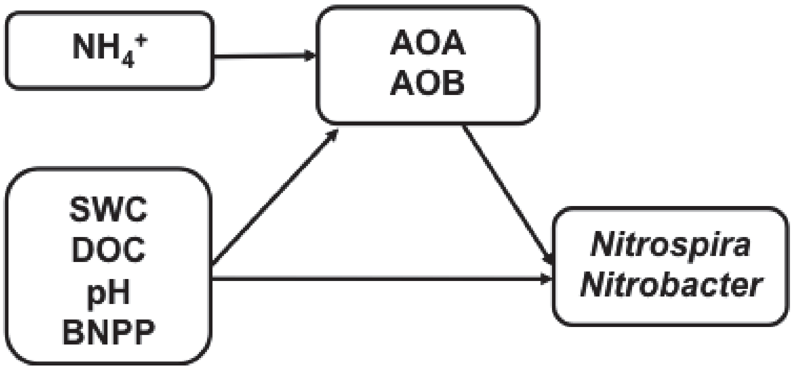

Fig. 41 Complete model used for identifying the main drivers of changes in the abundances of soil nitrifiers by structural equation modeling. Ammonia-Oxidizing Bacteria, AOB; Ammonia-Oxidizing Archaea, AOA. The variables included in the model are soil ammonium concentrations (NH_4^+), gravimetric soil water content (SWC), soil dissolved organic carbon content (DOC), soil pH (pH), and belowground net primary productivity (BNPP)

A χ^2 test was used to evaluate the model, *i.e.* whether the covariance structures implied by the model adequately fitted the actual covariance structures of the data (a non-significant χ^2 test with $P > 0.05$ indicates an adequate fit by the model). Within a model, the coefficients of paths indicate by how many standard deviations the explained variable would change if the causal variable was changed by one standard deviation.

4.4 Results

AOA abundances averaged across all treatments ranged from 2.08×10^5 copies g^{-1} soil to 3.89×10^6 copies g^{-1} soil copies g^{-1} soil and were higher than the abundances of other soil nitrifier groups except at 33 months after burning when *Nitrospira* abundance was the highest (Fig. 42). For each sampling date, the abundance of *Nitrospira* was higher than *Nitrobacter* abundance (from 4.66×10^5 to 1.26×10^6 copies g^{-1} soil for *Nitrospira*, and from 3.74×10^3 to 5.22×10^4 copies g^{-1} soil for *Nitrobacter*). The abundances of soil ammonia-oxidizers significantly varied between four sampling dates, whereas nitrite-reducers abundances were similar between the two first soil sampling dates (0.13 month before and 0.06 months after burning), but abundances tended to be lower at the 2 other dates and significant for *Nitrobacter*.

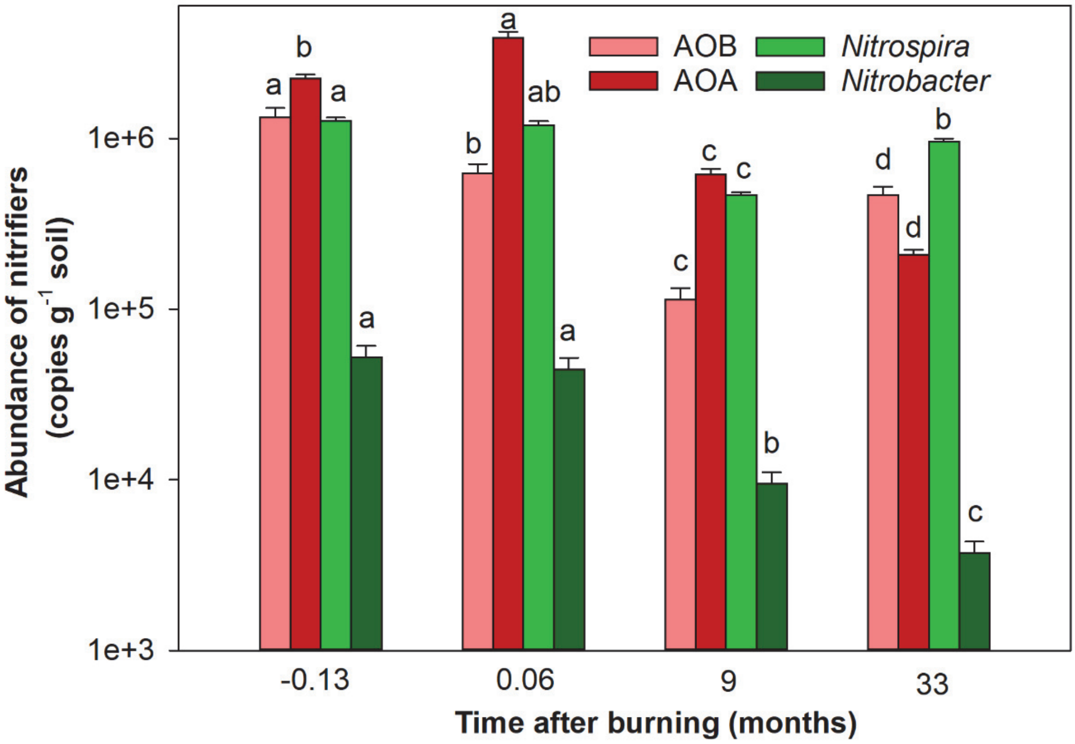

Fig. 42. The abundances of the four groups of soil nitrifiers averaged across all treatments at each of the four sampling dates: 4 days before burning (-0.13 month); 2 days after burning (0.06 month); 9 months after burning; and 33 months after burning. Bars are means + s.e. (n=128 minus outliers). Lowercases indicate significant difference between sampling dates of each group

4.4.1 Importance of the Main Effect and Interactive Effect of Fire and Other Global Change Factors on Nitrifier Abundances

Both significant main treatment effects and interactive treatment effects of global change factors including fire were observed on soil nitrifier abundances (Table 41). Overall and for the 3 sampling dates after burning and the 4 nitrifier groups considered, main treatment effects were the most commonly observed, followed by 3-way interactive effects (Fig. 43). I assessed to what extent the number of significant effects detected was linked to the number of tested possible effects. Overall, the percentage of significant effects across the 4 groups of nitrifiers and the 3 soil sampling dates after burning was 8.6%. Using this percentage and the number of tests performed, I computed an expected number of significant effects (black dots in Fig. 43) assuming an evenly distributed percentage of significant effects across the 1-, 2-, 3-, 4- and 5-way interactions. Focusing only on interactions including burning, and accounting for the number of possible interactions including burning, The expected number of significant effects including burning (red dot in Fig. 43) was also computed. The number of significant main effects was 1.8-fold higher than expected (Fig. 43). The number of significant 3-way interaction effects was close to the expected number based on the total percentage, whereas significant 2-, 4- and 5-way interactions were less numerous than expected (Fig. 43). The significant main treatment effects detected were dominated by N deposition, with a lesser importance of burning and precipitation. No significant main effect of CO_2 or heat was found (Table 41; Fig. 43). The number of significant main burning effects was higher than expected. For the 2-, 4 - and 5-way interaction effects including burning, the number of significant effects observed was lower than expected, whereas for 3-way interaction effects, the number of significant effects observed was as expected based on the mean percentage of significant effects (Fig. 43).

Table 41 Mixed model results summarizing the significant effects of global change factors (CO_2, C; heat, H; precipitation, P; N deposition, N; Burning, B) and their interactions on the abundances of soil nitrifiers detected at the 4 sampling dates: 4 days before burning (-0.13 month); 2 days after burning (0.06 month); 9 months after burning; and 33 months after burning. Ammonia-oxidizing oxidizers, AOB; ammonia-oxidizing archaea, AOA. Values between brackets are *P* values.

Nitrifiers	Effects	-0.13 month	0.06 month	9 months	33 months
AOB	Main effects	N(0.0021)	N(<0.0001)	B(0.0330) N(<0.0001)	B(0.0499) N(0.0324)
	Interactive effects including B		BCP(0.0395)	BN(0.0287)	BCHP(0.0254) BHN(0.0097)
	Interactive effects excluding B	HPN (0.0065)	HN (0.0199) CPN(0.0175) HPN (0.0394)		
AOA	Main effects	N(0.0082)	N(0.004)	N(0.0001)	
	Interactive effects including B			BCP(0.0374) BCHN(0.0428)	BHN(0.0265)
	Interactive effects excluding B				CN(0.0085)
nxrA	Main effects		P(0.0031) N(0.0466)	P(0.0014) N(0.0002)	N(0.0011)
	Interactive effects including B		BPN(0.0180)	BHPN(0.0018)	
	Interactive effects excluding B		CN(0.0094)	CH(0.0328)	
Ns	Main effects			P(0.0355)	B(0.0058)
	Interactive effects including B			BCH(0.0105)	
	Interactive effects excluding B	HPN(0.0056)		CN(0.0312) CPN(0.0245)	

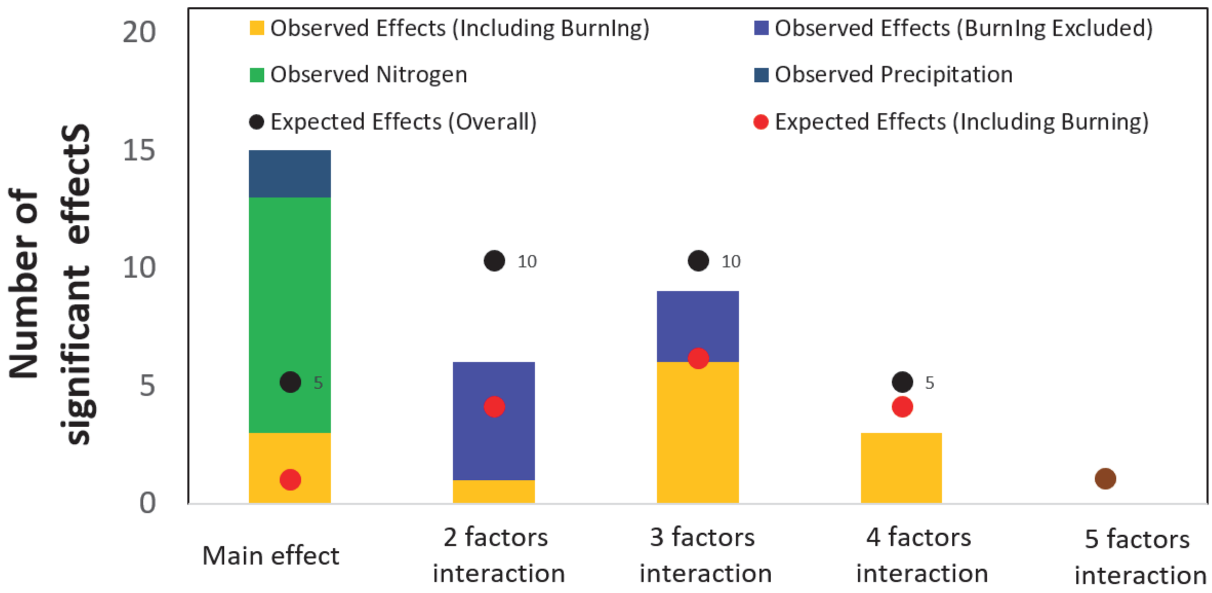

Fig. 43 Main and interactive effects of global change factors (CO_2, heat, precipitation change, and nitrogen deposition) and burning on the abundances of four groups of soil nitrifiers (Ammonia-Oxidizing Oxidizers, Ammonia-Oxidizing Archaea, *Nitrospira* and *Nitrobacter*) across the 3 sampling dates after burning. Columns indicate the observed number of significant effects. For multi-factor interactions, effects including burning (orange) or not (blue) were distinguished. Dots indicate the expected number of significant effects based on the observed mean percentage of effects that were significant (i.e. 8.6%) and the number of effects tested across the 4 groups of the nitrifiers and 3 sampling dates. Expected number of significant effects in total (black) or focusing only on effects including burning (red) were distinguished

4.4.2 Temporal trends in the Effects of Main Global Change Factors on Soil Nitrifiers

Among the four groups of nitrifiers, *Nitrospira* was the only one insensitive to the main effect of nitrogen (N) deposition, and was sensitive to precipitation (P) and burning (B) at one sampling data only (Fig. 44). Overall, a positive main N effects was observed on the three other groups of nitrifiers, the abundances of AOB and *Nitrobacter* being the most increased by N deposition (Table 41, Fig. 44-Left-Top).

