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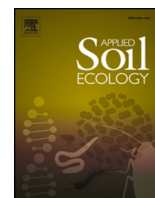
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# Loss in soil microbial diversity constrains microbiome selection and alters the abundance of N-cycling guilds in barley rhizosphere

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## ABSTRACT

Plant roots are shaping microbial communities that are distinct from the surrounding soil. These root-associated microbial communities can have both positive and negative effects on the host nutrient acquisition and thereby growth, yet how loss of soil microbial diversity will constrain the plant microbiome selection is relatively unknown. In this study, we manipulated the soil microbial community using a removal-by-dilution approach to examine how microbial diversity modulates microbiome selection in barley, including microbial guilds involved in nitrogen (N) cycling processes causing N loss, and its consequences for plant performance. We found that microbial diversity loss reduced the barley's ability to recruit specific microorganisms from the soil and only members of the Alphaproteobacteria and Bacteroidetes were enriched in both rhizosphere and root-associated compartments irrespective of dilution level. Loss in soil microbial diversity and the presence of plants affected the N-cycling communities, with the abundance of nitrous oxide reducers being 2–4 times higher in both barley compartments in the lower diversity soils. In these soils, the low abundance of bacterial ammonia oxidizers (close or below detection level in the barley compartments) was concomitant with an increase in leaf greenness (ca. 12%), an indicator of the plant N status. The reduction in soil microbial diversity was thus coupled to a change in functional traits of rhizosphere and root-associated communities, with consequences for plant performance. This work contributes to our understanding of plant-microbe interactions, which is needed to steer the crop microbiome towards increased N-use efficiency while minimizing negative environmental impact.

## 1. Introduction

Biodiversity is hypothesized to underpin ecosystem functioning (Balvanera et al., 2006; Lefcheck et al., 2015; Tilman, 1999) and whether biodiversity also supports ecosystem productivity is a major area of research in ecology (Gross et al., 2014; Naeem and Li, 1997; Tilman et al., 2006). While most studies addressing these questions in terrestrial systems have focused on plant communities, especially grasslands (see Duffy et al., 2017) for a meta-analysis), a growing body of literature suggests that microbial diversity loss can affect the functioning of soil ecosystems (Delgado-Baquerizo et al., 2016b; Hallin et al., 2012; Wagg et al., 2014). For example, Calderón et al. (2017) showed that microbial diversity loss resulted in alternative compositional states associated with lower nitrate pools and impaired nitrogen (N) cycle. Although microbial communities typically display some level of functional redundancy (Allison and Martiny, 2008), specialized functions carried out by phylogenetically constrained taxa are particularly

affected by diversity loss (Delgado-Baquerizo et al., 2016a; Levine et al., 2011). Deepening our knowledge on the link between soil microbial diversity and ecosystem functioning is particularly relevant to improve our understanding of how microbes can affect plant growth (Berendsen et al., 2012; Mendes et al., 2011). So far, the few studies that have investigated this link have shown effects on plant productivity (Hol et al., 2015), plant-herbivory interactions (Hol et al., 2010; Ourry et al., 2018), nutrient-based plant-soil feedbacks (Weidner et al., 2015) and bacterial interaction networks in the rhizosphere (Yan et al., 2017).

Plants recruit and shape, either directly or indirectly, the microbial communities surrounding the roots (Bulgarelli et al., 2013; Hu et al., 2018; Schulz-Bohm et al., 2018; Zhelnina et al., 2018). This results in the differential enrichment of taxa in the rhizosphere and the root-associated compartments compared to the bulk soil (Lundberg et al., 2012). Since microorganisms living inside, on and in proximity to root tissues are impacting plant growth and health, it has been proposed that the plant and its microbiota collectively form a holobiont

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**Table 1**

Effect of dilution on alpha-diversity indices (average  $\pm$  s.d.,  $n = 4$ ). Within-compartment differences are indicated with different letters. Sampling points before sowing (T42) and at the end of the experiment (T63) are indicated. At T63, all bulk soil samples except the control were compared.

	Dilution	Richness <sup>†</sup>	PD <sup>†</sup>	Pielou <sup>†</sup>	Shannon <sup>†</sup>
Bulk soil - T42	D1	1928 $\pm$ 68 <sup>a</sup>	178 $\pm$ 6 <sup>a</sup>	0.72 $\pm$ 0.01 <sup>a</sup>	5.44 $\pm$ 0.05 <sup>a</sup>
	D6	1080 $\pm$ 304 <sup>b</sup>	105 $\pm$ 29 <sup>b</sup>	0.57 $\pm$ 0.04 <sup>b</sup>	3.96 $\pm$ 0.23 <sup>b</sup>
Bulk soil T63 – unplanted	D1	1993 $\pm$ 135 <sup>a</sup>	185 $\pm$ 12 <sup>a</sup>	0.75 $\pm$ 0.01 <sup>a</sup>	5.70 $\pm$ 0.11 <sup>a</sup>
	D6	909 $\pm$ 75 <sup>b</sup>	91 $\pm$ 6 <sup>b</sup>	0.64 $\pm$ 0.03 <sup>b</sup>	4.36 $\pm$ 0.27 <sup>b</sup>
Bulk soil T63 - barley	D1	2004 $\pm$ 89 <sup>a</sup>	187 $\pm$ 7 <sup>a</sup>	0.76 $\pm$ 0.01 <sup>a</sup>	5.74 $\pm$ 0.08 <sup>a</sup>
	D6	1008 $\pm$ 88 <sup>b</sup>	100 $\pm$ 8 <sup>b</sup>	0.65 $\pm$ 0.05 <sup>b</sup>	4.50 $\pm$ 0.30 <sup>b</sup>
	Control <sup>‡</sup>	972 $\pm$ 72	97 $\pm$ 6	0.59 $\pm$ 0.06	4.06 $\pm$ 0.40
Rhizosphere T63	D1	1525 $\pm$ 250 <sup>a</sup>	143 $\pm$ 23 <sup>a</sup>	0.65 $\pm$ 0.04 <sup>a</sup>	4.78 $\pm$ 0.36 <sup>a</sup>
	D6	654 $\pm$ 106 <sup>b</sup>	68 $\pm$ 10 <sup>b</sup>	0.58 $\pm$ 0.06 <sup>a</sup>	3.77 $\pm$ 0.46 <sup>b</sup>
	Control	661 $\pm$ 48 <sup>b</sup>	70 $\pm$ 4 <sup>b</sup>	0.55 $\pm$ 0.05 <sup>a</sup>	3.58 $\pm$ 0.32 <sup>b</sup>
Root-associated T63	D1	1510 $\pm$ 129 <sup>a</sup>	151 $\pm$ 10 <sup>a</sup>	0.68 $\pm$ 0.02 <sup>a</sup>	4.98 $\pm$ 0.23 <sup>a</sup>
	D6	586 $\pm$ 108 <sup>b</sup>	67 $\pm$ 10 <sup>b</sup>	0.51 $\pm$ 0.11 <sup>b</sup>	3.27 $\pm$ 0.82 <sup>b</sup>
	Control	492 $\pm$ 47 <sup>b</sup>	59 $\pm$ 5 <sup>b</sup>	0.46 $\pm$ 0.03 <sup>b</sup>	2.83 $\pm$ 0.20 <sup>b</sup>

PD: Phylogenetic Diversity.

