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Amandine Laffite, Alessandro Florio, Kasaina Sitraka Andrianarisoa, Charline Creuzé Des Châtelliers, Brigitte Schloter-Hai, et al.. Biological inhibition of soil nitrification by forest tree species affects Nitrobacter populations. *Environmental Microbiology*, 2020, 22 (3), pp.1141-1153. <10.1111/1462-2920.14905>. <hal-02870271>

HAL Id: hal-02870271

<https://hal.science/hal-02870271v1>

Submitted on 17 Jun 2024

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Biological inhibition of soil nitrification by forest tree species affects *Nitrobacter* populations

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Running title: Biological inhibition of *Nitrobacter* by trees

Submitted to *Environmental Microbiology*

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/1462-2920.14905

Originality significance statement: This is the first study demonstrating biological nitrification inhibition (BNI) by tree species which directly affects the abundance of soil *Nitrobacter*. Before this work, BNI was thought to mostly affect ammonia oxidizers. This is an important breakthrough for understanding plant-microorganisms interaction processes that underlie niche construction by plants growing in environments with low soil N availability.

Summary

Some temperate tree species are associated with very low soil nitrification rates, with important implications for forest N dynamics, presumably due to their potential for biological nitrification inhibition (BNI). However, evidence for BNI in forest ecosystems is scarce so far and the nitrifier groups controlled by BNI-tree species have not been identified. Here we evaluated how some tree species can control soil nitrification by providing direct evidence of BNI and identifying the nitrifier group(s) affected. First, by comparing 28 year-old monocultures of several tree species, we showed that nitrification rates correlated strongly with the abundance of nitrite oxidizers *Nitrobacter* (50- to 1000-fold changes between tree monocultures) and only weakly with the abundance of ammonia oxidizing archaea (AOA). Second, using reciprocal transplantation of soil cores between low and high nitrification stands, we demonstrated that nitrification changed 16 months after transplantation and was correlated to changes in the abundance of *Nitrobacter*, not AOA. Third, extracts of litter or soil collected from the low nitrification stands of *Picea abies* and *Abies nordmanniana* inhibited the growth of *Nitrobacter hamburgensis* X14. Our results provide for the first time direct evidence of BNI by tree species directly affecting the abundance of *Nitrobacter*.

Keywords: *Ammonia oxidizing archaea (AOA); biological nitrification inhibition (BNI); comammox; Nitrobacter; soil nitrogen cycling*

Introduction

The oxidation of NH_4^+ to NO_3^- , i.e. nitrification, is a key pathway of the nitrogen (N) cycle in many terrestrial ecosystems. Nitrification is of key importance for soil quality, as it largely regulates the levels and types of mineral N forms, i.e. NH_4^+ and NO_3^- , available for plant nutrition, leaching of NO_3^- , and emission of the potent greenhouse gas N_2O (Baggs, 2011). The classical concept is based on the close interplay of two different groups of microbes: ammonia-oxidizing bacteria (AOB) and archaea (AOA) which oxidize NH_3 to NO_2^- (Kowalchuk and Stephen, 2001; Leininger *et al.*, 2006); and nitrite-oxidizing bacteria (NOB), with two main genera present in soils performing the oxidation of NO_2^- to NO_3^- (*Nitrobacter* and *Nitrospira*; Freitag *et al.*, 2005; Attard *et al.*, 2010). Recently this view was completed as microorganisms were detected which are capable to perform the complete nitrification process (COMplete AMMonia OXidisers, comammox; Daims *et al.*, 2015; Van Kessel *et al.*, 2015).

Nitrogen availability in soil and the balance between NH_4^+ and NO_3^- are the main factors limiting plant growth in most terrestrial ecosystems (Vitousek and Howarth, 1991; Stuart Chapin III *et al.*, 2011; Ma *et al.*, 2019). Plants thus depend on the activity of nitrifiers and can strongly compete with them for NH_4^+ (Vitousek *et al.*, 1982; Kuzyakov and Xu, 2013). However, it is increasingly recognized that plants can exert a major influence over soil N cycling rates (Chapman *et al.*, 2006). Plant species indirectly control soil nitrification or denitrification by altering the main environmental variables affecting nitrifiers and denitrifiers, i.e. NH_4^+ (Bengtsson *et al.*, 2003) and NO_3^- availability (Britto and Kronzucker; Norton and Firestone, 1996; Stark

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and Hart, 1997; Ashton *et al.*, 2010; Bardon *et al.*, 2018), organic C availability (Berks *et al.*, 1995), pH (Hobbie *et al.*, 2007), soil moisture (Le Roux *et al.*, 2013) and oxygen availability (Verstraete and Focht, 1977). In addition, some plant species directly inhibit nitrifiers (i.e. biological nitrification inhibition, BNI ; Jordan *et al.*, 1979; Paavolainen *et al.*, 1998; Lata *et al.*, 1999; Subbarao *et al.*, 2006) or denitrifiers (i.e. biological denitrification inhibition, BDI ; Bardon *et al.*, 2014, 2018) through the release of specific compounds to the soil *via* plant litter or root exudation. For instance, Subbarao *et al.* (2012) identified sakuranetin and sorgoleone as compounds responsible for BNI in sorghum. These mechanisms are of high importance mainly in ecosystems with low soil N availability as they minimize N losses and allow plants to effectively utilize the scarce N resource (Vitousek and Sanford, 2003; Boudsocq *et al.*, 2009).

Although BNI has been already observed in different ecosystems (Subbarao *et al.*, 2006; Srikanthasamy *et al.*, 2018), the underlying mechanisms have been identified only for a few perennial grass species, e.g. *Brachiaria spp.*, *Andropogon spp.*, and *Hyparrhenia diplandra* (Lata *et al.*, 1999, 2004; Subbarao *et al.*, 2009), as well as cultivated sorghum (Subbarao *et al.*, 2012) and rice (Sun *et al.*, 2016). In forest ecosystems, very low nitrification rates can occur in stands of particular tree species (Lodhi and Killingbeck, 1980; Degrange *et al.*, 1998), presumably due to the production of species-specific secondary metabolites including phenolics, alkaloids and terpenoids (Northup *et al.*, 1995; Erikson *et al.*, 2000; Castaldi *et al.*, 2008; Smolander *et al.*, 2012). However, evidence for direct BNI in forest ecosystems is

scarce to date. Both litter and root exudates seem to be important sources of BNI compounds in forest soils (Castaldi *et al.*, 2008). BNI exerted by monoterpenes produced by roots was observed in soils under Norway spruce stands, although indirect nitrification inhibition through N immobilization could not be excluded (Paavolainen *et al.*, 1998). Caffeic acid, chlorogenic acid and ferulic acid from *Quercus spp.* and *Pinus spp.* soil suspensions strongly inhibited the growth of *Nitrosomonas* but only marginally *Nitrobacter* model strains (Lodhi and Killingbeck, 1980; Rice and Pancholy, 2006).

