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Response of microbial functional groups involved in soil N cycle to N, P and NP fertilization in Tibetan alpine meadows

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ABSTRACT

The nitrogen (N) cycle is an important part of earth's biogeochemical cycles and N is a critical element for all life. Whereas the response to N - and more rarely phosphorus, P - fertilization of some microbial groups involved in soil N cycling has been studied, a comprehensive view of how the major microbial groups involved in soil N dynamics respond to combined N and P fertilization is lacking, which restricts our understanding of ecosystem responses to fertilization. Here we investigated the effects of different N, P and NP fertilizer levels (4 N levels without P; 4 P levels without N; and 4 P levels with constant N addition) on the abundances of 9 microbial groups involved in N dynamics. Real time PCR was used to target free N_2 fixers, nitrifiers (bacterial and archaea ammonia oxidizers, AOB and AOA, respectively; and the nitrite oxidizers *Nitrobacter* and *Nitrospira*), nitrate reducers, *nirK*- and *nirS*-nitrite reducers, and nitrous oxide reducers. Soil physical-chemical characteristics and potential nitrification, PNR, were also measured. N fertilization increased the abundances of AOB and *Nitrobacter* but did not affect the abundances of the other groups. P fertilization decreased the abundances of N_2 fixers, nitrate reducers and AOA, and increased the abundances of *Nitrobacter* and nitrous oxide reducers. NP fertilization decreased the abundances of AOA and *nirK*-nitrite reducers. Using a correlation network analysis, we demonstrate the strong coupling generally observed in these grasslands between N_2 fixers, AOA, *Nitrospira*, *narG*-nitrate reducers and *nirK*-denitrifiers (most of them responding to N/P availability, and being known to be favored by low oxygen availability); and between AOB and *Nitrobacter* (known to be favored by high oxygen and high N levels) that controlled changes in PNR. The observed (de)coupling between the responses of the different microbial groups may have major consequences for N cycling and N losses from fertilized Tibetan alpine meadows.

1. Introduction

Fertilization has been increasingly used to improve forage quality of grasslands degraded by overgrazing (Schellberg et al., 1999; Clark et al., 2007; Craine and Jackson, 2009; Bai et al., 2010). However, fertilization can strongly modify the characteristics of grassland soil (Pan et al., 2014). In particular, fertilization often influences soil nitrogen (N) cycle, inducing an increase of N_2O emission from soil (Kim et al., 2013; Mori et al., 2014), nitrate leaching and eutrophication of rivers and lakes (Glibert and Burkholder, 2006; Guo et al., 2013). Therefore, understanding how the main soil microbial groups involved in soil N cycle respond to fertilization is needed to understand the effects of different fertilization practices and ultimately select adequate practices and prevent undesirable N losses from fertilized ecosystems (Yao et al., 2011).

Several, functionally connected microbial groups play a crucial role in soil N cycling processes such as N_2 fixation, nitrification and denitrification (Kowalchuk and Stephen, 2001; Nannipieri and

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Eldor, 2009; Philippot et al., 2013). In grasslands without or with low amounts of legumes, biological N_2 fixation is carried out by free (non-symbiotic) prokaryotic organisms harboring the nitrogenase enzyme (Joerger et al., 1991). Nitrification is a two step process: the first step is driven by ammonia-oxidizing archaea, AOA (Könneke et al., 2005), and ammonia-oxidizing bacteria, AOB (Prosser, 1989), while the second step is driven by nitrite-oxidizing bacteria (NOB, especially *Nitrobacter* and *Nitrospira* in soil (Attard et al., 2010a)). Nitrate reduction is driven by nitrate reducers (Cheneby et al., 2003; Packman et al., 2004), while nitrite reducers (Zumft, 1997) and nitrous oxide reducers play a key role for denitrification (Zumft and Kroneck, 2006).

Many studies have reported the effects of fertilization on N cycle processes and associated microbial groups. However, a large majority of them focused on N fertilization, and on one or a few microbial groups. For instance, the effects of N fertilization was reported for N_2 fixation/fixers (Tan et al., 2003; Coelho et al., 2008, 2009; Hai et al., 2009), nitrification/nitrifier groups (Simonin et al., 2015a), and denitrification/some denitrifier groups (Hashida et al., 2013; Kastl et al., 2015), but the effect of N fertilization on the different major microbial groups involved in N cycling in a given ecosystem has been barely studied. Moreover, phosphorus, P, has been proven as an important factor for soil eukaryotes (Liu et al., 2012), but the response of prokaryotic organisms such as N-related microbial groups to P, and combined N and P fertilization has rarely been studied (Romero et al., 2012). In addition, the changes in soil environmental conditions induced by nutrient inputs in managed grasslands are likely not homogeneous at the microscale. For instance, contrasted responses of the total bacterial community to urea addition and plant clipping were reported according to bacteria micro-habitats (Attard et al., 2008). Because some microbial groups seem to have specific microhabitats in soil (e.g. *Nitrobacter* have been reported to occupy <10% of the soil volume, likely microhabitats with high N and oxygen levels: see (Grundmann et al., 2001; Le Roux et al., 2016)), the responses of different groups involved in N cycling may be linked or may be decoupled according to their preferred micro-habitats.

As one of the largest typical alpine grasslands in the world (He et al., 2006), the Tibetan Plateau meadows are high altitude and low nutrient ecosystems sensitive to management. In particular, the degeneration rate of the grassland has increased rapidly during the recent years due to overgrazing (Wang et al., 2005) and rat populations (Li et al., 2002; Harris, 2010), and fertilization was chosen as an effective way to improve the degenerated grassland. Former studies in these ecosystems reported the influence of fertilization on aboveground plant communities (Yang et al., 2011; Li et al., 2014) and soil eukaryotes (Liu et al., 2012). But how soil microorganisms involved in N dynamics respond to N/P fertilization remains to be studied.

Here we investigated the effects of 4 levels of 3 different fertilizer regimes (control without fertilization; 0, 5, 10, 15 g-N m^{-2} $year^{-1}$ for N only; 0, 2, 4, 8 g-P m^{-2} $year^{-1}$ for P only; and 0, 2, 4 or 8 g-P m^{-2} $year^{-1}$ with 10 g-N m^{-2} $year^{-1}$ for NP) on the main functional genes' abundances for free N_2 fixers, four nitrifier groups (bacterial and archaeal ammonia oxidizers, and the nitrite oxidizers *Nitrobacter* and *Nitrospira*), and denitrifiers (reducers of nitrate, nitrite and nitrous oxide) using a 3 year-fertilization trial in the Tibetan Plateau. Soil physical-chemical characteristics and potential nitrification rate, PNR, were also measured. The objectives of this study were to (1) assess the response of the abundances of the main microbial total groups involved in soil N dynamics to N, P and NP fertilization in these grasslands, (2) identify the main environmental drivers of the observed responses for each microbial group, and (3) analyse the coupling/decoupling between different microbial groups involved in soil N cycling that exist under the different types of fertilization. We hypothesized that N, P and NP fertilization would reveal coupling between the responses of microbial groups with similar N/P requirements and preferred micro-habitats, while strong decoupling would be observed between groups differing in their N/P requirements and preferred micro-habitats. We discuss the possible consequences of the coupling and decoupling between microbial groups observed in our study for the N cycling and N losses from these fertilized ecosystems.

2. Materials and methods

2.1. Site description and experimental design

The study site is situated in the eastern part of the Qinghai-Tibetan Plateau within the Maqu experimental site of Alpine Meadow and Wetland Ecosystem Research Station of Lanzhou University (33°39′N, 101°53′E; 3650 m a.s.l.) in Gansu Province, PR China. The mean annual temperature is 1.2 °C, ranging from −10 °C in January to 11.7 °C in July. The annual precipitation is about 672 mm, mainly falling during the short, cool summer (July and August). Cloud-free solar periods represent about 2580 h and there are less than 100 frost days in a year. The soil type of the study areas is mainly Mattic Cryic Cambisols (alpine meadow soil, Cambisols in FAO/UNESCO classification). The experimental site was used for grazing by sheep and yacks in the past and was fenced in 2011, being fertilized in May and grazed from November to April every year since 2011.

