The responses of NO2- and N2O-reducing bacteria to maize inoculation by the PGPR Azospirillum lipoferum CRT1 depend on carbon availability and determine soil gross and net N2O production

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Abstract

Seed inoculation by plant growth promoting rhizobacteria (PGPRs) is an agronomic practice that stimulates root carbon (C) exudation and nitrogen (N) uptake. Inoculation thus increases and decreases C and N availabilities to denitrifiers in the rhizosphere, respectively. Hence, denitrification rates in the rhizosphere can be positively or negatively influenced by root activity depending on the balance between these two processes. We assumed that inoculation effect on denitrifiers could strongly differ according to soil conditions. Would denitrifiers be mostly limited by C, inoculation would increase denitrifier abundance and activity through increased labile C availability. Would denitrifiers be limited by N rather than C, inoculation would decrease denitrifier abundance and activity through increased competition for N. Here we manipulated denitrification limitation by C and N (i) in a field trial through the use of different fertilization levels, and (ii) in a growth chamber experiment by mimicking root exudate inputs. We analyzed how the effects of maize inoculation by the PGPR *Azospirillum lipoferum CRT1* on potential gross and net N_2O production rates and NO_2^-- and N_2O-reducer abundances were related to C and N limitation levels. An increase in potential gross (up to +113%) and to a lesser extent net (+37%) N_2O production was observed for soils where denitrification was highly limited by C. This was explained by strong and moderate increases in the abundances of NO_2^-- and N_2O-reducers, respectively. In contrast, when denitrification was weakly limited by C, gross and net N_2O productions were negatively affected by inoculation (-15 and -40%, respectively). Our results show that the inoculation practice should be evaluated in term of possible increased crop yield but also possible modified N_2O emission, paying much attention to cropland soils where denitrifiers are highly limited by C.

Key-words: *nirK*, *nirS*, *nosZI*, *nosZII*, plant-microbes competition for nitrate, root exudates

1. Introduction

Denitrification is a microbial respiratory process during which soluble nitrogen (N) oxides (nitrate, NO_3^-, and nitrite, NO_2^-) are sequentially reduced by specific reductases into gaseous forms (NO, N_2O and N_2) (Tiedje et al. 1982). In particular, NO_2^- reductases, encoded by the *nirK* or *nirS* gene, catalyze the reduction of NO_2^- to NO, which is the first step of denitrification, leading to the production of a gaseous N oxide (Shapleigh, 2013). This is the key process for both NO and N_2O production as most NO-producing denitrifying cells convert efficiently NO which is a toxic compound (this explains why most *nir*-harbouring bacteria also harbor the *nor* gene catalyzing NO reduction; Graf et al., 2014). The reduction of N_2O to N_2 is catalyzed by N_2O reductases encoded by the *nosZI* gene or the recently discovered *nosZII* gene (Sanford et al. 2012; Jones et al. 2013; Domeignoz-Horta et al., 2016). Thus, the net soil emissions of N_2O (a potent greenhouse gas; Baggs, 2011) depend largely on the balance of its production and consumption, and on the responses of NO_2^-- and N_2O-reducers to changes in soil environmental conditions.

In particular, two main factors affect the ecology of NO_2^-- and N_2O-reducers in the plant rhizosphere, namely the availabilities of N and carbon (C). On the one hand, root growth and activity enhance soil NO_3^- uptake by plants, thus strengthening plant-denitrifiers competition for NO_3^-. This can decrease soil NO_3^- availability for denitrifiers and ultimately limit their growth (Kuzyakov and Xu, 2013). On the other hand, root exudation provides easily decomposable C sources to denitrifiers, which can increase their activity and abundance (Berks et al., 1995). Hence, denitrification rates in the rhizosphere can be positively or negatively influenced by root activity depending on the balance between these two processes. However, to what extent NO_2^-- and N_2O-reducers might display different sensitivities to C and/or N availability and how this might affect the responses of soil N_2O production and consumption to changed C and N availabilities remains unclear. For instance, we synthesized the results of previous studies analyzing the effects of soil amendments with labile C sources, mineral N, labile C plus mineral N,

60 or artificial root exudates (ARE) and we found that C or N addition increases potential gross and net N_2O
61 production (PGNP and PNNP, respectively) in only 50%-70% of the soils studied (Table 1). One
62 explanation is that the type of soil and in particular the soil C and N statuses influence the C or N addition
63 effects. This synthesis also shows that the effect of mineral N addition on the abundances of NO_2^-- and
64 N_2O-reducers has been analyzed in many studies, the effect being generally low, without any clear
65 difference between *nirK*-, *nirS*-, *nosZI*- and *nosZII*-bacteria (Table 1). In contrast, fewer studies reported
66 the effect of labile C amendment or of the addition of labile C plus mineral N, information being missing
67 for some groups (Table 1). This shows that it is still largely unknown whether these denitrifier groups
68 respond differently to changes in mineral N or labile C availabilities.
69 Inoculation of cereal seeds by plant growth promoting rhizobacteria (PGPRs) increases root C
70 exudation (Heulin et al., 1987; Shaw et al., 2006) and enhances N uptake by inoculated plants (Sarig et
71 al., 1988; Fallik et al., 1994; Mantelin and Touraine, 2004). Recently, Florio et al. (2017) analyzed how the
72 promoting activity of PGPRs may influence the activity and abundance of denitrifiers in rhizosphere soil
73 by modifying C and N availabilities. The authors reported contrasted effects of inoculation of denitrifiers,
74 between different soil types. They suggested that inoculation could increase and decrease *nirS*
75 abundance and consequently potential gross N_2O production when denitrification was highly and lowly
76 limited by soil C, respectively. However, soil type was a confounding factor with C availability in this
77 study which compared inoculation effects between different sites and soil types. Distinguishing the
78 effects of C and N availability from the effects of other soil characteristics actually requires to manipulate
79 C and N availabilities to denitrifiers using a same soil. Further, the authors studied inoculation effect on
80 potential gross but not net N_2O production.
81 Here we manipulated C and N availabilities for a same soil background by using different mineral
82 fertilization levels and mimicking different maize root exudation rates at the field and microcosm scales,
83 and we assessed the effects of maize inoculation by the PGPR *Azospirillum lipoferum CRT1* on potential

gross and net N_2O production rates, and on the abundances of NO_2^-- and N_2O-reducers, according to C and N availabilities. We hypothesized that when denitrification is highly limited by C, inoculation would increase the abundance and activity of NO_2^-- and N_2O-reducers as higher maize root exudation would have a key role (Fig. 1, red arrows). In contrast, when denitrification is less limited by C, the stimulating effect of root exudation would be less important than the effect of the increased competition between plants and denitrifiers for N, and inoculation would decrease denitrifier abundance and activity (Fig. 1, blue arrows). In addition, the sensitivity of the different denitrifier groups, in particular of NO_2^--reducers as compared to N_2O-reducers, to C and N availabilities, would determine the overall effect on gross and net N_2O production, which could not be easily predicted due to the lack of sufficient information on their ecology regarding C and N (Table 1). We discuss our results in term of possible implications of the inoculation practice for N_2O emission according to soil type.

2. Materials and Methods

2.1. Field experiment

The experimental site is located in Sérézin-de-la-Tour, southeast of France (45°37' N, 5°16' E). The soil is a Fulvic Cambisol (World Reference Base for Soil Resources, 2006), and its main physical and chemical characteristics are as follows: 34.7% clay, 26.9% sand, 38.3% silt; pH (H_2O 1:2.5) 7.1; SOC 31.6 g C kg^{-1}; TN 3.4 g N kg^{-1}; and Olsen P 0.153 g kg^{-1}. The experiment was set up as a randomized block design with 5 blocks, and treatments randomly assigned to one plot (12 m × 9.6 m) in each block. The experimental fields had been cultivated with wheat for three years previous to the experiment. Maize (*Zea mays,* cv. *Seiddi*) seeds were inoculated with *A. lipoferum CRT1* isolated from the rhizosphere of field-grown maize in France (Fages and Moulard, 1988). The targeted inoculum load was 10^6 CFU added per seed for inoculated plants, I, coated in a commercial peat-based Azo-Green™ formulation (Agrauxine, Beaucouzé, France). Coated but non-inoculated seeds, NI, were used as controls. Sowing

occurred on 30th April 2015 (95,000 seeds ha^{-1}). Five pairs of NI-I plots were not fertilized (nf plots) while two fertilization treatments were applied using a mineral fertilizer (NH_4NO_3) at a rate of 80 kg N ha^{-1} close to optimal N availability (f plots) or 40 kg N ha^{-1} for the reduced fertilization treatment (f/2 plots). Ten plots at an additional field site located in the proximity of the first site (45°57' N, 5°34' E) and managed under organic farming with feather meal used as organic fertilizer at a rate of 120 kg N ha^{-1} (F-org) were also included in the experimentation. The soil is a Calcisol (siltic) (World Reference Base for Soil Resources, 2006), and its main physical and chemical characteristics are as follows: 10.2% clay, 27.4% sand,62.5% silt; pH (H_2O 1:2.5) 8.1; SOC 20.0 g C kg^{-1}; TN 2.1 g N kg^{-1}; and Olsen P 0.134 g kg^{-1}. This led to a total of 40 plots, *i.e.* 4 treatments x 5 pairs of NI-I plots.