<sup>†</sup> Kruskal-Wallis test ( $p < 0.05$ ) followed by Dunn's test using *fdr* to correct for multiple comparisons.

<sup>‡</sup> ANOVA ( $p(F) < 0.05$ ), followed by Tukey's HSD test.

<sup>§</sup> When the effect of dilution was examined in the planted pots only (Bulk soil - T63 - barley), the control was statistically different from D1 but not from D6.

(Vandenkoornhuysen et al., 2015). Plants have in particular evolved specific types of interactions with N scavenging or transforming microorganisms to increase its availability (Moreau et al., 2019) since N is the most plant growth limiting nutrient in terrestrial ecosystems (LeBauer and Treseder, 2008). For example, mutualistic interactions with N<sub>2</sub>-fixing microorganisms are well-known, yet novel symbiotic associations are still being identified (Deynze et al., 2018). It has also been demonstrated that plants can inhibit microorganisms that cause N losses, notably through root exudation and modifications in N uptake rates (Hu et al., 2018; Moreau et al., 2019). Therefore, rhizosphere and root-associated microbial communities are key to plant available N. Yet, our understanding of the extent to which reduction in soil microbial diversity constrains plant-associated communities and the consequences of plant microbiome selection on plant performance remains limited.

Here, we examined how soil microbial diversity loss impacts (i) the rhizosphere and root microbiome and (ii) overall plant performance. Apart from the overall bacterial community, we specifically focused on the N-cycling microbial communities performing nitrification and denitrification that are responsible for N losses by leaching or nitrogenous gas emissions, including the greenhouse gas nitrous oxide (N<sub>2</sub>O). For our purpose, barley (*Hordeum vulgare* L.) was grown in pots filled with soil harboring different levels of microbial diversity and community composition generated by inoculating serial dilutions of soil suspensions into sterile soil (Salonius, 1981). We hypothesized that reduction in soil microbial diversity constrains plant microbiome selection, which, in turn, alters microbial communities involved in inorganic N-cycling processes causing N losses, and affects plant traits indicating growth and overall performance.

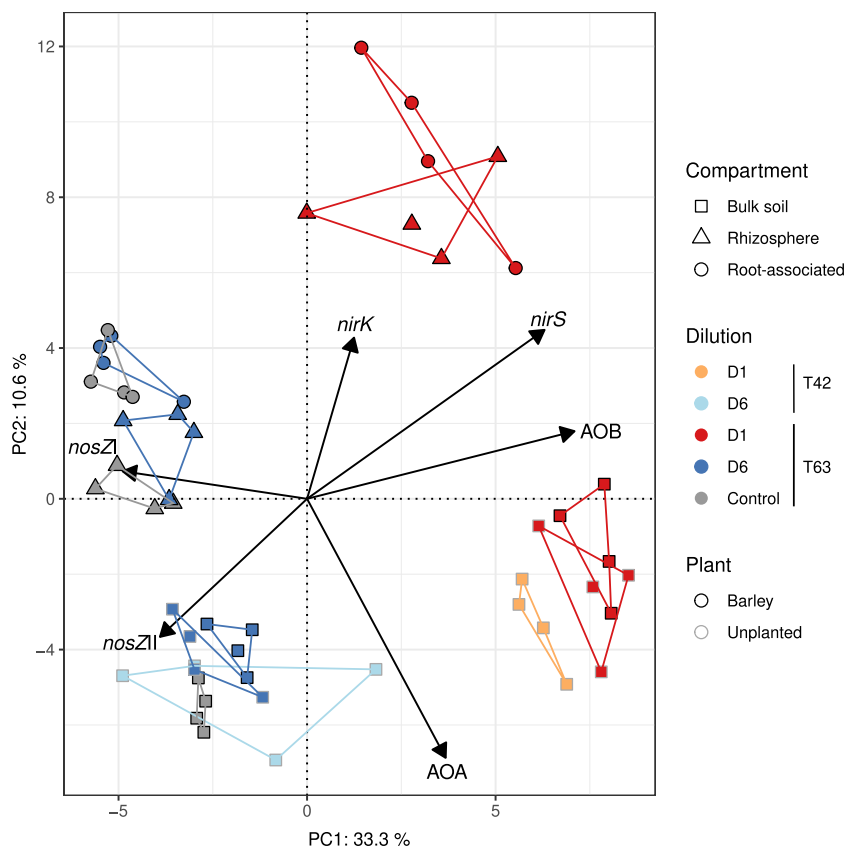
## 2. Material and methods

### 2.1. Soil sampling

Soil (0–15 cm depth) was collected in April 2017 in a bare field at Lövsta Research Station, Uppsala, Sweden (59°49'51.54"N; 17°48'25.77"E). This field has been under organic management since 1988, using a six-year rotation with two consecutive years of ley (grass/clover) followed by four years with annual crops (barley, oat, pea, potato or winter wheat). Crops were either fertilised with animal manure or not fertilised. Spring barley was grown in 2016, the year before sampling. The soil was sieved (4 mm) and then stored at 4 °C, except for the soil serving as inoculum, which was kept at –20 °C. The soil intended for sterilization was air-dried at room temperature for 7 days, homogenized and packed in plastic bags of ca. 875 g dry weight (DW). The bags were kept at –20 °C before shipping for sterilization by gamma-irradiation (>25 kGray, Scandinavian Clinics Estonia OÜ, Alliku, Estonia). The sterility of the soil was tested by spreading 0.5 g of soil onto plates with potato dextrose agar and trypticase soy agar media (5 replicates each, including the negative controls), in agreement with other studies (Griffiths et al., 2001; Yan et al., 2017, 2015). No growth was observed on any of the two media after five days of incubation at 25 °C. The water holding capacity (WHC) and the water content (oven-drying at 105 °C for 24 h) were estimated on three replicates of irradiated soil. These two measurements were used to estimate the volume of liquid (inoculum and/or water, see below) needed to reach 70% of WHC in the soil. Both irradiated and unirradiated soils were used to determine soil texture (Soil physics laboratory, SLU, Uppsala, Sweden), pH and nutrient content (Soil and plant laboratory, SLU, Uppsala, Sweden; Table S1).

