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ORIGINAL PAPER

Denitrifying bacterial communities display different temporal fluctuation patterns across Dutch agricultural soils

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Abstract Considering the great agronomic and environmental importance of denitrification, the aim of the present study was to study the temporal and spatial factors controlling the abundance and activity of denitrifying bacterial communities in a range of eight agricultural soils over 2 years. Abundance was quantified by qPCR of the *nir*S, *nir*K and *nos*Z genes, and the potential denitrification enzyme activity (DEA) was estimated. Our data showed a significant temporal variation considerably high for the abundance of *nir*K-harboring communities, followed by *nos*Z and *nir*S communities. Regarding soil parameters, the abundances of *nos*Z, *nir*S and *nir*K were mostly influenced by organic material, pH, and slightly by NO_3^-, respectively. Soil texture was the most important factor regulating DEA, which could not be explained by the abundance of denitrifiers. Analyses of general patterns across lands to understand the soil functioning is not an easy task because the multiple factors influencing processes such as denitrification can skew the data. Careful analysis of atypical sites are necessary to classify the soils according to trait similarity and in this way reach a better predictability of the denitrifiers dynamics.

Keywords Gene abundance · *nos*Z · *nir*K · *nir*S · Potential denitrification · Soil texture

Introduction

Denitrification is the major biological mechanism by which fixed N returns to the atmosphere from soil or

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water (López-Lozano et al. 2012), thereby completing the N cycle. The denitrification process promotes N loss from soil and might lead to the release of N_2O, a major greenhouse gas and air pollutant (Syakila and Kroeze 2011). Consequently there has been increasing concern about the factors that control denitrification as well as the size of denitrifying communities and how they relate to activity rates in intensive arable crop production systems. Moreover, the soil physical and chemical parameters influencing denitrification rates and N_2O emissions are poorly understood, and the prediction of their emission in the field remains problematic. A better understanding of the factors modulating abundance and activity of denitrifiers across a variety of soils in different climates and under different land uses may provide a key to better understand the variability of N_2O fluxes at the soil–atmosphere interface (Butterbach-Bahl et al. 2013). Multiple factors have been shown to influence denitrification rates. Mainly soil texture (D'Haene et al. 2003; Gu and Riley 2010; Gu et al. 2013), pH (Mørkved et al. 2007; Baggs et al. 2010; Cuhel et al. 2010; Ligi et al. 2014), organic material (OM) (Mosier et al. 2002; Barrett et al. 2016) and the amount of inorganic N (Niboyet et al. 2009) have a substantial impact. Based on these factors, beneficial management practices had been applied to reduce N_2O emissions including split fertilizer N applications, use of controlled release fertilizer (CRF) products and nitrification inhibitors (Smith et al. 1998; Akiyama et al. 2010). However, the effect of such practices can vary strongly with soil properties and climatic conditions (Burton et al. 2012; Rosa et al. 2014).

Previous studies have reported high rates of denitrification and N_2O emissions in agricultural soils, especially in areas that experience periods of lower oxygen availability (Bateman and Baggs 2005; Hofstra and Bouwman 2005; Gu and Riley 2010; Dandie et al. 2011; Ligi et al. 2014). In wetlands, it has been reported that temporal variation of denitrification is primarily dependent on temperature and nitrate concentration, with higher rates in early spring, compared to the other sampling periods (Song et al. 2012). However, seasonal variations in denitrification differ between ecosystems. Clément et al. (2002) found that the maximum denitrification rate was reached during the autumn in grasslands and during the summer in forests. In addition, Rasche et al. (2011) reported that seasonal dynamics displayed by nitrogen (N) cycling functional groups were tightly coupled with seasonal alterations in labile C of forest soil. These reports suggested that physiological responses of denitrifiers to environmental conditions might regulate denitrification rates. However, there are only few studies focusing on understanding the temporal dynamics of denitrification rates on a spatial scale (Bateman and Baggs 2005; Hofstra and Bouwman 2005; Gu and Riley 2010; Dandie et al. 2011; Marton et al. 2015).