Rhizosphere soil (0-20 cm) was sampled on 5th June at the 6-leaves stage. Six individual plants were randomly selected from each plot and removed using a spade to excavate the root system. Rhizosphere soil was collected by gently shaking the roots. Fresh soil retrieved from the 6 plants was pooled, sieved using 2-mm mesh size and stored at +4 °C a few days before activity measurements.

2.2. Growth chamber experiment

The soil (500 Kg) for the growth chamber experiment was collected from the surface layer (0-30 cm) of non-inoculated and non-fertilized plots following the end of field experiment in Sérézin-de-la-Tour. The soil was air-dried, homogenized by sieving (2-mm mesh size), pooled and stored a few days at room temperature before microcosm preparation. The experiment was conducted in pots (11.3 x 11.3 x 21.5 cm^3), each filled with 1.8 Kg soil. All pots were flooded with distilled water in order to leach excess mineral N, and kept to 70% of soil water holding capacity. Pots were transferred to an environmental growth chamber (photoperiod 16 h, temperature 19 °C night and 26 °C day, relative humidity 65%, PPFD: 350 µmol m^{-2} s^{-1}). One inoculated, I, or non-inoculated, NI, maize seed (same maize cv. and same inoculation load as for the field experiment) was sown in each pot at a depth of 1 cm. The experiment

was set up as a randomized block design with 6 blocks, and treatments randomly assigned to one pot in each block. Pots were kept in the chamber until maize plants reached the 6-leaves stage (25 days). During the first ten days, all maize plants were watered every other day with 10 ml H_2O_{dd}; then pots were amended with artificial root exudates C (ARE-C treatment) or distilled water (H_2O_{dd} control treatment). ARE-C solution was prepared with sterilized H_2O_{dd} and 9 C sources identified as main constituents of maize root exudates (Kraffczyk et al., 1984; Baudoin et al., 2003). The stock solution at C concentration of 25 mg C ml^{-1} contained 3 carbohydrates (66.75 mM glucose, 66.75 mM fructose and 79.75 mM arabinose), 3 carboxylic acids (49.5 mM succinic acid, 33.25 mM citric acid and 49.5 mM fumaric acid) and 3 amino acids (33.25 mM alanine, 25 mM aspartic acid and 19.5 mM glutamic acid). Working solutions consisted of 12.5 ml of stock solution mixed with H_20_{dd} in order to ensure an enrichment of 250, 100 or 20 µg C g^{-1} soil per pot. Furthermore, additional soil pots with 100 µg C g^{-1} were set up and fertilized with KNO_3 at 40 Kg N ha^{-1}. Half of the N fertilizer was added at sowing (day 0) and half at 3-leaves stage (day 10). At harvest (6-leaves stage), fresh rhizosphere soil was collected by gently shaking roots and stored at +4 °C or -20 °C before potential activities and molecular measurements. A ~10 g subsample was weighed and dried at 105°C during 24 h to determine gravimetric soil moisture. Soil nitrate concentration was measured using 5 g equivalent dry weight soil from I and NI plots after extraction with 20 ml of 2 mol l^{-1} KCl. The extraction solution was shaken at 10 °C for 1 h at 140 rpm, filtered at 0.2 µm and frozen at −20 °C until measurements of NO_3^- concentrations were made using an ion chromatograph (DX120 Dionex, Salt Lake City, USA) equipped with a 4 × 250 mm column (IonPac AS9 HC). The sum of substrate-induced respiration quantified by Community Level Physiological Profiles (CLPP) using the MicroResp™ system (Campbell et al. 2003) was used as an index of the heterotrophic microbial biomass. The MicroResp™ system consists of a 96-deep-well microplate (1.2-ml volume) filled with soil from I and NI plots and with the addition of a range of aqueous C substrates (SIR), as described by Bérard et al. (2012).

156

157 *2.3. Potential gross (PGNP) and net (PNNP) N_2O production measurements*

158 PGNP was measured for each defrosted fresh soil sample according to Patra et al. (2005), using 3.5 g
159 dw equivalent soil per 150 mL flask. The atmosphere of each flask was replaced by a 90:10 He-C_2H_2
160 mixture to provide anaerobic conditions and inhibit N_2O-reductase activity. PGNP was determined as the
161 linear rate of the gross production of N_2O during short-term (8 h) incubation using a gas chromatograph
162 (µGC R3000, Santa Clara, CA, USA), the headspace being sampled every 1.5 h. PNNP was determined for
163 each defrosted fresh soil sample except for F-org soils, using 3.5 g dw equivalent soil. PNNP was
164 determined as the linear rate of the N_2O production during short-term (8 h) incubation under anaerobic
165 conditions but without C_2H_2 addition. Glucose (0.5 mg C g^{-1} dry soil), glutamic acid (0.5 mg C g^{-1} dry soil)
166 and KNO_3 (50 µg NO_3^--N g^{-1} dry soil) were added to the soil samples and the soil moisture was brought to
167 100% water holding capacity. To test whether soil storage at -20°C affected denitrification, PGNP had
168 also been measured on each fresh soil sample, using the same protocol as detailed above. PGNP from
169 frozen soils were strongly correlated to those from fresh soils without any significant effect of storage
170 ($y=0.99x$, $R^2=0.65$, $p<0.0001$; regression not significantly different from the 1/1 line).

171 In addition, semi-potential gross N_2O production when either C (GNP_{C-}) or N (GNP_{N-}) was not added
172 were measured in order to evaluate the limitation of denitrification by C and N, respectively (Florio et al.,
173 2017). In these cases, denitrification depended on the availability of soil C (including ARE-C) and N,
174 respectively. Linearity of the N_2O production rate was always observed over 8 h, whatever the substrate
175 added. Activity measurements were performed at the AME platform (Microbial Ecology UMR5557,
176 Lyon).

177

178 *2.4. Quantification of the abundances of PGPR Azospirillum CRT1,* and NO_2^-- and *N_2O- reducers*

DNA was extracted from 0.5 g of soil using the FASTDNA SPIN Kit for Soil (BIO 101 Systems; Qbiogene, Carlsbad, CA, USA). DNA concentrations were determined using a Qubit® 2.0 fluorometer with Quant-iT^{TM} dsDNA broad range (BR) Assay Kit (Invitrogen, France).

The abundance of *Azospirillum CRT1* was measured for 6-leaves stage soil samples by quantitative PCR as described by Couillerot et al. (2010) but qPCR counts were always below detection limit (*i.e.* less than 10^2 g^{-1} soil).

The abundances of NO_2^-- reducers were measured by quantitative PCR targeting the *nirK* and *nirS* genes (encoding the copper and cd_1 nitrite reductases, respectively). Amplification was performed using primers nirK876/nirK1040 (Henry et al., 2006) or nirSCd3aF/nirSR3cd (Throbäck et al., 2004). The 20 µL final volume PCR mix contained (final concentrations) QuantiTect SybrGreen PCR Master Mix 1 x, 1 µM of each *nirK* primer or 0.5 µM of each *nirS* primer, 0.4 mg of T4 protein, and 5 ng or 12.5 ng of soil DNA extract for *nirK* or *nirS*, respectively. Quantitative PCR was performed as follows: 15 min at 95 °C, 40 (*nirK*) or 45 (*nirS*) amplification cycles (15 s at 95 °C, 30 s at 59 °C for *nirS* or 63 °C for *nirK*, 30 s at 72 °C, and 10 s at 40 °C).

The abundances of N_2O-reducers were measured by targeting the *nosZI* and *nosZII* genes (encoding the N_2O reductases corresponding to two distinct clades). Amplification was performed using primers nosZ2F/nosZ2R (Henry et al., 2006) or nosZ-II-F/nosZ-II-R (Jones et al., 2013). The 25 µL final volume PCR mix contained (final concentrations) QuantiTect SybrGreen PCR Master Mix 1 x (*nosZI*) or 1.2 x (*nosZII*), 1 µM of each primer, 0.8 mg of T4 protein (*nosZI*) or 2% BSA (*nosZII*), and 12.5 ng or 20 ng of soil DNA extract for *nosZI* or *nosZII*, respectively. Quantitative PCR was performed as follows: 15 min at 95 °C, 6 touchdown amplification cycles for *nosZI* (15 s at 95 °C, 30 s at 65 °C, 30 s at 72 °C, and 15 s at 80 °C) and 45 amplification cycles (15 s at 95 °C, 30 s at 60 °C for *nosZI*; or 53 °C for *nosZII*, 30 s at 72 °C, and 10 s at 40 °C).