### 2.2. Experimental design

Microbial communities were experimentally manipulated using a removal-by-dilution approach (Salonius, 1981). Briefly, one bag of sterile soil was added per pot (13 × 13 × 13 cm) with a 2 cm layer of sterilized clay pellets (Plantagen, Järfälla, Sweden; 8–14 mm) in the bottom. The soil in the pots was inoculated with two different soil suspensions (280 ml) obtained by serial dilution of fresh soil in sterilized, demineralized water. The suspension diluted to 10<sup>-1</sup> (Dilution 1, D1) was obtained by mixing 100 g of fresh soil in 1 l of sterilized water and was then serially diluted to 10<sup>-6</sup> (Dilution 6, D6). The soil suspensions were placed on a shaker for 5 min at 150 RPM between each dilution step. For the control, the equivalent volume of sterilized water was added. All pots received an additional 120 ml of sterilized water to reach 70% WHC. Each treatment was replicated four times, i.e. there were four pots for each treatment. The pots were incubated in a growth cabinet under controlled atmospheric conditions (20 °C with continuous light) for six weeks in order to let the microbial communities colonize the soil and reach comparable levels of biomass across the different dilution treatments prior to sowing (Timepoint T42). It has previously been shown that six weeks allows for colonization of sterile soil at similar abundances independent of dilution level (Philippot et al., 2013b). Colonization was checked by quantification of 16S rRNA gene copy numbers, see section 2.6 for methodological details. Barley (*Hordeum vulgare*, cv. Makof) seeds were first surface sterilized by two successive baths in 70% ethanol (3 min) and 1% sodium hypochlorite (3 min) followed by a thorough rinsing with sterilized water. Each pot contained four individual plants and was incubated with day/night temperatures of 20/15 °C and a day length of 18 h. The soil moisture was monitored throughout the experiment on three locations in each pot every two days with a HH2 moisture meter (Wet Sensor Kit, Delta, Cambridge, England). Sterile water was added when necessary to maintain a WHC of 50–60%. To solely assess the effect of soil microbial diversity and community composition on soil functioning and plant performance, nutrients were not added.



**Fig. 1.** Principal component (PC) analysis of the centered log-ratio abundances of the OTUs in the bulk soil, rhizosphere and root compartment for each dilution level (D1, D6), control, and time point (T42 and T63). Direction and length of the fitted vectors are proportional to the correlation between the two PCs and each functional gene ( $p < 0.001$ ), based on the abundances of the genes *amoA* in archaeal (AOA) and bacterial (AOB) ammonia oxidizers, *nirK* and *nirS* in denitrifiers and *nosZ* and *nosZII* in nitrous oxide reducers.

### 2.3. Harvesting and measurement of plant traits

When conducting the pot experiments, it was crucial to allow time for microbiome selection (Edwards et al., 2015), but at the same time avoid negative plant feedbacks that occurs due to growing plants in pots for too long, for example due to nutrient depletion. We also wanted to avoid later development stages, i.e. after flowering, since plants allocate less resources to the roots. The experiment was therefore terminated three weeks after sowing (T63), just before the barley plants would show signs of nutrient limitations (Graf et al., 2016). Bulk soil, rhizosphere and root samples were collected. We defined bulk soil as soil in the pots and soil that could be shaken off the roots, rhizosphere as the soil attached to the roots and collected by washing the roots, and root-associated communities as the microbes physically attached to the washed roots, both on and inside the roots. Bulk soil samples were homogenized and stored at  $-20^{\circ}\text{C}$  until DNA extraction. For each planted pot, roots from two individual plants were pooled in a falcon tube containing phosphate buffered saline (137 mM NaCl, 2.7 mM KCl, 10 mM  $\text{Na}_2\text{HPO}_4$  and 2 mM  $\text{KH}_2\text{PO}_4$ ). To separate the rhizosphere communities from the root-associated ones, the tubes were vortexed at maximum speed until the roots became white and centrifuged at 5000 g for 5 min. The supernatants were transparent (ocular inspection) and discarded, and the pellets, corresponding to the rhizosphere communities, were freeze-dried. Both pellets and washed roots were stored at  $-20^{\circ}\text{C}$  until DNA extraction.

The two remaining plants were independently used to measure plant traits, resulting in eight measurements per dilution level across the four replicates. Leaf greenness values for each plant were obtained by averaging three measurements on three different leaves (i.e.  $n = 9$  per plant) using a SPAD-502 meter (Konica Minolta Sensing, Tokyo, Japan). Leaf area was estimated by image analysis (ImageJ; Schneider et al., 2012) of leaf pictures taken with a Canon EOS 100D camera (Canon Inc., Tokyo, Japan). The roots were thoroughly washed with water and kept on paper

plates at  $-20^{\circ}\text{C}$ . They were later scanned with a flatbed Epson Perfection V800 scanner (Seiko Epson Corporation, Suwa, Nuagano, Japan) and the total root length was measured using the SmartRoot plugin (Lobet et al., 2011) implemented in ImageJ. Shoot and root dry weights were obtained after oven-drying at  $65^{\circ}\text{C}$  for 4 days.

### 2.4. DNA extraction, PCR amplification

DNA was extracted from bulk soil, rhizosphere and (washed) root samples that were cut in 1 cm pieces using the DNeasy PowerSoil kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions with 0.5, 0.25 and 0.20 g, respectively. Two independent extractions were done for each sample and pooled prior to further molecular analyses. Prokaryotic 16S rRNA gene fragments were amplified by a two-step PCR procedure and the first consisted of 10 ng extracted DNA,  $1 \times$  Phusion PCR Mastermix (Thermo Scientific, Waltham, MA, US), 1 mg/ml BSA and  $0.25 \mu\text{M}$  of the primers pro341F/pro805R (Takahashi et al., 2014) in  $15 \mu\text{l}$  reactions. Two independent PCRs were run under the following conditions: 3 min at  $98^{\circ}\text{C}$ , followed by 25 cycles of  $98^{\circ}\text{C}$  for 30 s,  $55^{\circ}\text{C}$  for 30 s and  $72^{\circ}\text{C}$  for 30 s and a final extension step of 10 min at  $72^{\circ}\text{C}$ . The PCR products were then pooled and checked by agarose gel electrophoresis. A single  $30 \mu\text{l}$  reaction was performed for the second PCR, using  $0.2 \mu\text{M}$  of primers with Nextera adaptor and index sequences, and  $3 \mu\text{l}$  of the pooled PCR product from the first PCR. Conditions were the same as the first step, except for an annealing temperature of  $55^{\circ}\text{C}$  and an extension time of 45 s, with 8 cycles. The final PCR products were purified using Sera-Mag purification beads (Merck KGaA, Darmstadt, Germany) following the manufacturer's instructions. The amplicon size was checked by gel electrophoresis and the quality control was done on a BioAnalyzer (Agilent, Santa Clara, CA, US). After quantification using a Qubit fluorometer (Invitrogen, Carlsbad, CA, US), two libraries were created by pooling equal amounts of purified amplicons from all bulk soil samples in one, and rhizosphere and root-associated samples in the

