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ORIGINAL PAPER

Compositional and abundance changes of nitrogen-cycling genes in plant-root microbiomes along a salt marsh chronosequence

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Abstract Disentangling the relative influences of soil properties and plant-host on root-associated microbiomes in natural systems is challenging, given that spatially segregated soil types display distinct historical legacies. In addition, distant locations may also lead to biogeographical patterns of microbial communities. Here, we used an undisturbed salt marsh chronosequence spanning over a century of ecosystem development to investigate changes in the community composition and abundance of a set of nitrogen-cycling genes. Specifically, we targeted genes of diazotrophs and ammonia oxidizers associated with the bulk and rhizosphere soil of the plant species *Limonium vulgare*. Samples were collected across five distinct successional stages of the chronosequence (ranging from 5 to 105 years) at two time-points. Our results indicate that soil variables such as sand:silt:clay % content and pH strongly relates to the abundance of N-cycling genes in the bulk soil. However, in the rhizosphere samples, the abundance of ammonia-oxidizing organisms (both bacteria and archaea, AOB and AOA, respectively) was relatively constant across most of the successional stages, albeit displaying seasonal variation. This result indicates a potentially stronger control of plant host (rather than soil) on the abundance of these organisms. Interestingly, the plant host did not have a significant effect on the composition of AOA and AOB communities, being mostly divergent according to soil successional stages. The abundance of diazotrophic communities in rhizosphere samples was more affected by seasonality than those of bulk soil. Moreover, the abundance pattern of diazotrophs in the rhizosphere related to the systematic increase of plant biomass and soil organic matter along the successional gradient. These results suggest a potential season-dependent regulation of diazotrophs exerted by the plant host. Overall, this study contributes to a better understanding of how the natural formation of a soil and host plants influence the compositional and abundance changes of nitrogen-cycling genes in bulk and rhizosphere soil microhabitats.

Keywords *nifH* · *amo*A · Rhizosphere · Ecological succession · Salt marsh · *Limonium vulgare*

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Introduction

Nitrogen (N) is an essential nutrient for all plants, and therefore N-cycling processes have been investigated in agricultural soils (Wartiainen et al. 2008; Hayden et al. 2010), forest soils (Levy-Booth and Winder 2010), arctic tundra (Walker et al. 2008), mangrove sediments (Romero et al. 2012) and salt marsh rhizosphere and bulk soils (Chaudhary et al. 2015). Microbial communities are responsible for key N transformations such as N_2-fixation, nitrification and denitrification. The capacity of nitrogen fixation, performed by either free-living or symbiotic diazotrophs, is widespread across distinct bacteria and archaea taxa and it contributes significantly to N increments in natural and agricultural systems (Galloway et al. 1995; Karl et al. 2002; Zhang et al. 2006; Roesch et al. 2008; Reardon et al. 2014). These diazotrophic communities are often studied by targeting the *nifH* gene that encodes the nitrogenase iron protein (Ueda et al. 1995; Lovell et al. 2000; Poly et al. 2001; Hsu and Buckley 2009; Hayden et al. 2010). Likewise, ammonia oxidation to nitrite, a rate-limiting step in soil nitrification, constitutes an important step within the N-cycle. This step is performed by organisms known as ammonia-oxidizing bacteria (AOB) and archaea (AOA) (Leininger et al. 2006; Nicol and Schleper 2006; Jackson et al. 2008; Prosser and Nicol 2008).

N-cycling associated microbial communities are often modulated by soil type and physicochemical properties given that (1) organisms are regulated by the concentration of nitrogen forms, and that (2) soil characteristics (e.g., pH, texture, organic matter) influence enzyme activities and therefore the overall community functioning. For instance, diazotrophic community activity, size and structure in soils have been related to distinct factors such as microbial biomass (Hayden et al. 2010), seasonal variation (Mergel et al. 2001; Pereira e Silva et al. 2011), and soil physicochemical properties (i.e., water content, texture, pH, carbon (C) and N quantity and availability) (Hsu and Buckley 2009; Hayden et al. 2010; Wakelin et al. 2010; Pereira e Silva et al. 2011). Similarly, the community structure and abundance of ammonia oxidizers are also influenced by soil factors such as temperature, CO_2 concentration, N fertilization, ammonium and salinity levels, and soil pH (Horz et al. 2004; Nicol et al. 2008; Tourna et al. 2008; Erguder et al. 2009; Prosser and Nicol 2012).

Plant species and phenology are also likely to influence the size and structure of diazotrophs and ammonia oxidizers in soils (Engelhard et al. 2000; Tan et al. 2003; Knauth et al. 2005; Roesch et al. 2006; Fan et al. 2011; Dias et al. 2012; Lauber et al. 2013). For instance, nitrogen fixers were shown to rapidly respond to artificial root exudates in a soil microcosm experiment, which led to higher activity in the rhizosphere than bulk soils (Bürgmann et al. 2005). In the case of ammonia oxidizers, it has been shown that the community structure of AOB and AOA vary according to the plant cultivars, which likely result from differential root exudation (Dias et al. 2012). Given the potential role of root exudation and the pool of C released in the rhizosphere, an important but unanswered question is whether plants exert a stronger effect on the selection on N-cycling associated microbial communities than soil physicochemical properties. Several studies have attempted to address this issue (Wartiainen et al. 2008; Cibichakravarthy et al. 2012; Li et al. 2014; Chaudhary et al. 2015), thus providing support for the predominant role of soil type over plant host in structuring these communities (Tan et al. 2003; Hai et al. 2009; Glaser et al. 2010; Mao et al. 2013). However, these conclusions may also be influenced by the fact that distinct soil types are spatially separated, likely reflecting in distinct soil properties, historical legacies and biogeographical patterns of soil microbiomes (Marschner et al. 2001; Garbeva et al. 2004; Salles et al. 2006; Lauber et al. 2008).

Here, we used an undisturbed salt marsh chronosequence—varying in soil texture, pH, organic matter, N content and salinity—to investigate the compositional and abundance changes of nitrogen-cycling genes in bulk soil and plant-root microbiomes. We selected the species *Limonium vulgare*, a typical perennial salt marsh plant, as the model species since it is broadly distributed across the distinct stages of soil formation in this chronosequence. We hypothesized that plants have a stronger influence on N-cycling communities than soil variables. Thus, whereas bulk soil communities will follow the consistent increment of fertility and changes in soil texture, pH and salinity, we hypothesized that the rhizosphere communities will be relatively stable along the soil gradient. Specifically, we made three predictions: First, the abundance of

N-cycling genes in the bulk but not rhizosphere soil samples are expected to consistently increase toward late successional stages, mirroring the progressive increments in plant biomass and soil nutritional status. Second, we expect N-cycling gene abundances to be higher in rhizosphere than in bulk soil samples across the successional stages, as nutrient availability is expected to be higher in the rhizosphere—the so-called 'rhizosphere effect' (Sorensen and Sessitsch 2007; Dotaniya and Meena 2015, Wang et al. 2016). Third, the structure of the N-cycling microbial communities is expected to be different between the rhizosphere and bulk soil samples, the latter varying according to three distinct successional phases—that is, initial, middle and late soil stages—following the soil and vegetation development along this salt marsh chronosequence (Schrama et al. 2012).