202 Standards were generated from PCR products obtained from soil DNA extracts as described by Florio
203 et al., 2017). Possible inhibitory effects of co-extracted humic compounds in soil extracts were checked
204 by dilution series, but no inhibition was observed. A melting curve analysis was performed to assess PCR
205 product specificity after amplification. The average real-time PCR efficiency for each of these genes was
206 97%, 100%, 86% and 84% for *nirK*, *nirS, nosZI* and *nosZII,* respectively. Gene copy number per gram of dry
207 soil was calculated from the copy number of each gene per ng of DNA multiplied by the amount of DNA
208 extracted from one gram of dry soil.
209
210 *2.5. Statistical analyses*
211 Significant effects of inoculation on microbial activities and abundances were identified using two-way
212 ANOVA with inoculation and C limitation as factors (JMP Pro 12, SAS Institute, Cary, North Carolina,
213 USA). Where necessary, data were log-transformed to ensure conformity with the assumptions of
214 normality and homogeneities of variances. For each pair of NI-I plots or microcosms, *i.e.* corresponding
215 to the same treatment within a given block, the effect of inoculation on a given variable V (*i.e.*, PGNP,
216 PNNP, and abundances of NO_2^-- and N_2O -reducers) was expressed as:

217 $$\%\ Inoculation\ effect = \left(\frac{V(I)}{V(NI)} - 1\right) * 100.$$

218 The limitations of denitrification by C or N were computed for NI treatments as follows:

219 $$Limitation\ of\ denitrification\ by\ C\ = \left(1 - \frac{GNP(C-)}{PGNP}\right) * 100.$$

220 $$Limitation\ of\ denitrification\ by\ N\ = \left(1 - \frac{GNP(N-)}{PGNP}\right) * 100.$$

221 Correlations were carried out to investigate the relationships (i) between the limitation of denitrification
222 by C and the amount of ARE-C amended to pots or SIR; (ii) between the limitation of denitrification by N
223 and the soil nitrate concentration; and (ii) between the inoculation effects on NO_2^--reducers, N_2O -
224 reducers, PGNP, PNNP, and C or N limitation.

225

226 **3. Results**

227 *3.1. Limitation of denitrification by C or N in the field and according to the amount of artificial root*

228 *exudates added to microcosms*

229 For field plots and non-amended microcosms, denitrification was strongly limited by organic C, with

230 potential gross N_2O production measured without C addition (GNP_{C-}, *i.e.* when denitrification activity

231 depends only on soil endogenous C supply, see 2.5 section) being reduced by 65-76% as compared to

232 PGNP (Supplementary Tab. S1). For microcosms, the limitation of denitrification by C significantly and

233 gradually decreased when the amount of ARE-C amended increased (Fig. 2). For the highest ARE-C level,

234 limitation of denitrification by organic C was reduced to 31% (Fig. 2). For amended microcosms,

235 fertilization did not affect the level of denitrification limitation by soil C (Fig. 2). Furthermore, organic

236 matter amendment according to the local organic farming practices led to significant reduction in

237 limitation by C down to 51% (Fig. 2).

238 The level of denitrification limitation by NO_3^- was always lower than the level of limitation by C for

239 field plots (29-35% and 51-76%, respectively; Supplementary Tab. S1), including non-fertilized plots, and

240 values for microcosms were not correlated with ARE-C amendment or fertilization levels (data not

241 shown). Furthermore, values of limitation of denitrification by N or C were negatively and significantly

242 correlated with soil nitrate content (Fig. S1a) and SIR (as a measure of microbial heterotrophic biomass

243 C; Fig. S1b), respectively.

244

245 *3.2. Effects of inoculation and C limitation on potential gross and net N_2O production rates, and on*

246 *denitrifier abundances*

247 Values of PGNP for field and non-amended microcosm samples ranged from 1.5 to 3.9 µg N g^{-1} h^{-1},

248 whereas PGNP values for amended microcosms significantly increased ($p<0.0001$) with increased ARE-C

levels from 3.8 to 7.1 µg N g^{-1} h^{-1} (Supplementary Tab. S1). Values of GNP_{C-} ranged from 0.74 to 4.64 µg N g^{-1} h^{-1} for F-org NI and ARE-C_{250} NI plots, respectively, whereas values of GNP_{N-} ranged from 0.80 to 5.07 µg N g^{-1} h^{-1} for ARE-C_{100} NI and ARE-C_{250} I plots, respectively (Supplementary Tab. S1). Values of PNNP for field and non-amended microcosms samples ranged from 1.5 to 3.4 µg N g^{-1} h^{-1}, whereas PNNP values for amended microcosms significantly increased with increased ARE-C levels from 2.1 to 4.9 µg N g^{-1} h^{-1} (Supplementary Tab. S1).

The abundance of *nirK*-harbouring NO_2^-- reducers was in the same order of magnitude as the abundance of *nirS*-bacteria for field plots, *i.e.* typically from 1.3 x 10^6 to 1.6 x 10^7 copies g^{-1} dry soil, but it was slightly higher for *nirK*- than *nirS*-bacteria for microcosms (*i.e.* from 1.8 x 10^8 to 2.4 x 10^8 and from 3.9 x 10^7 to 5.7 x 10^7 copies g^{-1} dry soil, respectively; Supplementary Tab. S2). The abundances of *nirK*- and *nirS*-bacteria were significantly and positively correlated ($p<0.0001$). The abundance of NO_2^--reducers as the sum of *nirK* and *nirS* abundances (*i.e.* total *nir* abundance) increased with increasing ARE-C levels ($p=0.0007$).

The abundance of *nosZI*-harbouring N_2O-reducers was in the same order of magnitude as the abundance of *nosZII* for field plots, *i.e.* typically from 4.9 x 10^5 to 1.5 x 10^7 copies g^{-1} dry soil, but it was higher for *nosZI* than *nosZII* for microcosms (*i.e.* from 2.4 x 10^6 to 4.4 x 10^6 and from 3.7 x 10^5 to 4.6 x 10^5 copies g^{-1} dry soil, respectively; supplementary Table S2). The total abundance of N_2O-reducers (sum of *nosZI* and *nosZII* abundances, *i.e. nosZ* abundance) increased with increasing ARE-C levels ($p<0.0001$).

Two-way ANOVA results showed a significant main effect of C limitation on *nosZI* and total *nosZ* abundances, and for PGNP (Table 2). Furthermore, a significant interaction effect between inoculation and C limitation was observed for the abundances of the NO_2^-- reducers ($p=0.026$, $p=0.047$ and $p=0.024$ for *nirK*, *nirS* and total *nir* abundances, respectively) and N_2O-reducers ($p=0.019$ and $p=0.030$ for *nosZII* and total *nosZ* abundances, respectively), and for PGNP and PNNP ($p=0.035$ and $p=0.023$, respectively)

(Table 2). This indicates that denitrifier abundances and activities were significantly affected by inoculation but with the effects varying depending on C limitation levels.

3.3. Relationship between the inoculation effects on PGNP and PNNP, and C limitation

A positive and exponential relationship was observed between the inoculation effect on PGNP and the level of denitrification limitation by C (R^2=0.92, p<0.0001; Fig. 3a), *i.e.* the higher the C limitation, the higher the inoculation-induced increase in PGNP. In particular, for field plots with the highest C limitation (70-76%), the increase of PGNP in response to inoculation was highest, reaching up to +113%. Values of C limitation for non C-amended microcosms (65%) were slightly lower than those observed for field plots, and were associated to a lack of inoculation effect on PGNP (Fig. 3a). Conversely, for microcosm soils amended with ARE-C, lower denitrification limitation by C were observed (ranging from 31 to 63%) and inoculation effect on PGNP was then neutral to negative (from +1% to -17%). No relationship was observed between the inoculation effect on PGNP and the level of denitrification limitation by N when considering the F and G treatments together (Supplementary Fig. S2a) or separately (data not shown)".

A positive relationship was observed between the inoculation effect on PNNP and the level of denitrification limitation by C (R^2=0.69, p=0.011; Fig. 3b). The inoculation effect on PNNP in field plots exhibiting the highest increase in PGNP was positive but reached only +37% (Fig. 3b). Conversely, for soils amended with ARE-C, inoculation decreased PNNP down to -46%. No relationship was observed between the inoculation effect on PNNP and the level of denitrification limitation by N (Supplementary Fig. S2b).

3.4. Relationships between the inoculation effect on denitrifier abundances and C limitation

The inoculation effect on the abundance of NO_2^--reducers was significantly and positively related to the limitation of denitrification by C. The best relationship was observed for the sum of *nirS*- and *nirK*-

296 harbouring bacteria (R^2=0.86, p<0.0001; Fig. 3c). In particular, inoculation increased *nir* abundance (up to
297 +91%) in field plots where limitation by C was the highest. In contrast, inoculation had a weak effect on
298 *nir* abundance (-7% to +5%) in microcosms and organic plots where C limitation was lower. No
299 relationship was observed between the inoculation effect on *nir* abundance and the level of
300 denitrification limitation by N (Supplementary Fig. S2c).

301 The inoculation effect on the total abundance of N_2O-reducers was significantly and positively
302 related to the limitation of denitrification by C (R^2=0.66, p=0.012, Fig. 3d). In particular, inoculation had
303 no or little effect on *nosZ* abundance (from -10 to +20%) in field plots that corresponded to the highest
304 values of limitation of denitrification by C, and it had a slightly negative effect in microcosms where C
305 limitation was lower (ranging from -19% for soil receiving the highest ARE-C dose to -6% for the medium
306 ARE-C dose and fertilized soil). No relationship was observed between the inoculation effect on *nosZ*
307 abundance and N limitation (Supplementary Fig. S2d).