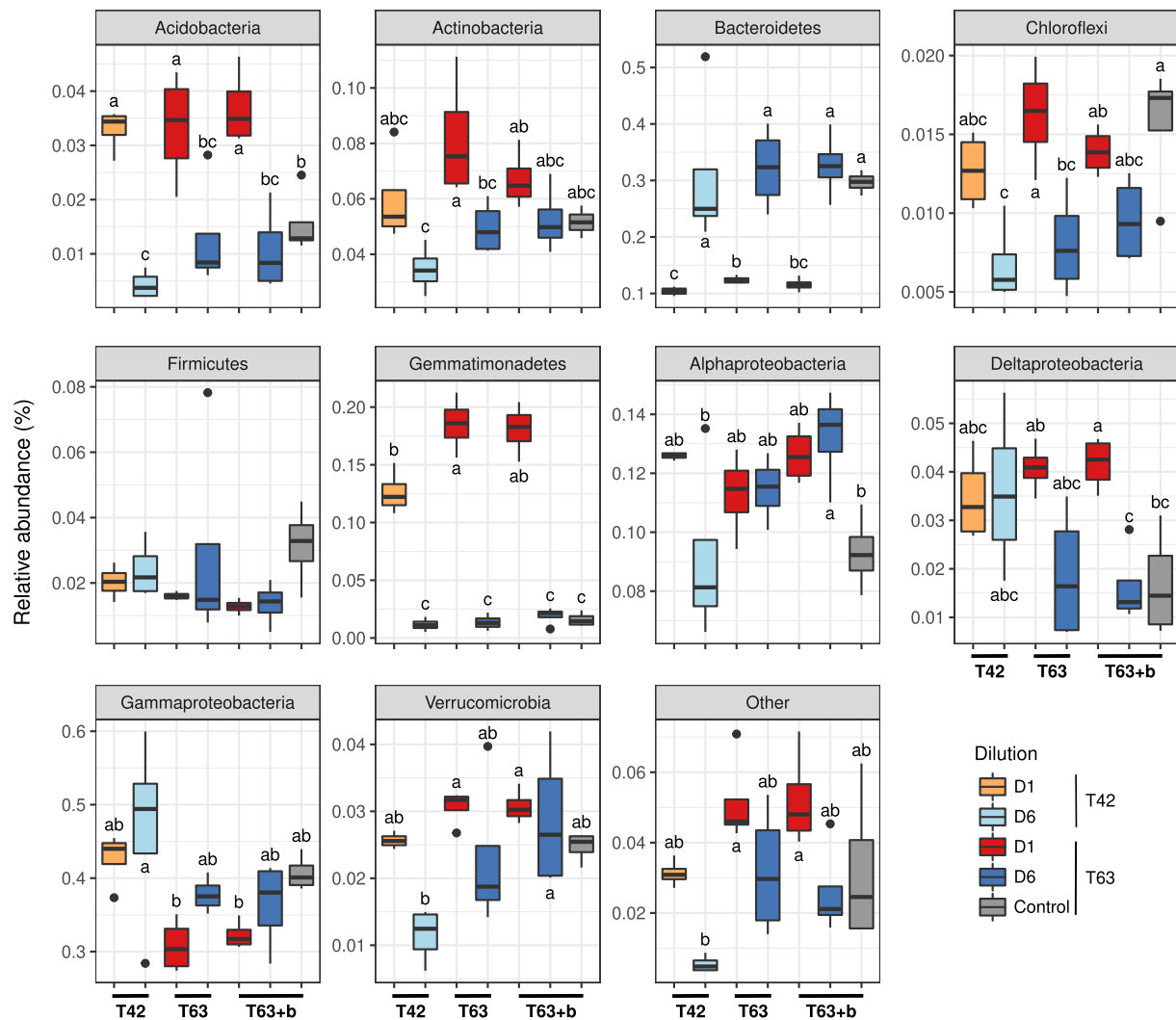


Fig. 2. Relative abundance of the main taxa in the bulk soil at time points T42 and T63 with (+) or without barley. Significant differences between dilution treatments and time points in each lineage are indicated with letters.

other. Sequencing was performed on an Illumina MiSeq instrument using the  $2 \times 250$  bp and  $2 \times 300$  bp chemistry for the bulk soil and rhizosphere/root-associated samples, respectively.

## 2.5. Sequence analysis

Forward and reverse reads were trimmed to remove non-biological bases upstream of the primers (FASTX toolkit, v. 0.0.14) and examined using FastQC (v. 0.11.7). The paired reads were subsequently merged using PEAR (v. 0.9.11; Zhang et al., 2014) with a minimum overlap of 20 bp. Reads with lengths lower than 300 bp and greater than 500 bp as well as those with uncalled bases were discarded. The maximum expected error ('ee', Edgar and Flyvbjerg, 2015) was then calculated for each merged read and those with an  $ee \geq 1$  were discarded using VSEARCH (v. 2.4.4; Rognes et al., 2016). The resulting high-quality sequences were then de novo clustered at 97% identity using the OTU clustering tool implemented in VSEARCH. Briefly, the de-duplicated merged reads were initially clustered at 97% identity and the singletons discarded. The validity of the OTUs was then screened using the de novo chimera checking tool and the reads mapped to the final set of OTUs. Finally, the representative sequence of each OTU was aligned to the SILVA reference database (SSU132 Ref NR) using the SINA aligner (v. 1.6.0; Pruesse et al., 2012) and classified using SINA's least common ancestor algorithm. OTUs classified as chloroplast or mitochondria were

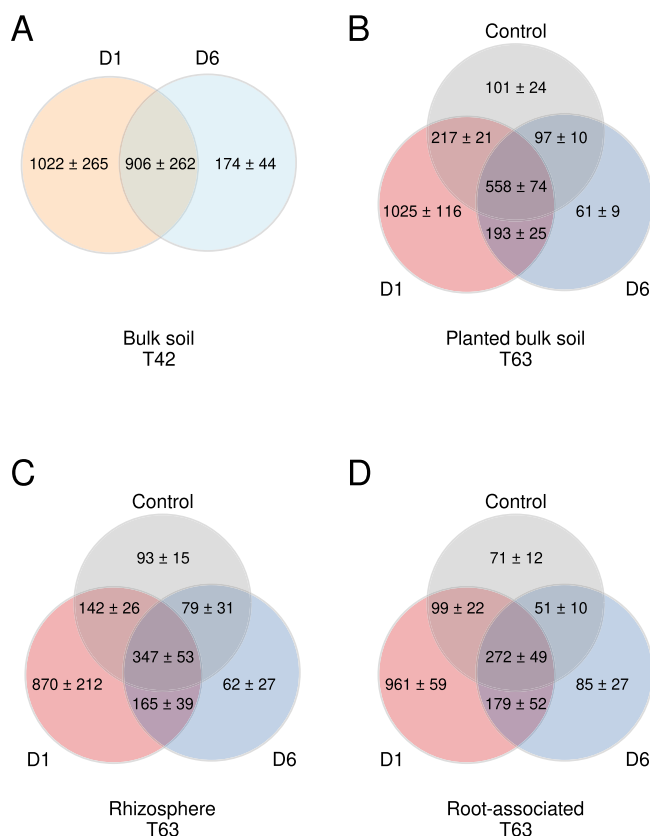
discarded.