Materials and methods

Study site and sample collection

Sampling was conducted at two time points (May and August) in 2014. Samples were collected at five soil successional stages in the salt marsh. This chronosequence encompasses over 100 years of primary succession and it is located at the island of Schiermonnikoog (53°30′N, 6°10′E), The Netherlands [for detailed information on sampling, see Wang et al. (2016)]. Previous work indicates that the sedimentation caused by the tidal regime led to modifications on the soil physicochemical conditions along the primary succession (Dini-Andreote et al. 2014). Importantly, the salinity level increases as the soil ages, due to an accumulative effect (Dini-Andreote et al. 2014; Wang et al. 2016). The subsequent development of the vegetation, which peaks in terms of biomass at the intermediate to late stage, leads to an increase in soil organic matter (Schrama et al. 2012; Dini-Andreote et al. 2014; Wang et al. 2016). In brief, along the course of the chronosequence, plant biomass increases from < 200 g/m^2 (at initial soil stages) to up to ca. 600 g/m^2 (at the intermediate and later soil stages; Schrama et al. 2012). In the current study, soil and rhizosphere samples were collected at successional stages with ages of 5, 15, 35, 65 and 105 years. For a description of the establishment of the plots and details on the chronosequence calibration, see Olff et al. (1997), Dini-Andreote et al. (2014), and Wang et al. (2016). Briefly, triplicate plots were established at the same base of elevation [vertical position relative to mean sea level at the initial elevation gradient on the bare sand flats with a base elevation of 1.16 m ± 2.2 cm (mean ± SE) above Dutch Ordinance Level]. Within each plot, four healthy-looking *L. vulgare* of similar sizes with attached soil adhering to the intact roots were collected and processed together constituting a composite sample per plot. Thirty composite samples in total were collected (5 stages × 3 plots per stage × 2 sampling point). Each sample was placed in a sterile plastic bag, sealed and transported to the laboratory < 24 h. From each composite sample, we collected the respective bulk and rhizosphere soils (see details below).

Pre-treatment of bulk soil and rhizosphere samples

Plant roots were carefully separated from the bulk soil by shaking the plants without damaging the integrity of the root system. The collected bulk soil samples were sieved using a 2 mm mesh sieve and stored at − 20 °C for DNA extraction, and at 4 °C for soil physicochemical measurements. Rhizosphere samples were collected by weighing ten grams of roots containing the tightly adhering soil particles (about 3 grams of rhizosphere soil). The collected root samples were transferred into 180 mL of sterile sodium pyrophosphate (0.1%) and shaken for 30 min at 200 rpm. A total of 0.5 mL of the suspension with rhizosphere soil was used as starting material for DNA isolation.

Measurement of soil chemical variables

Soil samples were subjected to the measurements of pH, soil water content (SWC), soil organic matter (SOM), nitrate (N-NO_3^-), ammonium (N-NH_4^+), soil exchangeable elements (Na, Mg, Ca, and K), phosphate (P), total carbon (TC) and total nitrogen (TN). Soil chemical analyses were carried out in collaboration with the department of community and conservation ecology at the University of Groningen (see Wang et al. 2016 for a detailed description of the methods). Soil sand:silt:clay % content were obtained from a previous study carried out in the same sites (see Dini-Andreote et al. 2014). Detailed information on

the soil physicochemical variables is provided in Table S1.

Total soil DNA extraction

Bulk soil and rhizosphere samples were subjected to DNA isolation using 0.5 g of soil and 0.5 mL of the suspension with rhizosphere soil as starting materials, respectively. Total DNAs were isolated using the MoBio PowerSoil DNA isolation kit (MoBio Laboratories, Carlsbad, CA, USA). Samples were processed following the manufacturer's instructions, except for the addition of glass beads (diameter 0.1 mm, 0.25 g) to the MicroBead tube and three cycles of bead beating (mini-bead beater, BioSpec Products, USA) for 60 s. The DNA samples were quantified using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies). Samples were standardized to the equal concentration of 5 ng μL^{-1} for further analyses.

Quantitative PCR (qPCR)

Quantitative PCR (qPCR) assays were used to quantify the abundance of the *nifH* gene, the archaeal and bacterial *amoA* gene, and the archaeal and bacterial 16S rRNA gene. For the *nifH* gene, the primer set FPGH19 (Simonet et al. 1991) and PolR (Poly et al. 2001) was used according to Pereira e Silva et al. (2011). The bacterial 16S rRNA gene was quantified using the primer set of 16SFP/16SRP (Bach et al. 2002). To quantify the abundance of the *amoA* gene for AOA and AOB, we followed the methodology previously described by Petersen et al. (2012). Briefly, for the AOA *amoA* gene, the primer set Arch-AmoAF and Arch-AmoAR was used (as in Francis et al. 2005). The primer set amoA-1F and amoA-2R was used for the AOB *amoA* gene (Rotthauwe et al. 1997). The archaeal 16S rRNA gene was quantified using the primer set Arch344 (Raskin et al. 1994) and Arch915 (Stahl and Amann 1991). Detailed information on thermo cycling conditions and primer sequences is provided in Table S2.

The standard curves for each gene were produced using tenfold dilution series of plasmids (10^2–10^7 gene copy numbers μL^{-1}) containing the respective cloned genes (Table S2). The efficiency was calculated using the formula Eff = $[10^{(1/\text{slope})} - 1]$. Efficiency values of the standard curves for the *nifH* gene, the archaeal and bacterial *amoA* gene, and the archaeal and bacterial 16S rRNA gene were 97.6, 93.4, 99.5, 96.1 and 110.3%, respectively. Quantitative PCR assays were carried out using triplicate bulk soil and rhizosphere samples on an ABI Prism 7300 Cycler (Applied Biosystems, Germany). Possible inhibitory effects were checked by mixing rhizosphere and bulk soil DNA samples with a range of known concentrations of the plasmid. No apparent inhibition was observed for any of the quantified genes. The specificity of the amplification products was confirmed by melting-curve analyses, and the expected sizes of the amplified fragments were checked using 1.0% agarose gel stained with ethidium bromide. The relative abundances of functional genes are shown as the ratio of the abundances of each N-cycling gene to the respective organismal abundance (either bacteria or archaea).

PCR-DGGE analysis

Fragment amplifications for the DGGE analysis were performed for the *nifH* gene, archaeal *amoA* gene, and the bacterial 16S rRNA gene specifically targeting AOB. PCRs of the *nifH* gene were performed using a nested protocol according to Diallo et al. (2004). The primer set used in the first reaction was FPGH19 (Simonet et al. 1991) and PolR (Poly et al. 2001), followed by a reaction with the primers PolF containing a GC clamp and AQER (Poly et al. 2001). The archaeal *amoA* gene was initially amplified using the Arch-amoAF and Arch-amoAR primer pair (Francis et al. 2005), followed by the second amplification with the primer set Arch-amoAF-GC and Arch-amoAR. The 16S rRNA gene of AOB was initially amplified with the primer set CTO189fAB, CTO189fC and CTO654r (Mobarry et al. 1996), followed by a second reaction using the primer set 341F-GC and 518R (López-Gutiérrez et al. 2004). Additional information on thermo cycling conditions is provided in Table S3.

