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Relationships between soil physicochemical properties and nitrogen fixing, nitrifying and denitrifying under varying land-use practices in the northwest region of Argentina

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Abstract: The aim of this study was to evaluate the response pattern of diazotrophic microbes, denitrifiers and nitrifiers to different types of land use management, such as soybean monoculture (M) during 5 and 24 years (M5 and M24) and soybean-maize rotation (R) during 4 and 15 years (R4 and R15) in two subsequent years at the time point of flowering. Soil samples from a site recently introduced into agriculture (RUA) and a pristine soil under native vegetation (NV) were used as controls. Abundances of different functional groups of microbes were assessed using the direct quantification of marker genes by quantitative real-time PCR using extracted DNA from rhizosphere samples. In addition, soil chemical and physical properties were analysed and correlated with the abundance data from the functional microbial groups under investigation. Overall, the results indicate that the abundance of *nif*H genes was higher under R treatments compared to M treatments. The abundance of ammonium monooxygenase genes *amoA* (AOA) was generally higher under rotation systems and decreased under M24. RUA evidenced a negative effect on the establishment and development of AOA communities. The influence of land use on *nirS* abundance was inconsistent. However, R treatments showed a high abundance of *nirK* genes compared to M treatments. In both growing seasons, the abundance of *nosZ* genes was higher under NV compared with the other treatments. Furthermore, M24 treatment was related to strongly changed chemical and physical soil properties compared with the other sites. As expected, soil samples from RUA showed the strong dynamics of measured parameters indicating the high sensitivity of soils under transition to environmental parameters. Our results also indicated that the long-term crop rotation modified the abundance of the investigated microbial groups compared to the monoculture and increased soil chemical and physical quality. Therefore, our results provide evidence for a stimulatory effect of the long-term crop rotation on the abundance of microbes involved in N transformation.

Keywords: agricultural management; denitrification; nitrification; nitrogen fixation; soybean

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Land-use changes belong among the main drivers for anthropogenic disturbances largely influencing the terrestrial ecosystem and acting as drivers for global climate change, loss of ecosystem services and species extinctions (Maharjan *et al*. 2017). Numerous studies in the past indicated that most of these observations are triggered by a direct effect of land use management on the soil microbiome. For example the conversion of native soils to agricultural soils is often associated with the loss of organic matter, which directly changes the structure and function of the soil microbiome and ultimately alters soil properties (Schloter *et al*. 2003). The crop rotation is an often proposed mitigation strategy as it modifies the composition of the microbial taxa in the rhizosphere, besides improving soil structure and increasing organic matter content, while inhibiting soil-borne pathogens. Thus in the long run crop management usually alters physical, chemical and microbiological processes, which subsequently influence mineralization and nutrient cycling. In this respect it has also been indicated that the crop rotation can improve biological nitrogen fixation (BNF) as the main source of meeting the N requirement of high-yielding crops (Rabbi *et al*. 2014).

Subtropical ecosystems in the northwestern region of Argentina are considered as important for their biodiversity and their various ecosystem services; despite the high vulnerability of these ecosystems to land degradation, in the last decades agricultural management has been characterized by land use intensification and implementation of monocultures like soybean, with so far unknown consequences for soil quality. In this study, we investigated the effects of land use change (crop rotation vs. soybean monoculture, under different land use intensities, with pristine soil as a basis) in the subtropical region of Argentina on microbes driving the inorganic nitrogen cycle using a molecular marker gene approach. The N cycle is one of the most significant biochemical cycles, as on the one hand N is a major element for all biota for growth and performance and on the other hand intermediates of the N cycle like nitrite or nitrate, improperly used, can cause groundwater and soil contamination in fragile ecosystems (Stone *et al*. 2015).

MATERIAL AND METHODS

Location and site description. Study sites were located in Anta Department (Las Lajitas), Salta, the northwestern subtropical region of Argentina (S 24°53'43.6''W, 65°27'58.6''S), 1100 m a.s.l. The selected sites were used for commercial soybean production and included sites with soybean monoculture [24 (M24) and/or 5 (M5) years under soybean monoculture] and soybean under rotation [15 (R15) and/or 4 (R4) years under rotation] with the following sequence: two years of soybean, one year of maize. Moreover, sites recently introduced into agricultural production (RUA) were additionally sampled. Native vegetation soils (NV) adjacent to each agricultural site were sampled as a control. The climate of the region is continental-subtropical, with low or no water deficit in January and February. The mean annual precipitation is 600–800 mm, concentrated in spring-summer, with a prolonged dry season in winter. The average annual temperature varies from 23°C in the north to 18°C in the south. The dominant soil type of the region has been characterized as loamy soil with 2.91% of organic matter (32% sand, 44% silt and 24% clay), classified as Ustorthent (Castrillo *et al*. 2014).