Fertilization treatments were conducted with nitrogen alone added as NH_4NO_3 (N fertilization gradient), phosphorus alone added as $Ca(H_2PO_4)_2$ (P fertilization gradient), or P mixed with N (NP fertilization gradient). We used 10 m × 20 m plots and six replicates per fertilization level for each fertilization gradient (i.e. increasing levels for N, P or NP fertilization) using a randomized complete block design. Each plot was separated from the others by a 1-m buffer strip. For the N treatment with N addition only, N fertilizer levels were 0, 5, 10 and 15 g-N m^{-2} $year^{-1}$. For the P treatment with P addition only, P fertilizer levels were 0, 2, 4, 8 g-*P*m^{-2} $year^{-1}$. The NP treatment corresponded to 10 g-N m^{-2} $year^{-1}$ NH_4NO_3 combined with 0, 2, 4 or 8 g-P m^{-2} $year^{-1}$ of $Ca(H_2PO_4)_2$.

2.2. Soil sampling and analyses of physical-chemical characteristics

Soils were sampled in August 2014, 3 years after treatments began, from each plot of the 10 treatments (n = 6 plots for each). Four soil cores (3.8 cm diameter, 10 cm depth) were randomly taken from each plot, mixed thoroughly to form one composite sample per plot (i.e. 60 composite samples). Soil samples were stored at 4 °C and brought back to the laboratory within a day. Fresh subsamples were used for measuring soil environmental variables and potential nitrification rates. Other sub-samples were stored at −20 °C for a few weeks before DNA extraction.

Soil moisture was measured gravimetrically, and soil pH was measured in 1 M KCL (1:5, w/v). Soil total N and organic C concentrations were analyzed using a CHNS-analyzer (Elementar Analysen systeme GmbH, Hanau, Germany). Soil NO^-_3-N and NH^+_4-N concentrations were analyzed by a FIAstar 5000 Analyzer (FOSS, Hillerød, Denmark) after extraction with KCl. Soil available phosphorus was extracted using a Mehlich-3 extractant (Mehlich, 1984) and measured using the molybdate-blue colorimetric method.

2.3. DNA extraction from soil and real-time PCR

DNA was extracted from 0.25 g of mixed soil using a PowerSoil DNA Isolation Kit (MO BIO laboratories, Inc, USA) according to the

manufacturer's protocol. Extracted DNA was stored at −80 °C before real time PCR assays. The abundances of free N_2 fixers (responsible for N_2 fixation into NH_4^+), AOB and AOA (responsible for the oxidation of NH_4^+ into NO_2^-), *Nitrobacter* and *Nitrospira* (NO_2^- oxidation into NO_3^-), nitrate reducers (NO_3^- reduction into NO_2^-), nitrite reducers (reduction of NO_2^- into N_2O) and nitrous oxide reducers (N_2O reduction into N_2) were quantified by real-time PCR targeting sequences of the genes *nifH* (coding for the nitrogenase), bacterial and archaeal *amoA* (coding for the ammonia monooxygenase), *Nitrobacter nxrA* (coding for the nitrite oxido -reductase) and *Nitrospira16S*, *narG* (coding the nitrate reductase), *nirK* and *nirS* (both coding for a nitrite reductase), and *nosZ* (coding the N_2O reductase), respectively.

The abundances of N_2 fixers, AOB, AOA, nitrate reducers, *nirK*-nitrite reducers and nitrous oxide reducers were quantified on an iCycler iQ5 thermocycler (Bio-Rad,USA), using 20 μl reaction volume with 32 ng of DNA templates, and 1.6 μl (0.8 μM) of each primer (see Table S1) and 10 μl SYBR Premix ExTaq™II (Takara, Japan). The abundances of *Nitrobacter, Nitrospira*, and *nirS*-nitrite reducers were quantified on a lightcycler 480 (Roche Dignostic, Meylan, France) using 20 μl reaction volume with 40 ng, and 25 μl with 10 ng and 12.5 ng of DNA templates, and 0.5 μM, 0.4 μM and 1 μM of each primer, respectively (see Table S1). Plasmids carrying sequences of the targeted genes were constructed by cloning the targeted gene fragments into plasmid pGEM-T Easy Vector (Promega, USA). Details of qPCR methodologies and standards used are presented in Table S1. Ten-fold serial dilutions of the linearized plasmid DNA were used to establish a standard curve for each gene. Copy numbers per ng DNA were transformed into gene copy numbers per gram of soil using the DNA amount retrieved per gram of dry soil.

2.4. Measurements of potential nitrification rates

Potential nitrification rates, PNR, were determined using triplicates for each soil sample. Soil samples (~180 g oven-dry mass equivalent sieved at 2 mm) were weighed into closed 250 ml flasks. The soils were amended with 100 mg NH_4-N kg^{-1} dry mass [as $(NH_4)_2SO_4$]. Additional deionized water was added to adjust soil moisture to 60% of water holding capacity and the flasks were sealed with a fresh-keeping film with some small holes. The soil was incubated at 25 °C. Subsamples were taken at days 0, 4, 7 and 14 after substrate addition. The soil nitrate was measured colorimetrically in a filtered soil extract (1 M KCl, 1:5 solid: liquid ratio, 2 h end-over-end shaking) (Smolders et al., 2001) using a continuous flow analyzer (SAN++,Skalar, The Netherland).

2.5. Statistical analyses

Data were log-transformed for normality when needed. The presence of potential outliers was tested by identifying, for each variable, values above (mean + 3 sigma) or below (mean − 3 sigma). This led to exclude one value (among 60) for each of the following variables: soil pH, AP, AN/AP, TN and OC.

To test the effects of fertilizer treatments on the soil environmental variables, one-way analysis of variance (ANOVA) was followed by Tukey's honest significant difference (HSD). $P < 0.05$ was considered to identify statistically significant differences. Possible changes in microbial abundances and PNR along each of the three fertilization gradients were tested by linear or non-linear regressions according to fertilization level. However, when complex, non monotonous responses were observed along a gradient, the effects of fertilizer treatments was tested by one-way ANOVA was followed by Tukey's HSD. Pearson's correlation coefficients (R) analyses were also used to test relationships between microbial abundances and soil characteristics. All these statistical analyses were conducted using SPSS v19.0 and Origin 8.0.

A triangular matrix was computed from the correlation coefficients observed between all pairs of microbial group abundances to identify to what extent the responses of different microbial groups where correlated or not. Significant (lack of significant) correlation between the changes in abundances of different groups was considered as an indication of a functional coupling (decoupling) between these groups. In particular, this could indicate that these groups share similar (different) ecological requirements and microhabitats, and thus drive N cycling steps tightly (weakly) coupled in soil. Correlation network mapping and analysis were performed using the open source Gephi software (Bastian et al., 2009). Two-dimension spatial mappings of the correlation networks were performed using the Force-Atlas algorithm in Gelphi. This algorithm creates a visual representation of nodes (microbial groups) connected by edges (level of the correlation observed between abundances). Two nodes are spatially closer if their abundances are strongly correlated. To examine the effects of fertilization types on the correlation network, maps were built for data retrieved for each of the three fertilization gradients. A map was also constructed using all data obtained across the three gradients.

3. Results

3.1. Soil physico-chemical properties

Fertilization treatments did not affect or affected weakly soil pH

Table 1
Changes in soil characteristics along the 3 fertilization gradients. Data are means ± SE (n = 6). OC, TN, NH_4^+-N, NO_3^--N and AP refer to concentrations of soil organic carbon, total nitrogen, ammonium, nitrate, and available phosphorus, respectively. AN/AP is the available nitrogen-to-available phosphorus ratio. For each soil parameter, letters indicate significant ($p < 0.05$) differences between fertilization levels (including control) for each fertilizer form. Note that the N0P0 and N10P0 treatments appear two times.