The DGGE gels were run using the Ingeny Phor-U apparatus (Ingeny International, The Netherlands). PCR products (i.e. 300 ng per lane) were loaded onto 6% (w/v) polyacrylamide gels, with denaturing gradients of 40–65, 30–50 and 40–60% (100% denaturant corresponded to 7 M urea and 40% (v/v) deionized formamide) in 0.5 × TAE buffer, for *nifH* gene, the archaeal *amoA* gene, and the 16S rRNA gene of AOB, respectively. Electrophoresis was performed at a constant voltage of 100 V at 60 °C for 16 h. The gels were stained with SYBR Gold (Molecular Probes,

Netherlands) in 0.5 × TAE buffer at room temperature for 60 min. Images of the gels were visualized and documented under UV light with Image Master VDS system (Amersham Biosciences, United Kingdom).

Data analysis

Significant differences in the genes abundance across sample types (i.e., bulk soil and rhizosphere), sampling time (i.e., May and August), and across distinct soil successional stages were identified using one-way analysis of variance (ANOVA), followed by Tukey HSD pairwise group comparisons. These analyses were carried out in R (http://www.r-project.org). To test for significant correlations between soil variables and the N-cycling gene abundances, we performed Spearman's rank-based correlational analysis (function *rcorr*) using the 'Hmisc' package (Harrell and Dupont 2017). The obtained results were visualized using the package 'corrplot' (Friendly 2002).

Digital images of the DGGE gels were analyzed using the software GelCompar (Applied Maths, Belgium). Each DNA band was located and quantified in relation to the marker on each gel. Bray–Curtis distance matrices were used to infer on the community dissimilarities based on the DGGE-band profiles. We applied permutational multivariate analysis of variance (PerMANOVA) (Anderson 2001), using the 'vegan' package (Oksanen et al. 2007), to test for significant community dissimilarities between different sample types, sampling time, and across distinct soil successional stages. Constrained analysis of Principal Coordinates (CAP) was applied on the Bray–Curtis distance matrix by constraining the influence of sample type and successional stages. This method investigates the results of a Principal Coordinates Analysis (function *cmdscale*) with linear discriminant analysis (lda), resulting in the best prediction of group identities of the sites (Anderson and Willis 2003). The significance of the differences observed across soil successional stages was tested using the function *pairwise.perm.manova* within the RVAideMemoire package. Only the stages showing significant differences among each other were separated by clusters. Different clusters separated by sample types and soil successional stages were visualized using the function *ordihull* in the package 'vegan'.

Results

Variation of N-cycling gene abundances (*nifH*, *amoA*-AOB and *amoA*-AOA) along soil succession

The distribution of the *nifH* gene abundance followed a gradual increasing pattern in the bulk soil for both sampling times (first sampling, Adj. $R^2 = 0.4451$, $P < 0.05$; second sampling, Adj. $R^2 = 0.6676$, $P < 0.001$), reaching the peak at the 105-year stage (first sampling, $10^{7.96} \pm 10^{0.71}$ gene copies gdw^{-1}; second sampling, $10^{8.79} \pm 10^{0.19}$ gene copies gdw^{-1}) (Fig. 1a). A hump-shaped pattern was observed for the rhizosphere samples in the second sampling (Adj. $R^2 = 0.6563$, $P < 0.001$), reaching the peak at the 65-year stage ($10^{5.31} \pm 10^{0.11}$ gene copies gdw^{-1}), whereas no significant pattern was observed for the first sampling time (peak in abundance at the 15-year stage, $10^{5.80} \pm 10^{0.25}$ gene copies gdw^{-1}).

The abundance of ammonia oxidizers in bulk soil also increased along the succession, peaking at different stages of this chronosequence. Hump-shaped distribution of *amoA*-AOB gene abundance were found consistently for both sampling times (first sampling, Adj. $R^2 = 0.3606$, $P < 0.05$; second sampling, Adj. $R^2 = 0.6320$, $P < 0.001$), however the highest abundance was variable during the two sampling times, peaking at the 35-year stage ($10^{8.34} \pm 10^{0.10}$ gene copies gdw^{-1}) and at the 65-year stage ($10^{8.99} \pm 10^{0.13}$ gene copies gdw^{-1}), for the first and the second sampling, respectively (Fig. 1b). For the *amoA*-AOA gene, however, distinct patterns were observed between two sampling times, i.e. the first sampling time showed a linear increase in abundance along the soil succession (Adj. $R^2 = 0.4708$, $P < 0.01$), which shifted to a hump-shaped distribution later in the season (Adj. $R^2 = 0.4703$, $P < 0.01$), reaching the peak at the 65-year stage in the second sampling ($10^{7.07} \pm 10^{0.12}$ gene copies gdw^{-1}) (Fig. 1c). The community size of ammonia oxidizers followed no specific distribution in the rhizosphere, being relatively constant along most of the successional stages ($P > 0.05$, with a more stable pattern found for *amoA*-AOA), albeit a significant difference in the abundance of *amoA*-AOB was found between the 5- to 15-year stage in the first sampling ($P < 0.05$). It is notable that both of the *amoA*-AOB and *amoA*-AOA gene abundances in the

rhizosphere were influenced by season, peaking in abundance at different stages across the two sampling times.

Assessment of the relative abundances of N-cycling genes (*nifH*, *amoA*-AOB and *amoA*-AOA) in the bulk soil and *L. vulgare* rhizosphere samples

Whereas the absolute quantification of these functional genes revealed an overall higher abundance in the bulk soil than in the rhizosphere samples (Figure S1), the relative numbers (abundance corrected by the number of bacteria and archaea present in the sample) provided a different picture (Fig. 2). Specifically, we observed a significant enrichment of the relative *nifH* gene abundance in the bulk soil samples across the succession in the first sampling (differences ranged from 0.74 to 41.53%, $P < 0.05$), whereas equal distribution was largely observed during the second sampling, except for the 105-year stage (Fig. 2a). In terms of ammonia oxidizers, the rhizosphere enrichment varied between bacterial and