Soil sampling. Sampling was performed at the flowering stage of soybean in February 2010 and 2011. All crops were managed using recommended production practices including pesticide applications and weed control measures. For the analysis of rhizosphere samples soybean roots were taken from the fields and rhizosphere soil was obtained by gently shaking off the adhering soil, which was placed in plastic bags and processed immediately. Each sample was well homogenized and divided into two parts; one was sent to the Laboratory of Soil and Water (EEA Salta INTA) to be processed for chemical and physical analyses, whereas the other field moist part was sieved through a 2mm mesh for microbial analyses and subsamples were immediately stored at –20°C until used and for further analysis. Six rhizosphere composite samples (each containing 3 plants) were taken per site, each 30 m apart from each other spanning an area of 900 m^2 per site.

Soil chemical and physical properties. Organic carbon (OC) was determined by wet oxidation following the Walkley and Black procedure (Black & Allison 1965); total N (tN) was measured using the micro-Kjeldahl method (Bremner 1996). Extractable phosphorus (Pe) was quantified as described by Bray and Kurtz (1945). Soil alkalinity (pH) and electrical conductivity (EC) were estimated potentiometrically in a 1 : 2.5 soil/water suspension. For the determination of ammonium (NH_4^+-N) and nitrate (NO_3^-), 3 g

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of soil was overhead shaken for 30 min with 12 ml of 0.01 M $CaCl_2$. Each extract was filtered through a Millex HV Millipore filter (Milipore, Ireland) (pore size 0.45 µm). Ammonium and nitrate measurements were performed on a Nanocolor 300D photometer (Macherey-Nagel, Germany) by using the commercial kits Nanocolor Ammonium 3 and Nitrate 50 according to the manufacturer's protocol. Aggregate stability (AS) was determined following the method of the micro-sieves (1–2 mm) according to Corvalán *et al*. (2000). Bulk density (BD) was measured by the core method (Blake & Hartge 1986).

Nucleic acid extraction and real-time PCR assay. DNA was extracted from 0.5 g of soil. Extraction was performed with the NucleoSpin® Soil Kit (Macherey-Nagel, Germany) using the manufacturer's protocol. DNA yield and purity were measured using a micro-volume fluorospectrometer (Thermo Scientific, NanoDrop Technologies, USA). The abundance of nitrifying microbes, denitrifiers and N-fixers was assessed by quantifying the respective maker genes via qPCR. The ammonia monooxygenase gene *amoA* was used as a proxy for nitrifying bacteria (AOB) and archaea (AOA); the functional redundant nitrite reductase genes *nirS* and *nirK* as well as the N_2O reductase gene (*nosZ*) as proxies for denitrifiers and the nitrogenase gene (*nifH*) as a marker for nitrogen-fixing microbes. All assays were performed in triplicates on the ABI Prism 7300 Cycler (Applied Biosystems, Germany). The reagents used for the real-time PCR assay were: bovine serum albumin (Sigma-Aldrich, Germany), primers (Metabion, Germany), dimethyl sulfoxide (Sigma, Germany), and Power SYBR Green PCR master mix (Applied Biosystems, Germany). All PCR runs started with an initial enzyme activation step performed at 95°C for 10 min. The PCR protocol for each gene differed; details can be found in Hai *et al*. (2009). Negative (ultrapure water) and positive DNA controls (*Pseudomonas aeruginosa*, 10-fold serially diluted) were also included. Melting curve analyses were carried out to ensure PCR specificity and the expected sizes of the amplified fragments were checked in a 1.5% agarose gel stained with ethidium bromide. Dilution series of plasmid DNA targeting the bacterial nitrogen cycle genes (*nifH*, AOB-*amoA*, *nirS*, *nirK* and *nosZ*) and the fosmid clone 54d9 targeting archaeal *amoA* genes were used to generate a standard curve for each of the five target genes (standard dilutions used for creating a standard curve ranged from 10^1 to 10^6 gene copies/µl). The amplification efficiencies (Eff) were calculated using the formula Eff = [10(–1/slope)–1] (Hai *et al*. 2009). All DNA extracts were diluted 1 : 30 to ensure no PCR inhibition.