To date, most of BNI compounds (generally produced by grass species) are known to inhibit ammonia monooxygenases (AMO) which catalyses NH_4^+ oxidation to NH_2OH , and some of them can effectively block the hydroxylamino-oxidoreductases (HAO) which is responsible for the second step of ammonia oxidation, namely the oxidation of NH_2OH to NO_2^- (Coskun *et al.*, 2017). Since NH_4^+ oxidation is traditionally assumed as the limiting-step of nitrification (Stevenson and Schmidt, 1982), the majority of studies analysing the BNI effect were conducted on AOA and AOB and did not take into account possible effects on NO_2^- oxidoreductase (NXR) and more generally on NOB (but see Lodhi and Ruess, 1988) as well as on the recently discovered comammox bacteria.

Here we evaluated how different temperate forest tree species can biologically control soil nitrifier activity by using a 3-step approach (Fig. 1). First, we compared the enzyme activities and abundances of nitrifiers (AOB, AOA, *Nitrospira*, *Nitrobacter* and Comammox from clades A and B), along with environmental

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conditions (moisture, mineral N concentrations and pH) between soils from 28-year-old monoculture stands (5 tree species) plus native forest. We assumed that AOA and *Nitrospira* abundances would be correlated with nitrification rates in these soils, as these groups are often reported to be better adapted than AOB and *Nitrobacter* to low N substrate levels and low pH values in soil (Hatzenpichler, 2012; Le Roux *et al.*, 2016). We also assumed that commamox bacteria might have an important role in nitrification, as their presence has been revealed in numerous environments (Daims *et al.*, 2015; Van Kessel *et al.*, 2015), including forest soils (Pjevac *et al.*, 2017). Second, we used a reciprocal soil core transplantation approach between low- and high-nitrification plots, i.e. putative BNI- and non BNI-tree species, respectively. The objective was to analyse the kinetics of changes in nitrifier activity and abundance in soils 16 and 28 months after transplantation. We assumed that soil cores transplanted from putative BNI- to non BNI-species would experience a release from inhibition, thus resulting in increased nitrifier abundance and activity. Conversely, we assumed that soil cores transplanted from putative non-BNI- to BNI-species would face inhibition, thus resulting in decreased nitrifier abundance and activity. More particularly, we expected AOA abundance to respond rapidly to this second type of transplantation assuming that BNI compounds would affect ammonia oxidizers rather than nitrite oxidizers. We also assumed that *Nitrospira* abundance would follow changes in AOA abundance as a result of changes in substrate availability. Third, to test whether low nitrification rates were due to the production of specific plant secondary metabolites, we incubated litter or soil extracts from BNI-tree species with a

strain chosen from the nitrifier group found as the most sensitive to BNI, and we measured its growth to more directly demonstrate the actual BNI capacity of the tree species associated to low soil nitrification rates. Our results jeopardize the classical belief that AOA would be the main nitrifiers targeted by BNI in these forest ecosystems.

Results

Relationships between nitrification and nitrifier abundances among the 28 year-old plots

In the 28 year-old plots, net nitrification and nitrifying enzyme activity (NEA, i.e. potential nitrification) were strongly and positively correlated ($R^2=0.90$, $p=0.039$), with very similar values observed for both net and potential rates (Fig. S1). Soil nitrification strongly differed between tree species, with highest values around $0.05 \mu\text{g-N h}^{-1} \text{ g}^{-1}$ soil observed for *P. nigra*, *P. menziesii* and *F. sylvatica*, and values lower than $0.01 \mu\text{g-N h}^{-1} \text{ g}^{-1}$ soil observed for *P. abies*, *A. nordmanniana* and the native forest (Fig. 2 and S2). No relationship was observed between soil nitrification and soil pH (Fig. S3). For the 3 low nitrification plots (L), net mineralisation was much higher than net nitrification, i.e. nitrification was low despite the availability of NH_4^+ (Fig. S2). For 2 of the 3 high nitrification plots (H), net nitrification was similar to net mineralisation, i.e. all the NH_4^+ formed was converted into NO_3^- (Fig. S2).

Differences in NEA across all stands were significantly and positively correlated to changes in the abundances of AOA and *Nitrobacter* ($p=0.0113$, $R^2=0.83$ and $p=0.0009$, $R^2=0.95$, respectively; Fig. 2, Top and middle left, respectively). However, differences in *Nitrobacter* abundance were particularly high (from 10^3 *nrrA* copies g^{-1} soil in the plot with the lowest NEA, up to 6×10^5 *nrrA* copies g^{-1} soil in the plot with the highest NEA) and the correlation between NEA and *Nitrobacter* abundance was particularly strong. In contrast, AOA abundance (from 6×10^5 to 10^6 *amoA* copies g^{-1} soil) did not significantly change between plots. No significant relationship was observed between NEA and the abundances of AOB, *Nitrospira* or Comammox from clades A and B (Fig. 2). Because net nitrification and NEA values were similar (Fig. S1), the same (lack of) relationships hold when considering net nitrification (not shown).

Relationships between changes in nitrification and changes in AOA and *Nitrobacter* abundances following soil core transplantation

Sixteen months after the soil core transplantation, net nitrification in the cores originating from one of the high nitrification (H) plots and transplanted to one of the low nitrification (L) plots, i.e. HL treatment, did not differ from nitrification in HH cores (Fig. 3). In contrast, net nitrification in LH cores was significantly higher than in LL cores (Fig. 3). However, nitrification for the LH treatment remained lower than for the HH treatment. Twenty-eight months after the soil core transplantation, net nitrification in the HL cores was significantly lower than in the HH cores, although it

remained higher than nitrification in the LL cores (Fig. 3). Net nitrification was much higher in the LH than in LL cores and not significantly different from nitrification in the HH cores (Fig. S4).

Sixteen months after the soil core transplantation, nitrification was strongly correlated to the abundance of *Nitrobacter* but only weakly correlated to the abundance of AOA ($p < 0.0001$, $R^2 = 0.80$ and $p = 0.046$, $R^2 = 0.34$, respectively; Fig. 4, top). In particular, the increase in nitrification observed for LH treatment was strongly linked to an increase in the abundance of *Nitrobacter*, whereas AOA abundance did not increase (Fig. 4). After 28 months, nitrification was well correlated to the abundances of AOA and *Nitrobacter* ($p = 0.0006$, $R^2 = 0.71$ and $p = 0.0002$, $R^2 = 0.78$, respectively; Fig. 4, bottom).