Fertilization gradient	Fertilizer level	Soil moisture (%)	pH	OC (%)	TN (%)	NH_4^+-N (mg/Kg)	NO_3^--N (mg/Kg)	AP (mg/Kg)	AN/AP
N	0	0.27 ± 0.01	5.29 ± 0.02ab	1.81 ± 0.04	0.28 ± 0.01	3.82 ± 0.37	18.14 ± 6.07a	7.18 ± 1.79	4.58 ± 1.71
	5	0.29 ± 0.01	5.37 ± 0.04a	2.04 ± 0.13	0.33 ± 0.02	3.17 ± 0.21	26.89 ± 2.17ab	7.93 ± 0.90	3.96 ± 0.35
	10	0.28 ± 0.01	5.25 ± 0.03b	1.94 ± 0.12	0.30 ± 0.02	4.68 ± 0.91	40.28 ± 2.57b	10.30 ± 1.16	4.54 ± 0.38
	15	0.28 ± 0.01	5.19 ± 0.02b	1.99 ± 0.07	0.31 ± 0.01	3.94 ± 0.58	42.71 ± 4.62b	10.32 ± 3.05	4.95 ± 0.98
P	0	0.27 ± 0.01	5.29 ± 0.02	1.81 ± 0.04ab	0.28 ± 0.01ab	3.82 ± 0.37	18.14 ± 6.07	7.18 ± 1.79a	4.58 ± 1.71a
	2	0.29 ± 0.00	5.24 ± 0.01	1.85 ± 0.06a	0.29 ± 0.01a	3.63 ± 0.46	13.58 ± 1.63	8.62 ± 1.14a	2.24 ± 0.43ab
	4	0.29 ± 0.00	5.27 ± 0.03	1.64 ± 0.02b	0.25 ± 0.00b	3.82 ± 0.28	9.96 ± 1.22	12.93 ± 2.60ab	1.57 ± 0.54ab
	8	0.28 ± 0.01	5.29 ± 0.04	1.73 ± 0.07ab	0.26 ± 0.01ab	3.18 ± 0.38	8.76 ± 1.23	19.05 ± 1.35b	0.64 ± 0.08b
NP	0	0.28 ± 0.01	5.25 ± 0.03	1.94 ± 0.12	0.30 ± 0.02	4.68 ± 0.91	40.28 ± 2.57	10.30 ± 1.16a	4.54 ± 0.38a
	2	0.30 ± 0.01	5.21 ± 0.05	1.86 ± 0.08	0.29 ± 0.01	4.78 ± 1.22	32.11 ± 3.46	12.28 ± 0.42b	3.01 ± 0.19b
	4	0.29 ± 0.01	5.20 ± 0.05	1.90 ± 0.10	0.30 ± 0.02	3.90 ± 1.19	29.54 ± 3.32	10.64 ± 1.11b	3.17 ± 0.27b
	8	0.29 ± 0.01	5.18 ± 0.02	1.78 ± 0.09	0.27 ± 0.01	4.72 ± 0.78	32.24 ± 3.60	20.42 ± 1.48b	1.84 ± 0.18c

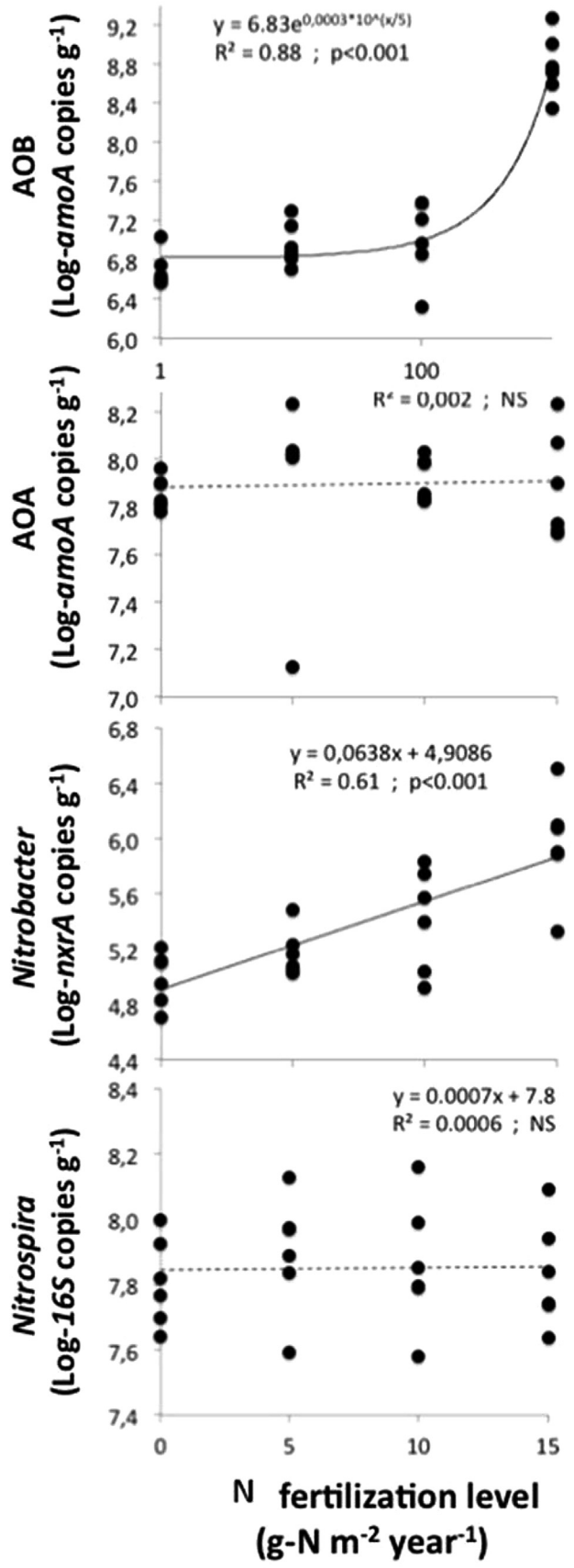


Fig. 1. Changes in the abundances of the four nitrifier groups (ammonia oxidizing bacteria, AOB; ammonia oxidizing archaea AOA; *Nitrobacter*; and *Nitrospira*) in soil along the N fertilizer gradient without P addition. Regression lines are significant for AOB and *Nitrobacter*, and not significant for AOA and *Nitrospira*.

(treatment mean values always around 5.18–5.37), organic C and total N concentrations (ranging from 1.64% to 2.04%, and from 0.25% to 0.33%, respectively), and the NH_4^+-N concentration (from 3.17 to 4.78 ppm) along each fertilization gradient (Table 1). No significant effects of fertilization type or level were observed on soil moisture (Table 1). In contrast, NO_3^--N concentration varied more strongly, ranging from 8.8 to 42.7 ppm (Table 1). The NO_3^--N concentration increased linearly from 18 to 43 ppm along the N fertilization gradient, and did not change significantly along the P and NP gradients (Fig. S1). Available P concentration did not change significantly along the N gradient, but increased linearly from 7.2 to 19.0 ppm and 10.3–20.4 ppm along the P and NP fertilization gradients, respectively (Fig. S1). The AN/AP ratio did not change significantly along the N gradient, but significantly decreased from 4.95 to 0.64 and from 4.54 to 1.84 along the P and NP fertilization gradients, respectively (Fig. S1).

3.2. Nitrifier abundances

The abundances of AOB and *Nitrobacter*-NOB increased exponentially from 1.0×10^7 to 7.7×10^8 copies g^{-1} ($P = 0.00$) and linearly from 1.0×10^5 to 1.2×10^6 copies g^{-1} ($P < 0.001$), respectively, with increasing N level along the N fertilization gradient (Fig. 1). In contrast, the abundances of AOA and *Nitrospira*-NOB did not change significantly (always within $7.3 \times 10^7 - 1.0 \times 10^8$ copies g^{-1}, and $6.7 \times 10^7 - 8.4 \times 10^7$ copies g^{-1}, respectively) along the same N fertilization gradient (Fig. 1).

The abundances of AOA and *Nitrobacter*-NOB decreased from 7.3×10^7 to 2.7×10^7 copies g^{-1} ($P = 0.00$) and increased from 1.0×10^5 to 2.9×10^5 copies g^{-1} ($P < 0.01$), respectively, with increasing P level along the P fertilization gradient (Fig. 2). The abundance of AOB responded in a non monotonous manner along this gradient, and was highest for the fertilization with 2 g-P m^{-2} $year^{-1}$ (9.9×10^7 copies g^{-1}; $p < 0.05$ when compared to abundance values observed for other P addition levels) (Fig. 2). In contrast, the abundance of *Nitrospira*-NOB did not change significantly (within $6.0 \times 10^7 - 9.9 \times 10^7$ copies g^{-1}) along the P fertilization gradient (Fig. 2).