To understand how soil microbial communities mediate N transformations a common approach is to determine the abundance of the functional groups associated with the process of interest, in addition to activity patterns. In the case of denitrification, three functional genes, i.e. *nirS*, *nirK*, and *nosZ*, coding for different enzymes in the denitrification pathway, are usually used. The conversion of nitrite (NO_2^-) to nitric oxide (NO) in the denitrification pathway is considered to be present in all denitrifiers. This transformation is catalyzed by at least two structurally dissimilar nitrite-reductases, a copper-containing nitrite reductase (encoded by *nir*K gene) and the cytochrome c nitrite reductase (encoded by *nir*S gene), and is the initial step for N output from the ecosystems (Butler and Richardson 2005). The last step of the denitrification cascade is the reduction of N_2O into N_2, performed by the multicopper homodimeric N_2O reductase (encoded by *nos*Z gene).

Little is known about what controls the abundance of denitrifiers in the environment and its relationships with denitrification enzyme activity (DEA) (Philippot et al. 2009; Butterbach-Bahl et al. 2013). Dandie et al. (2008) reported seasonal patterns of denitrifier gene numbers, which varied with the specific denitrifier community, but the denitrifier abundance were not related to any environmental parameter analised. Contrastingly, in other reports environmental factors had different effects on the abundances and proportions of *nirS*, *nirK*, and *nosZ* genes. For example, Ligi et al. (2014) observed that the abundance of the *nirS* gene and ratio of *nirK* and *nirS* genes were affected by soil pH, and that *nirK* gene proportions in the bacterial community were related to the NO3 concentration in soil. This finding suggests that microbes related to denitrification in soils of different wetland types do not respond similarly to the same environmental variables. In addition some studies have shown that the size of the denitrifying community was uncoupled from variations in DEA in agricultural soils (Henderson et al. 2010). However, evidences suggested that the abundance of denitrifiers determined using the genes

nirS, *nirK*, and *nosZ* as molecular markers is correlated with DEA, which was affected by the type of fertilization regimes (Hallin et al. 2009; Barrett et al. 2016) and spatial patterns (Philippot et al. 2009; Enwall et al. 2010). These conflicting results reflect the complexity of environmental parameters and how they affect the denitrifying community, which might vary in importance over different periods of the year and kinds of soils. It is necessary find a systematic method to analyze these complex data sets.

Thus, considering the agronomic and environmental importance of denitrification, the aim of the present study is to elucidate the temporal and soil related factors controlling the abundance and activity of denitrifying bacterial communities in a range of agricultural soils of different physical and chemical properties. Specifically, we were interested in understanding how the abundance of the following different subsets of the denitrifies, the *nir*S-, *nir*K- and *nos*Z-harboring communities, would fluctuate over time, and which soil parameters would have stronger influence over these communities and the denitrifying activity. To that purpose, we sampled eight representative agricultural fields across The Netherlands in the timespan of one year. These soils represent two types of textures, four of them are clayey and four of them are sandier. Community size was studied by quantifying the abundance of above-mentioned genes by quantitative polymerase chain reaction (qPCR) and their functioning was evaluated by measuring DEA. We hypothesized that temporal fluctuations in community size will occur, which will vary according to the gene targeted. Considering the higher sensitivity of *nir*K-harboring communities to environmental gradients (Smith and Ogram 2008; Dandie et al. 2011), we hypothesize that largest fluctuations in abundance will be observed for this group, whereas the communities containing the *nir*S gene will be more stable over time. Moreover, considering that bacteria containing *nir*K or *nir*S also harbor the *nos*Z gene, we could expect an intermediate behavior. Moreover, we hypothesize that soil type, classified by texture, will have a major influence on the abundances and also activity of denitrifiers, as texture has a direct influence on the oxygen diffusion in soil, thus establishing the conditions for optimal denitrifiers performance.