Statistical analysis. Statistical analyses were performed using InfoStat Professional (Di Rienzo *et al*. 2016). Differences between treatments of soil chemical and physical properties and gene copy were assessed by analysis of variance (ANOVA, LSD test, $P \leq 0.05$). In all cases, residuals were tested for normality by the Shapiro-Wilks test. A correlation analysis (Pearson coefficient) was also performed to evaluate the relationship between soil properties and the abundance of microbial communities involved in N cycling. Finally, a PCA analysis was done to determine the separation between the different treatments, and to analyse which of the chemical, physical and microbiological variables better contributed to the separation of field treatments.

RESULTS AND DISCUSSION

Soil chemical and physical properties. On both sampling dates, the different crop management systems affected both soil chemical and physical properties (Table 1). Both OC and tN showed the highest values under NV and R15, and the lowest under M24. Similarly, NV and R15 treatments showed higher NH_4^+-N and NO_3^--N contents, compared to M24, suggesting that long-term soybean monoculture affected nutrient cycling. The effect of RUA on soil chemical properties was inconsistent, with differing trends between both sampling years, indicating that mainly during the transition phase of sites from natural land to agricultural management effects are strongly mediated by climatic conditions and cannot be easily predicted. Soil under NV treatment showed the lowest pH, but the highest Pe, compared with the other treatments. At M24 evidence for a decrease of soil clay content and aggregate stability, but an increase of bulk density was visible. These results agree with numerous studies in which the continuous crop (monoculture) has progressively decreased both physical and chemical soil quality in comparison with conservation practices including rotation managements (Li *et al*. 2015).

Land use intensity and nitrogen fixation. Overall, in our study, the abundance of *nif*H genes was higher in R treatments compared to M treatments (Figure 1A), indicating that the abundance of N_2-fixers is sensitive to the management practice. This is supported by previous reports which stated that *nifH*

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Table 1. Soil properties under different land use types

	M24		M5		R4		R15		RUA		NV	
	2010–11	2011–12	2010–11	2011–12	2010–11	2011–12	2010–11	2011–12	2010–11	2011–12	2010–11	2011–12
Chemical parameters												
OC (%)	1.1^{c}	1.1^{c}	1.6^{b}	1.6^{b}	1.5^{b}	1.8^{b}	1.8^{a}	1.8^{b}	1.4^{b}	1.6^{b}	2.9^{a}	2.3^{a}
Pe (ppm)	16.6^{b}	15.0^{d}	28.3^{a}	27.0^{c}	28.5^{a}	29.9^{c}	30.6^{a}	35.6^{b}	29.3^{a}	27.0^{c}	38.8^{a}	59.0^{a}
pH	6.9^{a}	7.0^{a}	7.1^{a}	6.7^{a}	6.8^{a}	6.5^{a}	6.9^{a}	6.9^{a}	7.1^{a}	6.8^{a}	5.9^{b}	6.0^{b}
tN (%)	0.1^{c}	0.1^{c}	0.1^{b}	0.1^{b}	0.1^{b}	0.1^{b}	0.1^{a}	0.1^{b}	0.1^{b}	0.1^{c}	0.2^{a}	0.2^{a}
NH_4^+-N (mg N/kg soil)	2.8^{c}	2.6^{c}	3.7^{b}	3.5^{b}	4.0^{b}	4.3^{b}	4.7^{a}	4.3^{b}	3.7^{b}	3.6^{b}	4.9^{a}	5.2^{a}
NO_3^--N (mg N/kg soil)	60.0^{d}	57.1^{d}	78.0^{c}	80.1^{c}	83.6^{c}	87.0^{c}	97.0^{b}	100.0^{b}	87.0^{b}	86.2^{c}	136,9^{a}	124.4^{a}
Physical parameters												
Sand (%)	53.0^{b}	53.0^{b}	52.0^{b}	52.0^{b}	51.0^{b}	51.0^{b}	50.0^{b}	50.0^{b}	61.3^{a}	61.0^{a}	59.0^{a}	59.0^{a}
Silt (%)	39.6^{a}	39.0^{a}	38.5^{a}	39.0^{a}	38.6^{a}	39.0^{a}	38.0^{a}	38.0^{a}	28.0^{b}	28.0^{b}	27.7^{b}	29.0^{a}
Clay (%)	7.3^{c}	8.0^{c}	9.5^{b}	9.0^{b}	10.4^{b}	10.0^{b}	12.0^{b}	12.0^{a}	10.7^{b}	11.0^{ab}	13.3^{a}	12.0^{a}
EC (dS/m)	0.3^{b}	0.2^{c}	0.4^{b}	0.5^{b}	0.2^{c}	0.5^{b}	0.3^{c}	0.4^{b}	0.6^{a}	0.5^{b}	0.6^{a}	0.9^{a}
AS (%)	15.7^{d}	14.0^{d}	22.7^{c}	21.0^{c}	22.1^{c}	22.5^{c}	22.7^{c}	23.0^{c}	29.0^{b}	26.0^{b}	36.2^{a}	37.0^{a}
BD (g/cm^3)	1.6^{a}	1.7^{a}	1.5^{b}	1.5^{b}	1.3^{d}	1.3^{c}	1.4^{c}	1.3^{c}	1.4^{cd}	1.4^{c}	1.3^{cd}	1.3^{c}