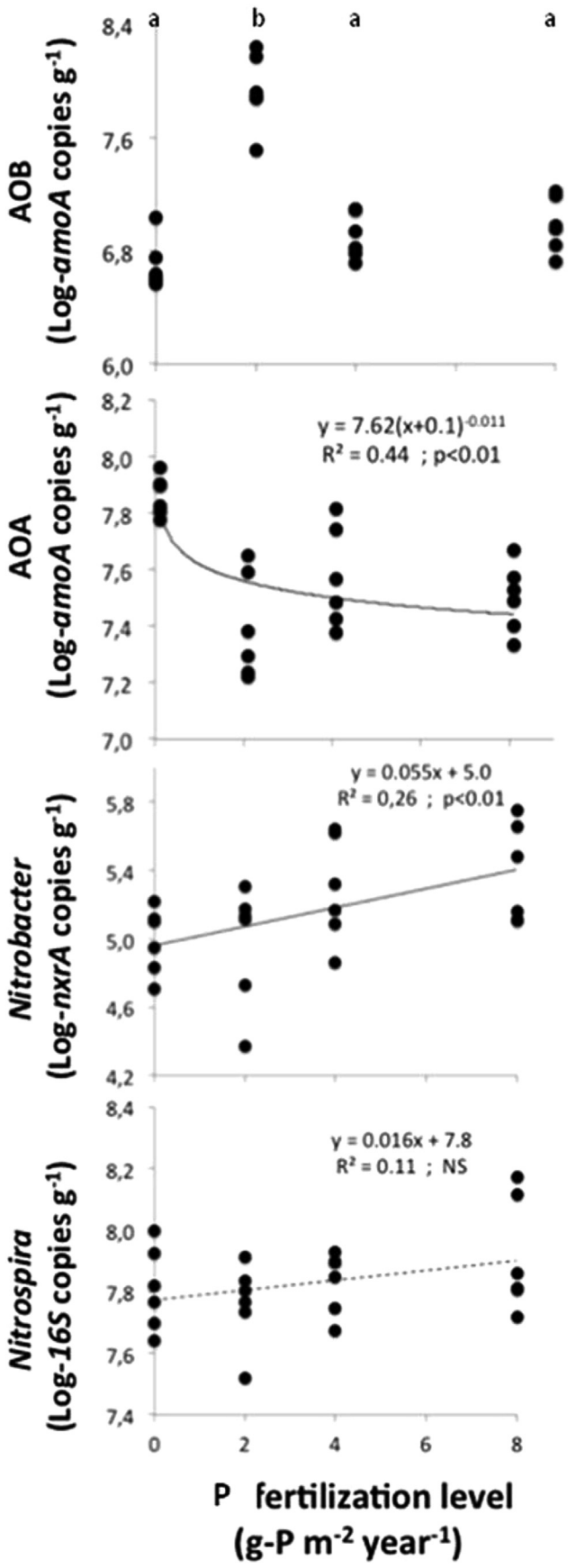
Along the NP gradient, the abundance of AOA decreased linearly from 8.5×10^7 to 3.4×10^7 copies g^{-1} ($P < 0.01$) with increasing P level, while the abundances of AOB and NOB (both *Nitrobacter* and *Nitrospira*) did not change significantly and remained within $9.2 \times 10^6 - 3.0 \times 10^7$, $3.5 \times 10^5 - 5.5 \times 10^5$ and $7.7 \times 10^7 - 7.9 \times 10^7$ copies g^{-1}, respectively (Fig. 3).

3.3. Abundances of N_2 fixers, nitrate reducers and denitrifiers

The abundances of free N_2 fixers, nitrate reducers, *nirK*- and *nirS*-like nitrite reducers, and nitrous oxide reducers did not change significantly with increasing N level along the N fertilization gradient (Fig. S2).

The abundances of free N_2 fixers and nitrate reducers decreased from 4.6×10^6 to 1.9×10^6 copies g^{-1} ($P = 0.00$) and from 3.3×10^7 to 2.0×10^7 copies g^{-1} ($P = 0.004$), respectively, and the abundance of nitrous oxide reducers increased from 2.4×10^7 to 4.8×10^7 copies g^{-1} ($P < 0.01$), with increasing P level along the P gradient (Fig. 4). The abundances of *nirK*- and *nirS*-like nitrite reducers did not change significantly along this P gradient (Fig. 4).

Along the NP gradient, the abundance of the *nirK*-like nitrite reducers significantly decrease 9.9×10^7 to 3.7×10^7 copies g^{-1} ($P < 0.01$) with increasing P level, while the abundances of free N_2



fixers, nitrate reducers, *nirS*-like nitrite reducers, and nitrous oxide reducers did not change significantly (Fig. S3).

3.4. Relationship between microbial abundances and soil environmental parameters

When considering all the data obtained across the 3 fertilization gradients, the abundances of AOA, free N_2 fixers, nitrate reducers and *nirK*-like nitrite reducers were all positively correlated to OC and TN and negatively correlated to available P ($P < 0.01$) (Table 2). The abundances of AOB, *Nitrobacter*-NOB and *nirS*-like nitrite reducers were positively, although weakly, correlated to total N and OC ($P < 0.05$) (Table 2). The abundance of nitrous oxide reducers was negatively correlated with pH (Table 2).

When focusing on the N fertilization gradient, the abundances of AOB and *Nitrobacter*-NOB were negatively correlated to soil pH, while the abundances of AOA and free N_2 fixers were positively correlated to OC and TN (Table S2). The changes in soil nitrate level were positively correlated to the changes in the abundances of both AOB and *Nitrobacter*-NOB (Table S2).

When focusing on the P fertilization gradient, AOB abundance was positively correlated to OC and TN, while the abundances of free N_2 fixers, nitrate reducers and *nirK*-like nitrite reducers were negatively related to available P or positively correlated to the AN/AP ratio (Table S3).

When focusing on the NP fertilization gradient, *Nitrobacter*-NOB abundance was positively correlated to soil pH, while the abundances of AOA and *nirK*-like nitrite reducers were negatively related to available P and positively correlated to the AN/AP ratio (Table S4).