308

309 **4. Discussion**

310 Over the last 20 years, strategies for sustainable agricultural development, including natural systems
311 agriculture and nature-based solutions (Eggermont et al. 2015), have been developed worldwide to
312 promote agroecosystem multi-functionality (Altieri, 1999). Major challenges are faced by farmers when
313 developing more sustainable agricultural systems less dependent on chemical inputs, and better use of
314 biotic interactions is part of their toolbox to promote the performance of agroecosystems under lower
315 chemical inputs (Barot et al., 2017). In this context, the practice of cereal inoculation with PGPRs is a
316 promising alternative to classical, intensive cropping systems for maintaining high yield while decreasing
317 fertilizer inputs (El Zemrany et al., 2006). Technical-economic acceptance by farmers and avoidance of
318 any negative side effects, including greenhouse gas emission from soils, are two major challenges
319 associated to cereal seed inoculation by *Azospirillum* (Bounaffaa et al., 2018). It has been reported that

320 the *Azospirillum* strains used for inoculation are unable to establish in soil, and that their abundance
321 generally drops below detection limit a few weeks after inoculation (Bashan, 1999) as also observed in
322 our study. Despite this, inoculation has been reported to indirectly affect soil microbial communities
323 (Baudoin et al. 2009) likely through lasting effects on plant and root growth and development. It is thus
324 important to assess whether inoculation can have some unintentional side effects on soil functioning, in
325 particular regarding greenhouse gas production.
326
327 *4.1. Fertilization and mimicking root exudation are effective in generating a range of levels of*
328 *denitrification limitation by C and N in the field and in microcosms*
329 In the rhizosphere, C and N availabilities are among the main factors modulating the interactions
330 between plant roots and denitrifying microorganisms (Philippot et al., 2007). Our hypothesis was that
331 maize inoculation by a PGPR would affect potential net and gross N_2O production rates and the
332 abundances of microbial groups playing a key role for N_2O production (NO_2^--reducers) and consumption
333 (N_2O-reducers) by altering two main biological processes which occur simultaneously in the rhizosphere
334 but act in an opposite way (see Fig. 1). The higher NO_3^- uptake by plants observed in response to
335 inoculation (Mantelin and Touraine, 2004) should strengthen roots-microbes competition for NO_3^-, which
336 should limit NO_3^- availability for microorganisms and thus decrease the abundance and activity of the
337 denitrifier groups mostly sensitive to NO_3^- availability. In contrast, the higher release of C exudates that
338 maize inoculation induces (Heulin et al., 1987; Shaw et al., 2006) should favor the activity and growth of
339 microbial heterotrophs, including denitrifiers. We also assumed that the relative importance of the two
340 processes would vary according to the importance of denitrification limitation by C and N. Testing this
341 hypothesis implied to manipulate the levels of denitrification limitation by C and N for the model soil
342 studied, which was achieved by adding mineral N fertilizer (in both field and growth chamber
343 experiments) and mimicking maize root exudate inputs (through addition of ARE-C in the microcosms).

344 This allowed us to generate a wide range of values for denitrification limitation by C and N for a given
345 soil. We observed that denitrification limitation by C was always higher than limitation by N for crop field
346 conditions, even for non-fertilized plots, and that limitations by N or C were negatively and significantly
347 correlated with soil nitrate content (Fig. S1a) and SIR (Fig. S1b), respectively. This could be due to a
348 rather high N and low C status of agricultural soils, in relation to previous years' fertilization practices
349 and to the important biomass export from annual cropping systems where soil C tends to decrease with
350 time (Recous et al., 1995). The addition of ARE-C to microcosms significantly decreased C limitation as
351 expected. We used realistic rates of daily ARE-C inputs, similar to rates used in other studies (Trofymow
352 et al., 1987; Iijima et al., 2000, Baudoin et al., 2003; Henry et al., 2008). Further, we applied ARE-C
353 repeatedly through low and recurrent additions rather than a large and single pulse, to better mimic the
354 exudation process. This probably allowed denitrifiers to grow and adapt to higher C availability
355 throughout the experiment, because a few days is sufficient to observe an increase in the size of
356 denitrifying communities following C addition (Henry et al., 2008). This can explain why a certain level of
357 denitrification limitation by C was still observed even at the highest ARE-C level. The treatment G-nf-ARE
358 250 not only decreased C limitation but also decreased N limitation (see Fig. S1), and it might thus be
359 possible that mineralization of the 3 exudate compounds that include N (i.e. the 3 amino acids, among
360 the 9 compounds used) fuelled N supply to denitrifiers. However, only 3 among the 9 exudate
361 compounds added to soil included N, the overall C:N ratio of the pool of artificial exudates used being
362 quite high (23.2); furthermore, no relationship was observed between N- and C-limitation levels,
363 considering either the whole data set or the data set from the microcosm experiment (not shown). Thus,
364 we did decouple N- and C-limitations in our study (even if the use of N-free exudates only could have
365 improved this decoupling). Overall, our approach allowed us to explore inoculation effects on denitrifiers
366 over a broad range of denitrification limitation by C (limitation level from 31 to 76%) and by N (from 29

367 to 69%) using a same, manipulated soil rather than comparing different soils to avoid the problem of
368 having confounding factors.
369
370 *4.2. The contrasted effects of inoculation on potential gross and net N_2O production are explained by*
371 *denitrification limitation by soil C*
372 Carbon availability is often recognized as the main determinant of denitrification in soil (Myrold and
373 Tiedje, 1985; Weier et al., 1993; Schaeffer et al., 2003), particularly in cropland soils (Chantigny et al.,
374 2010; Attard et al., 2011). Consistently, we observed that the effect of maize inoculation on potential
375 gross N_2O production was significantly and positively related to the denitrification limitation by C but not
376 to limitation by N. This shows that organic C rather than NO_3^- availability to denitrifiers controls
377 inoculation effects on potential gross N_2O production. Although measuring root exudation in soil is
378 challenging (Weixin et al., 1993) and was beyond the scope of our study, we can assume that the
379 increased root C exudation from inoculated plants drove the strong inoculation-induced increase (up to
380 +113%) in potential gross N_2O production for soils where denitrifiers were highly C-limited. Such an
381 amplitude of the stimulation of denitrification is consistent with a root exudation effect, because gross
382 N_2O production has been reported to increase by 16-250% in the rhizosphere as compared to bulk soil
383 (Stefanson, 1972; Vinther et al., 1982; Højberg et al., 1996; Mahmood et al., 1997) and by 50-660% in
384 response to ARE-C inputs to soil (Mounier et al., 2004; Henry et al., 2008; Langarica-Fuentes et al., 2018).
385 When denitrification limitation by C was decreased by recurrent root exudate inputs, the negative effect
386 of the competition between roots and denitrifiers for NO_3^- seemed to prevail, and the resulting outcome
387 of inoculation was a slightly negative effect on potential gross N_2O production (-17%).
388 The effect of inoculation on potential net N_2O production was also mainly related to denitrifier
389 limitation by C as it was positive (up to +37%) when level of denitrification limitation by C was high,
390 whereas it was negative (down to -46%) at low C limitation levels. However, when denitrification

391 limitation by C was high, the inoculation effect was lower for PNNP than PGNP (+37% and +113%,
392 respectively). The review of the literature concerning the effects of labile C and/or mineral N addition
393 provide no clue to explain these different amplitudes of the inoculation effects, as PNNP and PGNP seem
394 to respond similarly to labile C and mineral N additions (Table 1).
395
396 *4.3. The effects of inoculation on potential gross and net N_2O production are related to inoculation*
397 *effects on the abundances of* NO_2^-- and *N_2O-reducers*
398 The activity and abundance of denitrifiers are not necessarily tightly coupled, since the synthesis of
399 denitrifying enzymes is inducible (Zumft, 1997). Moreover, potential net N_2O production depends on the
400 balance between the activity and abundance of NO_2^-- and of N_2O- reducers (Chapuis-Lardy et al., 2007;
401 Assemien et al., 2019). Here we observed that inoculation-induced changes in potential gross N_2O
402 production were strongly and positively related to inoculation-induced changes in the abundance of *nir*-
403 harbouring bacteria (relationship between inoculation effects on PGNP and *nir* abundance: y=0.48x;
404 R^2=0.88; p<0.0001). Several authors have already reported such a coupling between changes in potential
405 gross denitrification and *nirS* and/or *nirK* abundances in agricultural soils (Čuhel et al., 2010; Enwall et al.,
406 2010; Attard et al., 2011; Jusselme et al., 2016; Assemien et al., 2019), although this is not necessarily the
407 case (Le Roux et al., 2013). This suggests that inoculation conditioned potential gross N_2O production in
408 the rhizosphere by mediating the build-up of NO_2^--reducers, probably due to changed C availability.
409 The inoculation effect on NO_2^--reducer abundance was concomitant to an inoculation effect on N_2O
410 reducer abundance, but the magnitude of the effect varied notably between these groups. Specifically,
411 the inoculation effect on total *nosZ* abundance was only slightly positive (up to +20%) when the effect on
412 *nir* abundance was highly positive (up to +91%). As the strong increase in NO_2^--reducers resulted in a
413 strong increase in PGNP, the moderate increase in N_2O reducers abundance partially dampened the
414 potential N_2O production induced by inoculation. This likely explains why inoculation increased PNNP but

not as highly as for PGNP under these conditions. These results suggest that N_2O emissions from maize croplands could be increased by the maize inoculation practice in the case soils are characterized by high levels of denitrification limitation by C. This calls for specific quantification of N_2O emissions from soils of inoculated and non-inoculated plots, in particular targeting croplands where limitation of denitrifiers by C is high.