OC – organic carbon; tN – total nitrogen; Pe – extractable phosphorus; EC – electrical conductivity; NH_4^+-N – ammonium concentration; NO_3^--N – nitrate concentration; AS – soil aggregate stability; BD – bulk density; different letters document statistically different means within each agricultural cycle ($P \leq 0.05$); M24 – 24 years under soybean monoculture; M5 – 5 years under soybean monoculture; R4 – 4 years under rotation; R15 – 15 years under rotation; RUA – sites recently introduced into agricultural production; NV – native vegetation soils adjacent to each agricultural site

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was differentially affected by plant species and cropping systems (Wang *et al*. 2012). In addition, the low abundance of *nif*H genes was found in NV soils compared with R treatments. The anthropic activities that allow incorporation of crop residues may stimulate microbial activities, promoting biodiversity and stability of ecosystems, while increasing the rate of biological nitrogen fixation (Jackson & Burger 2003). Although the soil under RUA presented a more recalcitrant deposit of litter and plant debris than the rest of agricultural treatments (with high OC), we observed lower *nif*H gene abundances compared with R and NV treatments. Some authors consider the crop rotation as an intermediate situation of anthropic disturbance, clearing an extreme disturbance that probably affects the establishment of diazotrophic microbes (Lauber *et al*. 2008). In fact, Prosser (2012) stated that the greatest diversity of soil microorganisms (N-fixing community) can still be maintained at intermediate levels of disturbance, as occurred in R15 treatment.

Land use intensity and nitrification. In our study, AOA revealed a strong response to land use intensity, especially in the first crop cycle where M treatments and RUA presented the lowest abundances (Figure 1B). Our results are in agreement with those of Martir-Torres and Bruns (2013), who observed that the size of AOA community was generally higher under conservation practices in relation to undisturbed situations (pristine soil). As expected, RUA evidenced a negative effect on the establishment and development of AOA communities. Interestingly, in the second crop cycle, the abundance of AOA genes in RUA was equivalent to M treatment, suggesting AOA re-establishment. Although NV soils are enriched with OC compared with M and R treatments, we observed a higher abundance of AOA genes in R treatments. However, in the second crop cycle the abundance of AOA genes was equivalent in the soil under NV and R.

In both years, our study also showed that the abundance of AOB was decreased under M24 (Figure 1C).