3.5. Level of coupling between microbial groups

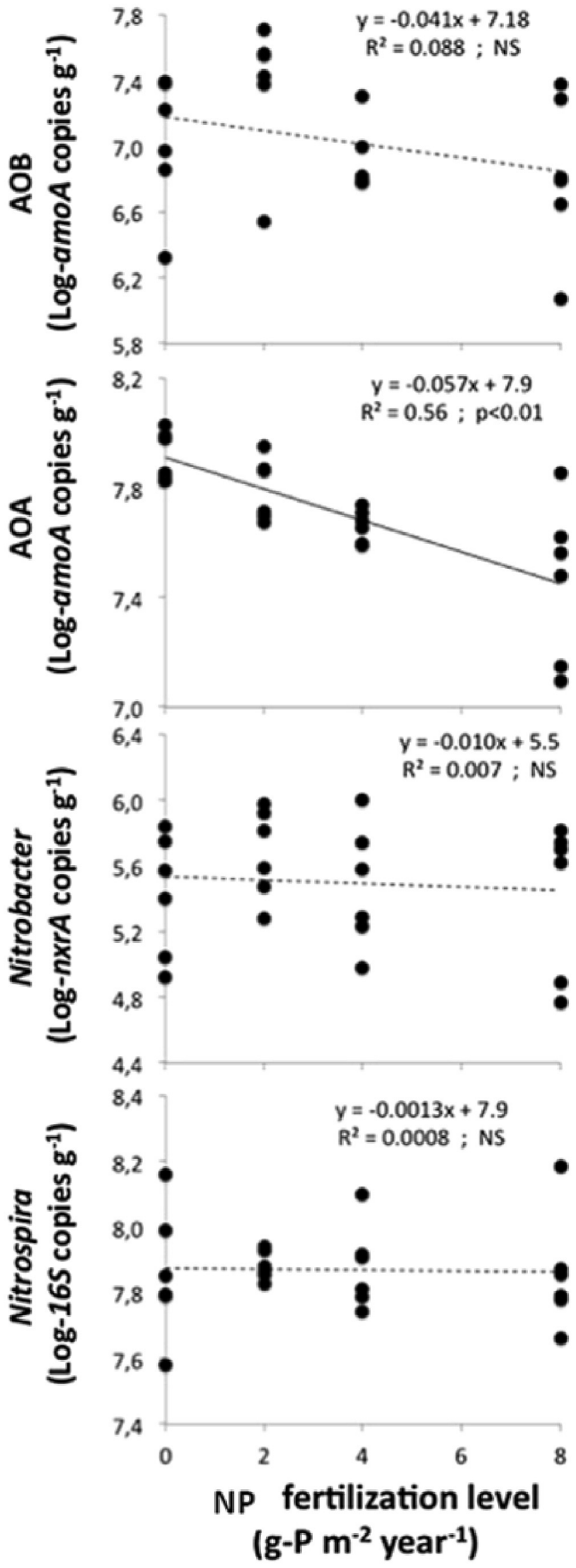
Strong correlation between the abundances of AOB and *Nitrobacter*-NOB was observed for soils of the N and NP gradients (Fig. 5), while the abundances of these two groups were not correlated to the abundances of other groups except *nirK*-nitrite reducers for the N gradient. Strong correlations ($P < 0.05$) were often observed between the abundances of N_2 fixers, AOA, *Nitrospira*-NOB, nitrate reducers and *nirK*-like nitrite reducers (Fig. 5). A lack of or weak correlation was observed between the abundances of different groups belonging to the same functional type, i.e. AOA and AOB (never correlated, each being correlated to different groups); *Nitrobacter*-NOB and *Nitrospira*-NOB (correlated only along the P gradient); and *nirS*- and *nirK*-like nitrite reducers (the abundance of *nirS*-nitrite reducers being often not or weakly correlated to the abundance of other groups, while the abundance of *nirK*-nitrite reducers was always strongly correlated with the abundances of AOA, N_2 fixers and nitrate reducers) (Fig. 5).

When considering all the soils from the 3 fertilization gradients, the abundances of AOB and *Nitrobacter*-NOB appeared strongly correlated, as were the abundances of N_2 fixers, AOA, *Nitrospira*-NOB, nitrate reducers and *nirK*-like nitrite reducers (Fig. S4).

3.6. Changes in PNR and relationship with nitrifier abundances

PNR significantly increased with N fertilization level, from 0.14 mg-N kg^{-1} d^{-1} in control soil to 0.30 mg kg^{-1} d^{-1} for the fertilization level of 15 g-N m^{-2} (Supplementary Fig. S5). PNR

Fig. 2. Changes in the abundances of the four nitrifier groups in soil along the P fertilizer gradient without N addition. Regression lines are significant for AOA and *Nitrobacter*, and not significant for *Nitrospira*. For AOB, the response is not monotonous, and different letters indicate that mean abundances are significantly different between P levels.



significantly increased with P fertilization without N addition, and tended to decrease with P fertilization level along the NP gradient, although the latter trend was not significant (Fig. S5).

PNR was correlated with the abundances of AOB and *Nitrobacter*-NOB along the N fertilization gradient ($p = 0.041$ and 0.046, respectively); with the abundances of *Nitrobacter*- and *Nitrospira*-NOB along the P fertilization gradient ($p = 0.003$ and 0.019, respectively); and with the abundances of AOB and AOA along the NP fertilization gradient ($p = 0.043$ and 0.002, respectively). Overall, the actual soil nitrate level was positively correlated to the abundances of AOB and *Nitrobacter*-NOB ($p = 0.001$ and 0.000, respectively).

4. Discussion

In this study, we quantified the effects of three types of fertilization (N, P and NP) on the abundances of 9 major microbial groups involved in soil N cycling, and evaluated the levels of coupling/decoupling that exist between the changes in the abundances of these different microbial groups. Such a comprehensive study on the responses of N_2 fixers, ammonia oxidizers, nitrite oxidizers, nitrate reducers, nitrite reducers and N_2O reducers to different fertilization types and levels in a same ecosystem had never been achieved so far. Although investigating active rather than total microbial populations (i.e. using RNA- rather than DNA-based quantifications) could be interesting, previous reports indicated that the abundances of N-related microbial groups can increase or decrease quite rapidly over several months following changes in environmental conditions (Attard et al., 2008; Le Roux et al., 2008). Our data allowed inference of the (dis)similarity of the N/P requirements and preferred micro-habitats between these 9 microbial groups, and the possible consequences for the N losses from these fertilized ecosystems.

4.1. Responses of soil nitrifiers to N, P and NP fertilization, and soil environmental drivers

N fertilization alone induced 142-fold and 12-fold increases in the abundances of AOB and *Nitrobacter*-NOB, respectively, but did not affect the abundances of AOA and *Nitrospira*-NOB. This supports the general view that soil AOB and *Nitrobacter*-NOB are favored by high soil N availability, whereas AOA and *Nitrospira*-NOB are less responsive to N availability (He et al., 2007; Wang et al., 2009; Attard et al., 2010b; Di et al., 2010; Shen et al., 2011; Wertz et al., 2012; Chen et al., 2014b; Simonin et al., 2015b). This could be explained because the AOB likely have a lower affinity for N substrate than AOA and are generally favored by higher N levels (Martens-Habbena, 2009). Similarly, *Nitrospira*-NOB and *Nitrobacter*-NOB have a high and low affinity for nitrite, respectively, so that *Nitrospira*-NOB outcompete *Nitrobacter*-NOB under conditions of low N availability (Schramm et al., 2000; Wagner et al., 2002; Le Roux et al., 2016). pH could be another driver of the changes in the abundances of AOB and *Nitrobacter* along the N gradient because long term N fertilization may have induced changes in soil pH. Both the abundances of AOB and *Nitrobacter* were negatively correlated to soil pH, which is surprising because another study (Nicol et al., 2008) reported that AOB and AOA abundances were not significantly affected when soil pH varied from 4.9 to 5.9. Furthermore AOA and AOB have been reported to be favored in acidic and neutral soils, respectively (Yao et al., 2011; Prosser and Nicol, 2012). Actually, in our study soil ammonium availability tended to be

Fig. 3. Changes in the abundances of the four nitrifier groups in soil along the P fertilizer gradient with constant N addition. Regression line is significant only for AOA.