Effects of soil and litter extracts from low nitrification stands on the growth of a model *Nitrobacter* strain

All the extracts of soil and litter from the low nitrification *P. abies* and *A. nordmanniana* plots decreased the growth of the nitrite-oxidizing strain *Nitrobacter hamburgensis* X14 (Fig. 5). The growth inhibition was strongly linked to the extract type, i.e. litter or root exudates ($p < 0.001$), with a low effect of the extract concentration ($p = 0.032$). The extracts from the litter derived from *A. nordmanniana* plots induced a decrease in *N. hamburgensis* growth of around -12% without any effect of extract concentration (Fig. 5, right). The extracts derived from the soil of plots grown with *A. nordmanniana* induced a decrease in *N. hamburgensis* growth of

around -16% and -26% for the lowest and highest extract concentration, respectively (Fig. 5, right). The extracts from both the litter and soil derived from *P. abies* plots induced a decrease in *N. hamburgensis* growth of -25 to -31%, respectively, without any significant effect of extract concentration (Fig. 5, left).

Discussion

Tree species effects on soil nitrification in old stands are mostly correlated to changes in *Nitrobacter* abundance

As previously reported (Moukoudi *et al.*, 2006; Zeller *et al.*, 2007; Andrianarisoa *et al.*, 2010), forest tree species strongly influenced soil nitrification. In the long term, here after 28 years, *P. abies*, *A. nordmanniana* and the native forest were associated with potential and net nitrification rates 10- to 1000-fold lower than the rates associated to *P. nigra*, *P. menziesii* and *F. sylvatica*. The differences between high and low nitrification stands were higher than within stand variations in nitrifier activity and abundances, and sampling a few soil cores per stand was adequate to capture within stand variability. This is likely due to the fact that in forest ecosystems one soil core includes roots from several tree individuals in relation to large horizontal root extension (e.g. Meinen *et al.*, 2009). Here, we demonstrated that these contrasted nitrification rates were correlated to the size of particular ammonia- and nitrite-oxidizing groups. Nitrification rates were not correlated to the abundances of AOB. In contrast, nitrification rates in the forest soils studied were significantly correlated to

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AOA abundances. This appears to be consistent with a large body of literature suggesting that AOA would be the main players of nitrification in forest soils (Stopnišek *et al.*, 2010; Zhang *et al.*, 2012). This would be due to better adaptation of AOA than AOB to low N-substrate availability and low pH, which are common conditions for forest soils (Verhamme *et al.*, 2011; He *et al.*, 2012; Hu *et al.*, 2015). However, our results indicated that the abundances of AOA and AOB were similar in the studied soils, i.e. 5.77×10^5 and 5.58×10^5 copies g⁻¹ dry soil on average, respectively. This finding is in contrast with several studies reporting higher abundances of AOA than AOB in forest soils (Szukics *et al.*, 2010; Yao *et al.*, 2011; Stempfhuber *et al.*, 2017; but see Wertz *et al.*, 2012; Zhang *et al.*, 2012). In addition, AOA abundances varied only by 8-fold while nitrification rates showed 100- to 1000-fold changes. Further, the low nitrification rates were observed despite high abundances of both AOA and AOB and rather high ammonification rates similar to those observed in high nitrification plots (Fig. S2), and were not explained by soil pH values (Fig. S3). All these results are thus not consistent with a tree species effect on nitrification mostly mediated by an effect on ammonia oxidizers, pH and/or N availability.

Concurrently, nitrification rates in soils of the 28-year-old stands were strongly correlated to the abundance of *Nitrobacter*, but not *Nitrospira*, although higher abundances were found for *Nitrospira* compared to *Nitrobacter*. Similarly, it has been shown that despite higher *Nitrospira* abundances, nitrification rates were mostly related to *Nitrobacter* abundances in agricultural (Attard *et al.*, 2010) and grassland

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soils (Le Roux et al., 2016). Thus, although the abundances of *Nitrospira* are often higher than *Nitrobacter* in forest soils, as observed here and as reported for soils under beech, spruce and pine (Wertz et al., 2012; Stempfhuber et al., 2017), our results suggest that *Nitrobacter* may have a more prominent role in nitrification. More specifically, *Nitrobacter* abundances showed 100- to 1000-fold changes (from 4.12×10^2 to 3.62×10^6 copies g⁻¹ dry soil, respectively) in parallel to the 100- to 1000-fold changes in nitrification rates. This means that in plots with no nitrification, high abundances of AOA, AOB and *Nitrospira* –along with high mineralization rates– were still observed, whereas only *Nitrobacter* abundances were strongly reduced. Furthermore, nitrification rates were not correlated to the abundances of neither Comammox clade A nor Comammox clade B. It has been suggested that comammox organisms may outcompete other nitrifier groups in acidic (Shi et al., 2018) and substrate-limited environments (Kits et al., 2017). However, little is known on the actual role of comammox on nitrification in forest ecosystems (but see Wang et al., 2019).

As ammonia oxidation is often assumed to be the rate-limiting step of nitrification, the majority of the studies published in the last decades have focused on ammonia oxidizers to better understand changes in nitrification rates, overlooking the possible importance of nitrite oxidizers. Hence, little information is available on the importance of NOB for determining nitrification rates in forest ecosystems. In addition, co-occurrences (relationships between abundances) of AOA and *Nitrospira* on the one hand, and of AOB and *Nitrobacter* on the other hand, have been previously

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reported for forest (Stempfhuber *et al.*, 2017), grassland (Ma *et al.*, 2016) and agricultural soils (Assémien *et al.*, 2017). This is traditionally explained by the fact that NO_2^- availability would be the main driver of niche partitioning between these groups, AOB and *Nitrobacter* being favoured under high N availability (Attard *et al.*, 2010). Here we did neither observe a strong correlation between the abundances of *Nitrobacter* and AOB or AOA, nor a relationship between *Nitrobacter* abundances and mineralization rates. These results strongly suggest that (i) *Nitrobacter* was the main nitrifier group restricting nitrification rates in low nitrification plots, and (ii) soil N availability and pH were not the main drivers of *Nitrobacter* abundances and consequently of nitrification.