A phylogenetic tree was built with the OTUs and a set of references from SILVA (SSU132 Ref NR) to calculate the Phylogenetic Diversity (PD; Faith, 1992) in each treatment/compartiment. The sequences were aligned using SINA. The alignment was manually checked with the ARB software (Ludwig et al., 2004), the vertical gaps removed, and the resulting alignment used to reconstruct a phylogenetic tree using the multi-threaded version of FASTTREE and the GTR + CAT model of nucleotide evolution (v. 2.1.3; Price et al., 2010).

## 2.6. Quantitative PCR

The abundances of total bacteria as well as N-cycling communities were determined by quantitative real-time PCR using the following genes as molecular markers: 16S rRNA (total archaea and bacteria), *amoA* (ammonia monooxygenase in bacterial [AOB] and archaeal [AOA] ammonia-oxidizers), *nirK* and *nirS* (dissimilatory nitrite reductase in denitrifiers), and *nosZI* and *nosZII* (nitrous oxide reductase in nitrous oxide reducers). The qPCR reactions were performed in duplicate runs in a reaction volume of 15  $\mu$ l using iQ<sup>TM</sup> SYBR Green Supermix (Bio-Rad, Hercules, CA, USA), 0.1% Bovine Serum Albumin, primers and 5 ng DNA on a CFX Connect Real-Time System (Bio-Rad). Primer sequences and concentration, qPCR conditions and amplification efficiencies can be found in Table S2. Standard curves were obtained by





**Fig. 3.** Venn diagrams showing the number of OTUs shared by, or specific to each dilution level in (A) bulk soil at T42, (B) planted bulk soil at T63, (C) rhizosphere and (D) root-associated compartments, both at T63 ( $\pm$  s.d.,  $n = 4$ ).

serial dilutions of linearized plasmids with cloned fragments of the specific genes. The amplifications were validated by melting curve analyses and agarose gel electrophoreses. Potential inhibition of PCR reactions was checked by amplifying a known amount of the pGEM-T plasmid (Promega, Madison, WI, USA) with the plasmid specific T7 and SP6 primers (Table S2) when added to the DNA extracts or non-template controls. No inhibition was detected with the amount of DNA used. Gene abundances were expressed in number of copies per ng of DNA to be comparable across compartments, since the root-associated 16S rRNA abundances likely contained more chloroplast sequences than the other compartments.

## 2.7. Statistical analyses

All statistical analyses were performed using the R software (v. 3.6.0, R Core Team, 2019). A rarefied table with 43,404 sequences per sample was obtained by averaging the OTU counts over 1000 computations, using the `rrarefy` function in 'vegan' (v. 2.5.5; Oksanen et al., 2018). Rarefaction curves were computed and indicated that the sequencing depth was sufficient to capture most of the diversity present in our samples (Fig. S1). Richness, Shannon's index, Pielou's evenness and PD were calculated using the rarefied OTU table and the 'vegan' and 'picante' (v. 1.8; Kembel et al., 2010) packages. Venn diagrams showing the number of OTUs shared by, or exclusive to, the different samples were generated using the 'gplots' package (v. 3.0.1.1; Warnes et al., 2016). Because sequencing data are compositional (Gloor et al., 2017), we examined the ratios between OTUs rather than their relative abundances. To avoid relying on pseudo-counts (Tsilimigras and Fodor, 2016), we first used a Bayesian-multiplicative replacement of the zero counts as implemented in the 'zCompositions' package (v. 1.40-2; Martín-Fernández et al., 2015). The centered log-ratio (clr)

transformation was applied to the zero replaced OTU table with the 'compositions' package (v. 1.2.0; van den Boogaart et al., 2018). The transformed dataset was used to compute a Principal Component Analysis (PCA) using the `rda` function in 'vegan'. Functional gene abundances were fitted onto the ordination using the `envfit` function in 'vegan' ( $p < 0.001$ , permutations = 9999). The effect of dilution and compartment on community composition were performed using permutational multivariate analysis of variance (PERMANOVA) from the `adonis2` function in 'vegan'. Pair-wise DESeq2 analyses (v. 1.24.0; Love et al., 2014) were conducted to detect differential OTU abundances between the three compartments ( $p < 0.01$ ) using the rarefied OTU table. Only OTUs absent in the control pots were included in the DESeq2 analysis. This was done to account for any extracellular and bacterial DNA left in the soil after sterilization (Yan et al., 2015). Gene abundances,  $\alpha$ -diversity indices and plant trait measurements were initially tested for normality using the Anderson-Darling test ('nortest' package, v. 1.0-4). Data not following a normal distribution were  $\log_{10}$ -transformed before further analysis. Gene abundance ratios were arcsine-transformed to account for the proportional nature of the data and ensure a normal distribution. Comparisons of treatment/time point means were performed using the Tukey's honestly significant difference (HSD) test implemented in the 'agricolae' package (v. 1.3.1; de Mendiburu, 2019). When underlying assumptions for ANOVA were violated, treatment comparisons were conducted using Dunn's tests and the false discovery rate correction available in the 'dunn.test' package (v. 1.3.5).