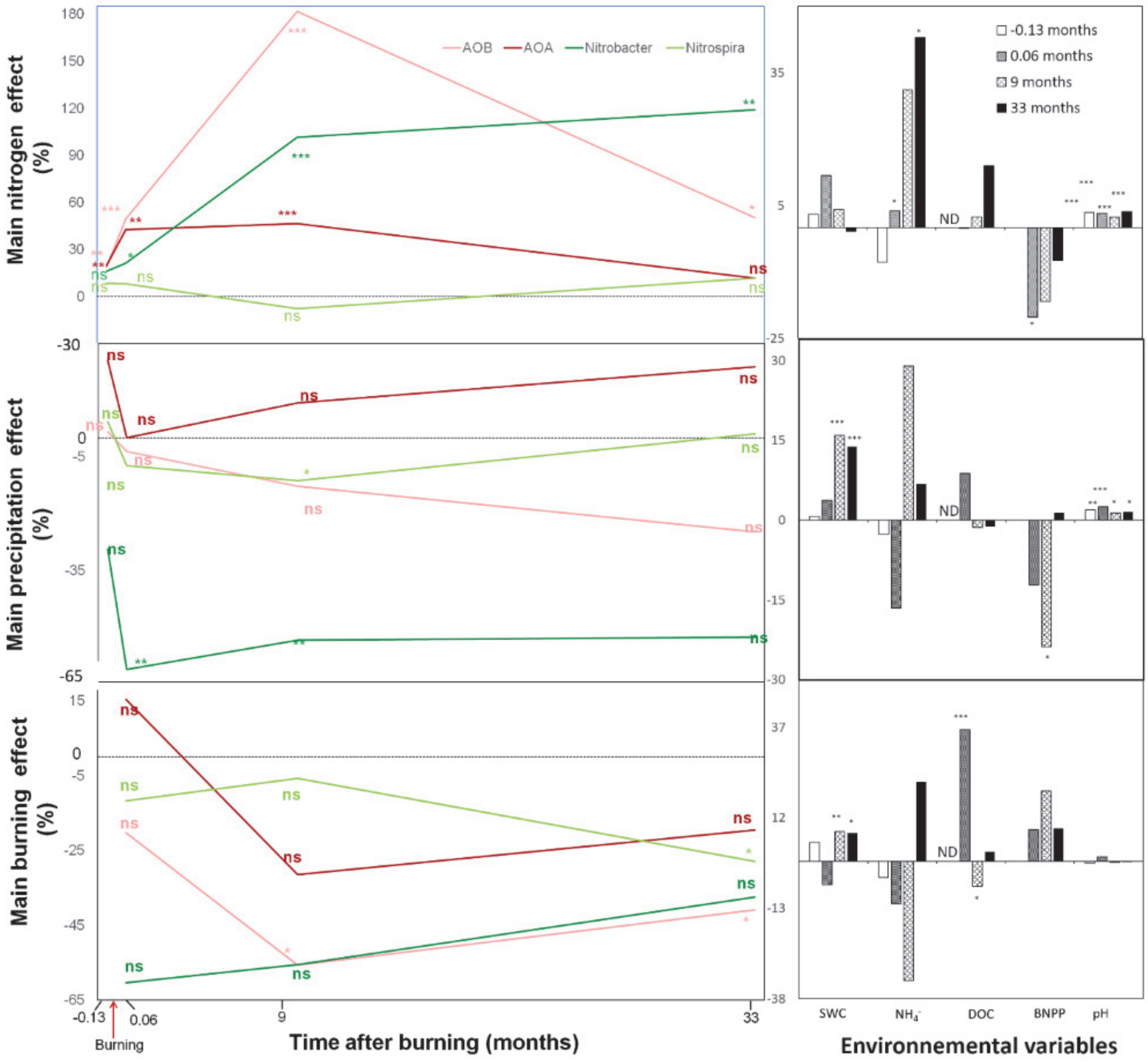

Fig. 44 Main effects of (Top) nitrogen addition, (Middle) precipitation change and (Bottom) burning on (Left) the abundances of soil nitrifiers (Ammonia-Oxidizing Bacteria, AOB; Ammonia-Oxidizing Archaea, AOA; *Nitrospira* and *Nitrobacter*) and (Right) environmental variables (soil water content ,SWC, soil ammonium concentration, NH_4^+, soil dissolved organic carbon, DOC, and plant belowground net primary productivity, BNPP) immediately prior to burning (-0.13 month, i.e. 4 days prior to the fire), and at the three sampling dates after burning (0.06 month, i.e. 2 days after burning, and 9 and 33 months after burning). ns, not significant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ND, no data

The N-induced increase in the abundance of AOB and AOA tended to be higher at the last two dates (corresponding to end of plant growing season) and lower at the first two dates (dry season). Concurrently, N deposition had a positive effect on NH_4^+ and soil DOC concentration at the last sampling date (+42.9% and +14.0% for NH_4^+ and soil DOC, respectively). In contrast, BNPP negatively responded to the main N treatment, especially in the burning year (2011) (7.4% decrease in response to the main N treatment; Fig. 43-Top- Right).

The main P effects were of lesser amplitude and more variable than the main N effect (Fig. 43-Middle). The main P effect tended to be positive (but was never significant) on the abundance of AOA, whereas the abundances of AOB and *Nitrospira* tended to decrease with P, the main P effect being significant only for *Nitrospira* 9 months after fire. In contrast, a strong and negative main P effect was observed on *Nitrobacter* abundance, ranging from -60% to -70% for the three dates after fire. Changed P also significantly increased SWC during plant peak biomass periods at 9 (by 16.0%) and 33 (by 13.8%) months after burning whereas the effect on soil NH_4^+ and DOC were variable. Further, changed P induced a significant decrease of BNPP 9 months after burning (-23.8%).

The main B treatment tended to negatively affect the abundances of the four nitrifiers groups except for AOA just after burning (Fig. 44-Left-Bottom), but effects were not significant, except 9 and 33 months after fire for AOB, and 33 months after fire for *Nitrospira*. A significant main B effect was observed on the abundance of AOB both 9 and 33 months after burning (-56% and -41%, respectively), and on the abundance of *Nitrospira* 33 months after burning (-28%). Across all treatments, burning significantly increased SWC by 8.1% and 7.8% after 9 and 33 months, respectively. The responses of NH_4^+ and DOC varied with sampling dates. In particular, B significantly increased DOC (+36.3%) just after burning but decreased DOC (-6.9%) 9 months after burning. BNPP did not significantly respond to the main B effect (Fig. 43-Right-Bottom)

4.4.3 Responses of Soil Nitrifiers to Burning according to Global Change Scenarios

4.4.3.1 Ammonia oxidizing bacteria and archaea

For both the abundances of AOB and AOA, the most significant effect including burning corresponded to the 3-way heat (H) × nitrogen (N) × burning (B) interaction 33 months after burning (*P* value of 0.0097 and 0.0265, respectively) (Table 41), so that this interaction effect was focused.

Under N deposition only, a significant positive burning effect on AOB abundance was found just after burning, but a negative larger burning effect 9 months after burning ($P < 0.01$) with a similar, not significant trend observed 33 months after burning. Under warming (H) only, burning significantly decreased AOB abundance 33 months after fire. A significant negative burning effect was also found under elevated H and N conditions 9 months after fire (Fig. 45-Top).

For AOA abundance, a significant burning effect was only observed under the H scenario 33 months after fire (9.90×10^4 copies g^{-1} soil and 2.29×10^5 copies g^{-1} soil under the HB and B treatments, respectively) (Fig. 46-Top).

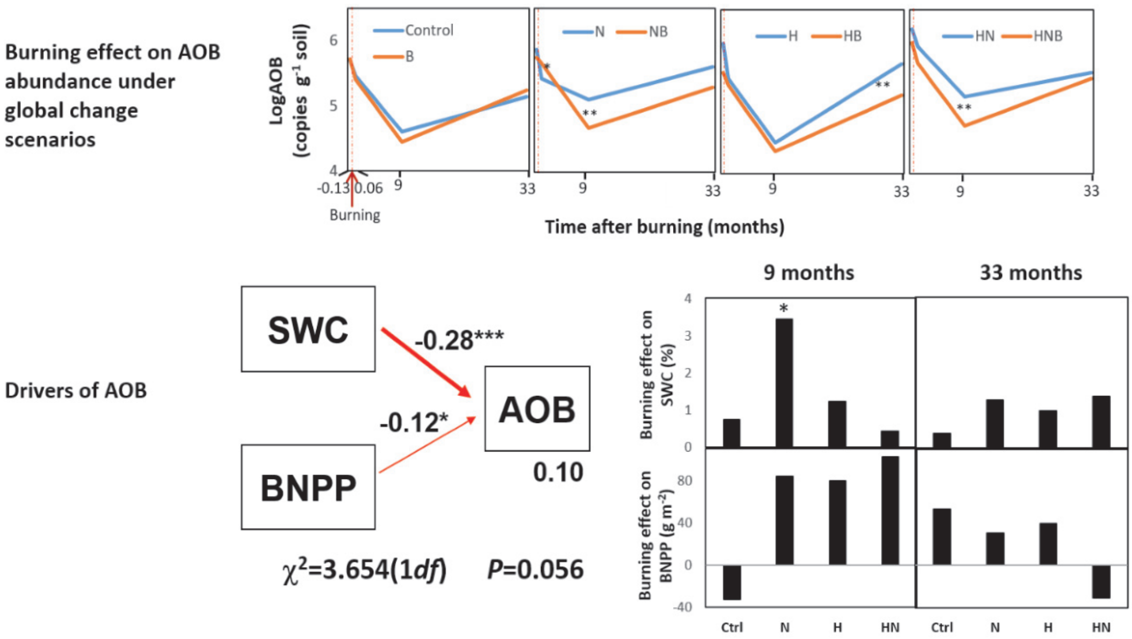

Fig. 45 (Top) Abundance of soil ammonia-oxidizing bacteria (AOB) under different global change scenarios (Control, Ctrl; N, N deposition; H, heat) with and without burning (B). (Bottom-Left) Structural equation model results relating AOB abundance to soil water content (SWC) and belowground net primary productivity (BNPP). Values near the arrows are path coefficients. The red arrows indicate negative correlations. The percentage of variance explained by the model is indicated at the bottom-right of the corresponding box. (Bottom-Right) Burning effect on SWC and BNPP under the relevant scenarios, 9 and 33 months after burning. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$

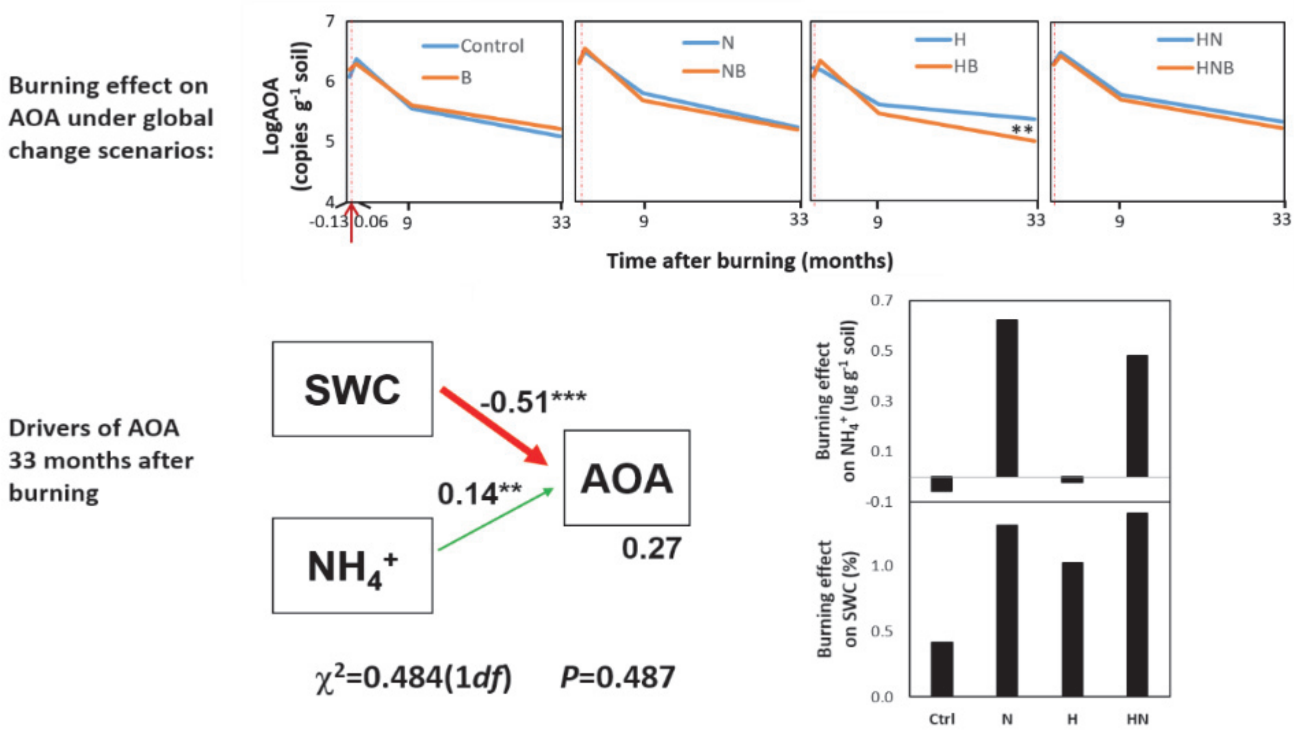

Fig. 46 (Top) Abundance of soil ammonia-oxidizing archaea (AOA) under different global change scenarios (Control, Ctrl; N, N deposition; H, heat) with and without burning (B). (Bottom-Left) Structural equation model results relating AOA abundance to soil water content (SWC) and ammonium content (NH_4^+). Values near the arrows are path coefficients. The green and red arrows indicate a positive and negative correlation, respectively. The percentage of variance explained by the model is indicated at the bottom-right of the corresponding box. (Bottom-Right) Burning effect on SWC and NH_4^+ under the relevant scenarios, 9 and 33 months after burning. **, $P < 0.01$; ***, $P < 0.001$

4.4.3.2 Nitrite oxidizing bacteria

Regarding *Nitrobacter* abundance, the most significant effect including burning corresponded to the H × P × N × B interaction 9 months after fire (P=0.0018) (Table 41). Burning significantly decreased *Nitrobacter* abundance under PN and HP conditions 9 months after fire, and under HPN conditions 2 days after fire.