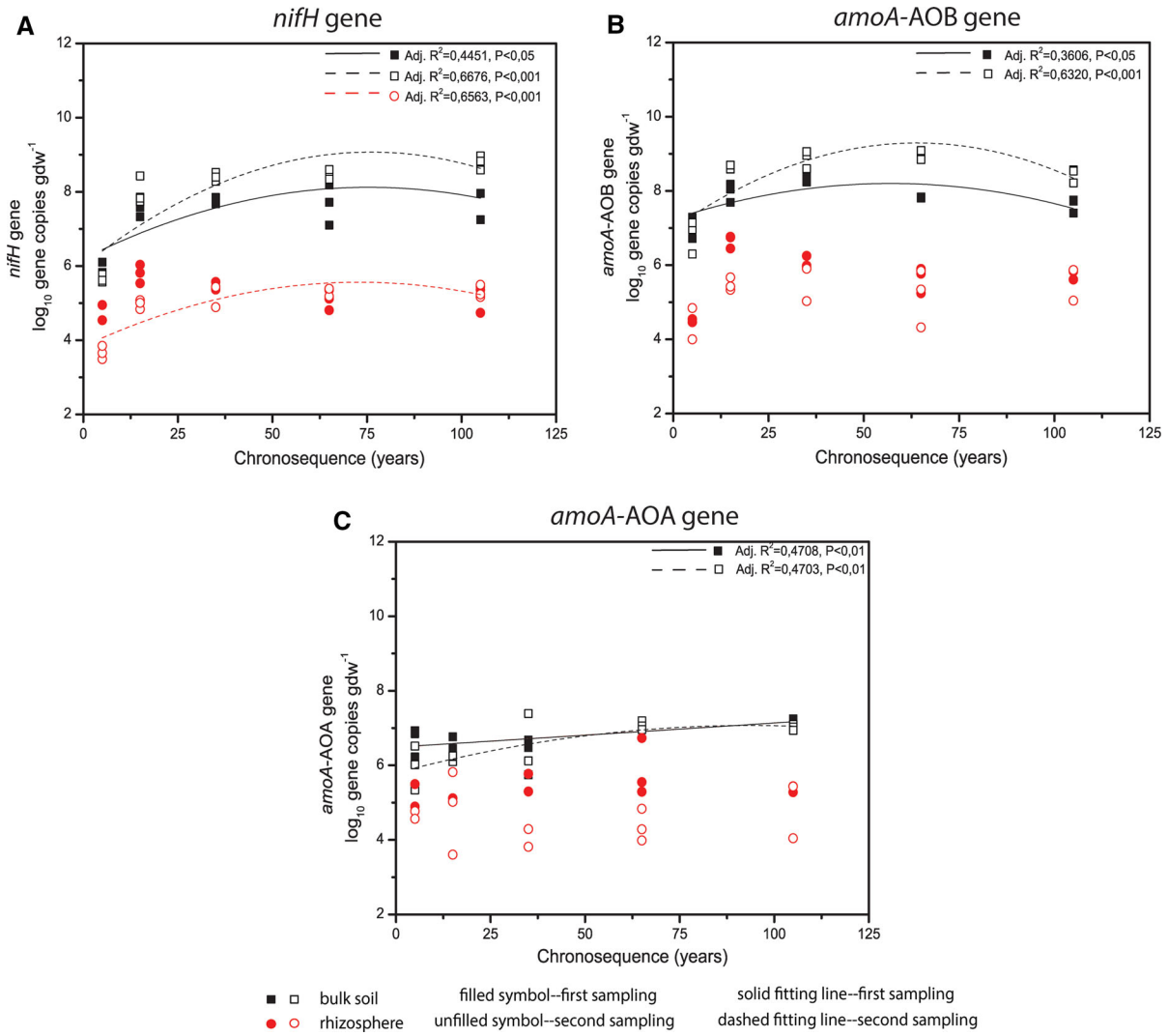


Fig. 1 Variation of the N-cycling gene abundances within rhizosphere and bulk soil samples along the chronosequence **a** *nifH* gene, **b** *amoA*-AOB gene and **c** *amoA*-AOA gene. Black symbols refer to bulk soil samples, and red symbols represent rhizosphere samples. Filled symbols refer to samples from the first sampling time, and open symbols represent samples from the second sampling time. For regression curves, solid and dashed lines represent the first and second sampling time, respectively

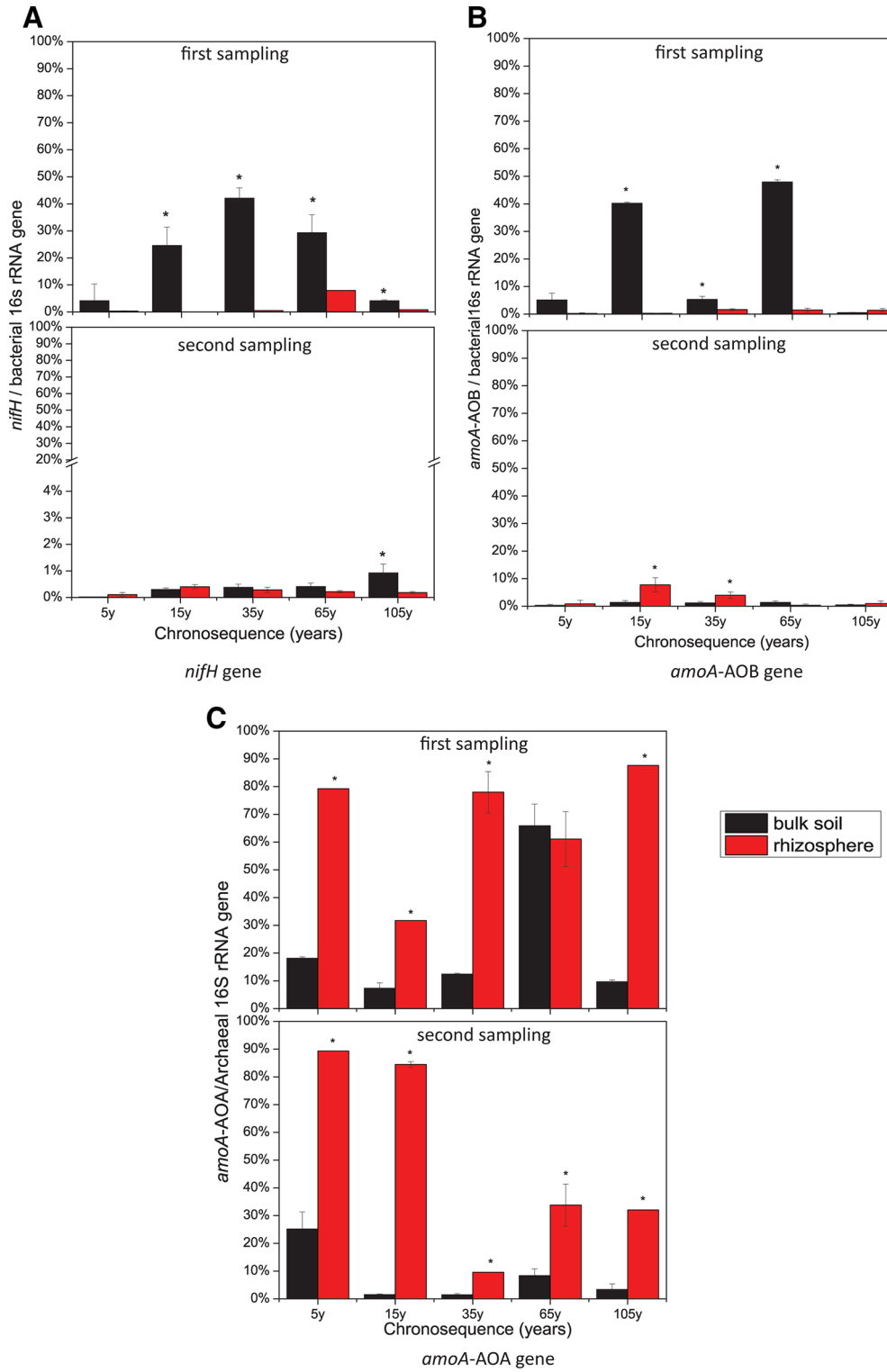


Fig. 2 Comparison of the relative abundances of N-cycling genes between rhizosphere and bulk soil samples **a** *nifH* gene, **b** *amoA*-AOB gene and **c** *amoA*-AOA gene. Black bars refer to bulk soil samples and red bars represent rhizosphere samples

archaeal counterparts. On one hand, significantly higher relative abundances of *amoA*-AOB genes were found in the rhizosphere than in the bulk soil samples at the middle stages in the second sampling (differences ranged from 2.79 to 6.39%, $P < 0.05$) (Fig. 2b). For the first sampling, however, enrichment of the *amoA*-AOB gene abundance was shown in the bulk soil rather than for the corresponding rhizosphere samples (differences ranged from 3.69 to 46.46%, $P < 0.05$), respectively at the 15-, 35- and 65-year stages. On the other hand, the relative *amoA*-AOA gene abundance were significantly higher in the rhizosphere than in the bulk soil samples (differences ranged from 8.10 to 77.92%, $P < 0.05$) during both sampling times, except for the 65-year stage in the first sampling ($P > 0.05$) (Fig. 2c).