Dynamics of nitrifier activity and abundances following soil core transplantation further suggests tree control of nitrification through *Nitrobacter* rather than AOA

Reciprocal transplantation of soil cores has already been used to investigate the effects of abiotic and biotic drivers on nitrifiers *in situ* (Bottomley *et al.*, 2004; Reed and Martiny, 2007; Kageyama *et al.*, 2013), and it has been shown that nitrifiers have the capacity to respond to changes in soil environmental conditions over a few weeks or months (Le Roux *et al.*, 2008). For instance, complete resilience of AOB abundance has been observed 2 years after a soil core transplantation experiment (Bottomley *et al.*, 2004). Here, the transplantation of soil cores amongst stands of different forest tree species allowed a better identification of the nitrifier group(s) mostly sensitive to tree species influence and determining nitrification rates. Assuming that the first step of

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nitrification would be the limiting one as commonly accepted, and that AOA would be the main functional players for nitrification in these forest soils as previously suggested (Stempfhuber *et al.*, 2017), we expected that soil core transplantation from low (L) to high (H) nitrification plots, i.e. LH treatment, would first result in increased AOA abundance, and then in subsequent increased NOB abundance due to an increase in their substrate availability (De Boer, *et al.*, 1991). Unexpectedly, our results showed that the LH treatment first led to an increase in *Nitrobacter* abundance in parallel to the observed increase in nitrification after 16 months, whereas no change in AOA abundance was observed. Only 28 months after the LH transplantation, an increase was also observed for AOA abundance. Overall, after 16 months, nitrification rates were strongly correlated to *Nitrobacter* abundances but only weakly to AOA abundances. This strongly suggests that *Nitrobacter* rather than AOA were the main nitrifier group restricting nitrification in low nitrification (L) plots, and that the driver of low *Nitrobacter* abundances in L plots was quickly released in H plots.

Interestingly, the responses of nitrification activity and both *Nitrobacter* and AOA abundances were slower for transplantation of soil cores from H to L plots than for the reverse LH treatment, a decrease in potential nitrification being observed only 28 months after HL transplantation. Similar asymmetric changes in nitrification rates and ammonia oxidizer abundances have been observed for grassland soils exposed to reversion of management (Le Roux *et al.*, 2008). Given that tree roots were cut when establishing the core transplantation experiment, one possibility is that the results of the soil core transplantation experiment would be due to a strong biological control of

Nitrobacter by roots. For LH treatments, cutting roots of trees that initially prescribed low nitrification rates, possibly through specific compounds, may have quickly relapsed this control depending, e.g. on the stability of the specific compounds involved. This could explain why a rather fast increase in *Nitrobacter* abundances was observed for LH treatments. In contrast, after establishment of HL cores, time would be needed for a sufficient colonization of soil cores by roots of the tree species able to inhibit *Nitrobacter*. However, this possible biological inhibition of *Nitrobacter* by specific compounds associated to some tree species required to be evaluated, which we tested through the laboratory experiment (without identifying any specific compounds).

Evidence that some tree species can inhibit *Nitrobacter*

Low pH and low N availability, typical for most forest (He *et al.*, 2012; Hu *et al.*, 2015), heathland (Bardon *et al.*, 2018) and humid savanna ecosystems (Le Roux *et al.*, 1995), harbor selected plants with diverse mechanisms for improving soil N availability (Chapman *et al.*, 2006). In particular, it has been shown that some plant species in these ecosystems can inhibit nitrifiers (Lata *et al.*, 2004; Subbarao *et al.*, 2009; Srikanthasamy *et al.*, 2018) and that this inhibition is due to the production of specific compounds, often by roots (Subbarao *et al.*, 2006, 2015; Coskun *et al.*, 2017). However, most plants previously identified as having a biological nitrification inhibition (BNI) capacity were grass species. Further, previous studies have reported that these species inhibited ammonia oxidizers, generally AOB (Subbarao *et al.*,

2012). Here, we cannot exclude that ammonia oxidizer populations were affected by BNI, as most of the nitrification inhibitors are bacteriostatic and not bactericidal (Amberger, 1989; Rodgers and Ashworth, 2011). This could be further explored in future research by determining to what extent AOB and AOA populations are functional using RNA-based approaches. However, the present study demonstrates for the first time a BNI capacity for tree species inducing inhibition of *Nitrobacter*. Indeed, our results clearly show that soil and litter extracts from spruce and Nordmann fir effectively inhibited the growth of the NOB strain *N. hamburgensis*. We have used *N. hamburgensis* as it is one of the few species of *Nitrobacter* isolated from soil for which the level of knowledge regarding its ecophysiology and ecology is very good, but further studies will be needed to detail BNI effects on a higher number of strains and species. The higher inhibition observed after incubation with soil rather than litter extracts from Nordmann fir suggests that this tree species might control *Nitrobacter* abundance and nitrification rates mainly through root exudation. In contrast, both soil and litter extracts from spruce were sources of BNI compounds. These findings thus demonstrate that the tree species associated to the lowest nitrification rates *in situ* can inhibit *Nitrobacter*, which explains the results obtained in the soil core transplantation experiment. Accordingly, NO_2^- accumulation in soil could be expected in soils under BNI tree species, but NO_2^- concentration was below detection limit during the nitrification assays, except for a very few soil samples (not shown). As NO_2^- accumulation might lead to plant, animal and microbial toxicity (Van Cleemput and Samater, 1996), alternative metabolic pathways, i.e. nitrifier-denitrification (Wrage *et*

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al., 2001), archaeal aerobic *nirK*-denitrification (Treusch *et al.*, 2005), production of nitrous acid through NO_2^- conversion (Su *et al.*, 2011), and NO_2^- incorporation into soil organic matter (Fitzhugh *et al.*, 2003) might take place and explain lack of NO_2^- accumulation.

The fast increase in *Nitrobacter* abundance 16 months after the LH transplantation suggests that the compounds responsible for BNI might be labile and might have been quickly degraded when roots of tree species with BNI capacity were cut and soil cores exposed to a new environment without BNI compound production. Consistently, some BNI molecules has been proved to lose their effectiveness after 100 days in soil (Subbarao *et al.*, 2008). In contrast, following HL transplantation, the slower response of *Nitrobacter* abundance might be due to the progressive colonization of the soil cores by BNI tree species roots. BNI molecules released at low concentrations during the early stages of root colonization might not be sufficient to exert a strong inhibiting effect. Overall, our findings demonstrate that the tree species associated with low nitrification rates *in situ* had the capacity to inhibit *Nitrobacter*. Yet, identification of the compounds responsible for BNI was beyond the scope of this study and remains to be investigated. Further, we worked on composite from 3 samples for each plot and each soil layer. This is sufficient to demonstrate the existence of inhibitors and for a first characterization of their action on *Nitrobacter*, but a more extensive sampling would be needed to characterize the possible spatial and temporal variation in BNI in forest stands.

Conclusion

It is increasingly recognised that many components of a plant's environment are determined by the plant itself, each plant individual shaping to some extent its own environment which ultimately influences its fitness (Schweitzer *et al.*, 2004). This ecological-evolutionary feedback loop is called 'niche construction' (Lewontin and Lewontin, 2000; Odling-Smee *et al.*, 2003). Plant niche construction abilities often concern plant-soil feedback loops (Schweitzer *et al.*, 2013). In particular, it was known that some grass species adapted to low soil N availability were able to inhibit nitrification via the exudation of specific compounds inhibiting ammonia oxidizers, mostly AOB (Subbarao *et al.*, 2009). Recently, Bardon *et al.* (2014, 2018) demonstrated that some forb and shrub species are able to inhibit denitrification, through the exudation of phenolic compounds by roots. Our results demonstrate for the first time that some tree species can also inhibit nitrification through knock out of the nitrite oxidizers *Nitrobacter*. This is an important breakthrough for understanding the range of plant-microorganisms interaction processes that underlie niche construction by plants growing in environments with low soil N availability.