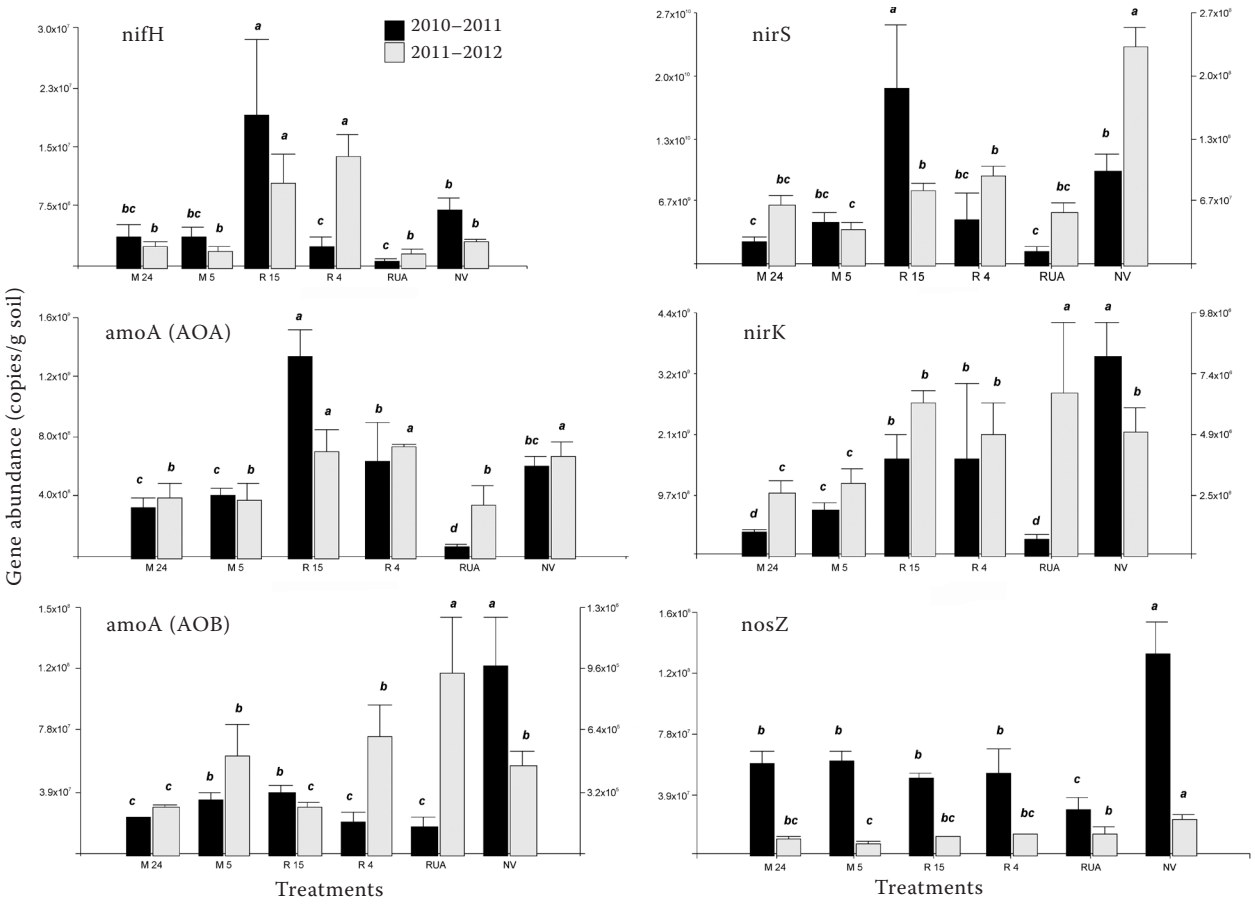

Figure 1. Copy numbers of functional microbial groups involved in nitrogen fixation, denitrification and nitrification in 2011 and 2012

Different letters document statistically different means within each agricultural cycle ($P \leq 0.05$)

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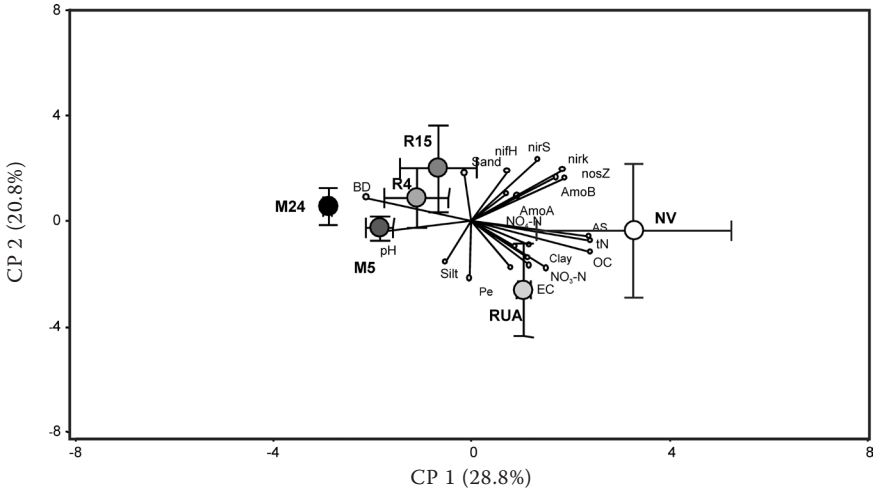