Regarding the relative abundances of bacterial and archaeal ammonia oxidizers, the abundance of *amoA*-AOA gene was lower than that of *amoA*-AOB gene within rhizosphere and bulk soil samples for both sampling times, except for the higher abundance of *amoA*-AOA over *amoA*-AOB gene in the rhizosphere at the 5-year stage (AOA/AOB ratio = 2.70) in the first sampling (Figure S1D). For the second sampling, the AOA/AOB ratio was significantly higher in the rhizosphere samples than in the bulk soil at the 15-, 65- and 105-year stages (respectively, differences of 0.30, 0.08 and 0.01, $P < 0.05$).

Relationship between soil variables and N-cycling gene abundances in the bulk soil

In order to identify the potential influence of soil properties on the abundance of N-cycling genes, Spearman's rank correlational analyses were performed including all measured variables (that is, soil physicochemical data) and the quantified N-cycling gene abundances (Fig. 3) for the bulk soil samples. We found the *nifH* gene to be significantly correlated with all measured soil variables ($P < 0.05$), with the exception of nitrate ($P > 0.05$). The soil pH and sand content negatively correlated with the abundance of

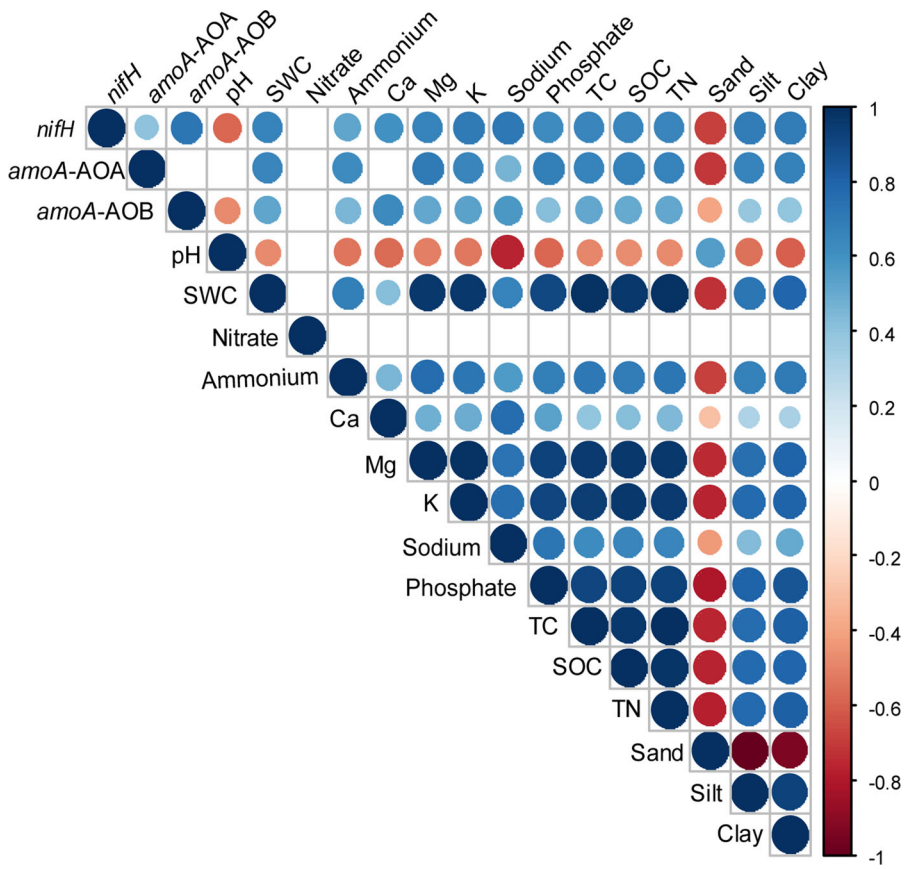


Fig. 3 Spearman correlation analysis of soil variables and the N-cycling gene abundances of bulk soil samples. The color intensity and the size of the circles represent the strength of the correlation. Blue colors refer to positive correlation, and red colors represent negative correlation

nifH, with sand content showing the strongest correlation ($\rho = -0.68$, $P < 0.001$). The other soil variables positively correlated with the abundance of *nifH*, with sodium and potassium having the highest correlation (Na: $\rho = 0.71$, $P < 0.001$; K: $\rho = 0.70$ $P < 0.001$) (Fig. 3). Similar correlations were found for the *amoA*-AOB gene, where pH showed the strongest negative correlation ($\rho = -0.47$, $P < 0.01$) while calcium and sodium showed higher positive correlation compared to other measured