The different responses of the abundances of NO_2^-- and N_2O reducers to inoculation might be due to their different sensitivity to C and N availability. Actually, our synthesis of results from previous studies regarding denitrifier abundances (Table 1) does not support the existence of any clear niche differentiation between both groups. It highlights that nearly no study has compared the responses of N_2O producers and reducers to labile C or ARE-C addition (Table 1), which should be better explored in the future.

4.4. Conclusions

Given that denitrification is a major source of N loss and N_2O emission in agroecosystems (Syakila and Kroeze, 2011), it is crucial to avoid practices that may increase this process. Our results, based on a field trial and a growth chamber experiment, show that the inoculation practice can have very strong effects of the activities and abundances of soil NO_2^-- and N_2O-reducing bacteria, but that the effects vary (and actually can be opposite) according to soil N and moreover C availability. More particularly, we showed that the level of denitrification limitation by C predicts well the resulting effect of inoculation on potential gross and net N_2O production. Inoculation by PGPRs can increase net N_2O production from cropland soils characterized by high C limitation by increasing the abundance of NO_2^--reducers more than the abundance of N_2O-reducers. In contrast, inoculation may increase the soil capacity to act as a sink for N_2O for soils where denitrifiers are not heavily limited by C. Because our results are based on potential (gross and net) N_2O production rates and abundances of key denitrifier groups, assessing the actual

439 effect of inoculation on N_2O emissions will require to quantify emission rates from inoculated and non-
440 inoculated plots across soils with contrasted C limitation levels. This could prove crucial for assessing and
441 mitigating the environmental consequences of such agricultural practice.
442
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448
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Table 1. A review of the effects of C and N amendments to soil on the abundances of *nirK*- and *nirS*-nitrite reducers, the abundances of *nosZI*- and *nosZII*-N_2O reducers, and potential gross (PGNP) and net (PNNP) N_2O production rates. Arrows indicate an increase (↑), a decrease (↓) or no change (→) in the variable considered as compared with non-amended control. Two arrows indicate two effects for half of cases each. G and F indicates growth chamber and field studies, respectively.

	Type of amendment	Reference	Land type	Type of experiment	Effects reported on denitrifier abundance and activity					
					nirK	***nirS***	***nosZI***	***nosZII***	**PGNP**	**PNNP**
Labile C amendment	Glucose	Myers and McGarity (1971)	Unplanted	G					↑	
	Glucose	Weier et al. (1993)	Unplanted	G					↑	↑
	Glucose	Dandie et al. (2008)	Unplanted	G					↑	
	Glucose	Miller et al. (2008)	Cropland	G					→	↓
	Glucose	Henderson et al. (2010)	Mixed crops	G		→	→		→	→
	Glucose	Miller et al. 2012)	Unplanted	G			↑		→	↑
	Glucose	Barrett et al. (2016)	Unplanted	G		↑				
					-	**↑50%**	**↑50%**	-	**↑50%**	**↑50%**
					-	**→50%**	**→50%**	-	**→50%**	→25%
					-	-	-	-	-	↓25%
Mineral N amendment	NO_3	Weier et al. (1993)	Unplanted	G					→	→
	NO_3	Enwall et al. (2005); Hallin et al. (2009)	Cropland	F	↑	→	↑		↑	
	NO_3	Gillam et al. (2008)	Unplanted	G					→	↑
	NO_3	Miller et al. (2008)	Cropland	G					→	↑
	NO_3	Miller et al. (2008)	Cropland	G					↑	↑
	NO_3	Miller et al. (2008)	Cropland	G					↑	↑
	$NO_3 + NH_4$	Niboyet et al. (2010)	Grassland	G					↑	
	$NO_3 + NH_4$	Clark et al. (2012)	Cropland	F	↑	→	→			
	$NO_3 + NH_4$	Clark et al. (2012)	Cropland		↑	↓	→			
	$NO_3 + NH_4$	Tatti et al. (2013)	Orchard	G	→	→	→		↑	↑→
	$NO_3 + NH_4$	Tatti et al. (2013)	Orchard	G	→	↑	→		↑	↑→
	$NO_3 + NH_4$	Kastl et al. (2015)	Grasslands	G	→	→	→			
	$NO_3 + NH_4$	Kastl et al. (2015)	Grasslands	G	→	→	→			
	$NO_3 + NH_4$	Kastl et al. (2015)	Grasslands	G	→	→	→			
	$NO_3 + NH_4$	Ma et al. (2016)	Grasslands	F	→	→	→			
	$NO_3 + NH_4$	Florio et al. (2017)	Cropland	F	→	→	→	→	→	
	$NO_3 + NH_4$	Krause et al. (2017)	Cropland	G	→	→	→	→	↑	→

	Type of amendment	Reference	Land type	Type of experiment	Effects reported on denitrifier abundance and activity					
					nirK	*nirS*	*nosZI*	*nosZII*	PGNP	PNNP
Mineral N amendment	NH_4	Enwall et al. (2005); Hallin et al. (2009)	Cropland	F	↓	↓	→		↑	
	NH_4	Torralbo et al. (2017)	Cropland	G	→	→	→	↓		
	NH_4	Torralbo et al. (2017)	Cropland	G	→	→	→	→		
					↑22%	↑7%	↑7%	-	**↑67%**	**↑62%**
					→71%	**→79%**	**→93%**	**→75%**	→33%	→38%
					↓7%	↓14%	-	↓25%	-	-
Labile C + mineral N amendment	Glucose + NO_3	Weier et al. (1993)	Unplanted	G					↑	↑
	Glucose + NO_3	Murray et al. (2004)	Unplanted	G					↑	↑
	Glucose + NO_3	Gillam et al. (2008)	Unplanted	G					→	→
	Glucose + NO_3	Miller et al. (2008)	Cropland	G					↑	↑
	Glucose + NO_3	Miller et al. (2008)	Cropland	G					→	↑
	Glucose + NO_3	Miller et al. (2008)	Cropland	G					↑	↑
	Glucose + NO_3	Miller et al. (2008)	Cropland	G					→	↓
	Glucose + NO_3	Miller et al. (2008)	Cropland	G					↑	→
	Glucose + NO_3	Miller et al. (2008)	Cropland	G			→		↑	↑
	Glucose + NO_3	Loick et al. (2016)	Unplanted	G						↑
	Glucose, cellulose + NO_3	Dendooven et al. (1996)	Unplanted						↑	↑
	Starch + NO_3	Murray et al. (2004)	Unplanted	G					↑	→
	Cellulose + NO_3	Murray et al. (2004)	Unplanted	G					→	→
	Organic acids, aminoacids + NO_3	Morley et al. (2014)	Unplanted	G					↑	↑
	Glucose, citric acid, glutamine + NO_3	Giles et al. (2017)	Unplanted	G					↑	↑
					-	-	-	-	**↑71%**	**↑66%**
					-	-	**→100%**	-	→29%	→27%
					-	-	-	-	-	↓7%
ARE-C amendment	Mucilage	Mounier et al. (2004)	Unplanted	G					↑	
	Artificial exudates	Henry et al. (2008)	Cropland	G	↑	↑	→		↑	↑
	Artificial exudates	Langarica-Fuentes et al. (2018)	Unplanted	G	↑	↑	↑	→	↑	→
					↑100%	**↑100%**	**↑50%**	-	**↑100%**	**↑50%**
					-	-	**→50%**	**→100%**	-	**→50%**
					-	-	-	-	-	-

660

661

662 **Table 2.** Overall effects of maize inoculation by *Azospirillum lipoferum* CRT1 on the abundances of *nirK*-, *nirS*- and *nir*- (*nirK*+*nirS*) NO_2^-- reducers,

663 the abundances of *nosZI*-, *nosZII*- and *nosZ*- (*nosZI*+*nosZII*) N_2O reducers, and potential gross (PGNP) and net (PNNP) N_2O production rates.

	nirK	***nirS***	***nir***	***nosZI***	***nosZII***	***nosZ***	**PGNP**	**PNNP**
Inoculation	NS	NS	NS	NS	NS	NS	NS	NS
C limitation	NS	NS	NS	**0.005**	NS	**0.003**	**<0.0001**	0.056
Inoculation x C limitation	**0.026**	**0.047**	**0.024**	0.060	**0.019**	**0.030**	**0.035**	**0.023**