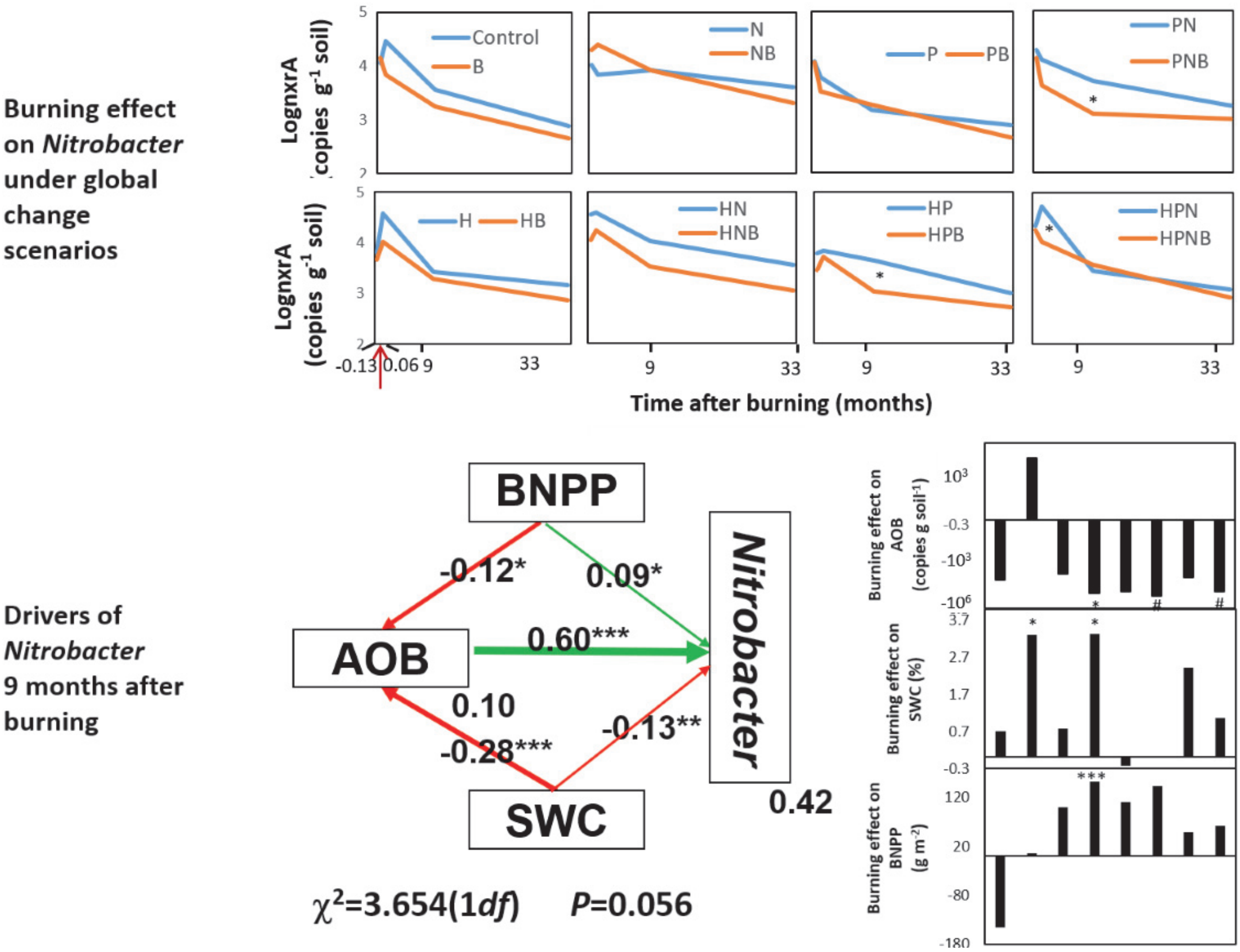

Fig. 47 (Top-Left) Abundance of soil *Nitrobacter* under different global change scenarios (Control, Ctrl; , N deposition; P, precipitation) with and without burning (B). (Bottom-Left) Structural equation model results relating *Nitrobacter* abundance to ammonia-oxidizing bacteria (AOB), soil water content (SWC) and below-ground net primary productivity (BNPP). Values near the arrows are path coefficients. The green and red arrows indicate a positive and negative correlation, respectively. The percentage of variance explained by the model is indicated at the bottom-right of the corresponding box. (Bottom-Right) Burning effect on AOB, SWC and BNPP under the relevant scenarios 9 months after burning. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$

Regarding *Nitrospira* abundance, the most significant effect including burning was the main B effect observed 33 months after burning ($P = 0.0058$) (Table 41). At this date, burning significantly decreased the abundance of *Nitrospira* from 1.03×10^{6} to 7.32×10^{5} copies g^{-1} soil (Fig. 48-Top-Left), whereas no significant effect was found at the other dates.

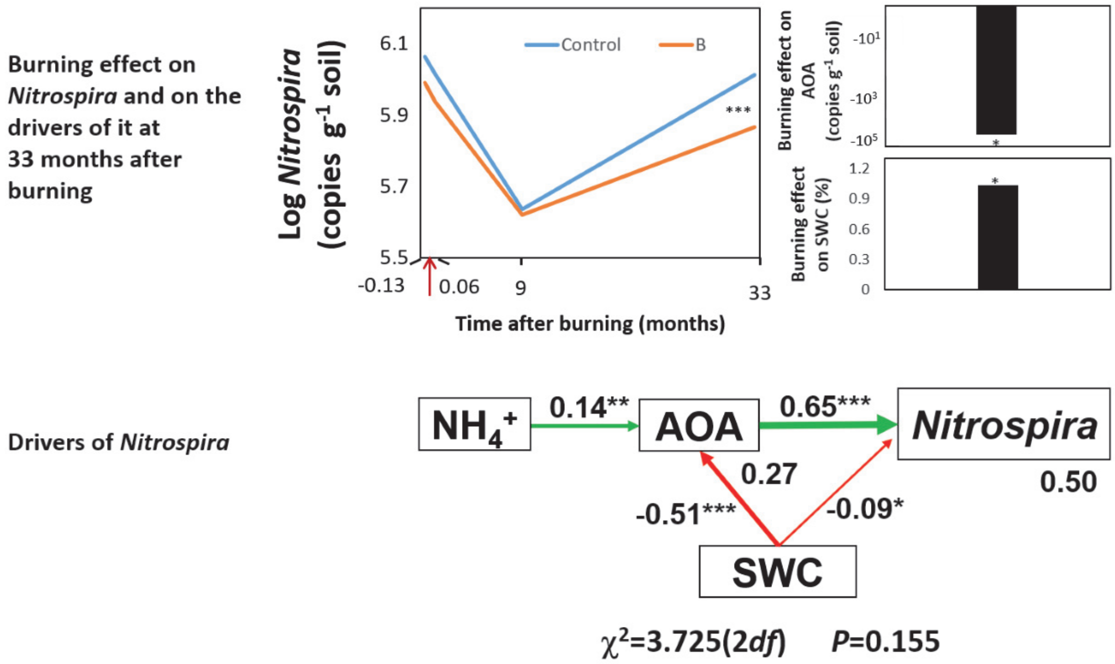

Fig. 48 (Top-Left) Abundance of soil *Nitrospira* under control (Ctrl) and burning (B) condition. (Top-Right) Burning effect on SWC and abundance of AOA under the relevant scenarios observed 33 months after burning. (Bottom) Structural equation model results relating *Nitrospira* abundance to soil water content (SWC), abundance of soil ammonia-oxidizing archaea (AOA) and ammonium concentration (NH_4^+). Details on graphical presentation and colours as in figure Fig. 47*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$

4.4.4 Identification of the Main Drivers of Nitrifier Abundances under the Burning and Global Change Treatments

Structural equation modelling (SEM) showed that AOB abundance was negatively related with SWC and to a lesser extent with BNPP (Fig. 45-Bottom-Left). SWC and BNPP however explained only 10% of the total variance in AOB abundance (Fig. 45-Bottom-Left). AOA abundance was also negatively related with SWC, and was positively related to NH_4^+. SWC and NH_4^+ concentration explained 27% of the total variance of AOA abundance (Fig. 46-Bottom-Left).

The abundance of *Nitrobacter* was explained (42% of total variance) by AOB abundance, BNPP and SWC, with a prominent role of AOB abundance and SWC (Fig. 47-Bottom-Left). The abundance of *Nitrospira* was positively related to AOA abundance and to a lesser extent negatively related to SWC. AOA and SWC explained 50% of the total variance in *Nitrospira* abundance (Fig. 4Bottom).

4.5 Discussion

4.5.1 Importance of Main Effects and Interactive Effects of Fire and Global Change Factors on Nitrifier Abundances

As compared to an even distribution of significant effects independently of the number of factors interacting (i.e. number of significant effects just proportional to the number of effects tested), the number of the main treatment effects that were significant was two-fold than expected, with a prominent role of the main nitrogen deposition (N) treatment, and to a lesser extent precipitation (P) and burning (B). This is consistent with previous research results for the same experimental site reporting that both plant and soil compartments in this grassland ecosystem are particularly responsive to the nitrogen supply [2, 232, 284, 396]. In addition, Niboyet et al. (2011b) [213] found significant response of soil nitrification rates to nitrogen and increased precipitation, but no response to CO_2 and warming. Regarding the other main effects of P and B, our results are also consistent with previous studies. In particular, a main effect of precipitation was observed on potential nitrification rate in addition to a main N effect, whereas no main effect of warming and CO_2 was found [284]. Similarly, Brown et al. (2012) observed a significant main effect of precipitation on N_2O emissions but no effect of warming and CO_2 [232]. This is likely due to the fact that the N treatment corresponded to a rather high N input applied over 13 years before our first soil sampling, inducing a high increase in soil mineral N as already observed [194, 214]. In addition, modified precipitation has been shown to have both direct effects on soil moisture [480] and cascading effects on plant growth and C and N cycling at Jasper Ridge, and all these effects can influence soil nitrifiers through altered oxygen, N and C availabilities. In contrast, heating intensity was weak at the study site, with a low increase of soil temperature initially with +0.8 to 1°C for 12 years and then +1.5 to +2°C [481], which is quite realistic but lower than warming treatments applied in other warming experiments on grasslands [205, 267]. Similarly, Zhang et al. (2013) reported no significant response of the abundance of soil ammonia-oxidizers to heating in a steppe ecosystem [259]. In addition, the +275 ppm increase in atmospheric CO_2 concentration could have only indirect effects on soil nitrifiers through plants because CO_2 concentration in the soil atmosphere has already reached ~ 400 ppm in 2013 [482]. This likely explains why no significant main effect of CO_2 was observed and that this factor rarely appeared in the significant interaction effects. This is consistent with previous studies reporting a lack of elevated CO_2 effect on the abundances of ammonia oxidizers in dryland ecosystem [260, 391], and in the rhizosphere of white clover and ryegrass cultured in dystric cambisol [219]. In addition, in the same experimental site, Horz et al. (2004) found that significant CO_2 effect on the abundance of AOB can only be found when combined with increased precipitation [214].

For interaction effects, the number of significant effects was lower than expected in particular for the 2-, 4- and 5-way factor combinations. This was likely due to the fact that the N and B treatments induced strong effects that appeared more as main effects than interaction effects. Still, some strong interaction effects were detected for some nitrifier groups, interaction effects being observed in more than half of the cases (nitrifier group × date) studied. This shows that global change effects on soil nitrifiers are not predictable from single factor studies. Below I first discuss the main effects of global change factors on the abundances of soil nitrifier groups that were significant. I then discuss how the effect of burning on nitrifier abundances was affected by global change scenarios involving N deposition, elevated CO_2, modified precipitation and/or warming.

4.5.2 Response of Soil Nitrifier Groups to Main Treatment Effect of Global Change Factors

4.5.2.1 Nitrogen Deposition

AOA and AOB abundances increased with N deposition, the positive N effect being greater on AOB than AOA. This supports the view that AOB rather than AOA are favored by N addition [20, 44-47, 320, 327, 338, 433]. The response of AOA to N addition is actually more variable, and AOA abundance can even decrease following N addition [307, 337]. Based on a meta-analysis, Carey et al. (2016) found that on average N addition increases the abundance of AOA and AOB by 27% and 326%, respectively [47]. Our results are thus explained. In addition, Nitrogen deposition increased the abundance of *Nitrobacter* but not *Nitrospira.* This is consistent with a previous report on N addition effect on NOB for the same site [26] and other grasslands [44, 46]. Overall, our results support the view that a niche differentiation exists between the nitrifier groups regarding N availability [10, 44, 432], and that AOB and *Nitrobacter* have a greater fitness and greater N oxidation rates at higher N level than AOA and *Nitrospira* [8, 14, 67, 69, 70], although functional diversity exists within each group [101]. Moreover, the SEM analysis showed that the changes of AOB abundance explained 60% of the changes in *Nitrobacter* abundance. Our results thus support the view that a close relationship often exists between the abundances of AOB and *Nitrobacter* [44, 433], likely because increased AOB abundance induced an increased NO_2^- availability.

4.5.2.2 Increased Precipitation

Increased precipitation (P) had no significant effect on soil AOA, AOB and *Nitrospira* (except a slight negative effect observed for *Nitrospira* at one date), but strongly decreased *Nitrobacter* abundance. Consistently, Le Roux (2016) reported that increased precipitation reduced soil potential nitrite oxidation rate at the same grassland site. This could be due to the fact that soil *Nitrobacter* bacteria, include chemolitotrophs and mixotrophs [26]. Whereas chemolitotrophic *Nitrobacter* are mostly positively related to oxygen and N availabilities, mixotrophic *Nitrobacter* are favored by increased organic carbon availability and can withstand well decreased oxygen level [26]. The decrease of the total *Nitrobacter* abundance with increased precipitation could thus result from the concurrent increase of SWC that induced lower O_2 availability (negative effect on chemolitotrophic *Nitrobacter*) and decrease of BNPP that likely induced lower organic C supply by roots (negative effect on mixotrophic *Nitrobacter*). Consistently, the SEM analysis showed that *Nitrobacter* abundance was positively related to BNPP and negatively related to SWC, in addition to the positive relation observed with AOB abundance.