Material and methods

Study site and soil sampling in 28 year-old plots

The study site is a long-term experimental site managed by INRA, located in the Breuil-Chenue forest ('SOERE F-OreT' site, Nièvre Morvan, France; 47°18'N and

4°44'E; elevation of 650 m). Mean annual temperature and precipitation are 9°C and 1280 mm, respectively. The native forest is a 150-year-old coppice dominated by beech (*Fagus sylvatica* L.) with *Quercus sessiliflora* Smith, *Betula verruosa* Ehrh, and *Corylus avenala* L. In the end of the 1970s, a 10-ha flat area was clear-cut, and bole wood and large branches were harvested. The area was planted in rows with tree monocultures of beech (*Fagus sylvatica*), Corsican pine (*Pinus nigra* Arn. Spp laricio Poirlet var corsicana), Douglas fir (*Pseudotsuga menziesii* Franco), Nordmann fir (*Abies nordmanniana* Spach.) and spruce (*Picea abies* Karst) (1000 m² for each species) (Andrianarisoa *et al.*, 2010). A reference plot of native forest was also studied. The humus in the native forest is a dysmoder and the soil developed from granite is classified as a Typic Dystrochrept (USDA 1999). The texture of the soil is sandy-loam (60% sand and <20% clay) and the soil is acidic (pH around 4).

Soil was sampled at 6 locations randomly chosen within each of the 6 plots with a corer (0-10 cm depth; 8 cm diameter). The 36 soil samples were sieved (4 mm) and visible roots were removed. Soil sub samples were stored during 4 days at 4°C before assays for pH and potential and net nitrification rates. Other sub samples were stored at -20°C for molecular analysis.

Soil core transplantation experiment and core sampling

Twenty eight years after plantation, 300 intact soil cores including the forest floor layer were collected with an auger (diameter 8 cm; depth 15 cm) along two parallel lines located 0.5 m away from a tree row (Fig S4), i.e. 60 cores for each pure stand

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plot. In the native forest, each of the 60 cores was located at 0.5 m from a beech dominant tree. Soil cores were wrapped in mesh bags (2 mm mesh size). Ten cores were placed back into their original location (i.e., 10 beech soil cores placed back in the beech plot, etc.) and 50 cores were placed into the empty holes in the five other plots (i.e. 10 beech soil cores translocated to the spruce plot, etc.). In each plot, half of the soil cores (i.e. 6 treatments x 5 replicates = 30) were collected 16 months after transplantation, and the other half after 28 months.

At each sampling date, the residual forest floor material from the original stand and the newly fallen litter from the host stand were collected before removing soil cores. For each core, soil was sieved (4 mm) and visible roots were removed. Soil sub samples were stored at 4°C for the nitrification assays, and frozen at -20°C for molecular analysis.

Measurements of soil environmental variables and nitrification activities

Gravimetric soil moisture and soil pH (using 1/1 vol/vol soil/water slurry) were measured for each of the 36 soil samples taken from the 28 year-old plots. On each sample, nitrifying enzyme activity (NEA) was measured using short-term laboratory incubations under non-limiting conditions according to (Dassonville *et al.*, 2011). Sub-samples of fresh soil (3 g equivalent dry soil) were incubated at 28°C with 6 ml of a solution of (NH₄)₂SO₄ (50 µg N-NH₄⁺ g⁻¹ dry soil), and distilled water was added in each sample to reach 24 ml of total liquid volume in flasks. Soil NO₃⁻ content was measured after 5, 24, 48 and 72 hours during an aerobic incubation under constant

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agitation (180 rpm) by ion chromatography (DX120, Dionex, Salt Lake City, USA). NEA was computed as the linear rate of nitrate production over 72h (no nitrite accumulation was observed, except on a very few soils). Net nitrification and net mineralization rates were also measured for each of the 36 samples according to Andrianarisoa *et al.* (2010). Sub-samples of 200 g fresh soil were placed into jars with airtight lids and incubated at 20°C in the dark for 42 days. The jars were opened for a few minutes twice a week. NH_4^+ and NO_3^- were extracted at the beginning and at the end of the incubation using 20 g of soil shaken in 1 M KCl for 1 h and then filtered. The NH_4^+ and NO_3^- concentrations of extracts were measured with a continuous-flow colorimeter (TRAACS, Bran and Luebbe). Net mineralization and nitrification rates were computed as the amount of total inorganic N and of nitrate, respectively, accumulated during the incubation. All concentrations and rates are presented on a dry soil mass basis.

For the soil core transplantation experiment, gravimetric soil moisture and net nitrification rate were measured as described above for each of the 360 fresh soil samples.

DNA extraction and quantification of nitrifier abundances

DNA was extracted from 0.5 g of soil for all soil samples using a PowerSoil DNA Isolation Kit (MO BIO laboratories, Inc, USA) according to the manufacturer's protocol. Extracted DNA was stored at -20°C until use. The abundances of AOB and AOA, *Nitrobacter*, *Nitrospira*, and *Nitrospira* comammox clades A and B were

quantified by real-time PCR targeting sequences of the bacterial and archaeal *amoA* (coding for the ammonia monooxygenase), the *nxrA* gene of *Nitrobacter* (coding for the nitrite oxido-reductase), the *16S rRNA* gene for *Nitrospira*, and the specific *amoA* genes for comammox clades A and B, respectively. All these 6 nitrifier groups were quantified for the 36 soil samples retrieved from the 28 year-old plots. Given the results obtained on old plots, only AOA and *Nitrobacter* were targeted for the 360 soil samples retrieved part of the core transplantation experiment. All samples were run in duplicate on a Lightcycler 480 (Roche Diagnostics, Meylan, France). Dilution series of the extracted DNA were performed to control for possible PCR inhibition by co-extracted compounds, and no inhibition was observed (data not shown).