664 Results were obtained using two-way ANOVA with inoculation and C limitation as fixed effects.

665

Figure legends

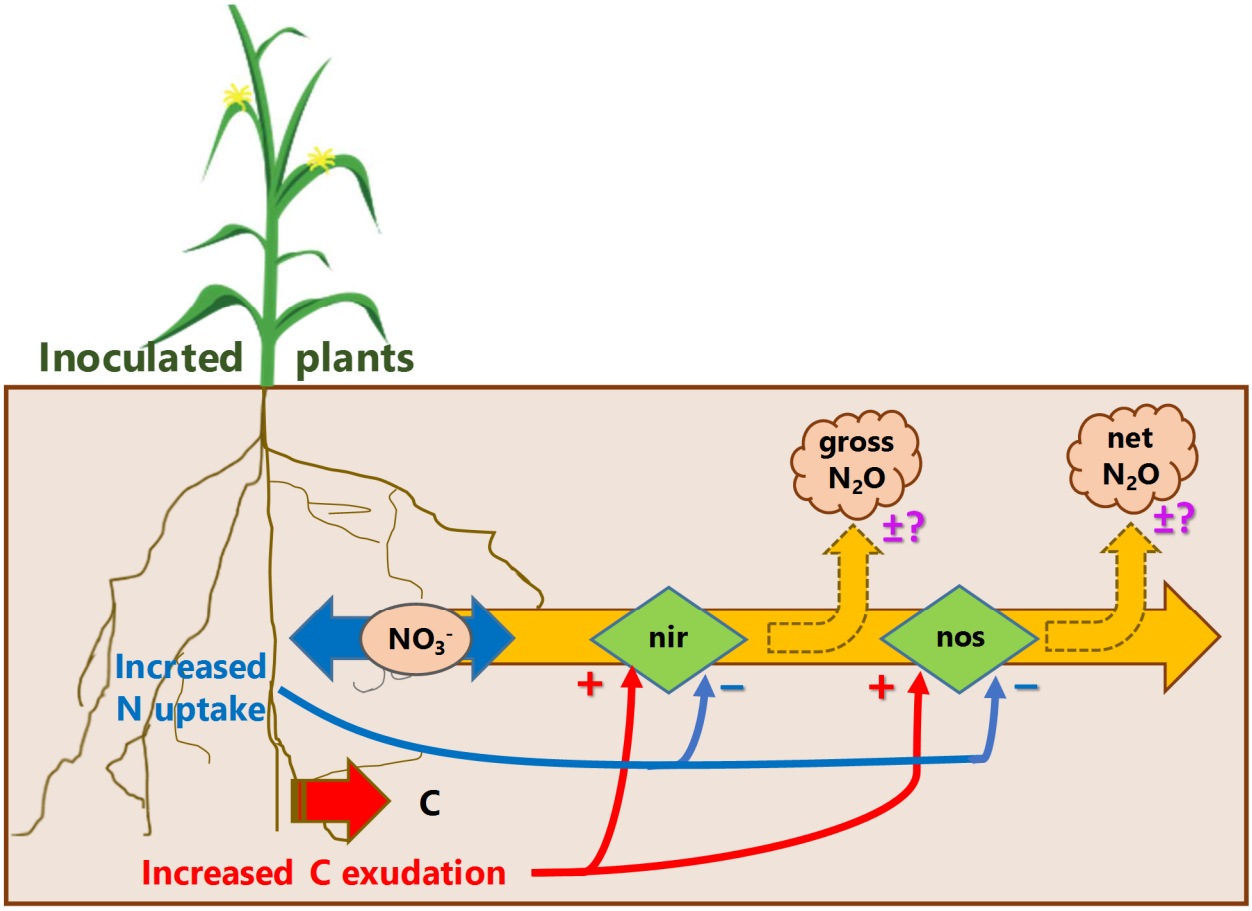
Fig. 1. We assumed that the effect of cereal inoculation with plant growth promoting rhizobacteria on potential gross and net N_2O production results from the balance between the inoculation-induced increase in root exudation and increased plant-microbes competition for NO_3^- which may differently affect the main denitrifier groups. In soils with low limitation of denitrification by organic C, exudation would play a minor role and enhanced competition for nitrate would lead to lower the abundance and activity of NO_2^--reducers (*nir*-harbouring bacteria) and N_2O-reducers (*nosZ*-harbouring bacteria). In soils with high limitation of denitrification by C, positive effect of increased exudation would prevail, and increased C availability would lead to higher denitrifier abundance and activity. The sensitivity of the different denitrifier groups, in particular of NO_2^-- reducers as compared to N_2O-reducers, to C and N availabilities would determine the overall inoculation effect on gross and net N_2O production.

Fig. 2. Relationship between the level of denitrification (*i.e.* potential gross N_2O production) limitation by soil organic carbon, C, and the amount of artificial root exudates-C (ARE-C) added to the microcosms under growth chamber (G) conditions (light and dark grey dots). White symbols corresponding to field (F) plots are presented for comparison. ARE-C_0, ARE-C_{20}, ARE-C_{100}, ARE-C_{250} refer to 0, 20, 100 and 250 µg C g^{-1} soil treatments. f/2, f, org and nf refer to reduced, optimal, organic and no fertilization, respectively.

Fig. 3. Relationship between the inoculation effects on (a) potential gross N_2O production, PGNP, (b) potential net N_2O production, PNNP, (c) the total abundance of NO_2^-- reducers, and (d) the total abundance of N_2O-reducers, and the level of limitation of denitrification by C. Each point corresponds to the mean of the inoculation effect calculated for each pair of NI-I plots or microcosms as described in section 2.5. Symbols for treatments are as in Fig. 2.

691 **Fig. 1**

692



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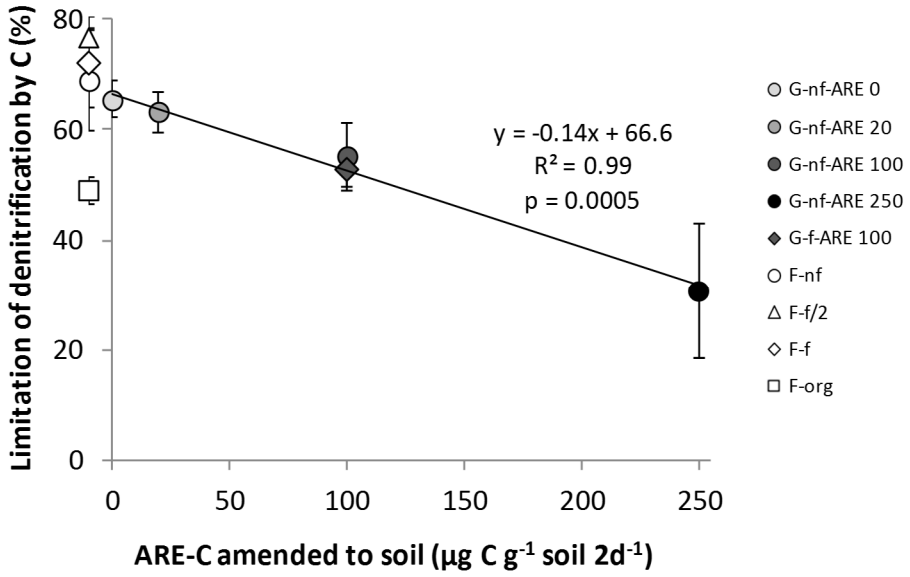
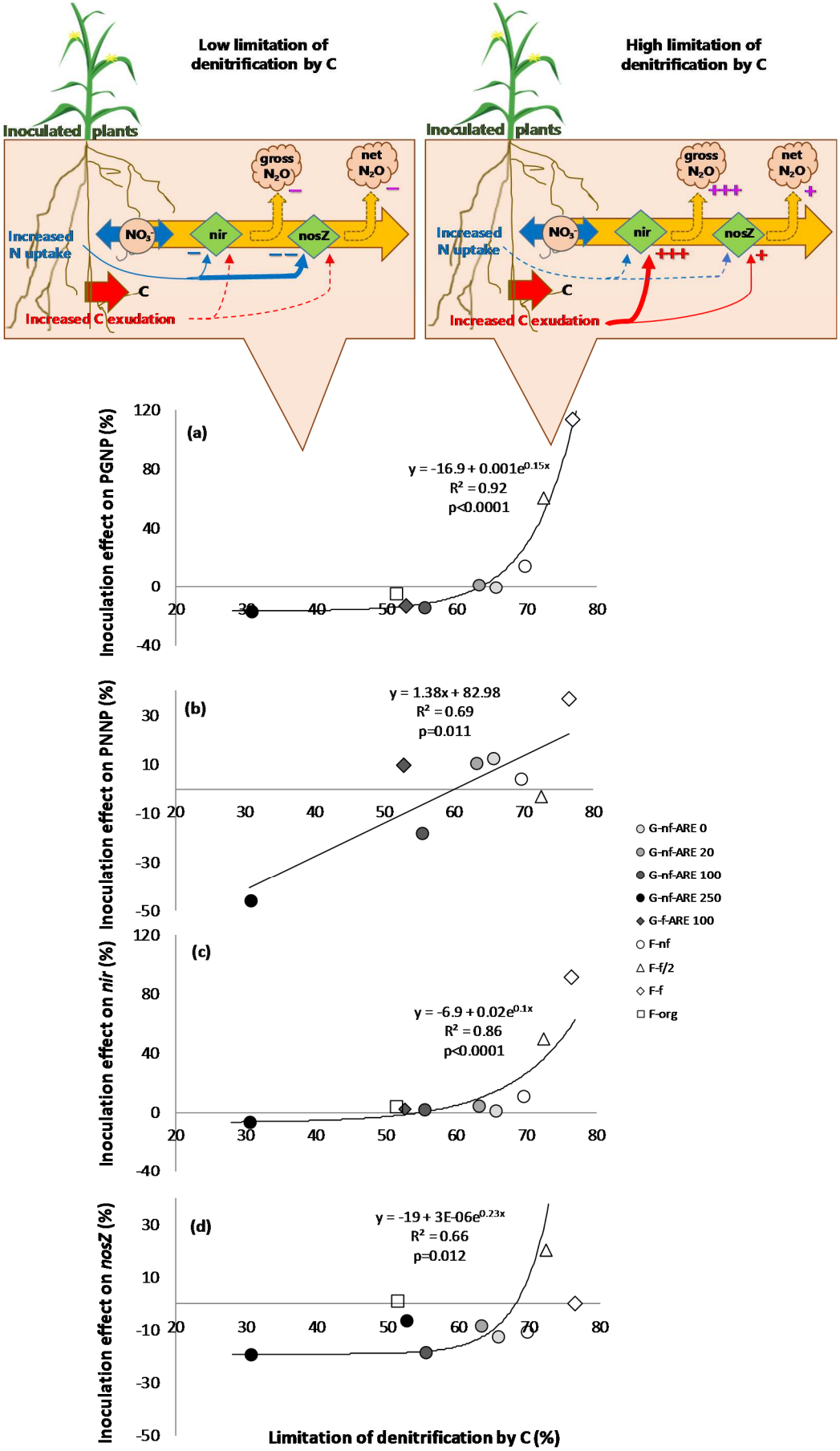
694