Figure 2. Principal component analysis (PCA) biplot based on chemical and physical soil parameters and microbial groups involved in nitrogen fixation, denitrification and nitrification in 2011 and 2012
OC – organic carbon; tN – total nitrogen; Pe – extractable phosphorus; EC – electrical conductivity; NH_4^+-N – ammonium concentration, NO_3^--N – nitrate concentration, AS – soil aggregate stability, BD – bulk density

This is in agreement with several authors who observed that soybean monoculture results in a great depletion of soil nutrients, low intake of crop debris and soil compaction, thereby affecting the abundance of AOB (Rabbi *et al*. 2014). Also, oxygen is an important factor for the autotrophic metabolism of AOB (Kowalchuk *et al*. 2000); thus, a reduction of oxygen that resulted from land use intensification (M24) might be another factor that contributed to the reduction of the AOB abundance in M24. In 2011, AOB gene abundance was significantly higher in NV compared with M, R and RUA. However, in 2012, the highest value of AOB genes was observed under RUA treatment. In this year, RUA presented lower levels of pH compared with the previous year (Table 1), possibly due to changes in the soil during the burning and deforestation of native vegetation.

Land use intensity and denitrification. The abundance of nitrite reducing microbes harbouring the *nirS* (encoding a cytochrome cd1-containing nitrite reductase) and *nirK* (encoding a copper-containing nitrite reductase) gene, respectively, were differentially affected by land use intensity, although the influence of land use on *nirS* abundance was inconsistent, with no clear effect between the two seasons (Figure 1D). In 2011, no significant differences were observed between M, R4 and RUA treatments; however, the abundance of *nir*S genes was higher under R15 and NV. In 2012, the highest and the lowest abundance of *nirS* genes was observed under NV and M5, respectively. In both seasons, the abundance of *nirK* genes significantly decreased under M treatments, especially in M24 (Figure 1E), probably related to the lowest OC found under this treatment. Because denitrification is a facultative anaerobic pathway, a decrease in the relative abundance of denitrifying bacterial communities could be a consequence of a competition for available O_2 between denitrifiers and total bacterial population (Barrena *et al*. 2017), principally in soils under a long-term history of conventional land use intensity, such as M24. In the first season, at RUA copy numbers of *nir*S and *nir*K genes were strongly decreased; however, in the second season, a significant increase was observed in the abundances of both genes. This response was probably due to changes in soil properties and edaphic conditions that occurred after these plots were introduced into agriculture. In both agricultural cycles, R treatments showed the high abundance of *nirK* genes. Finally, it is important to note that *nirS* abundance was approximately 1 to 2 orders of magnitude higher than *nirK* abundance, suggesting that *nirS* denitrifiers are dominant in this region. In both seasons, the abundance of *nosZ* genes was higher under NV compared with the other treatments (Figure 1F). The lower abundance of *nos*Z genes detected in our work compared with *nirK* genes has already been reported in subtropical agricultural soils by others authors (Henry *et al*. 2006).

Soil properties and N cycling. PCA analysis of the gene abundance data and selected chemico-physical soil properties revealed a shift in the soil functional

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Table 2. Correlation analysis between abiotic soil properties and selected functional groups of microbes in the soils under investigation