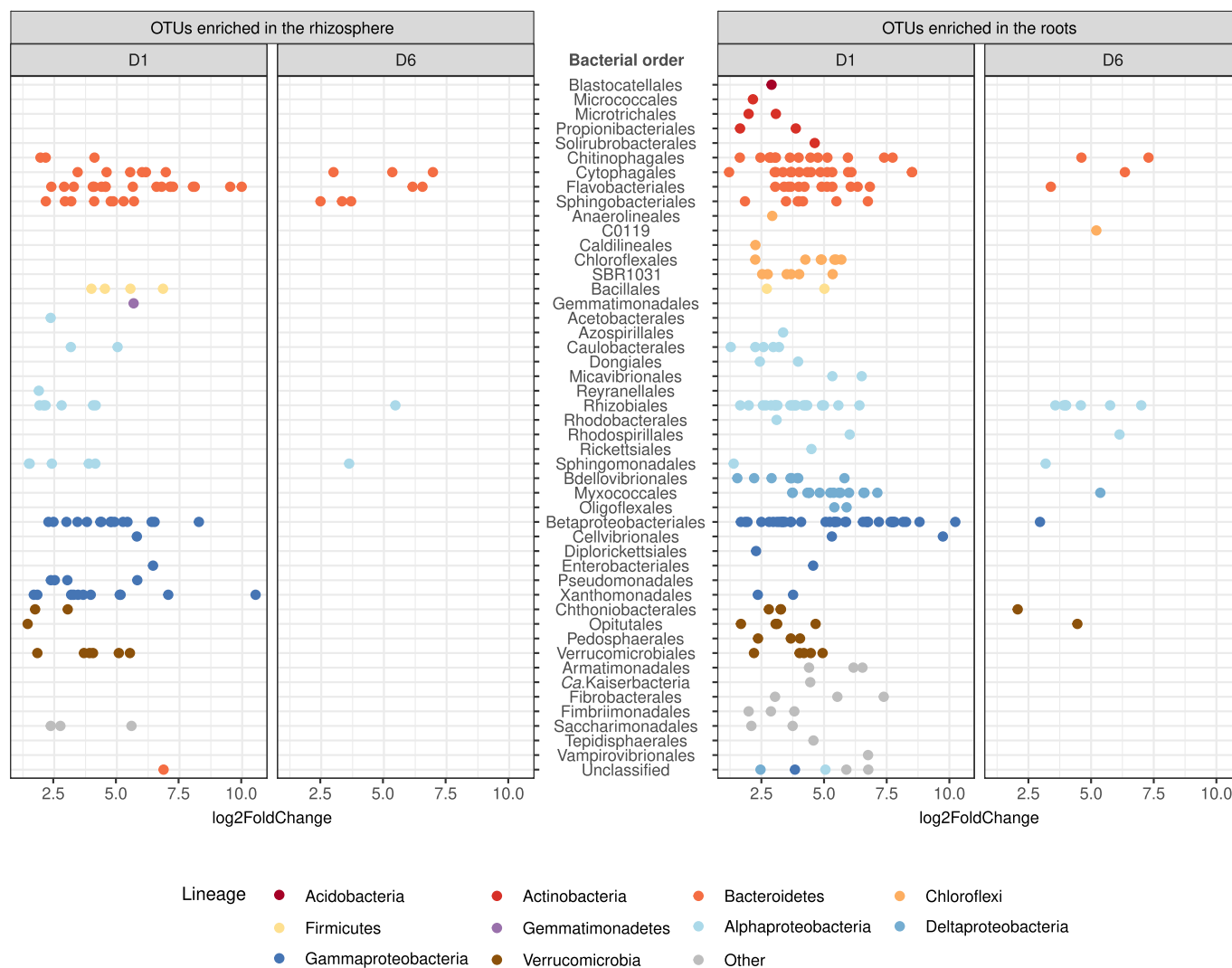
## 3. Results

### 3.1. Characterization of the initial bulk soil bacterial communities before sowing (T42)

The bacterial communities reached similar abundances in both dilution treatments, as determined by 16S rRNA copy numbers, at the end of the five-week incubation period prior to sowing (T42; Table S3) and were characterized by different levels of  $\alpha$ -diversity (Table 1). We observed the same pattern at the  $\beta$ -diversity level, with PC1 clearly separating D1 ( $10^{-1}$ ) from D6 (light orange and light blue squares, respectively; Fig. 1). Dissimilarities were attributed to differences across most high-rank taxa, but particularly Acidobacteria, Bacteroidetes and Gemmatimonadetes (Fig. 2). In addition, Venn diagrams revealed that each dilution level contained a set of unique OTUs (D1:  $53 \pm 14\%$  and D6:  $16 \pm 4\%$ ) (Fig. 3A). Archaea represented a minor fraction ( $< 1\%$  of the reads across all compartments) of the communities at both dilution levels and were therefore not considered further.

### 3.2. Bacterial community diversity and composition after 21 days (T63) with or without barley growth

In the bulk soil, little or no changes in the  $\alpha$ - and  $\beta$ -diversity of the bacterial communities were observed within the same dilution treatment between T42 and T63 days (Table 1 and Fig. 1), although the relative abundance of some phyla shifted slightly (e.g. Actinobacteria and Gammaproteobacteria in D1 and Alphaproteobacteria in D6) (Fig. 2). However, a gradient in evenness had established in both the planted and unplanted bulk soil with decreasing evenness from D1 to D6 (Table 1). For the bacterial communities in the rhizosphere and root-associated compartments, a similar effect of the dilution treatment on the  $\alpha$ - and  $\beta$ -diversity as in the bulk soil was observed (Table 1 and Fig. 1). Thus, the dilution level discriminated the bacterial communities along PC1, whereas the different compartments were separated along PC2 (Fig. 1). A two-way PERMANOVA analysis conducted on samples collected in planted pots confirmed this pattern, with a stronger effect of dilution ( $R^2 = 0.31$ ,  $P < 0.001$ ) than compartment ( $R^2 = 0.15$ ,  $P < 0.001$ ). However, in terms of  $\alpha$ -diversity, the overall trend was that diversity was lower in the two plant-associated compartments compared to the bulk soil, except for evenness in D6 where not statistical difference



**Fig. 4.** The log<sub>2</sub> fold change of the OTUs with a significantly different abundance in the rhizosphere (left panel) and root-associated (right panel) communities compared to the bulk soil, for each dilution level. Only OTUs absent in the control pots were included in the analysis.

was detected (Table S4). Venn diagrams showed that although a proportion of OTUs were shared between each dilution treatment and the control (25–39% and 55–72% in D1 and D6, respectively), a number of OTUs were also shared by, or exclusive to, the two dilution levels (Fig. 3B–D). The DESeq2 analyses indicated that the number of OTUs enriched in rhizosphere or root-associated compartments strongly decreased with the dilution level (Fig. 4). Most of them were enriched in both compartments and belonged to the Rhizobiales, Betaproteobacteriales and three orders of Bacteroidetes. Overall, the bacterial orders enriched in D6 were also enriched in D1.