695 **Fig. 2.**


Limitation of denitrification by C (%)
80
60
40
20
0
0
50
100
150
200
250
ARE-C amended to soil (µg C g-1 soil 2d-1)
y = -0.14x + 66.6
R² = 0.99
p = 0.0005
G-nf-ARE 0
G-nf-ARE 20
G-nf-ARE 100
G-nf-ARE 250
G-f-ARE 100
F-nf
F-f/2
F-f
F-org

696

697 **Fig. 3.**


Low limitation of denitrification by C
High limitation of denitrification by C
Inoculated plants
Inoculated plants
gross N2O
net N2O
gross N2O
net N2O
Increased N uptake
NO3-
nir
nosZ
Increased N uptake
NO3-
nir
nosZ
C
Increased C exudation
C
Increased C exudation
(a)
y = -16.9 + 0.001e0.15x
R² = 0.92
p<0.0001
Inoculation effect on PGNP (%)
(b)
y = 1.38x + 82.98
R² = 0.69
p=0.011
Inoculation effect on PNNP (%)
(c)
y = -6.9 + 0.02e0.1x
R² = 0.86
p<0.0001
Inoculation effect on nir (%)
(d)
y = -19 + 3E-06e0.23x
R² = 0.66
p=0.012
Inoculation effect on nosZ (%)
Limitation of denitrification by C (%)
G-nf-ARE 0
G-nf-ARE 20
G-nf-ARE 100
G-nf-ARE 250
G-f-ARE 100
F-nf
F-f/2
F-f
F-org

698

Supplementary Material

The responses of NO_2^-- and N_2O-reducing bacteria to maize inoculation by the PGPR *Azospirillum lipoferum CRT1* depend on carbon availability and determine gross and net N_2O production

by Alessandro Florio, Caroline Bréfort, Jonathan Gervaix, Annette Bérard & Xavier Le Roux

2 Supplementary Tables S1 & S2

2 Supplementary Figure S1 & S2.

709 **Table S1.** Potential gross N_2O production (PGNP), gross N_2O production when either C (GNP_{C-}) or N (GNP_{N-}) was not added to flasks, limitation of

710 denitrification by C or N, and potential net (PNNP) N_2O production rates in inoculated (I) and non-inoculated (NI) soils from growth chamber (G) and

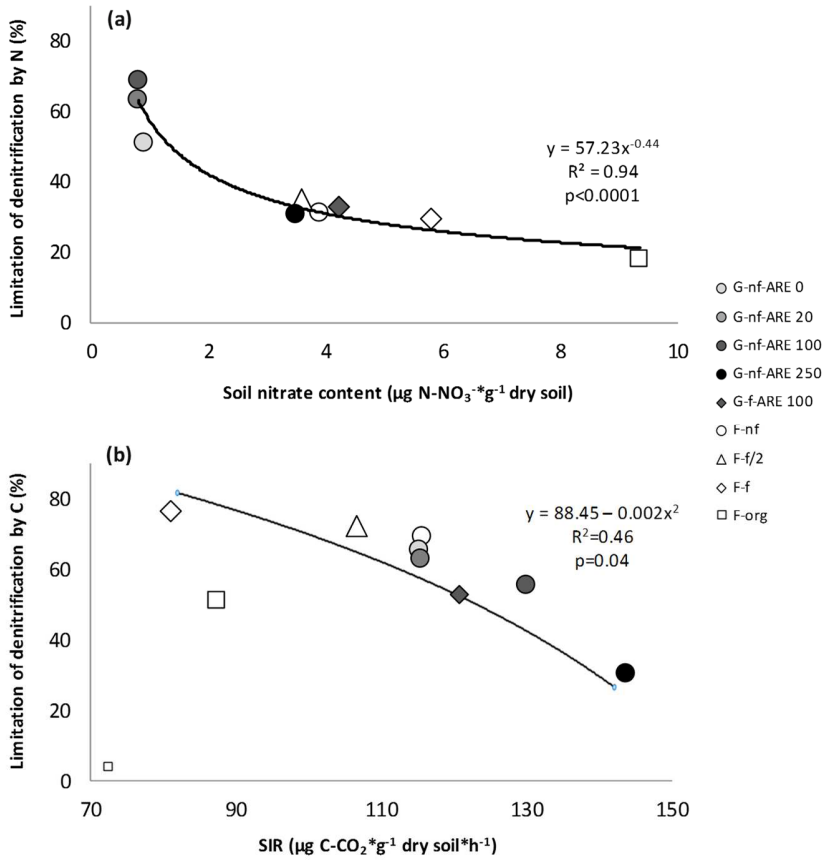
711 field (F) experiments. Acronyms for fertilization treatments are as in Fig. 2. Values are means ± s.e. (n=6 or 5 for G and F, respectively).

712

Type of experiment	Inoculation	Treatment	PGNP ($\mu g\ N_2O\text{-}N\ g^{-1}\ soil\ h^{-1}$)	GNP_{C-} ($\mu g\ N_2O\text{-}N\ g^{-1}\ soil\ h^{-1}$)	GNP_{N-} ($\mu g\ N_2O\text{-}N\ g^{-1}\ soil\ h^{-1}$)	Limitation of denitrification by C ((1-GNP_{C-}/PGNP)*100) (%)	Limitation of denitrification by N ((1-GNP_{N-}/PGNP)*100) (%)	PNNP ($\mu g\ N_2O\text{-}N\ g^{-1}\ soil\ h^{-1}$)
G	NI	ARE-C_0	3.70±0.18	1.07±0.14	2.00±0.67	70.65±4.04	46.73±16.54	3.40±0.24
		ARE-C_{20}	4.00±0.16	1.48±0.20	1.55±0.50	62.67±5.17	61.37±12.27	2.13±0.12
		ARE-C_{100}	4.99±0.23	1.94±0.20	0.80±0.20	60.38±5.26	84.35±3.47	4.20±0.18
		ARE-C_{250}	7.07±0.57	4.64±0.62	4.93±0.65	30.56±13.07	27.22±11.67	4.87±0.31
		ARE-C_{100}f	4.76±0.28	1.94±0.18	3.40±0.54	58.23±5.13	27.34±11.44	2.25±0.12
	I	ARE-C_0	3.66±0.17	1.24±0.08	1.71±0.54			3.45±0.15
		ARE-C_{20}	3.84±0.18	1.42±0.16	1.38±0.43			2.29±0.04
		ARE-C_{100}	4.58±0.35	1.98±0.18	2.51±1.25			3.46±0.26
		ARE-C_{250}	6.46±0.32	4.36±0.70	5.07±0.92			2.62±0.24
		ARE-C_{100}f	4.74±0.37	2.24±0.27	3.19±0.51			2.47±0.16
F	NI	nf	3.15±0.92	0.98±0.12	2.53±0.65	69.60±7.95	31.24±6.48	1.92±0.52
		f/2	2.63±0.74	0.85±0.07	2.57±0.60	72.45±6.08	35.03±5.71	2.24±0.65
		f	1.98±0.58	1.00±0.16	2.24±0.61	76.48±15.01	29.49±4.66	1.61±0.46
		org	1.54±0.05	0.74±0.03	1.31±0.13	51.43±2.19	18.15±5.19	n.d.
	I	nf	3.49±0.98	1.06±0.14	2.40±0.56			1.82±0.53
		f/2	3.92±0.89	1.08±0.10	2.55±0.46			1.69±0.31
		f	3.63±0.78	0.85±0.19	2.56±0.45			1.49±0.24
		org	1.46±0.06	0.79±0.05	1.17±0.07			n.d.