For AOA and AOB, amplification was performed using the primer sets CrenamoA23f and CrenamoA616r (Tourna *et al.*, 2011) and *amoA*_1F and *amoA*_2R (Rotthauwe and Witzel, 1997), respectively. Linearized plasmids containing cloned archaeal (54d9 fosmide fragment) and bacterial (*Nitrosomonas europaea*, GenBank accession number L08050) *amoA* genes served as standards. For *Nitrobacter*, the amplification was performed according to Attard *et al.* (2010) using the gene primers F1norA and R2norA (Wertz *et al.*, 2012). A linearized plasmid containing a cloned fragment of the *nxrA* gene of *Nitrobacter hamburgensis* X14 (DSMZ 10229) was used as standard. For *Nitrospira*, the amplification was performed according to Attard *et al.* (2010) using the gene primers Ns675f and Ns746r. Copies of a fragment of the *Nitrospira* 16S rRNA gene (GenBank accession number FJ529918) served as standard for quantification. For *Nitrospira* comammox clades A and B, the amplification was

performed according to Pjevac *et al.* (2017) using the gene primers comaA-244F/comaA-659R and comaB-244F/comaB-659R. A linearized plasmid containing cloned sequences from comammox clade A (DQ008369.1) and clade B (GenBank accession number AJ564438.1) *amoA* genes served as standard. Efficiency of qPCR assays was between 85 % and 100 %.

Quantification of the effect of soil and litter extracts on *N. hamburgensis*

In January 2014, three soil cores (10 cm depth) were randomly sampled from each plot for two low nitrifying stands (i.e. Spruce and Nordmann pine). For each of the six cores, the soil horizon O (organic layer made up mostly of leaf litter and humus) and the A horizon (topsoil of horizon A) were separated, and a composite sample was obtained for each layer per plot. Litter and mineral soils layers were subjected to sequential extraction with acetone, 70% methanol and water (3 times each, extracts pooled together for each sample). Supernatants were evaporated and re-suspended in 50% methanol/water at 50 mg ml⁻¹. The samples also contained roots just cut and the extracts could contain root compounds and exudates ; but retrieving root exudates from living tree roots is highly challenging and was beyond the scope of our study.

The *in vitro* activity of litter and soil extracts for BNI was tested using *Nitrobacter hamburgensis* X14 as a model by performing continuous absorbance measurements taken with a Bioscreen C system (LabSystems, Helsinki, Finland). Bacterial suspensions were prepared from cultures grown in DSMZ 756a media for 5 days at 28°C. Suspensions were adjusted to a final OD_{600nm} of 0.2. Honeycomb 100-well

microplates were filled with 888.9 μl of bacterial suspension and 111.1 μl of extract reaching the concentration of 0.25, 0.5 and 1 mg ml^{-1} . The plates were incubated in the Bioscreen C system at 28°C for 5 days and optical density was measured every 20 min with 5 s of prior shaking. The maximal growth μ_{max} was determined in day^{-1} . All growth curves were done in quadruplicates.

Statistical analyses

All statistical analyses were conducted using R software v3.4.2. Data were log-transformed for normality when needed (i.e. for abundance data). To test the effects of treatments on the microbial activities and abundances or *Nitrobacter* growth, one-way analysis of variance (ANOVA) was performed. This was followed by Turkey's honestly significant difference (HSD) at a $p=0.05$ level. Correlations between enzyme activity and abundance data were tested using the Pearson's product-moment correlation at a $p=0.05$ level.

Acknowledgments

This study was funded by the French Institute for agronomic Research, INRA (EFPA Department) and CNRS (INEE Department). We thank Vincent Gaillard and Gwenaelle Berrocal (Centre d'Etude des Substances Naturelles, CESN, LEM, Villeurbanne) for help during HPLC assays, and Claire Commeaux (LEM UMR INRA 1418) for help during qPCR assays.

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Figure captions

Fig. 1: Schematic representation of the three steps used in this work to analyse how tree species control soil nitrifier activity and abundance.

Fig. 2: Relationships between net nitrification and the abundances of (Top-Left) ammonia oxidizing archaea, AOA, (Top-Right) ammonia oxidizing bacteria, AOB, (Middle-Left) *Nitrobacter*, (Middle-Right) *Nitrospira*, (Bottom-Left) comammox clade A and (Bottom-Right) comammox clade B for 28 year-old plots. Each point corresponds to the mean value for a given tree species and bars are standard errors (n=6). NS: no significant relationship. Note that a common scale was used for all X axes. In the *Nitrobacter* panel, tree species are indicated: A n: *Abies nordmanniana* ; F s: *Fagus sylvatica* ; N f: Native forest ; P a: *Picea abies* ; P n: *Pinus nigra* ; P m: *Pseudotsuga menziesii*.

Fig. 3: Comparison of soil net nitrification (Top) 16 months and (Bottom) 28 months after the soil core transplantation. Cores were transplanted from high to high nitrification plots (HH1, HH2 and HH3), from high to low nitrification plots (HL1, HL2 and HL3), from low to high nitrification plots (LH1, LH2 and LH3), and from low to low nitrification plots (LL1, LL2 and LL3). See Table S1 indicating which label corresponds to which treatments ; for instance, LL1 includes the 3 treatments ‘Native forest to native forest’, ‘Native forest to *Picea abies*’ and ‘Native forest to

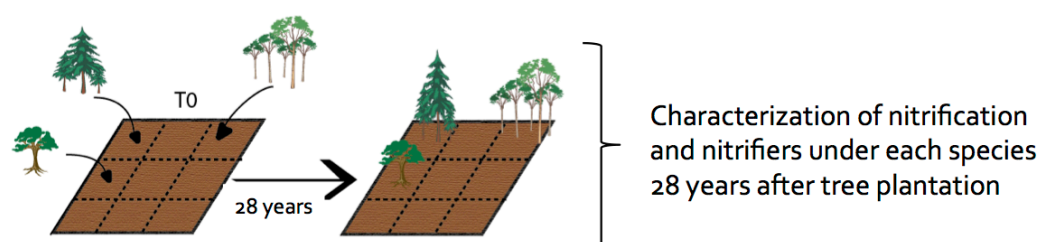
Abies nordmanniana'. Mean values are presented with standard errors (n=15). For each date, different letters indicate significant differences between treatments.

Fig. 4: Relationships between net nitrification and the abundance of (Left) *Nitrobacter* and (Right), ammonia oxidizing archaea, AOA, (Top) 16 months and (Bottom) 28 months after the soil core transplantation. Main treatment acronyms are as in figure 3. Mean values are presented with standard errors (n=15). Core transplantation treatments: HH, from high to high nitrification plots ; HL, from high to low nitrification plots ; LH, from low to high nitrification plots ; and LL, from low to low nitrification plots.