	*nif*H	AOA	AOB	*nir*S	*nir*K	*nos*Z
Chemical parameters						
OC (%)	0.11	0.37*	0.34*	0.21*	0.29*	0.28*
Pe (ppm)	0.14	0.06	0.06	0.14	0.08	–0.13
tN (%)	0.07	0.25*	0.43*	0.34*	0.43*	0.42*
NH_4^+-N (mg N/kg soil)	0.29*	0.08	0.01	0.28*	0.34*	0.02
NO_3^--N (mg N/kg soil)	0.08	0.40*	0.01	0.21*	0.06	0.06
Physical parameters						
Sand (%)	0.32*	0.09	0.11	0.24	0.21	0.12
Silt (%)	0.34*	0.05	–0.15	0.20	0.22	0.13
Clay (%)	0.03	0.26	0.03	0.12	0.03	0.03
AS (%)	0.10	0.23*	0.39*	0.24*	0.37*	0.34*
BD (g/cm^3)	–0.19	–0.27*	–0.28*	–0.24*	–0.25*	–0.23*

*significant P values ($P \leq 0.05$); OC – organic carbon; tN – total nitrogen; Pe – extractable phosphorus; NH_4^+-N – ammonium content, NO_3^--N – nitrate content; AS – soil aggregate stability; BD – bulk density; *nif*H – nitrogenase gene; AOA – ammonium monooxygenase genes *amoA*; AOB – ammonium monooxygenase genes for archaea; *nir*S, *nir*K – nitrite reductase genes; *nos*Z – N_2O reductase gene

microbial community related to N turnover under different land uses (Figure 2). PC1 and PC2 explained 28.8% and 20.6% of total variance, respectively. The biplot showed that M24 was negatively allocated along PC1 with NV being on the opposite side, clustering most distantly from the agricultural treatments. A clear separation was also found for RUA (along axe 1), being the treatment located between NV and the other agricultural treatments (M24, M5, R4, and R15). M5 also clustered distantly to M24 and close to R treatments. Some physical (AS), chemical (tN, OC), and molecular (AOB, *nirK* and *nosZ*) parameters were most important in the positive direction for PC1, while pH and BD were negatively correlated with PC1. The contents of sand, silt, Pe and *nifH* were correlated with PC2.

Our results indicate that both autotrophic nitrifiers and heterotrophic denitrifiers may be greatly influenced by both BD and AS (Table 2). Several authors have reported that soil physical characteristics were significant drivers of the community composition of both nitrifiers and denitrifiers (Ciccolini *et al*. 2016). In contrast, our results also revealed a poor relationship between copy numbers of *nifH* genes and selected soil chemical properties (Table 2). The abundance of *nifH* was correlated only with NH_4^+-N, suggesting that other microbial processes and chemical properties were involved in the dynamics of the communities of N fixers. However, the abundance of both nitrifiers (AOA and AOB) significantly correlated with OC, tN, and pH (Table 2). Moreover, AOA abundance was also positively correlated with NO_3^--N. Although several authors reported that archaeal AOA were inhibited by enhanced urea and ammonia concentrations (Hatzenpichler *et al*. 2008), in our study no significant correlation was observed between NH_4^+-N content and the abundance of AOA genes. A similar pattern was observed for the relationship between denitrifier abundances and soil chemical properties (Table 2). Denitrifier abundance (*nirK*, *nirS* and *nosZ*) positively correlated with both OC and tN, demonstrating the heterotrophic nature of denitrifying community (Zumft 1997), which was stimulated by the increase of OC content by crop residue inputs under R treatments. Consistent with this idea, abundance of denitrifiers was increased under NV, where high contents of OC, Pe, and tN were observed. Moreover, these genes responsible for denitrification also negatively correlated with pH, suggesting that these microorganisms may be greatly influenced by soil acidity, as was described by other authors (Bárta *et al*. 2010).

CONCLUSIONS

Our results revealed that the abundance of nitrifiers, denitrifiers and diazotrophs was differently affected by different forms of land management investigated in this study. The abundance of N-fixation microorganism was higher in soils under conserva-

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tion practices and lower in NV soils compared to sites under rotation. Furthermore, the abundance of nitrifiers (AOA-AOB) was generally higher under conservation practices in relation to pristine soil. The influence of land use on denitrifier (*nirS*–*nir*K) abundance was inconsistent throughout the growing seasons. The most important soil properties were several soil chemical (pH, OC and tN) and physical (AS and BD) parameters. Thus, increasing or decreasing the abundance of single microbial groups might need new forms of adopted management. Further it needs to be investigated if the abundance of a functional group of microbes also drives their activity, so a direct link between fluxes of nutrients and the corresponding microbial drivers can be made.

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