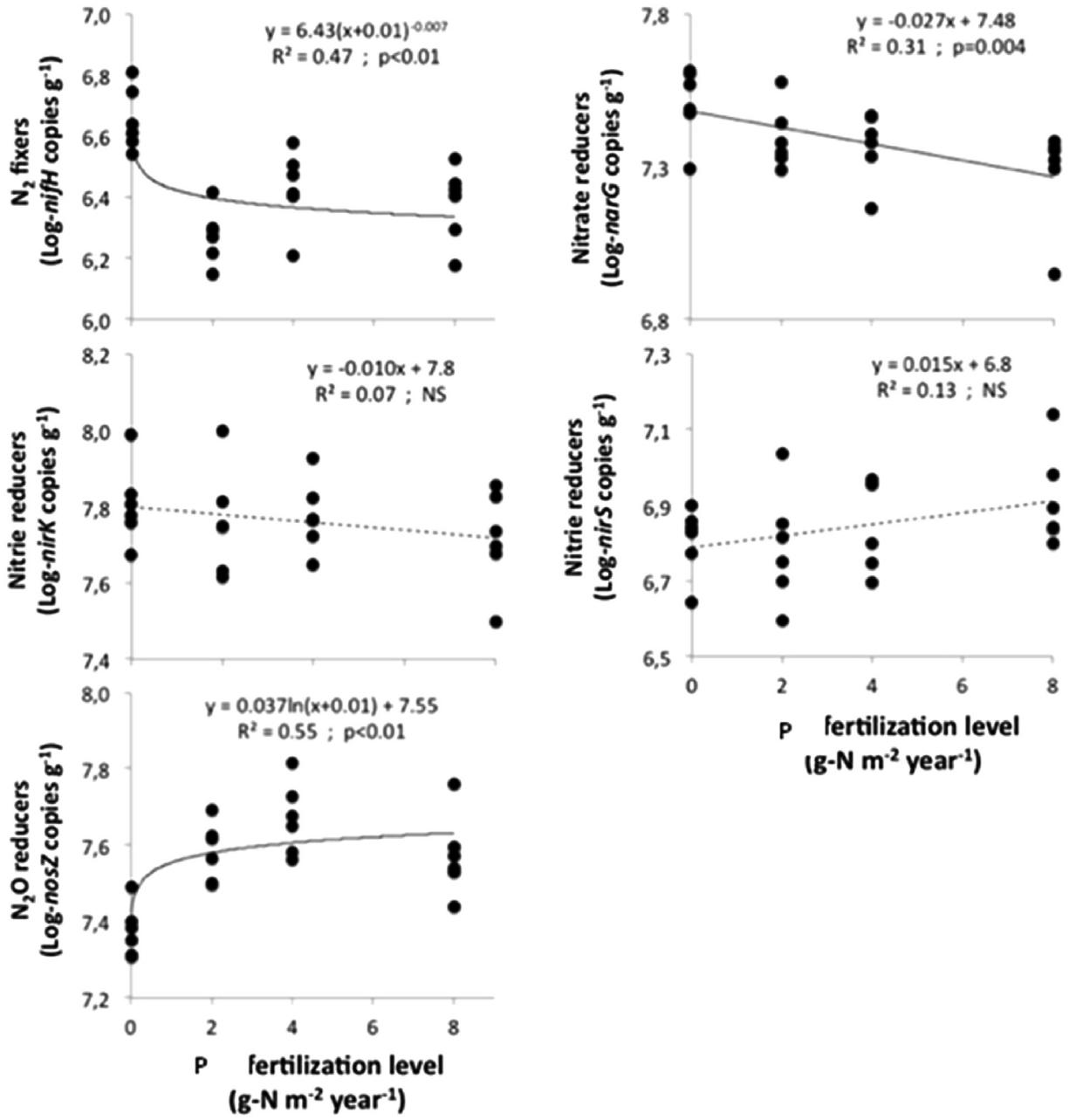


Fig. 4. Changes in the abundances of N_2 fixers (*nifH* gene copies), nitrate reducers (*narG* gene copies), nitrite reducers (*nirK* and *nirS* gene copies) and N_2O reducers (*nosZ* gene copies) in soil along the P fertilizer gradient without N addition. Regression lines are significant for N_2 fixers, nitrate reducers and N_2O reducers.

negatively correlated to pH along each fertilization gradient, the correlation being significant when considering the three gradients (see Table 2). It is thus possible that AOB (and *Nitrobacter*-NOB) were in fact more responsive to actual ammonium availability in soil despite the lack of correlation between their abundances and ammonium concentration. Snapshot measurements of ammonium concentration are indeed known to be questionable proxies of actual ammonium availability in soil (Robson et al., 2007), which could explain the poor relationships observed here between AOB and *Nitrobacter*-NOB abundances and ammonium concentration. In addition, the AOA abundance along the N gradient was correlated to soil organic carbon (OC) concentration. This could be due to the fact that AOA are likely more metabolically versatile, with possible heterotrophic/mixotrophic capacities, i.e. they may use soil organic carbon (Walker, 2010).

Addition of P decreased AOA abundance, both when no N was added and when a constant high N amount was added. This suggests a rather direct role of P addition, via the increased P availability and decreased AN/AP ratio observed along the P and NP fertilization gradients. The significant, positive relationship observed between the AOA abundance and the AN/AP ratio along the NP gradient, and the same positive trend (not significant) observed along the P gradient, support this view. Another study (Chen et al., 2014b) reported that P and NP fertilization also tended to decrease AOA abundance in a temperate steppe soil at one of the two dates studied. In contrast, adding P without N increased the abundance of *Nitrobacter*, suggesting that *Nitrobacter*-NOB could be particularly sensitive to P limitation. However, P addition did not

Table 2
(Left-bottom half part of the table) R values retrieved from Pearson correlations between soil parameters and gene copy abundances; (Right-top half of the table) P-values for the same correlations. Significant ($p < 0.05$) correlations are indicated in bold. NS: not significant. * and ** indicate $p < 0.05$ and $P < 0.01$, respectively. Abbreviations for soil parameters are as in Table 1.

	NH_4^+-N	NO_3-N	OC	TN	AP	AN/AP	SM	pH	AOA *amoA*	AOB *amoA*	NOB *nxrA*	NOB *Nitrospira*	N_2 fixers *nifH*	Nitrate reducers *narG*	Nitrite reducers *nirS*	Nitrite reducers *nirK*	N_2O reducers *nosZ*
NH_4^+-N		NS	NS	NS	NS	NS	NS	0.003	NS	NS	NS	0.012	NS	NS	NS	NS	NS
NO_3-N	0.136		0.002	0.003	NS	0.000	NS	0.031	0.002	0.001	0.000	NS	0.034	0.036	NS	0.004	NS
OC	−0.123	**0.362****		0.000	0.003	0.008	NS	NS	0.001	0.036	0.026	NS	0.000	0.002	0.048	0.001	NS
TN	−0.154	**0.346****	**0.965****		0.000	0.004	NS	NS	0.001	0.041	0.034	NS	0.001	0.001	0.023	0.001	NS
AP	−0.026	−0.065	**−0.345****	**−0.410****		0.000	NS	NS	0.001	NS	NS	NS	0.023	0.044	NS	0.000	NS
AN/AP	0.199	**0.508****	**0.310****	**0.332****	**−0.661****		NS	NS	0.002	NS	NS	NS	0.01	NS	NS	0.000	NS
SM	−0.038	0.15	−0.049	0.029	0.038	0.02		0.014	NS	NS	NS	NS	NS	NS	NS	NS	NS
pH	**−0.343****	**−0.254***	0.024	0.031	−0.109	−0.075	**−0.289***		NS	NS	NS	NS	NS	NS	NS	NS	0.003
AOA	0.045	**0.354****	**0.386****	**0.394****	**−0.381****	**0.365****	−0.142	0.03		0.012	0.037	0.029	0.000	0.000	NS	0.000	NS
AOB	−0.042	**0.386****	**0.248***	**0.241***	−0.194	0.208	−0.126	−0.186	**0.294***		0.000	NS	NS	NS	NS	0.016	NS
nxrA	−0.074	**0.488****	**0.262***	**0.250***	−0.101	0.185	−0.058	−0.141	**0.246***	**0.830****		NS	NS	NS	NS	0.012	NS
Nitrospira	**0.294***	0.11	0	−0.005	0.044	0.107	−0.092	0.014	**0.258***	0.06	0.098		0.000	0.000	0.013	0.001	NS
nifH	0.15	**0.250***	**0.411****	**0.400****	**−0.268***	**0.301***	−0.039	0.146	**0.670****	0.036	0.01	**0.430****		0.000	0.004	0.000	NS
narG	0.215	**0.248***	**0.367****	**0.376****	**−0.238***	0.213	0.005	−0.142	**0.531****	0.009	0.051	**0.403****	**0.735****		0.006	0.000	0.000
nirS	−0.113	0.079	**0.233***	**0.268***	0.104	−0.055	0.091	0.049	0.151	0.11	0.178	**0.292***	**0.331****	**0.321****		0.011	0.02
nirK	0.164	**0.336****	**0.397****	**0.377****	**−0.402****	**0.461****	−0.162	0.123	**0.689****	**0.283***	**0.296***	**0.369****	**0.662****	**0.580****	**0.299***		NS
nosZ	0.084	0.155	0.089	0.132	0.181	−0.15	0.207	**−0.340****	0.021	−0.089	0.094	0.17	0.142	**0.483****	**0.274***	0.118	

significantly influence *Nitrobacter* abundance when a constant and high N amount was added, which suggests that P availability has a complex effect on *Nitrobacter* abundance, rather than just alleviating P limitation. Actually, P fertilization may have indirect effects on plant growth and root functioning (Shi et al., 2015) that may influence the functional relationships between *Nitrobacter*-NOB and plant roots differently if N is also added or not, but no information is available in the literature on this issue.