Materials and methods

Soil sampling

Soil samples were collected at eight potato fields across the Netherlands. Four sandy soils named Buinen (B), Valthermond (V), Droevendaal (D) and Wildekamp (W), and four clayey soils, Kollumerwaard (K), Steenharst (S), Grebbedijk (G) and Lelystad (L) were selected, all of them subjected to crop rotation, except for Wildekamp which is permanent grassland (Table S1). Bulk soil samples were collected five times: in September and November of 2009, and in April, June, and October of 2010. For each soil four replicates were taken. Each replicate consisted of ten subsamples (15–20 cm deep) collected between plots, away the roots with a spade. A total of 2 kg of each soil were thus collected in plastic bags and thoroughly homogenized before further processing in the lab. A 100-g subsample was used for measuring denitrifying enzyme activity and soil chemical properties on each replicate.

Denitrification enzyme activity

Potential denitrification enzyme activity (DEA) was measured over a short period by making all factors affecting denitrification non-limiting (Smith and Tiedje 1979; modified by Patra et al. 2005). Fresh soil (10 g equivalent oven-dried) was placed into 150 ml plasma flasks. The flasks were sealed with rubber stoppers and their atmosphere was evacuated and replaced by a 90:10 He:C_2H_2 mixture to provide anaerobic conditions and inhibit N_2O^- reductase activity. In each flask, 3 ml distilled water-containing KNO_3 (50 μg N-NO_3^- g^{-1} dry soil), glucose (0.5 mg C g^{-1} dry soil) and sodium glutamate (0.5 mg C g^{-1} dry soil) was added. N_2O efflux was measured in this flask after 2, 3, 4, 5 and 6 h, to make sure no *de novo* synthesis of denitrifying enzyme took place. Negative controls were performed without addition of soil. N_2O concentrations were analysed on a gas chromatograph equipped with a katharometer detector (microGC RS3000, SRA instruments, Marcy l'Etoile, France).

Soil chemical analysis

The soil pH was measured in a water suspension 1:4.5 (g/v) using an Inolab Level 1 pH-meter (WTW GmbH, Weilheim, Germany). Nitrate (NO_3^-) was determined colorimetrically in a solution of 0.01 M $CaCl_2$ with an Autoanalyzer II (Technicon Instrument Corporation, Tarrytown, New York) (samples from 2009) and in $CaCl_2$ extracts by a colorimetric method using the commercial kits Nanocolor Nitrat50 (detection limit, 0.3 mg N kg^{-1} dry weight, Macherey–Nagel, Germany) (samples from 2010). Briefly, for the extraction, 20 g of soil was overhead shaken for 45 min with 20 ml of 0.01 M $CaCl_2$. The extracts were filtered through a Millipore filter (pore size, 0.45 µm) and measured as described by the manufacturer. Water content was measured by comparison of fresh and dried (65 °C; 48 h) weight of samples. Organic matter (OM) content is calculated after 4 h at 550 °C.

Soil DNA extraction

For extraction of soil DNA, the PowerSoil DNA extraction kit (Mo Bio Laboratories Inc., New York) was used with 0.5 g of soil, according to the manufacturer's instructions, with the exception that the cells were disrupted by bead beating (glass beads diameter 0.1 mm; 0.25 g, mini-bead beater; BioSpec Products) three times for 60 s. To assess the quantity and purity, the crude DNA extracts were run on 1.5% agarose gels at 90 V for 1 h in 0.5 TAE buffer (20 mM Tris, 10 mM acetate, 0.5 mM EDTA; pH 8.0) using a fixed amount (5 ml) of a 1-kb DNA ladder (Promega, Leiden, the Netherlands) as the molecular size and quantity marker. After staining with ethidium bromide, DNA quality was determined based on the degree of DNA shearing (average molecular size) as well as the amounts of coextracted compounds.