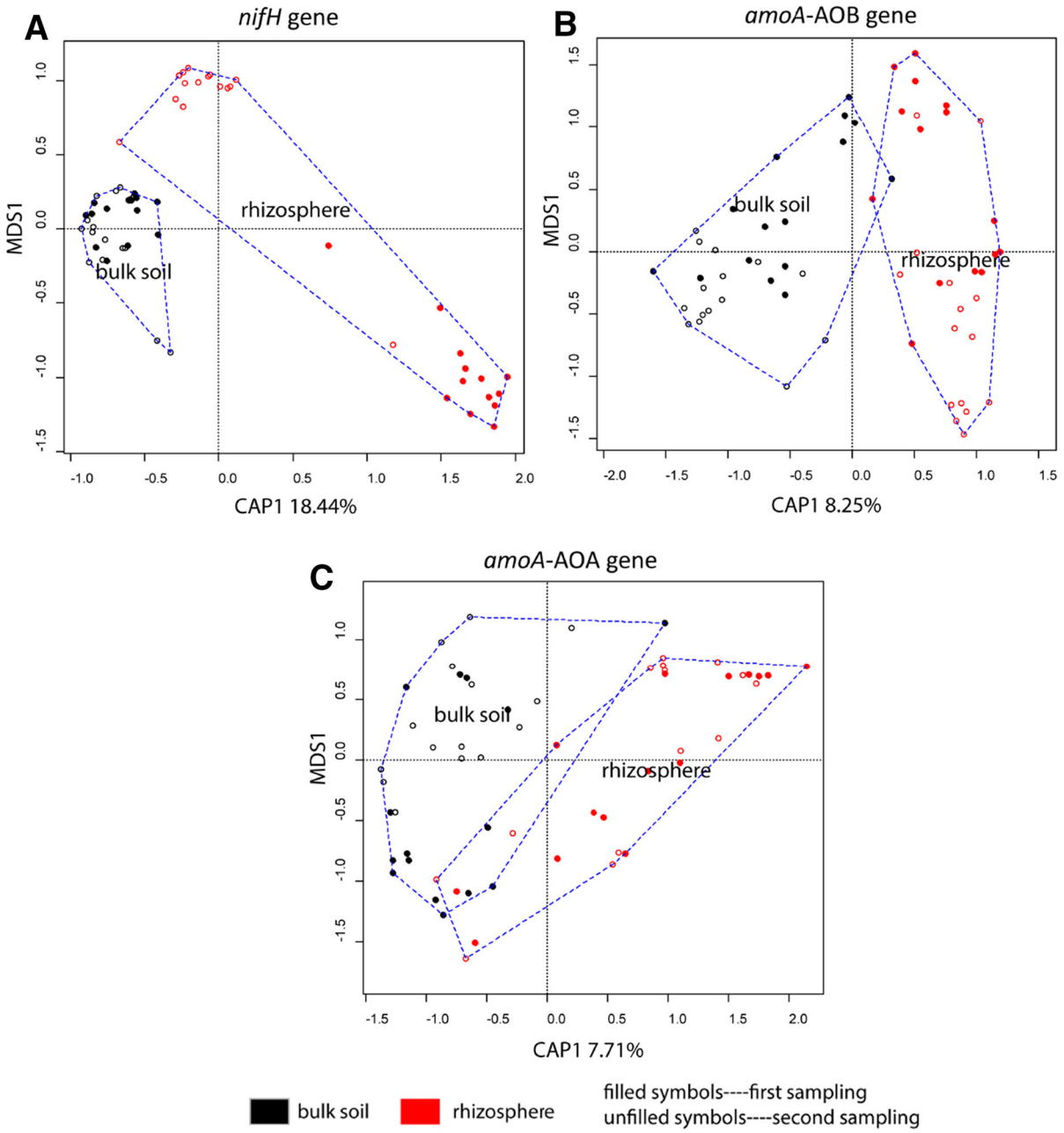


Fig. 4 Differences in the structure of the N-cycling communities between bulk soil and rhizosphere samples collected along the salt march chronosequence **a** *nifH* gene, **b** *amoA*-AOB gene and **c** *amoA*-AOA gene. Black color refers to bulk soil samples, and red color represents rhizosphere samples. Filled symbols refer to samples from the first sampling time, and open symbols represent samples from the second sampling time

variables (Ca: $\rho = 0.64$, $P < 0.001$; Na: $\rho = 0.58$, $P < 0.001$). The abundance of the *amoA*-AOA gene had similar patterns as that of the *amoA*-AOB gene, with the exception of not being significantly correlated with soil pH and calcium content ($P > 0.05$). For additional detail see Fig. 3.

Assessment of the community structure of N-cycling genes (*nifH*, *amoA*-AOB and *amoA*-AOA)

The analyses of the community structure of N-cycling related genes consistently segregated the samples between bulk soil and rhizosphere. We found a significant effect of the sample type (i.e. bulk soil and rhizosphere) on the structures of the *nifH* (Pseudo-$F = 15.55$, $R^2 = 0.290$, $P < 0.01$), *amoA*-AOB (Pseudo-$F = 5.55$, $R^2 = 0.118$, $P < 0.01$) and *amoA*-AOA (Pseudo-$F = 5.37$, $R^2 = 0.086$, $P < 0.01$) genes (Fig. 4).

By analyzing the bulk soil and rhizosphere samples separately, we found that the structure of the diazotrophic community clustered according to the successional phase (that is, initial, middle and late) for bulk soil samples (pairwise comparisons, $P < 0.01$), but similar clustering was not observed for the rhizosphere samples (Fig. 5a). Likewise, the community structure of ammonia oxidizers was significantly influenced by soil successional stages in both bulk soil (AOB: Pseudo-$F = 2.63$, $R^2 = 0.26$, $P < 0.01$; AOA: Pseudo-$F = 1.78$, $R^2 = 0.19$, $P < 0.01$) and rhizosphere samples (AOB: Pseudo-$F = 3.05$, $R^2 = 0.27$, $P < 0.01$; AOA: Pseudo-$F = 3.69$, $R^2 = 0.36$, $P < 0.01$) (Fig. 5b, c).