The abundance of AOB peaked at a low level of P added when no N was added, whereas P addition had no effect on AOB abundance when a constant N amount was also added. This AOB response to P fertilization level can explain the lack of effect of P fertilization on AOB abundance reported in another study (Chen et al., 2014a) where the single P fertilization level studied was high (5 g P_2O_5 m^2 $year^{-1}$) for this temperate steppe soil.

4.2. Responses of the other N-related microbial groups to N, P and NP fertilization, and soil environmental drivers

The abundances of N_2 fixers, nitrate reducers, nirK- and nirS-nitrite reducers, and nitrous oxide reducers did not change significantly along the N gradient, and the abundance of each group could not be related to any soil environmental variable, except the N_2 fixer abundance that was positively related to soil organic C and total N concentrations. The lack of significant effect of the N treatment on reducers is consistent with a previous study (Miller et al., 2008). This may indicate that N_2 fixers, nitrate reducers, nirK- and nirS-nitrite reducers, and nitrous oxide reducers were not much limited by N in these grasslands. In contrast, the abundances of N_2 fixers and nitrate reducers significantly decreased along the P fertilization gradient, while the abundance of nitrous oxide reducers significantly increased. The abundance of *nirK*-nitrite reducers decreased with P addition under constant N addition, which is consistent with a previous study reporting that *nirK* abundances were significantly correlated with soil P (Graham et al., 2010). Actually, available P and moreover the AN/AP ratio was a major driver of changes in the abundances of N_2 fixers, nitrate reducers and *nirK*-nitrite reducers along the P gradient; and the AN/AP ratio was also the main driver of changes in the abundances of *nirK*-nitrite reducers along the NP gradient (the same, non significant, trend being observed for N_2 fixers and nitrate reducers). In contrast, no relationship was observed between the abundances of *nirS*-nitrite reducers and nitrous oxide reducers and P availability or AN/AP ratio. All these results suggest an important role of the N:P stoichiometry for N_2 fixers, nitrate reducers and *nirK*-nitrite reducers, and not for *nirS*-nitrite reducers and nitrous oxide reducers, although this aspect of their ecology largely remains to be studied. To date, the role of C:N:P stoichiometry for microbial groups involved in N cycling has been mostly studied for aquatic environments (e.g., (Deutsch et al., 2007) for N_2 fixers; and (Tyrrell and Lucas, 2002) for denitrifiers). In contrast, the importance of C:N:P stoichiometry for soil microorganisms has been investigated mainly for the total microbial biomass, which has revealed that the microbial C/N ratio is more constrained (ranging from 3 to 24) than the N/P ratio (from 1 to 55) between soils (Cleveland and Liptzin, 2007). This may be an indication that different microbial groups have different requirements in term of N:P balance, but this remains to be studied. Studies of C:N:P stoichiometry for the different groups of the N cycle are thus highly needed to advance our understanding of their responses to fertilization —and more generally environmental- gradients.

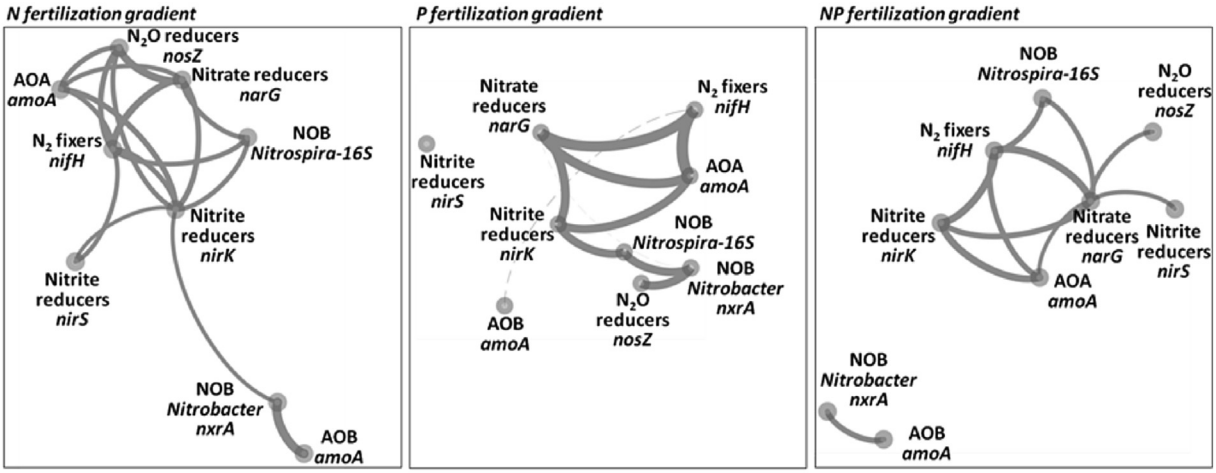


Fig. 5. View of the coupling observed between changes in the abundances of the 9 microbial groups involved in soil N dynamics studied along each fertilization gradient: (Left) N gradient; (Middle) P gradient; and (Right) NP gradient. Dark grey links indicate significant and positive relationships (link thickness proportional to correlation coefficient) while the two light grey, dotted links indicate significant negative relationships. Correlation coefficient values are presented in Tables S2–S4.

4.3. Levels of (de)coupling between the nine microbial groups: possible drivers and possible implications for N losses

Our study offers a comprehensive view of the responses of 9 major microbial groups involved in soil N cycling along N, P and NP fertilization gradients, and thus allows identification of the level of (de)coupling that exists between these groups (Fig. 6). In particular, the correlation network analysis highlights that the changes in the abundances of different groups belonging to the same functional type (i.e. AOA and AOB; *Nitrobacter* and *Nitrospira*; or *nirS*- and *nirK*-like nitrite reducers) were weakly or not correlated, which demonstrates that ecological requirements vary strongly between different groups within each of these 3 functional types. The decoupling observed between AOB and AOA is consistent with the recent report by (Simonin et al. (2015a)) who found that N fertilization induced a decoupling between AOB and AOA abundances. Similarly, other studies (Attard et al., 2010a; Simonin et al., 2015a) showed that changes in the abundances of *Nitrobacter* and *Nitrospira* in soil were not correlated along environmental gradients. With regards to denitrifiers, a recent study (Xie et al., 2014) reported that on average, the abundances of *nirS*- and *nirK*-like nitrite reducers responded differently to grazing pressure, and (Le Roux et al., 2013) reported that changes in the abundances of *nirS*- and *nirK*-like nitrite reducers were only weakly correlated in a grassland soil. Our results confirm the existence of a high functional diversity between the main groups from the ammonia oxidizing, nitrite oxidizing and nitrite reducing communities, which may increase the adaptation capacity of key N cycling functions (i.e. nitrification and denitrification) to environment pressures.

Our results also show that some groups belonging to different functional types were tightly coupled in these soils: this is true for AOB and the *Nitrobacter*-NOB on the one hand; and for N_2 fixers, AOA, *Nitrospira*-NOB, nitrate reducers and *nirK*-nitrite reducers on the other hand (Fig. 6). Strong coupling of AOB and *Nitrobacter*-NOB has already been reported by (Simonin et al. (2015a)) who analyzed the effects of N addition and elevated CO_2 on soil nitrifiers. Interestingly, the actual soil nitrate concentration was positively correlated to the abundances of both AOB and *Nitrobacter*-NOB along the N gradient. This supports the view that AOB and *Nitrobacter*-NOB are two tightly coupled guilds that play a major role in the response of nitrification to N fertilization (Fig. 6), as suggested in previous studies (Erguder and Boon, 2009; Jia and Conrad, 2009; Attard et al., 2010a; Di et al., 2010; Long et al., 2012; Petersen et al., 2012; Wertz et al., 2012). In contrast, AOA and *Nitrospira*-NOB likely have a major role for ammonia- and nitrite-oxidation, respectively, in low N environments (Offre, 2009; Gubry-Rangin, 2010). These results may be explained by the N:P stoichiometric constraints specific of these nitrifier groups (which should be studied in the future), and by the preferred soil micro-habitats for these different nitrifier groups. *Nitrobacter*-NOB have been reported to occupy ca. 6% of the soil volume (Grundmann et al., 2001) and perform well in microhabitats with high N and oxygen levels, but are outcompeted by *Nitrospira*-NOB in microhabitats with lower N and oxygen levels (Le Roux et al., 2016). Our results (Fig. 6) suggest that the responses of AOB and *Nitrobacter*-NOB to N and NP fertilization are coupled due to their shared, preferred micro-habitats that are characterized by high oxygen levels and N availability levels that vary strongly with fertilization level. In contrast, the responses of AOA, *Nitrospira*-NOB, nitrate reducers, and *nirK*-nitrite reducers are often coupled (Fig. 6) likely because they share similar micro-habitats characterized by low and possibly more stable resource levels. This should be further explored by studying the spatial distribution of these groups at the micro-scale in soils from fertilization gradients.