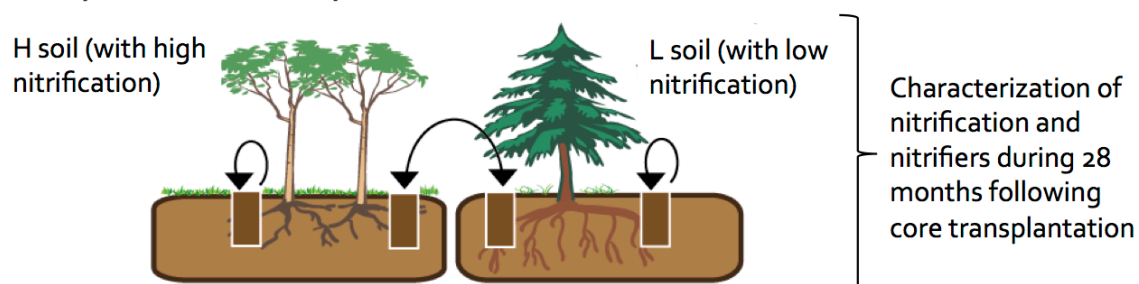
Fig. 5: Level of the decrease in *Nitrobacter hamburgensis* maximum growth rate, μ_{\max} , induced by the extracts of litter and soil from the –low nitrification– *Picea abies* and *Abies Nordmanniana* plots, as compared to the control without any extract. For each tree species and type of extract, 3 concentrations were tested. Mean values are presented with standard errors (n=4). Different letters indicate significant differences between treatments.