Quantification of denitrifying communities

The abundance of denitrifiers was quantified by real time PCR (qPCR) targeting *nir*S, *nir*K and *nos*Z genes. The primer details and specific amplification conditions for each gene are show in Table S2 (Henry et al. 2006; Liu et al. 2003; Throbäck et al. 2004). Absolute quantification was carried out from each of the three soil replicates on the ABI Prism 7300 Cycler (Applied Biosystems, Germany). The specificity of the amplification products was confirmed by melting curve analysis, and the expected size (425 pb for *nir*S, 348 pb for *nir*K and 226 pb for *nos*Z) of the amplified fragments was checked in a 1.5% agarose gel stained with ethidium bromide. The standard curves were obtained using serial dilutions of the fragment vector plasmid, containing a cloned fragment of each gene. Inhibition in the PCR reactions was tested mixing serial dilutions of DNA extracted from soil with a known amount of standard DNA before qPCR. The absence of severe inhibition was confirmed when the Ct values of the standard DNA did not change in the presence of the diluted soil DNA.

Statistical analysis

All statistical analyses were conducted using R (R Development Core Team 2008). We assessed soil type and time dependent variation of soil parameters (pH, OM, NO_3^-, DEA and denitrifier abundances) with repeated measures ANOVA on the same subjects (sampling sites). The data of DEA and denitrifier abundances were log transformed to improve model fit. Normality of residuals was checked with the Shapiro–Wilk Test. Post hoc tests (Tukey Tests) were applied to determine group differences for significant factors. Moreover, to understand the variation of the data, we analyzed four dependent variables, DEA (in µg N_2O-N g^{-1} h^{-1} dw) and the log-transformed abundances of each gene (in gene copies g^{-1} dw). For each of these dependent variables, a general linear model was determined. The explanatory variables considered in these models were as follows: soil type (grouped by texture), date, location, nitrate in mg Kg^{-1} (NO_3^-), pH and organic material in % (OM). The starting model for each dependent variable included all explanatory variables. For each dependent variable, the corresponding model that best fits data was obtained by removal of non-significant variables using backward deletion tests. Normality of residuals was checked with the Shapiro–Wilk test. Furthermore, correlation coefficients between the soil characteristics and the relative abundances of the denitrifiers or DEA were calculated. Pearson correlations were used for normally distributed variables (according to the Shapiro–Wilk test) and Spearman rank correlations for non-normally distributed variables. For all analysis significance level was 0.05 and results show mean and standard error of the mean.

Results

General physicochemical characteristics of the soils

Soil chemical analyses were performed across a year (Table S3). The pH, OM and NO_3^- showed small but non-significant variations over time. Soil pH was significantly higher ($p < 0.001$) in the clayey (6.9 ± 0.20) than in the sandy soils (4.7 ± 0.08). The NO_3^- and OM were significantly higher ($p < 0.05$) in the sandy soils (50.0 ± 7.45 mg kg^{-1} and $6.2 \pm 1.18\%$, respectively) than in the clay soils (31.2 ± 6.36 mg kg^{-1} and $4.5 \pm 0.32\%$, respectively). This difference was apparently due to higher NO_3^- in site S, compared to the average in clayey soils, and higher OM in site V, compared to the average of sandy soils. Considering these sites as "outliers" in the sandy-clay classification, we repeated the analysis removing their data. As a result we still found higher values of NO_3^- in the sandy soils (43.1 ± 8.34 mg kg^{-1}) but without the data of site V, higher values of OM were observed in the clayey soils ($4.0 \pm 0.36\%$). Hence we decided to analyze the data with and without these sites carefully in the next sections.

Potential denitrification enzyme activity

The values of DEA ranged from 0.3 to 3.6 μg N_2O-N g^{-1} h^{-1} dw over all samples, being the values in the clayey soils higher ($p < 0.05$) at all sampling dates (mean values for clayey soils 1.6 ± 0.20 and 0.7 ± 0.10 μg N_2O-N g^{-1} h^{-1} dw for sandy soils) (Fig. 1). Throughout the sampling period there was no time effect, independently whether the analyses were done with the overall data or separately by soil type (Table 1). The sites with significantly higher DEA were V (sandy), G (clayey) and S (clayey) (Fig. S1).