4.5.2.3 Burning

Published studies about fire impact on soil nitrifier abundances are rare in grassland ecosystems [4]. More studies focused on forest ecosystems [4, 365]. Considering studies on burning effects on total soil microbial abundance, variable effects were reported, from negative [365, 483] to positive [484-486] suggesting that multiple direct and indirect mechanisms underlie the burning effects. The most direct effect of burning is heating and increased soil temperature linked to fire intensity and duration [487]. This can lead to heat-induced mortality of soil microbes [362, 363]. Previous studies have indicated that fatal temperatures for soil microbes can be lower than 100°C [488]. However, in grasslands, with fires of similar intensity as observed in our study (fire speed of 2-5 m/min and flames height of 1-2 m), the heat wave did not penetrate very deep in soil. For instance, Delmas et al. (1995) reported that during a fire event soil temperature reached 100°C at the surface, 75°C at 1 cm depth, 50°C at 3 cm depth [364]; below no change in temperature was observed [364]. As we sampled the 0-7 cm soil layer, the direct effect of burning through heating is thus expected to be marginal. This is supported by the lack of any significant burning effect observed just after fire for AOA and *Nitrospira*, while only one (interaction) effect including burning was observed for AOB and for *Nitrobacter* but with a lower significance ($P > 0.01$) as compared to other effects. Similarly, no significant effect of a low intensity fire was found on bacterial abundance in a sclerophyll forest [25]. In contrast, the abundance of AOB was significantly reduced by the burning treatment 9 months after fire, while both AOB and *Nitrospira* abundances decreased in response to burning 33 months after fire. This demonstrates a lasting effect of burning on soil nitrifiers, likely through indirect effects, including on SWC as observed here and likely on the vegetation status. Similarly, increased soil water content [368, 489] and BNPP [367] after a burning event were reported in grassland ecosystems. Fire can also affect N inputs and losses and more generally the grassland N balance [4, 284], which could impact soil nitrifiers. For instance, burning can induce large amount of NH_3 volatilization, and more generally induces a loss of N from the aboveground plant parts. Here, no significant main effect of burning on soil NH_4^+ but only a negative trend, which could contribute to the limitation of AOB growth, was observed.

A striking result is the lasting effect of burning on soil nitrifiers, two groups remaining impacted by burning 33 months after fire. In contrast, Vázquez et al. (1993) reported a short-term stimulation of burning on soil microbial abundance [490], whereas the effect of burning disappeared one year after burning. In contrast, Mabuhay et al. (2004) found that burning effect on soil microbial biomass and abundance can last at least 25 years [491], illustrating the long-term fire effects of burning on soil microbes in forest system.

4.5.3 Responses of Soil Nitrifiers to Fire Disturbance Are Modified by Global Change Scenarios

Because a major novelty of our work is to combine burning with 16 global change scenarios combining N deposition, increased precipitation, elevated CO_2 and warming, and because interaction effects including burning were often observed, we analyzed how and why the responses of nitrifier abundances to burning are modified according to global change scenarios.

For both AOB and AOA, the strongest interaction effect including burning was the N × H × B effect. Burning never affected AOB abundance under ambient N and H conditions, but significantly decreased AOB abundance under elevated N and N × H conditions 9 months after fire, and under H conditions 33 months after fire. These effects were linked to burning effects on SWC and BNPP. The negative effect of burning under N deposition after 9 months was mostly explained by a particularly positive effect of burning on SWC when combined with N deposition but not heating. The reason for such a strongly positive effect of burning on SWC mostly under N only conditions remains unclear. However, this increase in SWC probably lead to lower soil O_2 availability, and AOB maybe more sensitive to environmental change because soil AOB are largely monophyletic on the basis of 16S rRNA and their diversity is relative low as compared with other functional groups [27]. In addition, the negative burning effect on AOB under N × H conditions after 9 months was mostly linked to an observed trend for increased BNPP. Higher BNPP may have induced an increased competition between plant roots and AOB for ammonium, and AOB are weak competitors for ammonium as compared to plant roots [492]. The interaction effects were not stable with time, as after 33 months the burning effect was significant only under H conditions. This is explained because the burning effect on SWC and BNPP changed with time: for instance, 33 months after fire, no burning effect on SWC was observed under N conditions and a negative trend was observed for burning effect on BNPP under H × N conditions. This is consistent with previous reports showing that burning effect on soil moisture [371] and plant biomasses [368, 377] can vary with time following burning either because of cascading effects that evolve with time after fire or because of different climatic conditions. Previous results reporting significant positive burning effect on the abundance of AOB were published by Long et al. (2014) [263, 372] and Zhang et al.

(2018) [370]. The difference response of the abundance of AOB to fire disturbance maybe resulted from the intensity and duration of burning event as discussed in 4.5.2.3, and also soil nutrient state.

AOA abundance responded to burning only under H condition 33 months after fire. Under H conditions, burning increased SWC without modifying soil NH_4^+ concentration, which explained the decrease in AOA abundance induced by burning likely through decreased O_2 level. This contrasts with control (ambient N and H) conditions where burning did not increase much SWC and did not affect soil NH_4^+ concentration. Further, under N and H × N conditions, the burning-induced increase in SWC (negative for AOA according to the SEM analysis) was counterbalanced by increased soil NH_4^+ concentration (positive for AOA according to SEM results).

For the abundances of *Nitrobacter*, the most significant effect including burning was the P × N × H × B interaction after 9 months. A significant negative burning effect was found only under the H × P and P × N conditions. The significantly negative response of AOB abundance to burning under the PN scenario was explained by the combination of decreased AOB abundance (thus likely decreased nitrite supply) and increased SWC (increased anoxia), which were the main two drivers of AOB abundance according to the SEM analysis. In contrast, whereas a similar burning effect on SWC was observed under N only conditions, this was counterbalanced by a positive effect on AOB abundance. Similarly, whereas decreases in AOB abundance due to burning were similar under H × × N and H × P × N conditions than under P × N, no concurrent increase in SWC were observed under these two other global change scenarios. Our results show that change in only AOB abundance or only SWC was insufficient to lead to a decrease of *Nitrobacter* abundance in response to burning, but that the synergy between decreased AOB abundance and increased SWC induced a negative response of *Nitrobacter*. When burning induced a decrease in SWC only, without altered N supply to NOB, this likely counter selected autotrophic *Nitrobacter* that are sensitive to O_2 level, but mixotrophic *Nitrobacter* could then take over [26]. Only concurrent decreases of SWC and AOB abundance would induce conditions (low N and O_2 availabilities) unfavorable for the two main trophic types of *Nitrobacter*.

Overall, based on a unique comparison of 32 treatments (16 global change scenarios, with or without burning), our results support three major conclusions:

- Due to niche differentiation, the four groups of nitrifiers responded differently to the 32 global change treatments. This indicates that the balance between different nitrifier groups may be altered in the future;

- Soil nitrifiers were particularly sensitive to the main effects of N deposition, and to a lesser extent increased precipitation and burning, whereas no main effect of elevated CO_2 and warming was observed.

- Besides the importance of main effects, many interaction effects, many of them including 3 or 4 factors, and many of them including burning, were observed. This demonstrates that the effects of global change x disturbance scenarios on soil N cycling cannot be predicted simply by studying the effects of one or two factors. Global change research thus increasingly requires the use of experimental designs manipulating many factors and their possible interactions. Our results also highlight the strong interactions observed between global change conditions and fire disturbance. The good news is that we were often able to relate these interaction effects on soil nitrifiers to effects observed on a few soil environmental drivers like moisture, N availability and plant growth. Modelling and evaluating the generality of these complex interaction effects is a high priority for future global change research.

4.6 Summary

We studied the effects of 32 treatments mimicking global change scenarios based on the manipulation of N deposition, atmospheric CO_2, warming, precipitation and a fire disturbance (accounting for all their possible combinations) on soil nitrifier abundances in a Mediterranean grassland. Four major groups of soil nitrifiers were quantified by quantitative PCR: ammonia-oxidizing bacteria (AOB), ammonia-oxidizing archaea (AOA), *Nitrobacter* and *Nitrospira*. Overall, nitrogen deposition increased the abundance of AOB and to a lesser extent AOA and *Nitrobacter*, but not *Nitrospira*. *Nitrobacter* abundance was mostly affected by global change factors through their effects on AOB abundance, whereas *Nitrospira* abundance was more related to changes of AOA. Precipitation had a negative main effect on *Nitrobacter* and no clear effect on the three other nitrifier groups. Burning had a negative main effect on AOB. No main effect of elevated CO_2 and heating was observed. However, many interactive effects between global change factors were observed, often including burning. Our results show that the effects of global change × disturbance scenarios on soil N cycling cannot be predicted simply by studying the effects of one or two factors. They also highlight the strong interactions observed between global change conditions and fire disturbance. We were often able to relate the interaction effects on soil nitrifiers to effects observed on a few soil environmental drivers (moisture, N availability and plant growth). Modelling and evaluating the generality of these complex interaction effects is a high priority for future global change research.

5 Response of Soil Denitrifiers to Multiple Global Change Factors and Fire Disturbance in a Mediterranean Grassland

5.1 Introduction

Denitrification is a microbial respiratory process during which soluble nitrogen (N) oxides (nitrate, NO_3^-, and nitrite, NO_2^-) are sequentially reduced by specific reductases into gaseous forms (nitric oxide NO, nitrous oxide N_2O and N_2) [493], N_2O being a potent greenhouse gas [231]. The abundance and activity of soil denitrifiers depend on environmental conditions like organic carbon, moisture, oxygen, pH and N availability, which can all be affected by global changes. Thus, understanding the response of soil denitrifiers to global change is important to anticipate possible feedbacks to climate change.

Previous studies reported that N_2O emissions depend on the balance between different denitrifier groups [81, 446, 494], in particular (1) nitrite-reducers (harboring the *nirK* or *nirS* gene) that reduce NO_2^- to NO but also have a key role for N_2O production as most NO-producing denitrifying cells convert efficiently NO which is a toxic compound [495]; and (2) N_2O-reducers (harboring the *nosZI* gene or the recently discovered *nosZII* gene) that transform N_2O into N_2 [93, 94, 496]. Thus, understanding how net soil N_2O emissions may be affected by global change factors requires to analyze the responses of NO_2- and N_2O-reducers to changes in soil environmental conditions induced by global change. In particular, global changes such as rising atmospheric CO_2, warming, changes in precipitation regime, increased N deposition, as well as disturbances such as fire could all alter the soil environmental conditions to which soil denitrifiers are sensitive. These global changes all prone to change in the next decades [149] and can have interactive effects [232]. However, the effects of co-occurring multiple global change factors and fire disturbance on soil denitrifiers have not been studied (most studies have focused on 1 or 2 global change factors only: see Florio et al. 2019 [446] for a review of N addition effects on denitrifiers; and Hartmann et al. 2013 [300]; Li et al. 2017 [303]).

We studied the responses of four major groups of soil denitrifiers (*nirK*-, *nirS*-, *nosZI*-, and *nosZII*-harboring bacteria) to a prescribed fire disturbance under 16 global change scenarios, based on the manipulation of warming, atmospheric CO_2, N deposition and altered precipitation regime and all their combinations, at the Jasper Ridge Global Change Experiment in California (Shaw et al. 2002) [109]. The 32 treatments at this experimental site (16 global change scenarios, each with or without fire) were replicated 4 times. The abundances of the four denitrifier groups were quantified by quantitative PCR just before fire, just after fire, and 9 and 33 months after the fire. The objectives were to:

(1) evaluate the importance of the main effect of each factor and of 2-, 3-, 4- and 5-way interaction effects on denitrifier group abundances using same way as for nitrifiers, see section 4.1;

(2) analyze how these effects on denitrifiers change with time following the fire disturbance. We assumed that the effects of main treatments like N deposition would be strong and visible at all dates and across all treatments for *nirK, nirS* and *nosZI*, as these groups are known to be particularly sensitive to altered N supply but not *nosZII* (see Table 15; also see review table of Florio et al. 2019[446]. In addition, as the five global factors manipulated here are known to often influence ecosystems through indirect effects involving modified plant growth and soil properties and C and N cycling [284, 368, 377, 388]and as these effects can evolve with time after fire [368], we expected that complex interaction effects could arise when different global change factors and fire are combined, and that these effects could evolve according to time after fire. In particular, we assumed that fire effect on soil denitrifiers could differ depending on the global change scenarios considered and on the time since the initial fire disturbance. Further, we hypothesized that fire effects would be dampened with time after the fire disturbance if fire had mostly a direct effect through increased soil temperatures killing bacteria in the top soil [497], but that lasting effects would be observed if fire had mostly indirect effects influencing water/carbon/nutrient cycling and vegetation dynamics as often reported [365];

(3) identify the main drivers explaining the effect of burning on the four groups of soil denitrifiers and possibly the most significant interaction effects between burning and global change factors with soil environmental variables (soil moisture, nitrate, organic C and pH) and abundance of nitrifiers. We used structural equation modeling to identify the main drivers of the abundance of each denitrifier group across all treatments, and explore whether interactive effects between fire and global change treatments on the abundance of each denitrifier group could be explained by interactive effects on the main drivers identified.

5.2 Study Site

See chapter 4.2

5.3 Materials and Methods

5.3.1 Experimental Design

See section 1.4.2.

5.3.2 Sampling and Measurement

5.3.2.1 Plant/Soil Variables

Plant and soil variables were measured as part of a project former to the present Ph.D. and will be used here as putative drivers of the abundances of the denitrifiers groups. See section 4.3.2.1.

5.3.2.2 Soil DNA extraction and quantification of the abundance of nitrite-reducers (*nirK*- and *nirS*-harboring bacteria) and N_2O-reducers (*nosZI*- and *nosZII*-harboring bacteria)

DNA extraction method is the same as Chapter 4.3.2.2. The abundances of nitrite-reducers were quantified by measuring the fragments of the *nirK* and *nirS* genes encoding the copper and cd1 nitrite reductases, respectively [498]. For *nirK*, the primers used are *nirK*876 and *nirK*1040 [477]. The final reaction volume was 20 μl and contained (final concentration) 1 μM of each primer, 1 × of Quanti Tect SybrGreen PCR Master Mix (Qiagen, Courtaboeuf, France), 0.02 μg 0.1% of T4 gene protein 32 (Qbiogene, Carlsbad, CA USA) and 5 ng of sample DNA or 10^2–10^7 copies of standard DNA (linearized plasmid with the *nirK* gene of *Sinorhizobium meliloti* 1021). PCR cycles were as follows: first with 15 min at 95°C for initial denaturation step; 45 amplification cycles (15s at 95°C, 30s at 63°C, and 30s at 72°C); one cycle melting step (1s at 95°C, 20s at 68°C and continuous 98° C); and one cycle cooling step with 10s at 40°C; Samples were analyzed on a Lightcycler 480 (Roche, Diagnostics, Meyland, France). For *nirS*, the sets of primers used were *nirS*Cd3aF [83] and *nirS*R3cd [453]. The final reaction volume was 25 μl and contained (final concentration) 1 μM of each primer, 1 × of Quanti Tect SybrGreen PCR Master Mix (Qiagen, Courtaboeuf, France), 0.02 μ g 0.1% of T4 geneS protein 32 (Qbiogene, Carlsbad, CA USA) and 12.5 ng of soil DNA extract or 10^2–10^7 copies of the targeted DNA sequence, using a linearized plasmid containing the *nirS* gene of Pseudomonas stutzeri Zobell DNA as standard. The samples were run on a Lightcycler 480 (Roche, Diagnostics, Meyland, France) as follows: first with 15 min at 95°C for initial denaturation step; 45 amplification cycles (15s at 95°C, 30s at 59°C, 30s at 72°C and 30s at 80°C); one cycle melting step (15s at 95°C, 30s at 78°C and continuous 95°C); and one cycle cooling step with 10s at 40°C; Samples were analyzed on a Lightcycler 480 (Roche, Diagnostics, Meyland, France).