Fig. 1

Step #1: Tree species plantation



Step #2: Soil core transplantation



Step #3: Effect of soil and litter extracts on *Nitrobacter*

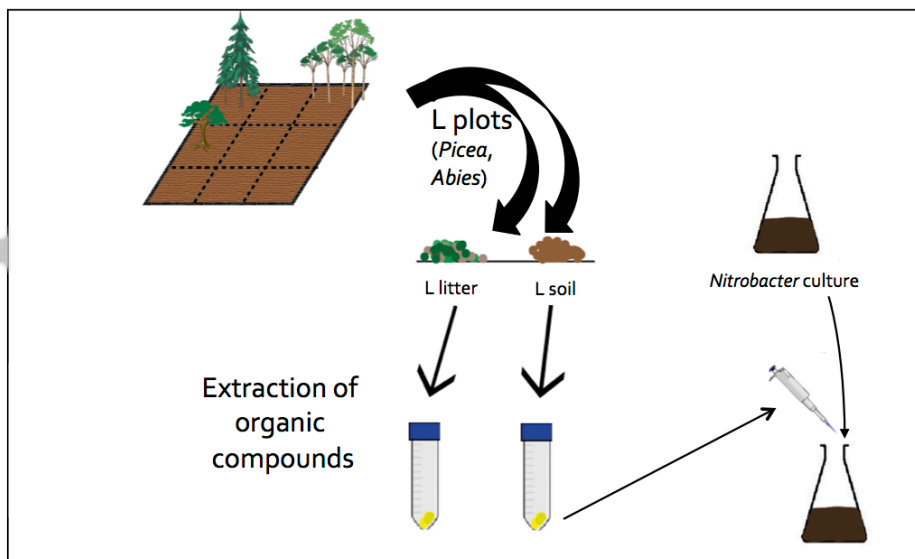


Fig. 2

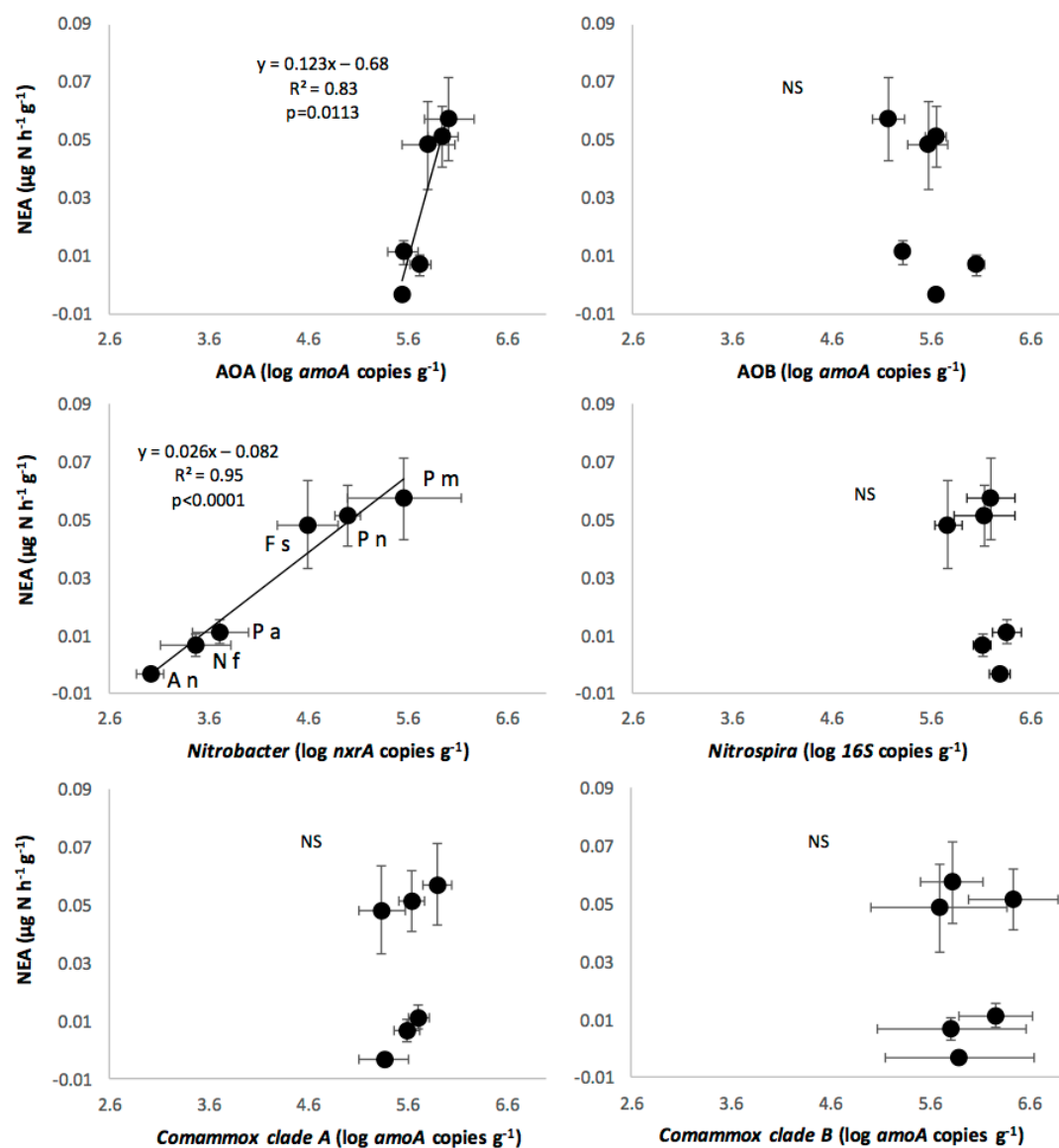


Fig. 3

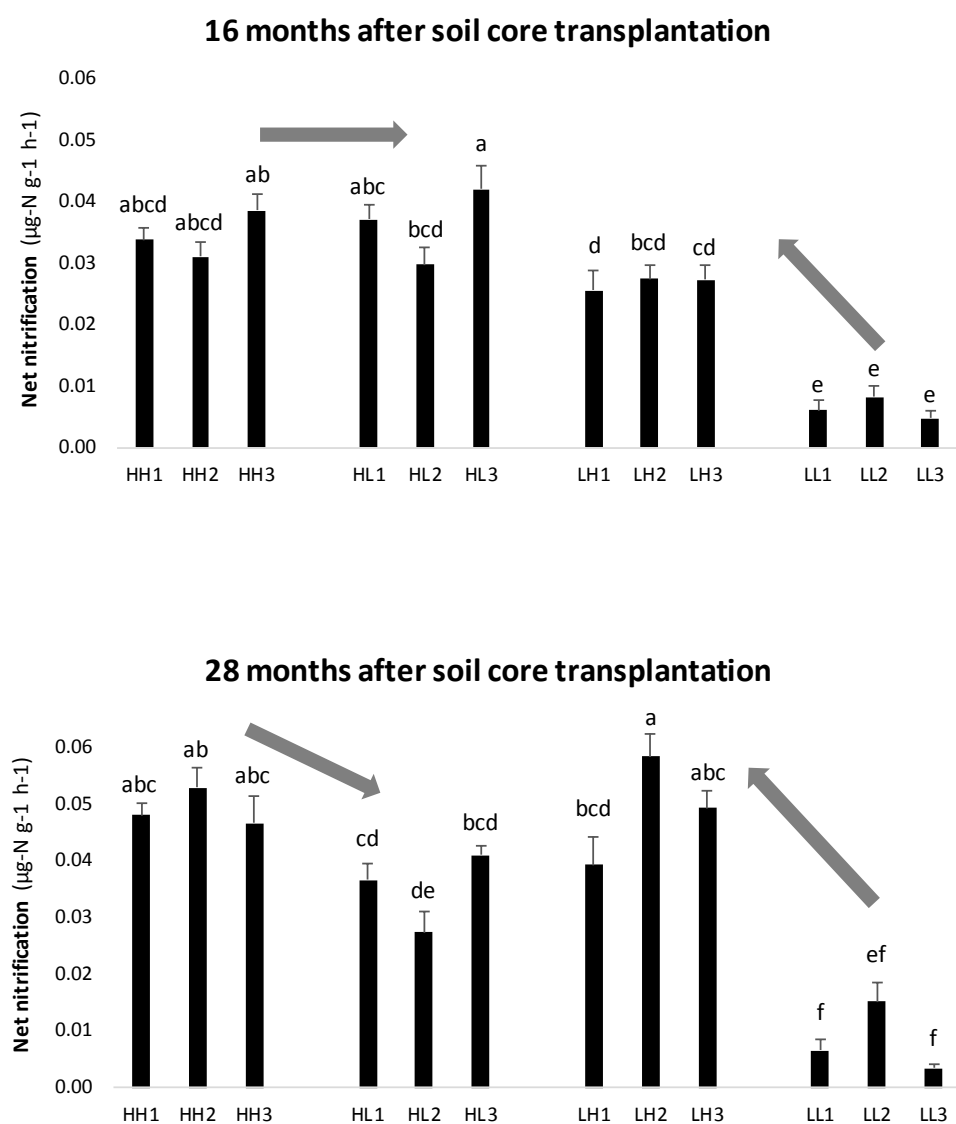


Fig. 4

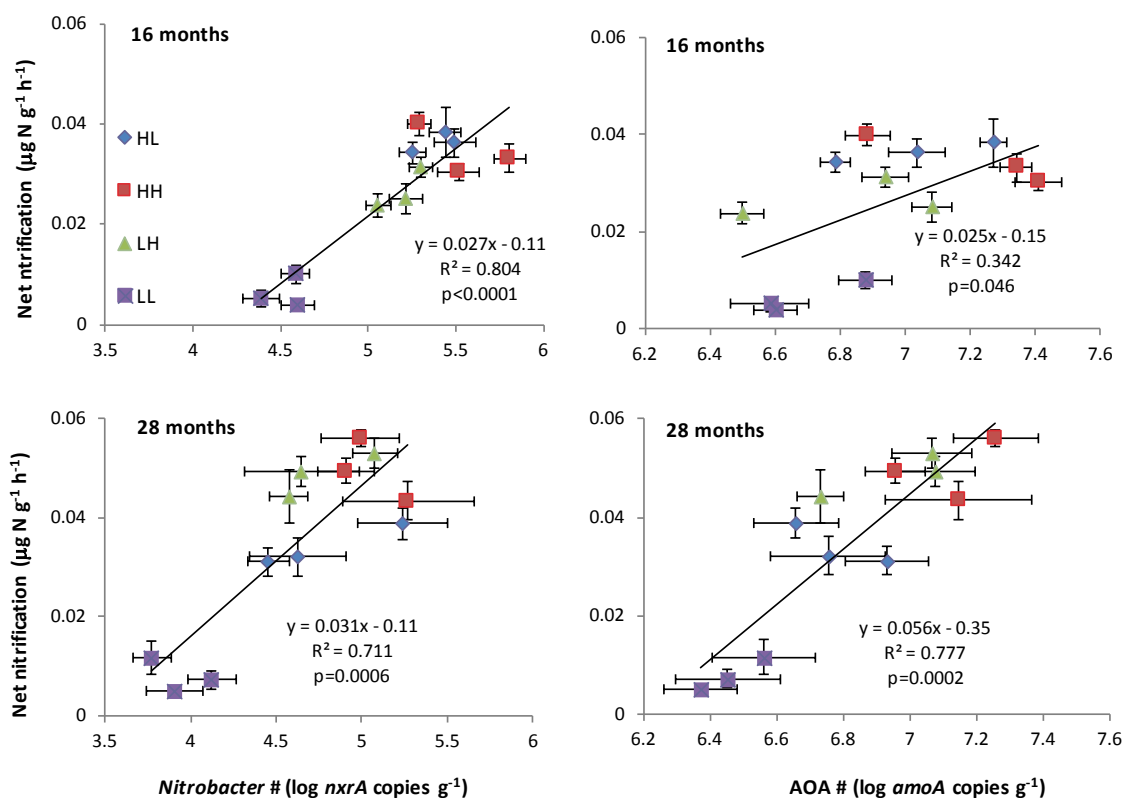


Fig. 5