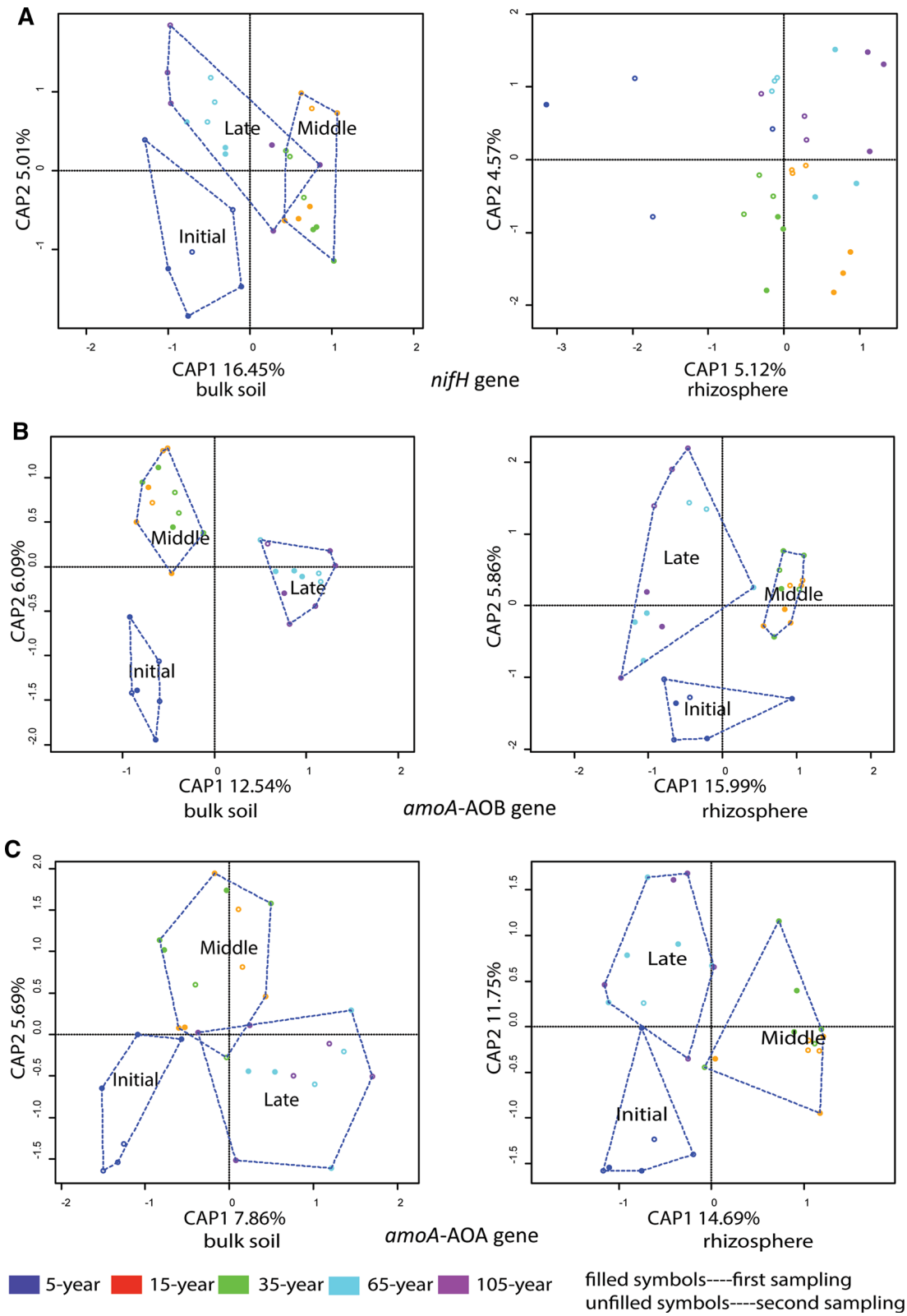
Discussion

In this study, we aimed at understanding the relative influences of soil properties and plant host on root-associated microbiomes in a natural ecosystem. To do this, we focused on investigating the potential drivers of microbial communities related to N-cycling processes (i.e. N fixation and nitrification) in bulk soil and rhizosphere samples of the plant species *L. vulgare*. This plant species was chosen given its broad occurrence across the different stages of soil formation in the chronosequence. Moreover, we argue that the use of a naturally evolving chronosequence minimizes the potential effect of the meta-community and geo-climatic conditions influencing potential differences in root-associated microbiomes.

Fig. 5 Segregation of the N-cycling communities associated with bulk soil and rhizosphere corresponding to the distinct soil successional stages **a** *nifH* gene, **b** *amoA*-AOB gene and **c** *amoA*-AOA gene. Dark blue, red, green, sky blue and purple colors refer to samples collected at the stages 5, 15, 35, 65 and 105-year, respectively. Filled symbols refer to samples from the first sampling time, and open symbols represent samples from the second sampling time

Changes in soil physicochemical properties relate to changes in N-cycling communities in bulk soil but not in the rhizosphere

Overall, we found that soil variables and plant biomass significantly relate to the abundance of N-cycling genes in bulk soil samples, an effect that was absent in the rhizosphere. The systematic increments in soil nutrient status and the elevated plant biomass likely contribute to the enrichment of available 'niches' for diazotrophs and ammonia oxidizers in the soil (Kowalchuk and Stephen 2001; Okano et al. 2004; Reardon et al. 2014). The gradual increase in the abundances of N-cycling genes associated with the bulk soil samples collected along the chronosequence followed previously observed plant biomass patterns (Schrama et al. 2012), which also correlated with the amount of nitrogen in the soil (Dini-Andreote et al. 2016). However, only ammonium, and not nitrate, significantly correlated with the abundance patterns of N-cycling genes. This is likely a result of the low variation in soil nitrate, and in the abundance of *nifH* and *amoA* genes, particularly at the initial and intermediate stages. Thus, collectively our results partially corroborate the notion that N availability relates to changes in the abundance of genes involved in nitrification (AOA and AOB), and that plant diversity and soil structure are important drivers of nitrogen fixation in bulk soils (Salles et al. 2017). By investigating the successional patterns of key genes involved in the nitrogen cycle in the same chronosequence on bulk soil communities, Salles et al. (2017) showed that the abundance of the diazotrophic communities observed from stage 35 years onwards corresponded to the high primary productivity at the intermediate to late successional stages, which were supported by significant correlations with plant


A
CAP2 5.01%
CAP1 16.45%
bulk soil
Late
Middle
Initial
CAP2 4.57%
CAP1 5.12%
rhizosphere
nifH gene
B
CAP2 6.09%
CAP1 12.54%
bulk soil
Middle
Late
Initial
CAP2 5.86%
CAP1 15.99%
rhizosphere
amoA-AOB gene
C
CAP2 5.69%
CAP1 7.86%
bulk soil
Middle
Initial
Late
CAP2 11.75%
CAP1 14.69%
rhizosphere
amoA-AOA gene
5-year
15-year
35-year
65-year
105-year
filled symbols----first sampling
unfilled symbols----second sampling

biomass. Moreover, we found the abundance of N-cycling genes in bulk soil to correlate with most of the soil variables, including soil texture and pH in the case of the *nifH* gene abundance. This finding is consistent with the study conducted by Pereira e Silva et al. (2011), who found that soil type was the major factor influencing N-fixing community abundance across different agricultural soils. Such relation is likely explained by the soil clay fraction, which is important in distributing specific physical properties, forming micro- and macro-aggregates (Gupta and Roper 2010), and providing microaerophilic or anaerobic conditions that favour N fixation. Similarly, the strong correlation between the ammonia-oxidizing community sizes and soil variables corroborate studies showing that ammonia-oxidizing communities are affected by soil type (Philippot et al. 2009), pH level (De Boer et al. 1995; De Boer and Kowalchuk 2001; Nicol et al. 2008; Lehtovirta-Morley et al. 2011), temperature (Avrahami and Conrad 2005; Tourna et al. 2008) and ammonium concentration (Prosser and Nicol 2008; Di et al. 2010).

In the rhizosphere, increments in soil nutrients can lead to the enrichment in the abundance of N-cycling genes, as demonstrated by Hai et al. (2009)—albeit the effect is dependent on the functional group. For instance, it has been shown that soil nutrient status significantly increased the gene abundance of *nifH* and *amoA*-AOB in the rhizosphere, whereas AOA were relatively more stable and did not respond at a similar extent to urea amendment as their bacterial counterparts (Santoro et al. 2008). Similarly, by exploring the effects of various forms of mineral N fertilizer on the AOB and AOA community dynamics in the barley rhizosphere, Glaser et al. (2010) showed that whereas AOA fluctuated in abundance irrespective of ammonia availability, AOB were more responsive to increasing fertilizer amendment. Despite the potential variation in abundance of N cycling genes in the rhizosphere with respect to nutrient amendment, our data revealed that the abundances of N-cycling communities associated with the rhizosphere of *L. vulgare* were moderately constant across most of the soil stages (except for the abundance of *amoA*-AOB at the 5- and 15-year stages in the first sampling), regardless the progressive increment in soil nutrient status along succession. It is likely that the growth of microbial populations was consistently stimulated by the constant secretion of a diverse range of root exudates (Hinsinger et al. 2009; Nelson et al. 2015), overcoming the variation in the soil nutritional status. We speculate that this lack of variation might be a common trend in microbial communities associated with non-agricultural, perennial plants, such as *L. vulgare*, whose long-standing presence in this natural, non-fertilized field, could select for stable microbial densities that are optimized for the soil conditions where they naturally occur. Interestingly, a general increase in the *nifH* gene abundance in the rhizosphere at the second sampling time was observed, indicating that diazotrophs might be more sensitive to variation in plant exudation (Knauth et al. 2005; Soares et al. 2006; Chaudhary et al. 2015). In this case, the diazotrophic community might be affected by the flowering phonological stage of this plant species.