For *nosZI*-harboring N_2O reducers, the sets of primers used were nosZ2F and nosZ2R [377]. The final reaction volume was 25 μl, with (final concentration) 1.25 μM of each primer, 1 × of QuantiTect SybrGreen PCR Master Mix (Qiagen, Courtaboeuf, France), 0.016 μg 0.1% of T4 gene protein 32 and 12.5 ng of soil DNA extract or 10^2–10^7 copies of the targeted DNA fragment (linearized plasmid with the targeted *nosZ* gene of Pseudomonas stutzeri). Assays were run on a Lightcycler 480 (Roche, Diagnostics, Meyland, France), as follows: first with 15 min at 95°C for initial denaturation step; 6 cycles of touchdown (95°C for 15s, 65°C for 30s and 72°C for 30s and 80°C for 15s), 40 amplification cycles (95°C for 15s, 60°C for 15s, 72°C for 30s and 80°C for 15s); one melting cycle (15s at 95℃, 15s at 60℃ and continuous 95℃); and one cycle cooling step with 30s at 40°C. For *nosZII*-harboring N_2O reducers, the sets of primers used were *nosZII*F and *nosZII*R2 [93]. The final reaction volume was 25 µl and contained (final concentration) 1 µM of each primer, 1.2 × of QuantiTect SybrGreen PCR Master Mix (Qiagen, Courtaboeuf, France), 0.3 mg ml^{-1} of bovine serum albumin (BSA) and 20 ng of soil DNA extract or 10^2–10^7 of targeted fragments (linearized plasmid with the *nosZ* gene of uncultured bacterium clone CJEAb111). Assays were performed in on a Lightcycler 480 (Roche Diagnostics, Meyland, France) according to the following steps: first with 15 min at 95°C for initial denaturation step; 45 amplification cycles (30s at 95°C, 1min at 53°C, and 1min at 72°C); one cycle fusion step (15s at 95℃, 15s at 60℃ and continuous 95℃); and one cycle cooling step with 30s at 40°C.

Prior to qPCR assays, two types of tests were performed to evaluate possible inhibition of PCR. First, for all soils, a dilution approach was used: qPCR assays were performed with 5, 10 and 20 ng of soil DNA to evaluate possible PCR inhibition according to the amount of co-extracted compounds (*nirK* used as a target). Second, we compared the qPCR results obtained when using (i) the standard DNA plasmid leading to a concentration of 10^7 *nirK* copies and (ii) the standard DNA plasmid at the same concentration plus 5 ng of extracted DNA for each soil. No inhibition was observed. The amplification efficiencies were 93%, 79%, 86 %, and 93%, for *nirK*, *nirS*, *nosZI* and *nosZII*, respectively. Abundances were expressed as gene copy numbers per *g* equivalent dry soil.

5.3.3 Analysis

Methods and steps of outliers detection and block effect assessment were the same as for nitrifiers, see section 4.3.3 for details. The outlier numbers of *nirK* are 6, 4, 1 and 1, respectively -0.13, 0.06, 9 and 33 months after fire; the corresponding outlier numbers of *nirS* are 5, 4, 0, and 1; of *nosZI* are 9, 2, 0, 3. Note that, for *nosZII*, no data available before fire (-0.13 months), the outlier numbers are 12, 8, and 1 at 0.06, 9, and 33 months after fire disturbance, respectively.

Data were analyzed to assess the overall effects of the burning disturbance and other global environmental changes treatments on the abundance of the four groups of soil denitrifiers (*nirK*, *nirS*, *nosZI* and *nosZII*-harboring bacteria) using PROC MIXED in SAS 9.4 using a full factorial split-plot analysis of variance in order. At each sampling date, the CO_2, warming and burn treatments were included as whole-plot factors, and the precipitation and N treatments as split-plot factors. The normality of the residuals and the independence of the residuals related to the predicted values were analyzed. If these criteria were not met, data were transformed with Box-Cox using Minitab® 18.0 (Minitab Inc., PA, USA) software. Effects with $P < 0.05$ are referred to as significant.

Based on the results of the mixed-model analysis obtained for the four sampling dates, the most significant interaction effect observed between burning and global change factors was selected for each denitrifier group. Accordingly, the effect of burning was compared under the different relevant global change scenarios using T-test. For example, the precipitation (P) × nitrogen (N) × burning (B) interaction effect was the most significant interaction effect involving burning observed across the sampling dates for *nirS* gene. Therefore, the burning effect was compared between the control, P, N and PN conditions, using *T-tests* (Fig. 55) and checking data normality and homogeneity of variance after data transformation if needed. For graphical representation, main treatment effect size was calculated as: *Effect* = *T* – *C,* see section 4.3.3 for details.

In order to explore and hierarchize the links between changes in the abundances of the four soil denitrifier groups following fire and environmental variables (SWC, pH, DOC, NO_3^- and ANPP and BNPP), as well as nitrifier group abundances (AOA, AOB, *Nitrobacter*, and *Nitrospira*), path analysis was performed using Amos25® (Amos Development Corporation, Crawfordville, FL, USA). The complete model with all possible causal relationships considered is presented in Fig. 51.

A χ^2 test was used to evaluate the model, *i.e.* whether the covariance structures implied by the model adequately fitted the actual covariance structures of the data (a non-significant χ^2 test with $P >$ 0.05 indicates an adequate fit by the model). Within a model, the coefficients of paths indicate by how many standard deviations the explained variable would change if the causal variable was changed by one standard deviation.

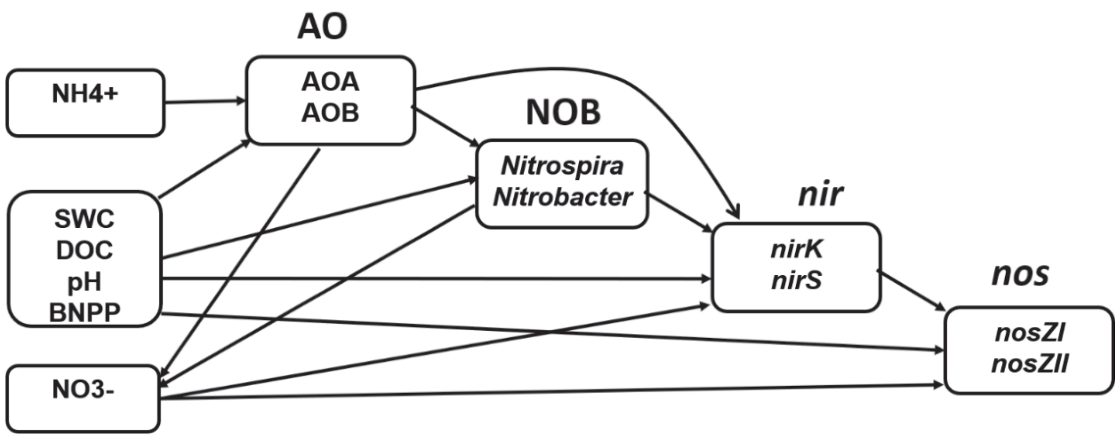

Fig. 51 Complete model used for identifying the main drivers of changes in the abundances of soil denitrifiers (*nirK*, *nirS*, *nosZI* and *nosZII*-harbouring bacteria) by structural equation modelling. The variables included in the model are the abundances of soil nitrifiers (AO, Ammonia-oxidizers; AOB, Ammonia-Oxidizing Bacteria; AOA, Ammonia-

Oxidizing Archaea; NO, Nitrite-Oxidizers), soil ammonium concentrations (NH_4^+), gravimetric soil water content (SWC), soil dissolved organic carbon content (DOC), soil pH (pH), belowground net primary productivity (BNPP), and soil nitrate concentrations (NO_3^-)

5.4 Results

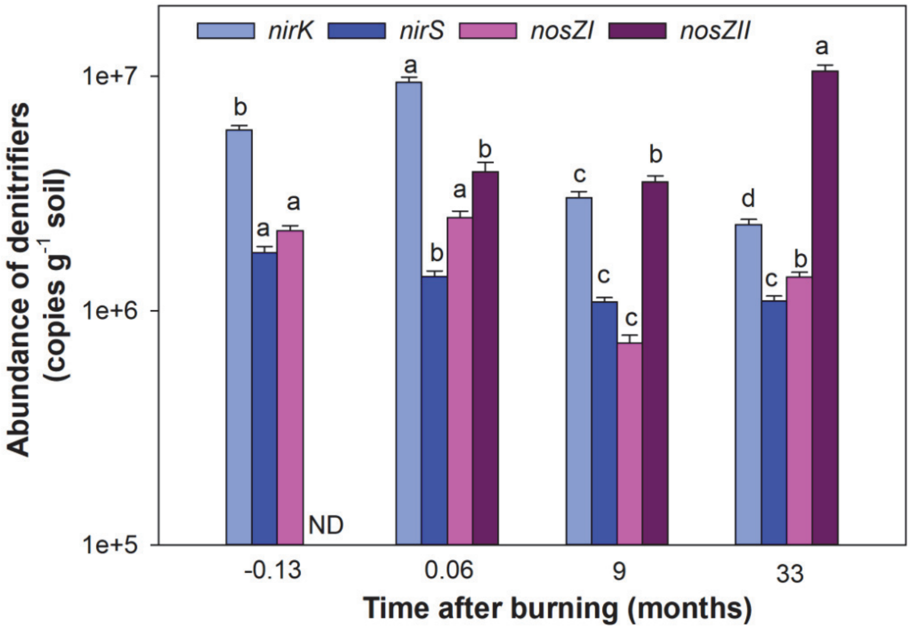

Fig. 52 The abundances of the four groups of soil denitrifiers averaged across all treatments 4 days before burning (-0.13 month); 2 days after burning (0.06 month); 9 months after burning; and 33 months after burning. Bars are mean + s.e. (n=128 minus outliers). Different letters indicate significant differences of the abundance of a given group between sampling dates. ND indicates no data available

The *nirK* abundance dominated over *nirS* abundance for each sampling date, ranging from 2.33×10^6 to 9.40×10^6 copies g^{-1} soil and 1.09×10^6 to 1.77×10^6 copies g^{-1} soil, respectively (Fig. 52). The *nosZII* abundance was always higher than *nosZI* abundance at the three dates for which *nosZII* data were available. *nosZI* and *nosZII* abundances ranged from 7.27×10^5 to 2.50×10^6 copies g^{-1} soil and 3.54×10^6 to 1.05×10^7 copies g^{-1} soil, respectively.

5.4.1 Importance of the Main Effect and Interactive Effect of Fire and Other Global Change Factors on Denitrifier Abundances

Both significant main treatment effects and interactive treatment effects of global change factors, often including fire, were observed on soil denitrifier abundances (Table 51). Overall, for the 3 sampling dates after burning and the 4 denitrifier groups considered, main treatment effects were the most commonly observed, followed by 3-way interactive effects (Fig. 53). We assessed to what extent the number of significant effects detected was linked to the number of effects tested.

Overall, the percentage of significant effects across the 4 groups of denitrifiers and the 3 soil sampling dates after burning was 8.3%. Using this percentage and the number of tests performed, the expected number of significant effects (black dots in Fig. 53) was computed assuming an evenly distributed percentage of significant effects across main effects and the 2-, 3-, 4- and 5-way interactions. Focusing only on interactions including burning, and accounting for the number of possible interactions including burning, the expected number of significant effects including burning was also computed (red dot in Fig. 53). The number of significant main effects was 1.4-fold higher than expected (Fig. 53). The number of significant 3-way interaction effects was close to the expected number based on the total percentage, whereas significant 2-, 4- and 5-way interactions were less numerous than expected (Fig. 53). The significant main treatment effects detected corresponded mostly to N deposition effect, with a lesser importance of main burning effect and precipitation effect. No significant main effect of CO_2 or heat was found (Table 51; Fig. 53). The number of significant observed main burning effects was 2-fold higher than expected. For the 3-, 4- and 5-way interaction effects including burning, the number of significant effects observed was lower than expected, whereas for 2-way interaction effects, the number of significant effects observed was as expected based on the mean percentage of significant effects (Fig. 53).