In order to understand the effect of the sampling time, location, soil type (texture) and soil properties on DEA, general linear models were applied, and the corresponding models that best fit data are reported. The model considering the whole data set (DEA = pH + NO_3^-+OM + date + location + type of soil), showed that, soil type, location and OM explained a significant amount of the variation in DEA, in sum 62.02% (32.50, 15.72 and 13.80%, respectively), and pH, date and NO_3^- together explained only 26.90% of the variation (9.50, 8.90 and 8.50% respectively; Table 1). However, when the data were separated by soil type, variation in DEA in the clayey soils was explained by all parameters except NO_3^- (DEA clay soils = date + location + type of soil + pH + OM), but interestingly pH explained 29.30% of the variation (a higher percentage in comparison to the model considering all data). Besides, variation in DEA in the sandy soils was explained mostly by OM (44.20%) (DEA sandy soils = NO_3^-+OM + Location) (Table 1).

When we removed the data of sites V and S from our analysis we found that the trend of higher DEA values in the sandy soils still hold true (mean values for clayey soils 1.4 ± 0.19 and 0.4 ± 0.03 μg N_2O-N g^{-1} h^{-1} dw for sandy soils). However, results from the general linear models showed that, considering only six soils (without V and S), the effect of soil type was not

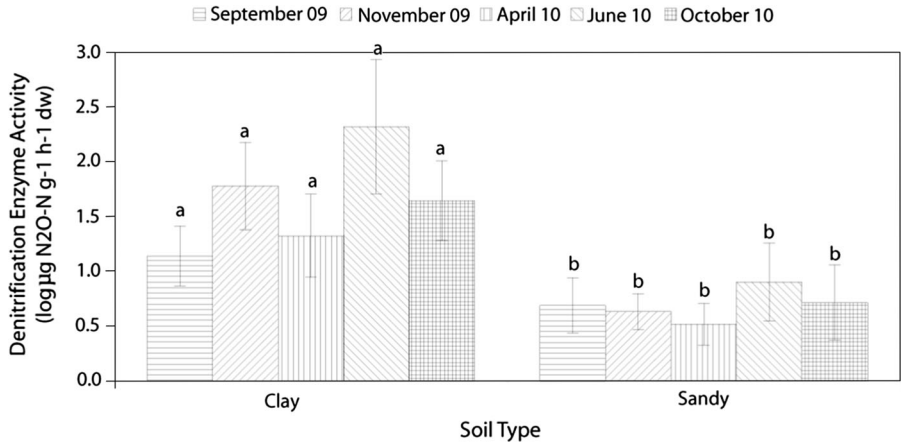

Fig. 1 Potential denitrification enzyme activity (DEA) for sandy and clay soils from potato fields across the Netherlands. Average values and standard *error bars* are shown for all sampling dates

Table 1 Summary of stepwise multiple linear regression results to determine which soil properties predict the denitrifiers abundance and activity considering the overall data (eight soils) and per type of soil