AOB rather than AOA dominate the size of ammonia-oxidizing communities in bulk soil and rhizosphere of *L. vulgare*

The relative abundances of AOA and AOB in soils represent an important index, as these groups have distinct physiological characteristics and ecological niches (Prosser and Nicol 2012), and are expected to respond differently to environmental disturbances (Bannert et al. 2011; Brankatschk et al. 2011; Banning et al. 2015). Previous studies have shown that *amoA*-AOA are more abundant than *amoA*-AOB in many environments, including terrestrial (Leininger et al. 2006), semi-arid agricultural soil (Banning et al. 2015), hot spring sediment (Hatzenpichler et al. 2008), coastal (Wuchter et al. 2006) and marine ecosystems (Beman et al. 2008). We found, however, the abundance of *amoA*-AOA gene to be lower than that of *amoA*-AOB in both bulk soil and rhizosphere samples along this salt marsh chronosequence, except for the 5-year stage. This finding is consistent with a previous study performed in the same plots, which showed that AOB outnumbered AOA (AOB:AOA ratios of > 1; Dini-Andreote et al. 2016). The prevalence of *amoA*-AOB over *amoA*-AOA has generally been observed in estuaries (e.g. Caffrey et al. 2010; Wankel et al. 2011; Li et al. 2015). In these systems, as in the studied chronosequence, high salinity seems to be a main factor accounting for the dominance of AOB community (Bernhard et al. 2005; Jung et al. 2011). In addition to salinity and levels of trace elements that selectively promote autotrophic activity of AOB or

AOA in estuarine systems, the sensitivity of AOA to elevated ammonium concentrations may also explain the dominance of AOB (see Martens-Habbena et al. 2009; Verhamme et al. 2011; Li et al. 2015). This is of particular importance at the intermediate and later stages of soil in this chronosequence.

The community structure of N-cycling genes in bulk soil and rhizosphere are mostly related to changes in soil physicochemical properties

Different community structures of diazotrophs, AOB and AOA were found in the bulk soil and rhizosphere of *L. vulgare*, a result that corroborates with the phylogenetic diversity of total bacterial communities in these samples (Wang et al. 2016). This finding supports our hypothesis that the pool of microorganisms selected by the plant root differs (phylogenetically and functionally) from those present in the bulk soil, regardless of soil properties. Moreover, these results are in line with the study conducted by Chaudhary et al. (2015), who demonstrated that the microbial community structure and functional diversity were influenced by the rhizosphere effect of halophytes (i.e., the genera *Salicornia*, *Aeluropus* and *Suaeda*). Thus, there is evidence that root physiology and exudates are strong modulators of both the taxonomic and functional assembly of the associated root microbiome across divergent plant species (Knauth et al. 2005; Soares et al. 2006; Garbeva et al. 2008; Chaudhary et al. 2012).

Given the significant role of plant host in the abundance of N-cycling genes in the rhizosphere of *L. vulgare*, we expected the community structure of these genes to be similar along the gradient of soil formation. However, our data revealed that (1) the structure of ammonia-oxidizing communities in the soil and rhizosphere segregated into clusters following the nutrient status and vegetation development, and (2) differences in diazotrophic community structure were only found in bulk soil samples. This lack of clustering indicates a potential overriding effect of the rhizosphere over the soil properties in structuring the rhizosphere diazotrophic community. Changes in the community structure of rhizosphere-associated diazotrophs seem to depend on the plant species. For example, no variation has been reported in rice (Wartiainen et al. 2008), but significant changes were found to occur in maize (*Zea mays* L.) (Rodríguez-Blanco et al. 2015). In addition, the amendment of fertilizers was reported to sustain a larger and diverse diazotrophic community in the rhizosphere (Yim et al. 2009). With respect to changes in the community structure of AOB and AOA, Liu et al. (2015) reported that the community structure of ammonia oxidizers was influenced by the climate change simulations (i.e. elevated CO_2 and warming), regardless the side effect of warming on soil properties such as moisture, pH and ammonium concentrations (Horz et al. 2004). Besides these differential variation observed in the community structures of N-fixers and ammonia-oxidizers, it is notable that for the particular case of N-fixers, the diazotrophic communities in the rhizosphere of *L. vulgare* was also found to vary between the two sampling times. This supports a potential seasonal/phenological effect of the plant-host on the structure of these communities.

The rhizosphere of *L. vulgare* is enriched in AOA but not in AOB and N-fixers

Despite the observed differences in AOB:AOA ratio, correcting the abundances by the total number of bacteria or archaea revealed, to large extent, an enrichment of AOA abundance in the rhizosphere and of AOB in the bulk soil. This finding corroborates our hypothesis that N-cycling gene abundances are higher in the rhizosphere of *L. vulgare* than in bulk soil samples. This observed enrichment of AOA in the rhizosphere seems to be a common feature of plants presenting an exploitative strategy for nutrient acquisition and relatively high N demand (a common feature of grasses) (Thion et al. 2016), albeit the proper determination of N demand in *L. vulgare* remains elusive. We thus speculate that, given the growing season, the increased root exudates may likely have improved the availability of easily degradable organic matter, stimulating particular AOA types that might have a propensity for mixotrophic metabolism (Tourna et al. 2008).

In our study system, the diazotrophs and AOB abundances were significantly enriched in the bulk soils. This stands in contrast to those findings reported by Cibichakravarthy et al. (2012) and Chaudhary et al. (2015), where the abundance of the *nifH* gene was reported to be enriched in the rhizosphere. These authors suggested that their findings are supported by the fact that diverse root exudates might act as

essential nutritional sources for N-fixers. However, as part of our results oppose to the literature, it is tempting that in our study system (1) there might be no strong symbiotic relationship between the *L. vulgare* and diazotrophic populations in the rhizosphere. Thus, the decrease of N-fixers in the rhizosphere can be a result of competitive exclusion; (2) the root exudates of *L. vulgare* do not favour (or either antagonizes) N-fixers and AOB communities; and (3) the seasonal/phenological plant status at the sampling times may not have been a stage that favours the enrichment of N-fixers and AOB at the rhizosphere of *L. vulgare*.

Concluding remarks

Overall, this study provides important information on the variation in abundance and structure of N-cycling microbial communities associated with the rhizosphere of *L. vulgare* and in bulk soil samples collected along a salt marsh chronosequence. Interestingly, we showed that *L. vulgare* exerts a stronger influence than soil on the abundance of AOA, consistently enriching this community size in the rhizosphere along the chronosequence development. However, this enrichment effect in the rhizosphere was not observed for AOB and diazotrophs, thus indicating a weak influence of the plant host on the abundance patterns of these communities, whose variation was mostly season-dependent. Notably, the effect of the season resulted in differences in the structure of diazotrophic communities in the rhizosphere of *L. vulgare*, whereas changes in the community structure of the ammonia-oxidizers were mostly related to the variations in soil properties along the chronosequence. Taken together, we argue this study advances fundamental knowledge on the distributional patterns of N-cycling related genes along a naturally evolving ecosystem. We advocate further studies are needed to improve our comprehension on the degree at which distinct plant species modulates the abundance of root-associated communities in natural ecosystems. Moreover, the use of chronosequences provides useful model systems that minimize the effect of spatial variation and geo-climatic conditions, thus favouring the disentangling of the relative contributions of soil properties and host physiology on the assembly and dynamics of rhizosphere communities in naturally occurring plant species.

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Conflict of interest The authors declare no conflict of interest.

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