Table 51 Mixed model results summarizing the significant effects of global change factors (CO_2, C; heat, H; nitrogen deposition, N; precipitation change, P; Burning, B) and their interactions on the abundances of soil denitrifiers (*nirK-, nirS-, nosZI-* and *nosZII*-harboring bacteria) at the 4 sampling dates: 4 days before burning (-0.13 month); 2 days after burning (0.06 month); 9 months after burning; and 33 months after burning. Values between brackets are *P* values

Denitrifiers	**Effects**	**-0.13 month**	**0.06 month**	**9 months**	**33 months**
nirK	Main effects	B(0.0385) N(0.0245)	N(<0.0001)	N(0.0005)	B(0.0004) N(<0.0001)
	Interactive effects including B	BHP(0.0038)		BHPN(0.0413)	
	Interactive effects excluding B				
nirS	Main effects	P(0.0078) N(0.0441)	N(0.0010)	P(0.0056)	P(0.0206) N(0.0316)
	Interactive effects including B		BPN(0.0213)		BC(0.0314) BP(0.0484)
	Interactive effects excluding B	CN(0.0049) PN (0.0179) CPN(0.0125)	CPN(0.0257)	CN(0.0384) HPN(0.0305)	HN(0.0368) PN(0.0162) HPN(0.0054)
nosZI	Main effects	N(0.0.0011)	N(<0.0001)	N(0.0009)	B(0.0018) N(<0.0001)
	Interactive effects including B		BPN(0.0462)		BP(0.0317) BPN(0.0031)
	Interactive effects excluding B	CN(0.0181)		HPN(0.0318)	
nosZII	Main effects				
	Interactive effects including B	BHPN(0.0407)	BPN(0.0145)	BHPN(0.0115)	BC(0.0069)
	Interactive effects excluding B		CPN(0.0131)		HPN(0.0343)

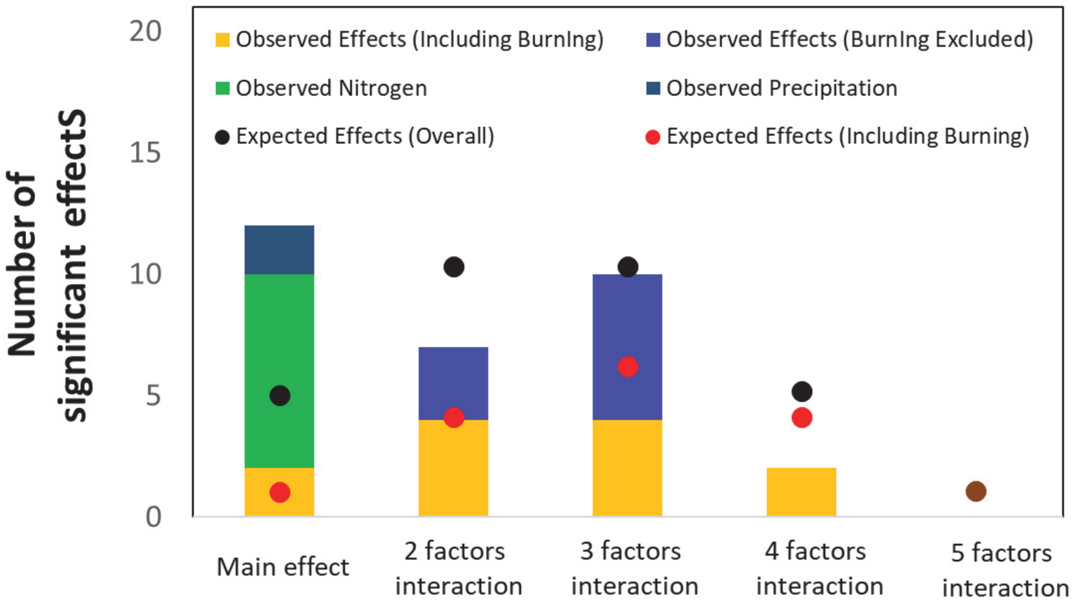

Fig. 53 Main effects and interactive effects of global change factors (CO_2, heat, precipitation change, and nitrogen deposition) and burning on the abundance of four groups of soil denitrifiers (*nirK*- and *nirS*-harboring nitrite-reducers; and *nosZI*- and *nosZII*-harboring N_2O reducers) at the 3 sampling dates after burning (2 days after burning; 9 months after burning; and 33 months after burning). Columns indicate the observed number of significant effects. For significant multi-factors interactions, we distinguished those including burning (orange) or not (blue). Black and red dots indicate the expected number of significant effects based on the ratio between the number of observed significant effects and the number of total interactions tested in the model for 4 groups of the denitrifiers of 3 sampling dates

5.4.2 Main Interaction Effect of Global Change Factors on Soil Denitrifiers

Overall, positive main N effects were observed on the abundances of denitrifier groups except *nosZII*-harboring bacteria, the abundances of *nirK* and *nosZI* being increased by 14.7% to 41%, and 27.1% to 46.1% by N deposition, respectively; whereas *nirS* abundance increased only by 10.1% to 26.4% (Table 51, Fig. 54-Left-Top). Concurrently, a very strong and positive N deposition effect was observed on soil NO_3^- concentrations (Fig. 54-Left-Top). In addition, *nirS*-harboring bacteria was the only group that responded to the main precipitation (P) effect, being significantly increased by 16.5% to 26.0% by elevated P at 3 among the 4 sampling dates (Fig. 54). Increased P also significantly increased SWC during plant peak biomass periods (9 and 33 months after fire, by 16.0% and by 13.8%, respectively), whereas the effects of increased P on soil NO_3^- and DOC were variable and mostly not significant. Further, increased P significantly decreased BNPP by 23.8% at 9 months after burning.

Significant main burning effects (B) were observed 33 months after burning for *nirK* and *nosZI*

(-37.2% and -29%, respectively) (Fig. 54). Across all treatments, burning also significantly increased SWC by 8.1% and 7.8% after 9 and 33 months, respectively. The responses of NO_3^- and DOC varied with sampling dates. In particular, B significantly increased DOC (+36.3%) just after fire but decreased DOC (-6.9%) 9 months after burning. BNPP did not significantly respond to the main B effect (Fig. 43-Right-Bottom).

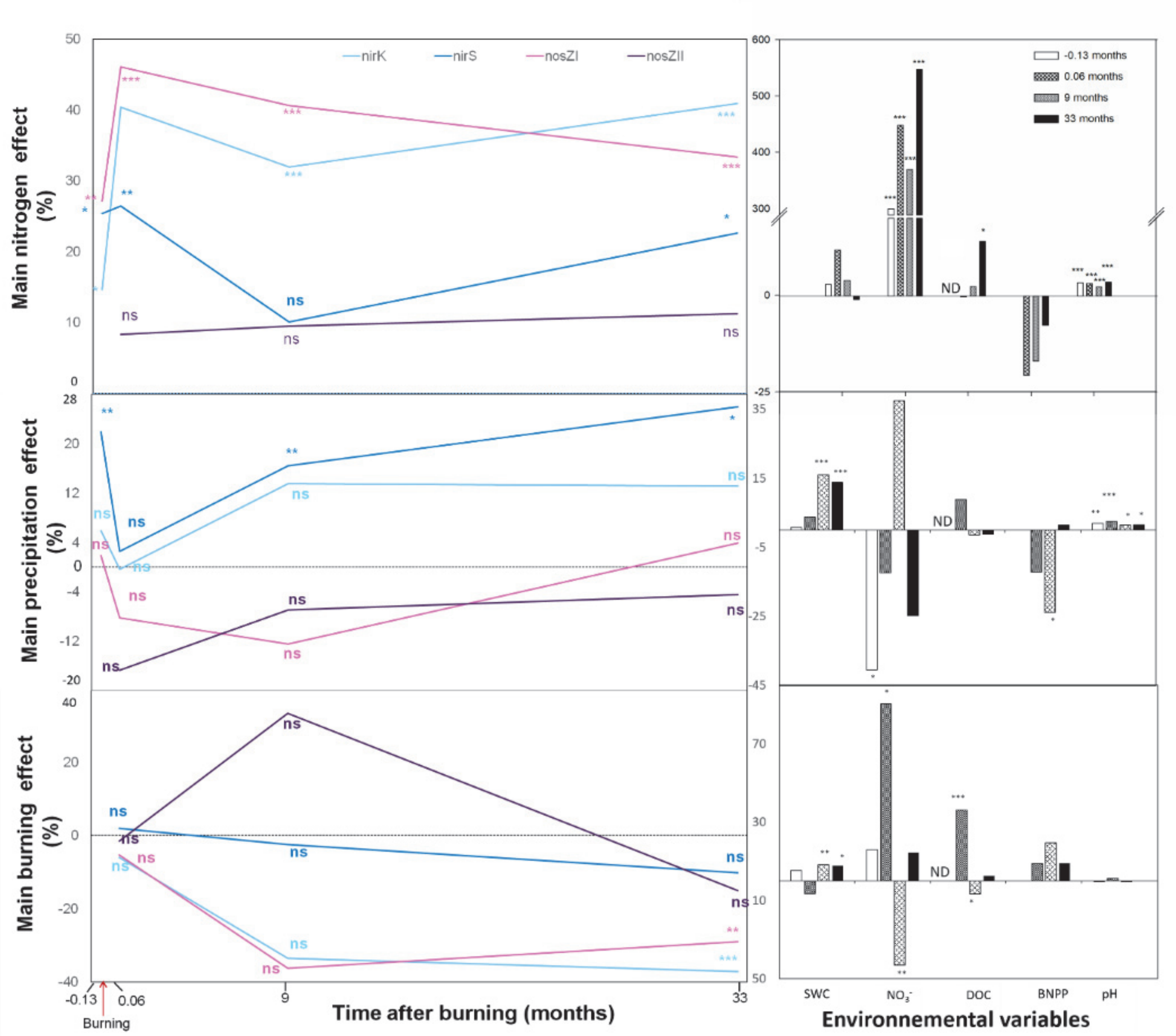

Fig. 54 Main effect of nitrogen addition, increased precipitation and burning on the abundances of soil denitrifiers (*nirK* -, *nirS*-, *nosZI*- and *nosZII*-harboring bacteria) (Left) and (Right) on environmental variables : soil water content (SWC), soil nitrate concentration (NO_3^-), soil dissolved organic carbon (DOC) and plant belowground net primary productivity (BNPP) at the four sampling dates (-0.13 month, that is 4 days before burning; 0.06 month, 2 days after burning; and 9 and 33 months after burning). ns, not significant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ND, no data; Values of BNPP for 2011 is presented at 2 days after burning

5.4.3 Responses of Soil Denitrifiers to Fire Disturbance: Interactions with Global Change Scenarios

5.4.3.1 Nitrite-reducers

Significant burning effects (main and interactive) were found before fire for *nirK* (Table 51). This might be due to a legacy of the 2003 fire on the abundance of *nirK* and we thus did not analyze *nirK* response to fire.

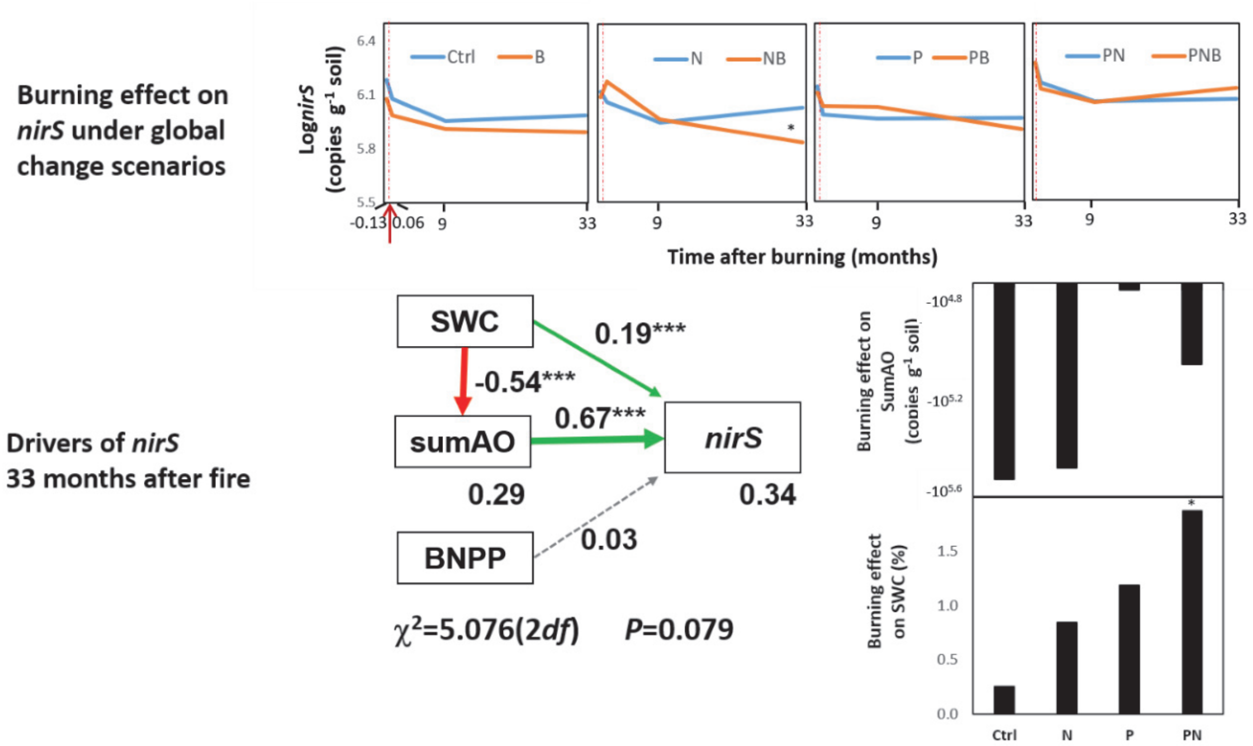

Fig. 55 (Top) Abundance of soil *nirS*-harboring nitrite-reducers (*nirS*) under different global change scenarios (Control, Ctrl; N, N deposition; P, precipitation; whatever the warming and elevated CO_2 treatments) with – orange lines- and without – blue lines- burning (B). (Bottom-Left) Structural equation model results relating *nirS* abundance to soil water content (SWC), total abundance of ammonia oxidizers (SumAO), and belowground net primary productivity (BNPP). Values near the arrows are path coefficients. The green arrows indicate positive correlations. The red arrows indicate negative correlations. The percentage of variance explained by the model is indicated at the bottom-right of the corresponding box. (Bottom-Right) Burning effect on SumAO and SWC under the relevant scenarios at 33 months after burning. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$

Regarding the abundance of *nirS*-harboring nitrite reducers, the most significant interactive effect including burning corresponded to the 3-way interaction of precipitation (P) × nitrogen (N) × burning (B) (Table 51). Therefore, this 3-way interaction effect was further analyzed (i.e. across the other warming and elevated CO_2 treatments). Burning significantly decreased *nirS* abundance only under the N deposition scenario 33 months after fire (6.80×10^5 and 1.08×10^6 copies g^{-1} soil under the N × B and N treatments, respectively), whereas no burning effect was observed under control (no N deposition and ambient P), P and P × N conditions (Fig. 55-Top).