Another important result from a functional point of view is that the abundance of nitrous oxide reducers appeared to be weakly correlated to the abundances of both types of nitrite reducers, at least for the two P fertilization gradients (Fig. 6). Moreover, the N_2O reducers-to-nitrite reducers ratio tended to increase with increasing P fertilization levels along the P and NP gradients (the ratio *nosZ*/(*nirS* + *nirK*) increasing from 0.23 to 0.94, and from 0.28 to 1.82, respectively). Nitrite reducers contribute to the generation of NO and N_2O, major radiative forcing and stratospheric ozone-depleting gas (Ravishankara et al., 2009; Smeets et al., 2009), while nitrous oxide reducers can convert N_2O into the inert compound N_2. The different responses of these microbial guilds to P fertilization may thus decrease the potential of soils to act as N_2O emitters. Because the existence of two distinct clades of *nosZ*-harbouring bacteria was recently revealed (Jones et al., 2014) and only the response to fertilization of the first clade was characterized here using the existing molecular tools, characterizing the response of the second clade will be needed to properly infer P fertilization effect on the soil N_2O reduction potential. However, our results could explain why alleviating the P as compared to N limitation

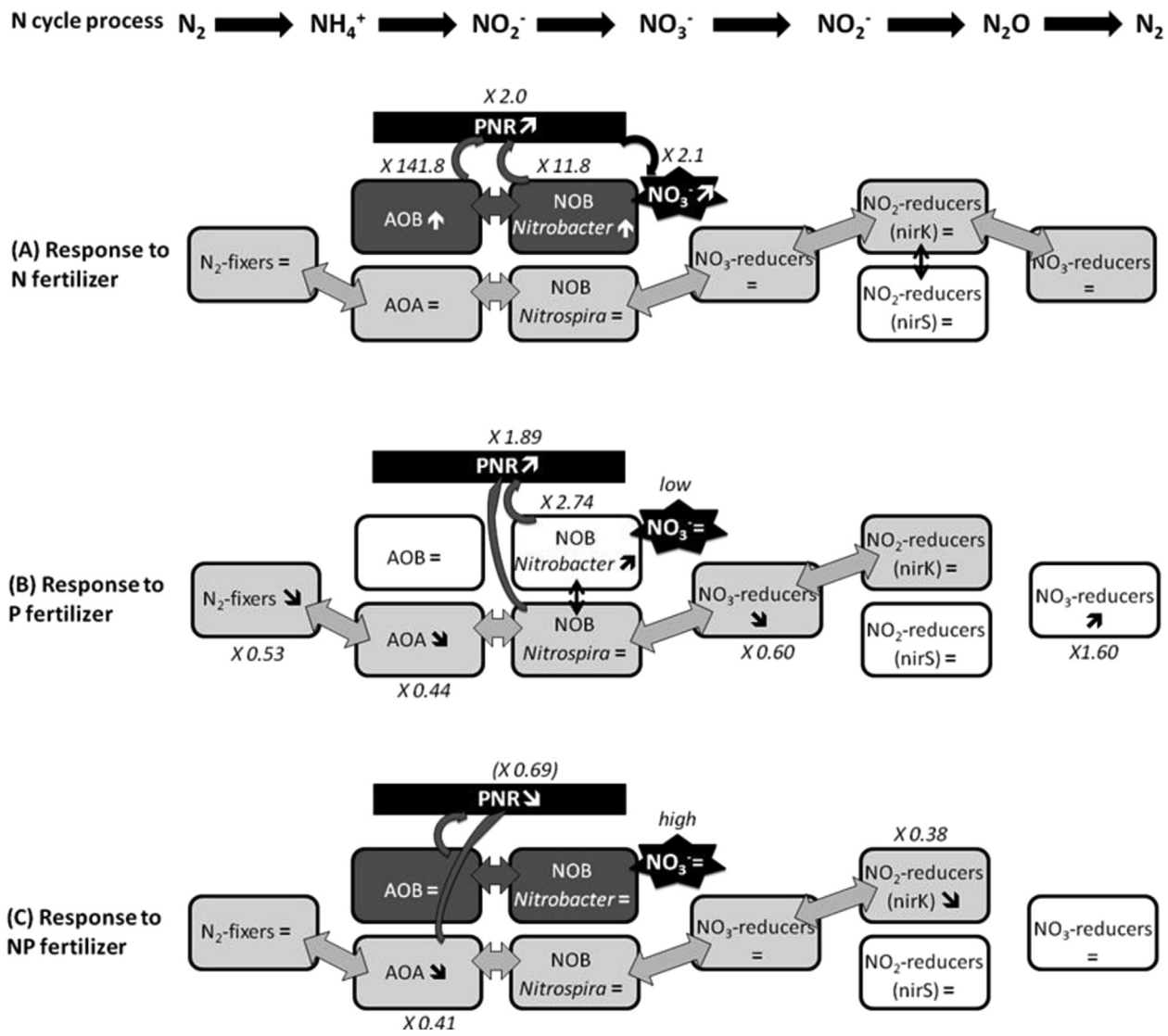


Fig. 6. Synthesis of the effects of the three types of fertilization on the abundances of the different microbial groups involved in soil N dynamics, potential nitrification rate, PNR, and soil nitrate levels: (Top) N fertilization gradient without P addition; (Middle) P fertilization gradient without N addition; and (Bottom) P fertilization gradient with constant N addition. Grey colours indicate groups for which changes in abundances are correlated; white indicates groups not well correlated to other groups; and black refers to PNR and nitrate levels. Numbers nearby boxes (e.g. *X 1.89*) indicate the amplitude of abundance changes for the microbial group from the control, non-fertilized plots to the plots with the maximum fertilizer level.

reduces N_2O emissions from soils, as reported for a tropical leguminous plantation (Mori et al., 2013) and forest ecosystems (Wang et al., 2014).

5. Conclusion

Our study reports for the first time the responses of 9 major microbial groups involved in soil N cycling to three types of fertilization (N, P and NP). First, it shows that the changes in the abundances of different groups belonging to the same functional type (i.e. AOA and AOB; *Nitrobacter*- and *Nitrospira*-NOB; and *nirS*- and *nirK*-nitrite reducers) were weakly or not correlated, highlighting that ecological requirements vary strongly between groups within each of these 3 functional types. Second, it demonstrates that some groups belonging to different functional types were tightly coupled: this is true for AOB and the *Nitrobacter*-NOB on the one hand, which could be explained by their shared preference for soil microhabitats with high oxygen and N levels; and for N_2 fixers, AOA, *Nitrospira*-NOB, nitrate reducers and *nirK*-nitrite reducers on the other hand, which could be explained by their preference for microhabitats with low oxygen levels and their frequent dependency on the AN/AP ratio. Third, our results demonstrate an important role of the N:P stoichiometry for *nirK*-nitrite reducers, and not for *nirS*-nitrite reducers and nitrous oxide reducers, which induces changes in the balance between nitrite- and nitrous oxide reducers and can explain that the alleviation of the P as compared to N limitation can reduce N_2O emissions from soils. All these results call for reinforcing our knowledge on (i) the micro-scale distribution of the main microbial groups involved in soil N cycling along fertilization gradients, and (ii) the C:N:P stoichiometric requirements for these different groups, as it has been achieved for microbial communities in aquatic environments.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.soilbio.2016.07.023.

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