### 3.3. Nitrogen-cycling microbial communities before and after barley growth

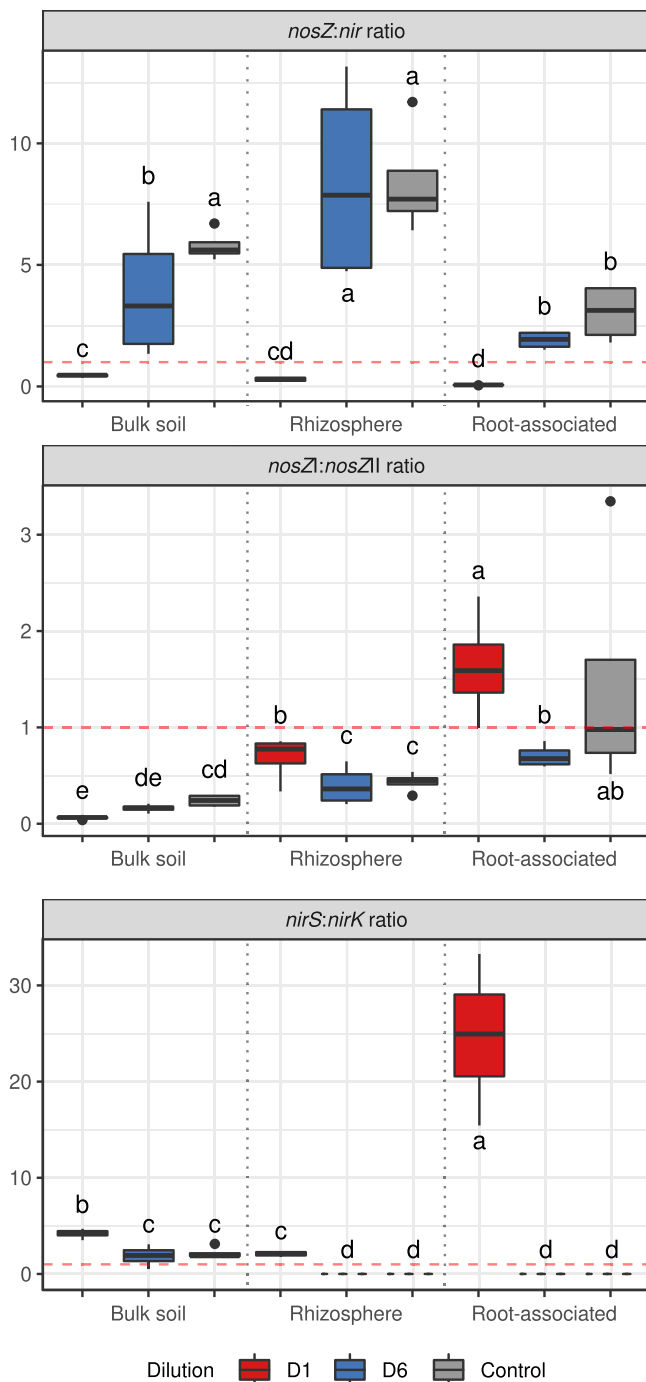
In the bulk soil communities prior to sowing barley (T42), ammonia-oxidizing bacteria (AOB) were more abundant in D1 than in D6, whereas ammonia-oxidizing archaea (AOA) were equally scarce in both dilution treatments (Table S3). Denitrifiers (*nirK* and *nirS*) were more abundant in D1 than in D6, while the opposite was observed for the nitrous oxide reducers (*nosZI* and *nosZII*).

At T63, the abundances of the different N-cycling communities were significantly correlated with the two main PC ( $p < 0.001$ ), the direction and length of the fitted vectors indicating that specific N transformations

were affected both by dilution level and compartment (Fig. 1). Similar to what was observed in the bulk soil at T42, *nir* (*nirK* + *nirS*) and *nosZ* (*nosZI* + *nosZII*) genes tended to be more abundant in D1 and D6, respectively. (Fig. 1 and Table S3). This resulted in higher *nosZ/nir* ratios in D6, independent of compartment (Fig. 5). Several N-cycling genes also showed distinct abundance patterns in the presence of plants. For example, the abundance of *nosZI* was higher in the rhizosphere than in the root-associated and the bulk soil compartments whatever the dilution level (Table S3). Compared to the bulk soil, a significantly lower abundance of AOA and AOB was also observed in both rhizosphere and root-associated compartments and in the root-associated communities, respectively (Fig. 1 and Table S3). In D6, *nirS*-denitrifiers were only detected in the bulk soil whereas in D1 the *nirS/nirK* ratio was lowest in the rhizosphere and highest in the root (Fig. 5). Overall, the *nosZI/nosZII* ratio consistently increased from bulk soil to root compartments.

### 3.4. Plant traits indicating plant performance

Leaf greenness was the only measured plant trait affected by the dilution level, with a significantly higher greenness in D6 compared to D1 (Table 2). Further, leaf greenness values were negatively correlated to the abundance of AOB in the rhizosphere ( $R^2 = 0.48$ ,  $p < 0.001$ ; Fig. S2). None of the other plant traits showed any significant effect of



**Fig. 5.** Abundance ratios of functional genes in the planted pots at the end of the experiment (T63) in bulk soil, rhizosphere and root compartments, for each dilution level (D1, D6) and the control. The *nosZ/nir* ratios were calculated as the sum of *nosZI* and *nosZII* divided by the sum of *nirS* and *nirK*. Different letters indicate significant differences between treatments across the three compartments. The horizontal red line indicates a ratio of 1:1. Black dashed lines indicate a ratio of 0 (due to *nirS* being below detection limit; Table S3).

the dilution treatment.

#### 4. Discussion

In agreement with the experimental design, the removal-by-dilution approach caused a decrease in  $\alpha$ -diversity and alterations in  $\beta$ -diversity of the bulk soil communities at T42. Further, these communities showed

**Table 2**

Effect of dilution on plant traits (mean  $\pm$  s.d.,  $n = 8$ ). Significant differences within dilution treatments are indicated with letters.

	D1 <sup>#</sup>	D6	Control
Leaf greenness <sup>†</sup>	43.57 $\pm$ 1.91 <sup>b</sup>	48.69 $\pm$ 2.10 <sup>a</sup>	48.98 $\pm$ 1.86 <sup>a</sup>
Specific leaf area <sup>‡</sup> (cm <sup>2</sup> g <sup>-1</sup> )	333.94 $\pm$	279.16 $\pm$	301.29 $\pm$
Total root length <sup>‡</sup> (cm)	51.25 <sup>a</sup>	39.74 <sup>a</sup>	21.90 <sup>a</sup>
Specific root length <sup>‡</sup> (m g <sup>-1</sup> )	158.94 $\pm$	126.36 $\pm$	107.86 $\pm$
	42.68 <sup>a</sup>	55.73 <sup>a</sup>	28.54 <sup>a</sup>
Shoot biomass <sup>§</sup> (g)	52.81 $\pm$ 8.53 <sup>a</sup>	45.56 $\pm$ 12.93 <sup>a</sup>	41.98 $\pm$ 13.25 <sup>a</sup>
Root biomass <sup>§</sup> (g)	0.26 $\pm$ 0.03 <sup>a</sup>	0.24 $\pm$ 0.09 <sup>a</sup>	0.26 $\pm$ 0.04 <sup>a</sup>
	0.03 $\pm$ 0.01 <sup>a</sup>	0.03 $\pm$ 0.01 <sup>a</sup>	0.03 $\pm$ 0.01 <sup>a</sup>

<sup>†</sup> ANOVA,  $F$ -ratio = 19.24,  $p(F) < 0.001$ .

<sup>‡</sup> ANOVA,  $p(F) > 0.05$ .

<sup>§</sup> Kruskal-Wallis test,  $p > 0.05$ .

<sup>#</sup> For greenness, specific leaf area and shoot biomass, the missing value was replaced by the mean value for the corresponding dilution level.

minimal changes between the two sampling dates, which suggests that they had reached a stable state whatever the dilution level. However, the initial microbial biomass loss prior to full recolonization and decreased biodiversity in our highest dilution, D6, might have favored the invasion and establishment of airborne species (van Elsas et al., 2012), causing the partial similarities observed between D6 and the control. Still, the control and D6 communities showed little or no overlap in the ordination and we identified a set of OTUs enriched in the rhizosphere and root-associated compartments in D6 after removal of those also present in the control pots. This indicates that our approach allowed us to investigate how reduction in microbial diversity affects the selection of microorganisms by plants as well as the associated consequences on nitrogen cycling guilds in the microbial community and plant performance.