Structural equation modelling (SEM) showed that *nirS* abundance was mostly explained (34% of total variance) by the total abundance of ammonia oxidizers (SumAO) and SWC (Fig. 55-Bottom -Left) (Fig. 55-Bottom-Left).

5.4.3.2 N_2O-reducers

Regarding *nosZI* abundance, the most significant effect including burning corresponded to the P × N × B interaction observed 33 months after fire (*P*=0.0031) (Table 51).

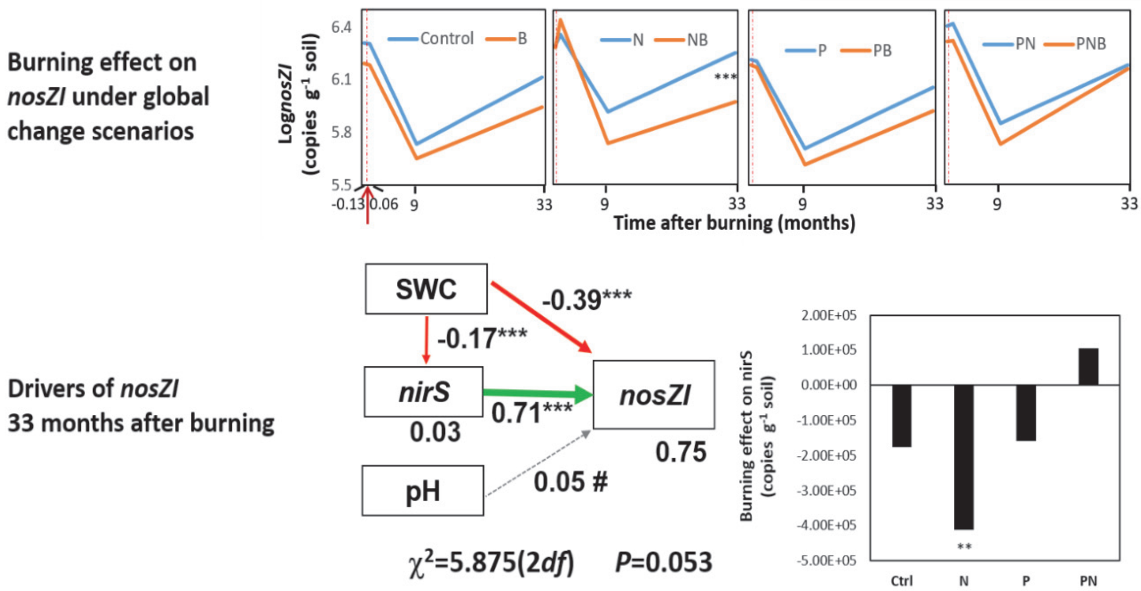

Fig. 56 (Top) Abundance of soil *nosZI*-harboring nitrite-reducers (*nosZI*) under different global change scenarios (Control, Ctrl; P, precipitation; N, N deposition;) with －orange lines- and without －blue lines- burning (B) (B). (Bottom-Left) Structural equation model results relating *nosZI* abundance to soil water content (SWC), *nirS* abundance (*nirS*) and soil pH. Values near the arrows are path coefficients. The green arrows indicate positive correlations. The red arrows indicate negative correlations. The percentage of variance explained by the model is indicated at the bottom-right of the corresponding box. (Bottom-Right) Burning effect on *nirS* abundance under the relevant scenarios at 33 months after burning. #, 0.05< *P* <0.1; *, *P* < 0.05; **, *P* < 0.01; ***, *P* < 0.001

At this date, burning significantly decreased *nosZI* abundance only under N conditions (9.40×10^5 and $\times10^6$ copies g^{-1} soil under the N and NB treatments, respectively) (Fig. 56-Top). The abundance of *nosZI* was related to *nirS* abundance and SWC, and marginally to soil pH. These variables explained 75% of the total variance of *nosZI* abundance. Consistently with the PNB interaction effect observed on *nosZI* abundance, burning only decreased *nirS* abundance under N conditions (Fig. 55).

For *nosZII* abundance, the most significant effect including burning corresponded to the C × B interaction 33 months after fire (P=0.0069) (Table 51). Under ambient CO_2 conditions, burning decreased *nosZII* abundance (1.18×10^7 and 6.87×10^6 copies g^{-1} soil under the ambient and B treatments, respectively), whereas burning increased *nosZII* abundance at elevated CO_2 (7.26×10^6 and 9.18×10^6 copies g^{-1}) under the C and CB treatments, respectively) (Fig. 57-Top).

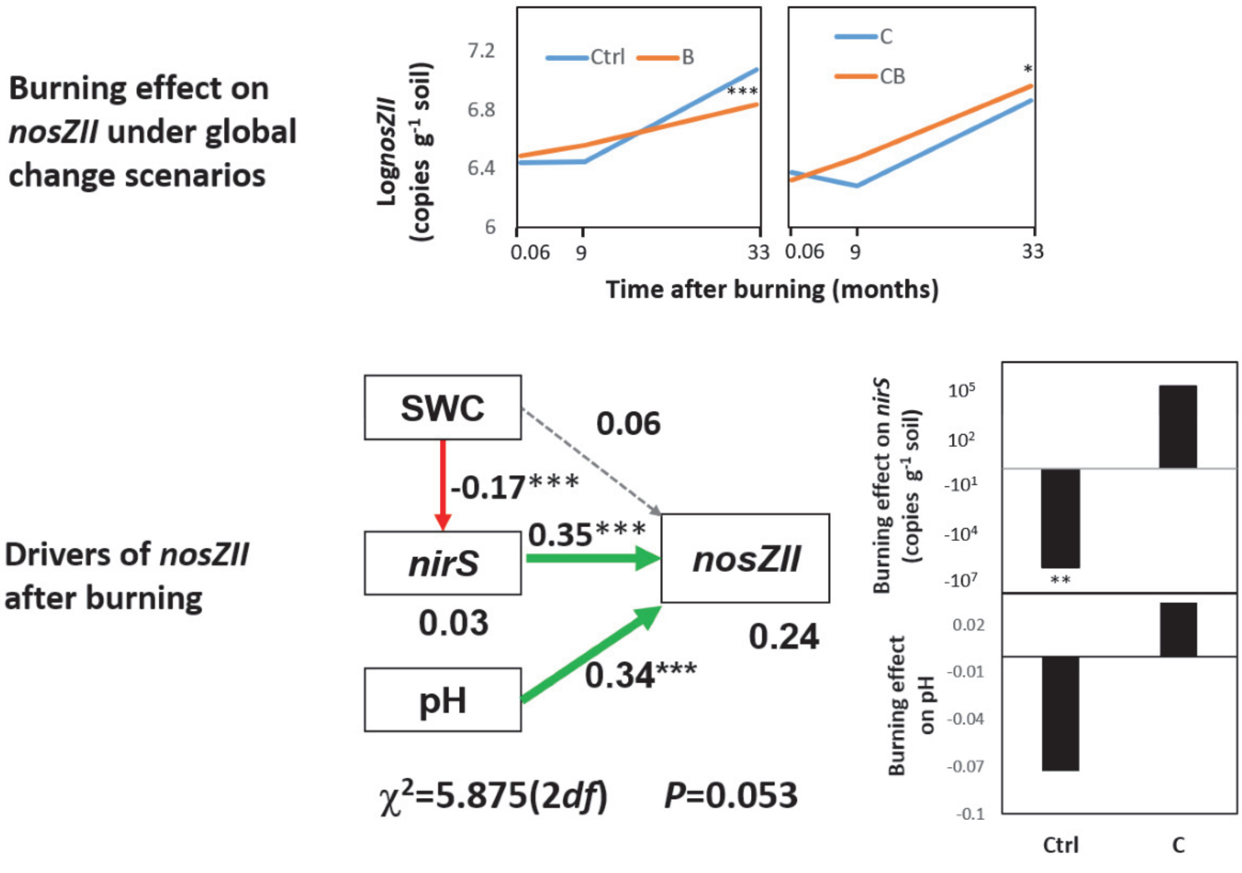

Fig. 57 (Top) Abundance of soil *nosZII*-harboring nitrite-reducers (*nosZII*) under different global change scenarios (Control, Ctrl; C: elevated CO_2) with －orange lines- and without －blue lines- burning (B). (Bottom-Left) Structural equation model results relating *nosZII* abundance to soil water content (SWC), *nirS* abundance (*nirS*) and soil pH. Values near the arrows are path coefficients. The green arrows indicate positive correlations. The red arrows indicate negative correlations. The percentage of variance explained by the model is indicated at the bottom-right of the corresponding box. (Bottom-Right) Burning effect on *nirS* abundance and soil pH under the relevant scenarios at 33

months after burning. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$

The abundance of *nosZII* was mostly related to *nirS* abundance and soil pH. These variables explained 24% of the total variance of *nosZII* abundance (Fig. 57-Bottom-Left).

5.5 Discussion

5.5.1 Importance of Main Effects and Interactive Effects of Fire and Global Change Factors on Denitrifier Abundances

Consistent with results obtained for soil nitrifier abundances (see 4.5.1), the number of the main treatment effects that were significant on soil denitrifiers abundance was two-fold than expected, with main positive nitrogen deposition (N) treatment effect being the most often observed, and followed by main positive precipitation (P) and negative burning (B) effects, and with no main effect of warming and CO_2. This is consistent with previous studies for the same experimental site reporting a main effect of N addition and a main effect of elevated P which both increased denitrifying enzyme activity DEA, whereas elevated CO_2 and warming had no significant effect on DEA [213]. Brown et al. (2012) also observed a significant and positive main effect of P on soil N_2O emissions and showed that denitrification was the main driver of soil N_2O emissions at this site [232]. In addition, a main B treatment effect on N_2O emissions from soil was also reported after the 2003 fire at the same study site but this effect was positive [284] which contrasts to the main B treatment effect reported in the present study that was negative on both the abundances of *nirK* and *nosZI*-harboring bacteria. In contrast, weak heating intensity (initially with +0.8 to 1°C and then +1.5 to +2°C,) which is quite realistic but lower than warming treatments applied in other warming experiments on grasslands [205, 267]. In addition, the +275 ppm increase in atmospheric CO_2 concentration could have only indirect effects on soil nitrifiers through plants because CO_2 concentration in the soil atmosphere has already reached ~ 400 ppm in 2013 [482]. This likely explains why no significant main effect of CO_2 was observed and that this factor rarely appeared in the significant interaction effects.

For interaction effects, as for soil nitrifiers, the number of significant effects was lower than expected in particular for the 2-, 4- and 5-way interactions, which may be due to the fact that the N and B treatments induced strong effects that appeared more as main effects than interactive effects. However, some strong interaction effects from two- to four-way factors were observed for denitrifier groups. This is consistent with previous studies reporting that global change factors can elicit complex interactive responses in plant and/or soil compartments [109, 232, 284, 377], and in particular with a previous study reporting interactive effects of fire and global change factors on DEA and soil N_2O emissions [284]. Our results thus further indicate that the effects of global change factors and fire cannot be simply predicted by single effects in isolation.

5.5.2 Response of Soil Denitrifier Groups to Main Treatment Effect of Global Change Factors

5.5.2.1 Nitrogen Deposition

N addition effect on soil denitrifiers are reported to vary with intrinsic soil nutrient state [44, 312] and other soil environmental variables like soil pH [345]. In limited N conditions, which is our case in JRGCE [377], the abundances of soil denitrifiers are generally increased by N addition directly by increased mineral N substrate availability [312]. As expected, the N treatment induced positive responses in the abundance of nitrite-reducers. We observed that *nirK* abundance was more (positively) responsive to N deposition than *nirS*. This is consistent with previous studies reporting that *nirK* communities are more sensitive than *nirS* communities to environmental gradients [499]. Other studies also reported that *nirS*-harboring bacteria responded more than *nirK* to increased N deposition in winter wheat grown system [312], and that *nirS*-harboring bacteria in moist savanna and steppe soils are mainly favored by higher soil NO_3^- availability whereas *nirK*-harboring bacteria would depend more on soil organic carbon [81]. The N addition also increased the abundance of *nosZI-* but not *nosZII*-harboring bacteria. This illustrates the niche differentiation that exists between these two groups of N_2O-reducers, as highlighted by Assemien et al. (2019) [461], who also identified *nosZI*-harboring bacteria as mainly driven by NO_3^- availability, whereas *nosZII*-harboring bacteria would be more favored by soil organic carbon in West African cultivated soils.

5.5.2.2 Increased Precipitation

Generally, the response of soil denitrifier abundance to precipitation amount not only depends on changes in soil moisture but also on changes in soil N and C substrate availabilities [290] through changing plant growth and/or litter decomposition. In our study, the *nirS* abundance responded positively to increased P, which is consistent with results of Ding et al. (2015) showing that *nirS* abundance significantly increased with increasing soil moisture, and consistent with the observed increase in soil water content under increased precipitation at our site [287]. The difference in phylogenetic diversity between *nirK* and *nirS* denitrifiers explained the variable response of *nirK*- and *nirS* abundance to P treatment. In contrast, the abundances of soil N_2O-reducers did not respond to elevated P, which is consistent with previous results [259, 289, 290]. For instance, Zhang et al. (2013) reported no watering effect on soil N_2O-reducers as part of a long-term global change experiment in a steppe ecosystem of Inner Mongolia [259]. Microbial response to global changes varied from year to year and microorganisms might adapt to environmental changes after long-term treatments [2], indicating that the denitrifiers (except *nirS*-harboring bacteria) may adapt to the P treatments in our long-term simulated treatment.