Table S2. Abundances of *nirK*-, *nirS*- and *nir* (*nirK*+*nirS*) NO_2^--reducers, and of *nosZI*-, *nosZII*- and *nosZ*- (*nosZI*+*nosZII*) N_2O-reducers in inoculated (I) and non-inoculated (NI) soils from growth chamber (G) and field (F) experiments. Acronyms for fertilization treatments are as in Fig. 2. Values are means ± s.e. (n=6 or 5 for G and F, respectively).

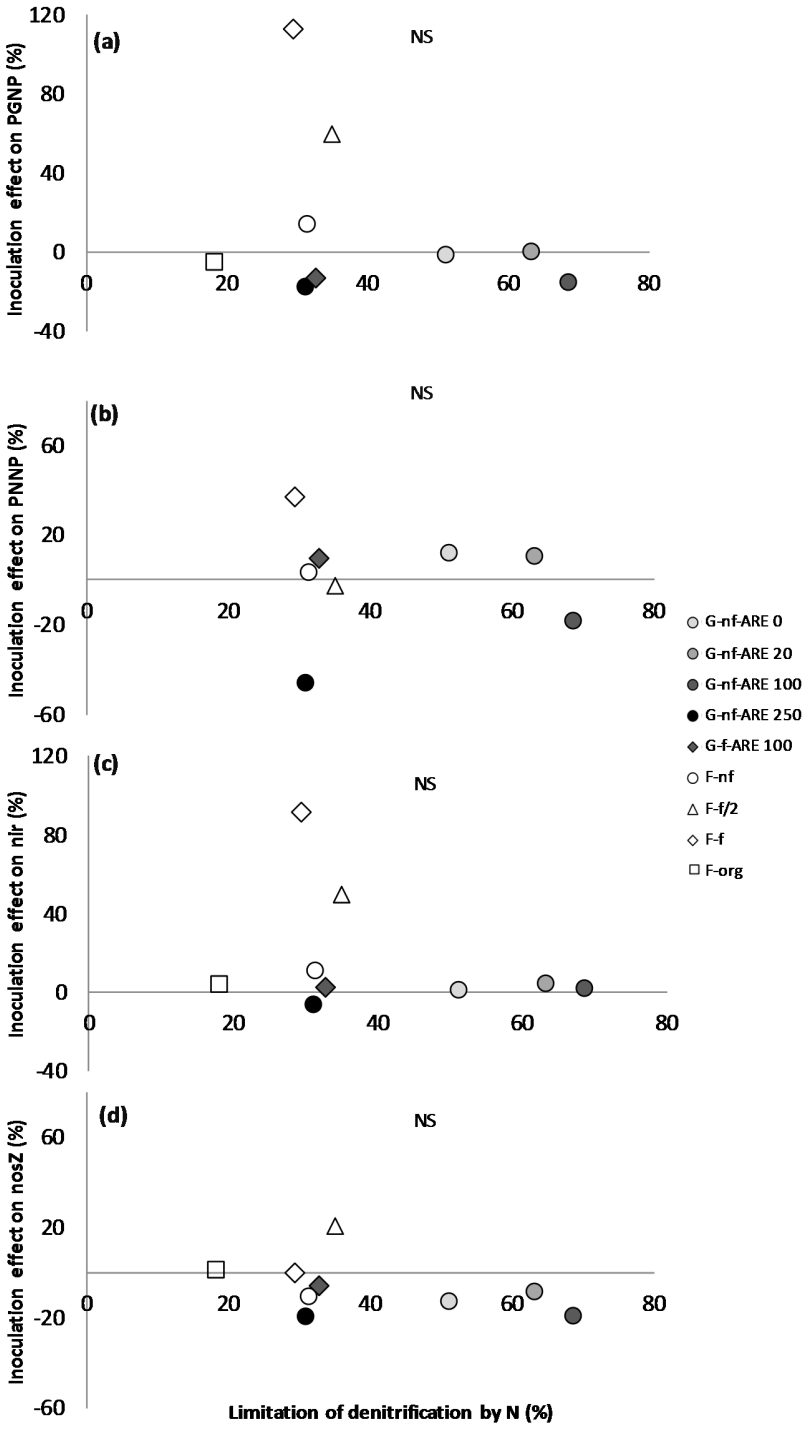
Type of experiment	Inoculation	Treatment	*nirK* (*nirK* copies g^{-1} soil)	*nirS* (*nirS* copies g^{-1} soil)	*nir* (*nirK+nirS* copies g^{-1} soil)	*nosZI* (*nosZI* copies g^{-1} soil)	*nosZII* (*nosZII* copies g^{-1} soil)	*nosZ* (*nosZI+nosZII* copies g^{-1} soil)
G	NI	ARE-C_0	1.87E+08±1.31E+07	3.93E+07±7.51E+06	2.26E+08±1.72E+07	2.91E+06±2.17E+05	4.46E+05±9.24E+04	3.36E+06±3.30E+05
		ARE-C_{20}	1.83E+08±9.11E+06	4.22E+07±5.24E+06	2.25E+08±1.86E+07	2.91E+06±1.73E+05	4.28E+05±6.74E+04	3.34+06±2.05E+05
		ARE-C_{100}	2.04E+08±1.14E+07	4.82E+07±7.78E+06	2.52E+08±1.08E+07	3.62E+06±2.07E+05	4.62E+05±7.74E+04	4.08E+06±2.12E+05
		ARE-C_{250}	2.25E+08±2.43E+07	5.66E+07±1.10E+07	2.82+0.8±5.14E+07	4.44E+06±4.38E+05	4.49E+05±9.75E+04	4.89E06±3.51E+05
		ARE-C_{100}f	2.01E+08±8.01E+06	4.52E+07±8.31E+06	2.46E+08±1.43E+07	3.24E+06±1.42E+05	3.99E+05±8.24E+04	3.64E+06±2.05E+05
	I	ARE-C_0	1.82E+08±1.04E+07	5.24E+07±1.29E+07	2.34E+08±1.83E+07	2.37E+06±2.20E+05	3.68E+05±7.49E+04	2.74E+06±1.87E+05
		ARE-C_{20}	1.94E+08±8.90E+06	4.20E+07±3.48E+06	2.36E+08±1.40E+07	2.48E+06±2.90E+05	4.31E+05±7.75E+04	2.91E+06±2.48E+05
		ARE-C_{100}	2.04E+08±1.23E+07	4.94E+07±9.07E+06	2.53E+08±3.43E+07	2.76E+06±3.20E+05	4.43E+05±1.20E+05	3.20E+06±3.68E+05
		ARE-C_{250}	2.39E+08±1.98E+07	5.57E+07±8.48E+06	2.95E+08±2.59E+07	3.73E+06±3.55E+05	4.53E+05±9.60E+04	4.18E+06±2.88E+05
		ARE-C_{100}f	1.88E+08±5.27E+07	7.14E+07±1.15E+07	2.59E+08±6.90E+07	3.54E+06±7.63E+05	4.01E+05±8.28E+04	3.94E+06±1.87E+05
F	NI	nf	8.45E+06±1.51E+06	1.15E+07±3.03E+06	1.99E+07±3.87E+06	8.54E+05±1.69E+05	1.22E+06±1.55E+05	2.08E+06±1.08E+05
		f/2	1.19E+07±3.44E+06	1.28E+07±3.19E+06	2.47E+07±5.56E+06	9.17E+05±1.88E+05	1.08E+06±1.92E+05	2.00E+06±1.20E+05
		f	9.68E+06±3.95E+06	1.07E+07±2.38E+06	2.04E+07±5.75E+06	8.43E+05±1.73E+05	1.35E+06±3.27E+05	2.20E+06±1.83E+05
		org	7.38E+06±1.86E+06	2.15E+06±2.16E+05	9.53E+06±1.88E+06	5.14E+05±3.77E+04	1.55E+07±1.91E+06	1.62E+07±1.92E+06
	I	nf	9.42E+06±2.75E+06	1.32E+06±3.42E+06	1.07E+07±5.84E+06	7.56E+05±1.14E+05	1.08E+06±2.16E+05	1.83E+06±1.57E+05
		f/2	1.47E+07±2.78E+06	1.56E+07±3.10E+06	3.03E+07±3.99E+06	1.03E+06±1.85E+05	1.06E+06±2.77E+05	2.35E+06±3.10E+05
		f	1.42E+07±3.44E+06	1.45E+07±2.65E+06	2.87E+07±5.70E+06	9.32E+05±1.39E+05	1.20E+06±2.33E+05	2.13E+06±1.22E+05
		org	6.03E+06±8.78E+05	1.95E+06±2.14E+05	7.98E+06±1.17E+06	4.91E+05±6.77E+04	1.47E+07±1.74E+06	1.53E+07±1.79E+06



718

719 **Fig. S1**. Relationship between (a) the limitation of denitrification by N and soil nitrate content, and

720 between (b) the limitation of denitrification by C and SIR. Symbols for treatments are as in Fig. 2.



721

722 **Fig. S2**. Relationship between the inoculation effects on (a) potential gross N_2O production, PGNP, (b)

723 potential net N_2O production, PNNP, (c) the total abundance of NO_2^--reducers (*nir*), and (d) the total

724 abundance of N_2O-reducers (*nosZ*), and the level of limitation of denitrification by N. Symbols for

725 treatments are as in Fig. 2.