According to our first hypothesis, the results indicate that a reduction in diversity in the bulk soil, considered as the soil microbial seed bank, constrained plant microbiome selection. The number of OTUs enriched in the rhizosphere and root-associated compartments compared to the bulk soil strongly decreased between the low and high dilution treatments. This could partly be due to the fact that many of the barley-enriched OTUs in D1 were not detected anymore in the bulk soil at the higher dilution and therefore the plants' possibility to recruit specific microorganisms was reduced. Within the same dilution level, the separation between the compartments (bulk, rhizosphere and root-associated) confirms that plants are shaping bacterial communities as previously reported (Berg and Smalla, 2009; Ofek-Lalzar et al., 2014; Zhalnina et al., 2018) and indicates that acquisition of root-associated microbiomes from soil is rapid as effects were detected within three weeks from germination. This aligns with work with time series showing assembly of a root-associated microbiome within the first day after germination and approaching steady state within two weeks (Edwards et al., 2015). Previous studies also showed that root exudate levels are likely to be substantial in barley (Giles et al., 2017; Suku et al., 2014) during the first 24 days, suggesting a large impact on the root microbiome during this period. In agreement, the DESeq2 analyses showed that many of the taxa enriched in the barley compartments are known to have the capacity to utilize a range of carbon compounds exuded by the roots (Philippot et al., 2013a). Overall, it was mainly an enrichment of Proteobacteria and Bacteroidetes in the rhizosphere and root-associated communities, which was consistent with previous work with cereals including barley (Bulgarelli et al., 2015; Edwards et al., 2015; Peiffer et al., 2013; Turner et al., 2013). Some of them are also known to contribute to protect plants against soil-borne pathogens (Mendes et al., 2011). The observed enrichment in the rhizosphere and root environments supports the existence of a strong link between microbial functional traits and the assembly of plant-associated communities (Yan et al., 2017).



The targeted N-cycling microbial communities causing N loss from soils were equally or more abundant in D1 than D6 after the dilution-recolonization processes except the nitrous oxide reducers. This counter-intuitive increase of nitrous oxide reducers could be explained by the loss of microbial taxa by dilution that affect the fitness of the remaining ones during soil recolonization with, for example, poor competitors being favored by the loss of the strong ones. This mechanism was recently experimentally demonstrated by Romdhane et al. (2021). It is also supported by the increase in Bacteroidetes in D6 compared to D1. Within this phylum, microorganisms harboring a *nosZ* gene (*nosZII*) without possessing any *nir* gene are overrepresented (Graf et al., 2014). Thus, the loss of microbial biodiversity led to an increase in nitrous oxide reducers and also of the nitrous oxide reducers in relation to denitrifiers ( $(nosZI + nosZII)/(nirK + nirS)$ ) which, if verified at large scale, can have consequences for climate regulation. Indeed, nitrous oxide reducers have the capacity to reduce the greenhouse gas  $N_2O$  to harmless dinitrogen gas and therefore play a key role in the soil  $N_2O$ -sink capacity (Hallin et al., 2018; Jones et al., 2014). The significant increase of *nosZI*-bacteria in the rhizosphere compared to the bulk soil is consistent with recent results showing a relatively higher abundance of *nosZI* in the rhizosphere of barley, sunflower, wheat and birch trees (Ai et al., 2020; Graf et al., 2016; Truu et al., 2017) and aligns with the root environment being a “hotspot” for denitrification (Moreau et al., 2019; Philippot et al., 2009). In the present study, we observed a tendency for contrasting patterns for *nirS* and *nirK* type denitrifiers, with *nirS* below detection in both barley compartments in D6 whereas *nirK* abundances remained comparable. This is in agreement with the observed enrichment of Rhizobiales, of which many are denitrifiers carrying *nirK* (Graf et al., 2014). Significant differences in the *nirS/nirK* ratio between the three compartments in D1 could reflect distinct niche preferences of denitrifiers carrying either of these genes (Enwall et al., 2010; Jones and Hallin, 2010; Wittorf et al., 2016).

The abundances of ammonia-oxidizing communities decreased with dilution and in the barley compartments, especially in the root-associated compartment. The AOA were even below the detection limit in the rhizosphere and root-associated compartments in both dilution treatments and the AOB in the latter in D6. It has been evidenced that plants could engage not only in exploitative competition with ammonia-oxidizers for ammonium, but also in interference competition by exuding secondary compounds that specifically inhibit ammonia-oxidizers, with both processes leading to lower nitrification rates and  $N_2O$  emissions (Cantarel et al., 2015; Subbarao et al., 2009; Thion et al., 2016). This decrease in ammonia-oxidizers in the rhizosphere and root-associated compartments should therefore result in an increased N-availability for the plant, similar to the effect of nitrification inhibitors (Subbarao et al., 2015). Accordingly, a higher leaf greenness - an indicator for the crop N nutrition status - was observed in D6, which exhibited the lowest abundances of ammonia oxidizers. Recent work addressing the relationships between microbial community structure and plant productivity support our findings. For example, experimental manipulation of microbial communities has been shown to affect various plant traits such as above-ground biomass, chlorophyll content, flowering time and number of flowers (Panke-Buisse et al., 2015; Wagner et al., 2014). There are several putative mechanisms by which rhizosphere microbes can impact plant performance (Vandenkoornhuyse et al., 2015). In our study, it is however not possible to distinguish the mechanistic forces underlining the observed shifts in plant performance beyond the importance of bacterial diversity and the abundance of microorganisms involved in N availability.

## 5. Conclusions

Altogether, our results showed that the reduction of microbial diversity in the bulk soil constrained plant microbiome selection, and only Alphaproteobacteria and Bacteroidetes were enriched in the barley compartments irrespective of dilution level. This constrained

recruitment was associated with changes in functional traits within the microbial community. The increased abundance of nitrous oxide reducers in rhizosphere and root-associated compartments in the low diversity soils can have consequences for the soil  $N_2O$  sink capacity. By contrast, the bacterial ammonia oxidizer abundances decreased in the plant-associated compartments in the low diversity soils, which could have led to changes in the plant nutritional status, as reflected by the higher greenness. The characterization of the mechanisms behind these changes, as well as their impact over the plants' life cycle, was however beyond the scope of this work. Future research should also be undertaken to provide a more thorough understanding of how the interplay between N-cycling microorganisms and plants is modulated by the diversity of the soil microbial seed bank and its implications for ecosystem functioning.

## Data availability

The raw sequence dataset is available under BioProject accession number PRJNA637634. The newick tree, qPCR data and scripts used to conduct this study are available at Zenodo (<https://doi.org/10.5281/zenodo.5336204>).

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.apsoil.2021.104224>.

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