5.5.2.3 Burning

No direct burning effect was found on all 4 groups denitrifiers immediately after fire, consistently with the effect of fire observed on soil nitrifiers (see section 4.5.2.3). This further illustrates that the low intensity fire disturbance in this grassland ecosystem did not exert direct effect on soil microbes through heating during the fire event in our study site. The abundances of *nirK*- and *nosZI*-harboring bacteria were reduced by burning 33 months after fire, illustrating the indirect burning effect by increasing of SWC. This is consistent with previous studies reporting burning changed soil water content [368, 378, 489], available N [135], available carbon [135, 371], and soil pH [371]. Consistent with this, we have found that Burning significantly altered soil moisture, soil nitrate and DOC contents at our site, with a remaining effect of fire on soil moisture almost 3 years after the initial disturbance. Fire-induced changes in soil properties may last for several years. For instance, Alcaniz et al. (2016) reported that the burning effects on soil pH, N, available phosphorus, potassium, calcium and magnesium still existed 9 years after fire in a Mediterranean forest [371]. Consistently, Mabuhay et al. (2004) found that responses of soil microbial biomass and abundance to burning can last at least 25 years [491], illustrating the long lasting fire effects of burning on soil microbes. In addition, over the long-term, burning may modify the soil microbial community by altering plant community composition via plant-induced changes (e.g. C allocation) in the soil environment [500].

5.5.2.4 Responses of Soil Denitrifiers to Fire Disturbance Are Modified by Global Change Scenarios

Similar with the analysis of soil nitrifiers (see section 4.5.3), we found that the responses of denitrifier abundances to burning were modified by the global change scenarios.

The strongest interactive effect including burning was the P × N × B 3-way interaction on the abundances of *nirS*- and *nosZI*-harboring bacteria and the C × B 2-way interaction on the abundance of *nosZII*-harboring bacteria. Burning decreased the abundance of *nirS* only under high N conditions, 33 months after the initial fire disturbance. The main drivers of the abundance of *nirS* were the total abundance of ammonia-oxidizers and to a lesser extent soil moisture. The burning induced decrease in the abundance of ammonia-oxidizers – though not significant – may partly explain this decrease in the abundance of *nirS* in the burned plots due to lower substrate availability. Contrary to expectations, this decrease in substrate availability for *nirS*-harboring bacteria might have been exacerbated under high N conditions – as the N treatment stimulates plant growth which might elicit higher competition between plants and microbes for mineral N [109, 396].

Similarly, burning also decreased the abundance of *nosZI* only under high N conditions 33 months after the initial fire disturbance. This effect likely resulted from the decreased in *nirS* abundance induced by burning at high N conditions. Indeed, the abundance of *nirS* was identified as the main driver of changes in the abundance of *nosZI* by structural equation modelling and a decrease in the abundance of *nirS* in the burned and fertilized plots would lead to a decrease in the availability of substrates for *nosZI*-harboring bacteria in these plots. This is consistent with a previous study conducted in West African cultivated soils that showed that the *nirS*- and *nosZI*-harboring bacteria tended to covariate and have similar niche distribution [81].

Burning decreased the abundance of *nosZII*-harboring bacteria at ambient CO_2 but increased it at elevated CO_2 33 months after the fire disturbance. These treatment effects might be at least partly explained by changes in the abundance of *nirS* induced by burning, and by subsequent changes in substrate availability for *nosZII*-harboring bacteria. Indeed, under ambient CO_2 conditions, burning significantly reduced the abundance of *nirS* which might have led to the observed decrease in the abundance of *nosZII* resulting from lower substrate availability, while at high CO_2 conditions, the trend for higher abundance of *nirS* in the burned plots – though not significant – may have contributed to the increase in the abundance of *nosZII* with more substrate availability. Changes in soil pH in the burned plots – though not significant – may further explain the observed effects as it has been reported that the abundance of *nosZII*-harboring bacteria is positively related to soil pH [501].

5.6 Summary

We studied the combined effects of 16 global change scenarios based on the manipulation of atmospheric CO_2, warming, precipitation and N deposition and a fire disturbance (i.e. 32 treatments in total) – on the abundances of soil denitrifiers in a Mediterranean grassland. Four groups of soil denitrifiers were quantified by quantitative PCR: *nirK*-, *nirS*-, *nosZI* and *nosZII*- harboring bacteria at several sampling dates across the three years following the fire. Several main effects of the treatments were reported: overall, enhanced nitrogen deposition increased the abundance of *nirK* and *nosZI* and to a lesser extent *nirS*, but not *nosZII*, and increased precipitation increased the abundance of *nirS*, while no main effect of CO_2 and warming was found. Burning did not affect the abundances of soil denitrifiers immediately but decreased the abundance of *nirK* and *nosZI* almost three years after the initial disturbance. In addition, several interactive effects between fire and global change factors were observed, the Burn × Precipitation × N and the Burn × CO_2 interactions affecting the most the abundances measured. Our results thus indicate that the effects of multiple global changes and fire on soil denitrifying microbial communities are not additive and thus cannot be predicted by studies on single global change factor.

6 Conclusions and Perspectives

6.1 Conclusions

Based on my results, I conclude that:

(1) In both grasslands, the responses of different groups of soil (de)nitrifiers to global change scenarios differed strongly. In the Monsoon grassland, AOB mostly responded to N, either directly for N deposition, or indirectly when other global change factors like altered precipitation amount or frequency affected N availability. In contrast, AOA were more sensitive to soil water than N dynamics. The *nirK*- and *nirS*-harboring nitrite reducers and *nosZI*-harboring N_2O reducers were more sensitive to N deposition than *nosZII*-harboring N_2O reducers, and *nirK*- and *nirS*-bacteria responded to reduced precipitation in the opposite direction.

(2) In the Mediterranean grassland, the effects of multiple global changes and fire disturbance on soil denitrifying microbial communities are not additive. The interactive effects were explained by key environmental variables like soil moisture, mineral N availability, pH and belowground plant growth. This indicates that it is impossible to predict how (de)nitrifiers and (de)nitrification respond to global change scenarios involving multiple factors only from the knowledge of single factor. Modelling and evaluating the generality of these complex interaction effects is thus a high priority for research to predict the responses of soil N cycling processes to global change and feedbacks on climate in the future.

6.2 Novelty and Limitation of My Work, and Perspectives

Numerous researches have documented the effects of global change factors on soil N cycling process, but they have rarely investigated the combined effects of multiple global change factors and/or disturbances, which is a main novelty of the work presented here. In particular, several novelties of this Ph.D. work can be highlighted:

This study performed in China is the first one assessing the effects of changes in the amount of precipitation, the frequency of precipitation and the co-occurring chronic wet N deposition on soil N cycling process, recognizing that wet N deposition is coupled to individual precipitation events. However, a limit of the study is that our conclusions are based on one sampling date only and that the study was conducted in mesocosms and not directly in the field. A perspective of this work would be to further study the combined effects of changes in precipitation regime and associated wet deposition over the long-term in an *in situ* experiment, but this raises feasibility concerns regarding the manipulation of precipitation and wet N deposition. Further, it is also needed to compare how N deposition affects soil nitrous oxide emission when N is added as chronic N deposition small N amount associated to each rainfall event) and when only a few N addition events (typically 1 or 2 events per year, maximum one event per month) as traditionally used in many studies to simulate N deposition.

Our study is also the first one assessing the effects of a fire disturbance under 16 long-term global change scenarios applied *in situ* on the abundances of soil nitrifiers and denitrifiers (AOA, AOB, *Nitrobacter*, *Nitrospira* and *nirK*-, *nirS*-, *nosZI*- and *nosZII*-harboring bacteria, respectively). Changes in the abundances of the soil nitrifiers and denitrifiers may alter N_2O emissions from grassland soils. As we found that fire altered the abundances of soil nitrifiers and denitrifiers 33 months after the initial disturbance, a longer-term survey of fire effects would allow assessing the duration of a fire event on soil N cycling microbial communities and associated processes.

More generally, our results have revealed significant interactive effects of multiple global change factors and disturbances on soil nitrifiers and denitrifiers and they thus highlight the need for long-term *in situ* global change experiments manipulating multiple global change factors and disturbances. This is highly needed if we want to better predict the response of soil N cycling to ongoing global change.

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Supplementary

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Postscript

In the fall of 2015, with passion and expectation, I was fortunately enough to pass the assessment application of the Northeast Normal University to be a member of the Ph.D. In the twinkling of an eye, the four valuable years passed away. Now, at the moment of approaching the end of my Ph.D. study, the feeling of joy, affection, bitterness, and happiness are intertwined. This moment of the complex emotions condenses the sweat and hardship during these four years, but also contains too much care without expressing in words and dedication of too many people around me. At the time of graduation, please allow me to express my gratitude in the simplest words.

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At this moment, I will make a perfect point to end my study lasting for twenty-four years, and I will open the next chapter of my life. Once again, I am grateful to all those who helped me, and I wish you all the best! At last, I also would like to express great appreciation to myself with a big hug: thank you for your hard work, darling, all the effort will be paid, continue to work hard and do not miss the spring and your own! Good luck!

Yujie SHI 2019.11.8

List of Publication During Ph. D. study period

Title	**Journal**	**Year**	**Journal Type**	**Author Order**
Tradeoffs and synergies between seed yield, forage yield and regulation of nitrogen losses disservices for a typical semi-arid perennial grassland under different nitrogen fertilization strategies	Biology of Fertilized Soil	2019	SCI (IF 4.829 top journal)	1
Fall nitrogen application increases seed yield, forage yield and nitrogen use efficiency more than spring nitrogen application in *Leymus chinensis*, a perennial grass	Field Crops Research	2017	SCI (IF 3.868 top journal)	1
Seed-germination response of *Leymus chinensis* to cold stratification in a range of temperatures, light and low water potentials under salt and drought stresses	Crop & Pasture Science	2017	SCI (IF 1.804)	1
Long-term summer drought decreases *Leymus chinensis* productivity through constraining the bud, tiller and shoot production	Journal of Agronomy and Crop Science	2019	SCI （IF 2.96）	2
Strategies for lead distribution in organs of *Phragmites australis* (Cav.) Trin. ex Steud. (Common reed) subjected to Pb pollution in flood and drought environments.	Hydrobiologia	2018	SCI （IF 2.165）	4
Salt-alkali tolerance during germination and establishment of *Leymus chinensis*, in the Songnen Grassland of China.	Ecological Engineering	2016	SCI （IF 3.406）	3
The tolerance of growth and clonal propagation of *Phragmites australis* (common reeds) subjected to lead contamination under elevated CO_2 condition.	Rsc Advances	2015	SCI (IF 3.049)	7

Effects of water and fertilizer treatment on pollen, germination and seed setting rate of *Leymus Chinensis*	Journal of Northeast Normal University	2019	Chinese Core Journals (In Chinese)	2
Microbial groups involved in soil N cycling respond differently to a fire disturbance under different global change scenarios	17th *ISME*	2018	Conference	1
Effects of salt stress, storage time, seed mass on seed germination rate and germination vigor of *Leymus Chinensis*	Ecological Society of China	2017	Conference	2
Ratio of seed yield to nitrogen loss, an effective approach for assessing nitrogen benefits and risks in perennial grasses seed production in semi-arid regions	International Rangeland Congress	2016	Conference	2
Relationships between nitrogen fertilizer application and nitrous oxide emission in *Leymus Chinensis* grassland	The *5th* China-Japan-Korea Grassland Conference	2014	Conference	1
N addition modulates N_2O response to rainfall regimes in semi -arid perennial grassland	Global Change Biology	Submitting	SCI	1
Fire disturbance effect on abundance of soil (de)nitrifiers under global change scenarios	-	In preparation	SCI	1

List of Research Funding During Ph. D. Study Period

Funding Title	Funding Source	Period	Role
Study on key technologies for the establishment of degraded and desertified grassland	National Science and Technology Plan Project National Key R&D Program （2016YFC0500705）	2016-2020	Major Participator
Effects of nitrogen-water coupling effects on soil nitrous oxide emission in grassland	Jilin Province Science and Technology Department Jilin Province Natural Science Foundation Project (20170101163JC)	2017-2019	Major Participator
Formation mechanism and regulation pathway of productivity of artificial grassland, a key biological mechanism for the formation of typical grassland productivity	National Program on Key Basic Research Project (973 Program) (2015CB150801)	2015-2016	Major Participator
The response mechanism of nitrous oxide emission to nitrogen deposition and rainfall regime in Songnen grassland.	National Natural Science Funds for Distinguished Young Scholar (31300410)	2014-2016	Major Participator
Rainfall regime, nitrogen deposition and coupling effects on the trade-off between sexual and clonal reproduction of Leymus chinensis	National Natural Science Foundation of China (31370432)	2014-2017	Participator
Physiological and Molecular Mechanisms of the birth time development and spike formation of *Leymus chinensis* daughter shoots.	National Natural Science Foundation of China (31172259)	2012-2015.	Participator

List of Participation to Conferences

During the period of my Ph. D study, I participated 3 times international Conference in total. The details as follows:

1) August 2018, "17th ISME" Germany (Leipzig)-Poster Presentation
   Yujie Shi, Audrey Niboyet, Nona Chiariello, Charline Creuzet des Chatelliers, Bruce Hungate, Chris Field, Xavier Le Roux (2018) Microbial groups involved in soil N cycling respond differently to a fire disturbance under different global change scenarios.
2) July 2017, "International Congress of Ecology" China Beijing-Oral Presentation
   Junfeng Wang, Yunna Ao, Yujie Shi, Mengxing Liu, Donghao Zhou (2017). Effects of salt stress, storaged time, seed mass on seed germination rate and germination vigor of *Leymus Chinensis*. Ecological Society of China
3) August 2014, "The 5th China-Japan-Korea Grassland Conference China Changchun-Poster Presentation
   Yujie Shi, Song Gao, Yanping Cui, Junfeng Wang, Chunsheng Mu (2014) Relationships between nitrogen fertilizer application and nitrous oxide emission in *Leymus chenisis* grassland. The 5th China-Japan-Korea Grassland Conference