Source	*nos*Z				*nir*S				*nir*K				DEA			
	df	F	P	%SS	df	F	P	%SS	df	F	P	%SS	df	F	P	%SS
Overall data[a]																
pH	1,25	0.015	0.90		1,27	30.14	**<0.001**	16.60	1,26	3.56	0.07		1,25	21.64	**<0.001**	9.50
NO_3^-	1,33	5.26	**0.03**	5.70	1,26	1.73	0.20		1,27	11.7	**0.002**	2.93	1,25	19.30	**<0.001**	8.50
OM	1,33	12.32	**0.001**	13.40	1,25	0.067	0.80		1,25	0.47	0.49		1,25	31.20	**<0.001**	13.80
Date	4,33	10.40	**<0.001**	44.70	4,27	9.47	**<0.001**	20.86	4,27	78.34	**<0.001**	78.47	4,25	5.04	**0.004**	8.90
Soil location	7,26	1.13	0.37		6,27	8.72	**<0.001**	28.80	7,27	6.74	**<0.001**	11.80	6,25	5.94	**<0.001**	15.72
Type of soil	1,25	0.006	0.94		1,27	34.30	**<0.001**	18.90	1,27	0.58	0.45		1,25	73.70	**<0.001**	32.50
Clay[b]																
pH	1,9	0.03	0.85		1,15	19.52	**<0.001**	42.30	1,9	1.48	0.25		1,10	20.70	**0.001**	29.30
NO_3^-	1,14	4.30	0.06		1,9	3.04	0.10		1,10	17.30	**0.002**	9.50	1,9	0.43	0.52	
OM	1,10	0.0001	0.99		1,14	2.56	0.13		1,10	15.70	**0.002**	8.60	1,10	12.12	**0.006**	17.20
Date	4,15	7.15	**0.002**	65.60	4,10	2.98	0.07		4,10	17.90	**<0.001**	39.40	4,10	3.60	**0.04**	20.60
Soil location	3,11	0.83	0.50		3,15	3.80	**0.03**	24.90	3,10	22.40	**<0.001**	36.90	3,10	4.40	**0.03**	18.70
Sandy[c]																
pH	1,9	1.90	0.20		1,14	38.02	**<0.001**	54.70	1,10	32.60	**<0.001**	17.10	1,13	1.59	0.23	
NO_3^-	1,10	4.40	0.06		1,10	2.83	0.12		1,10	6.20	**0.03**	3.30	1,14	35.60	**<0.001**	18.90
OM	1,11	2.70	0.13		1,90	0.07	0.80		1,90	4.70	0.06		1,14	83.30	**<0.001**	44.20
Date	4,12	8.10	**0.01**	54.70	4,14	4.30	**0.01**	25	4,10	23.70	**<0.001**	49.70	4,90	0.75	0.57	
Soil location	3,12	4.90	**0.002**	25	3,11	1.70	0.22		3,10	15.70	**<0.001**	24.70	3,14	18.50	**<0.001**	29.40

The significant terms are in bold

%SS percentage of the total sum of squares explained by each variable

[a] Adjusted R^2: *nos*Z, 0.58; *nir*S, 0.78; *nir*K, 0.90; DEA, 0.82

[b] Adjusted R^2: *nos*Z, 0.56; *nir*S, 0.59; *nir*K, 0.89; DEA, 0.73

[c] Adjusted R^2: *nos*Z, 0.68; *nir*S, 0.72; *nir*K, 0.90; DEA, 0.90

significant and instead pH was the most explanatory variable (60.98%), followed by soil location (14.60%), OM (8.56%) and date (7.09%) (DEA = pH + OM + date + location). Separating by soil type, this dataset yielded also contrasting results, with soil location explaining 35.17% and OM 31.17% of variation in the clay soils (DEA clay soils = OM + location). Besides, pH explains 34.61% and soil location 27.69% of the variation in the sandy soils (DEA sandy soils = pH + Location; Table S4; Fig. S2). Differences observed in the results with and without extreme data suggest that the range of soil variation could interfere in the interpretation. However, it is worth noting that, independently, location explains around 30% of the variation in DEA, and the trend of a positive relationship between DEA and OM remains (Fig. S2).

Abundance of denitrifying communities

Overall, the population sizes for *nos*Z varied from 2.9×10^5 gene copies g^{-1} dw to 6.7×10^7 gene copies g^{-1} dw. The *nos*Z abundance increased significantly ($p < 0.001$) only from June to October of 2010 (5.4×10^6 gene copies g^{-1} dw to 1.7×10^7 gene copies g^{-1} dw; Fig. 2). However there were not significant differences between June 2010 and September–November of the previous year (2009; 3.3×10^6 and 2.7×10^6 gene copies g^{-1} dw, respectively). Comparing the soil types, there were not significant differences between clayey (2.9×10^6 gene copies g^{-1} dw) and sandy soils (3.1×10^6 gene copies g^{-1} dw), either considering all the eight soils or the six soils, without soils V and S (data not shown).

General linear models were also used to evaluate the effect of sampling time, location, soil type and physico-chemical variables on the abundance of denitrifiers, whereas correlation tests were used to evaluate the relationship between DEA and gene abundance. DEA is measured with acethylene to inhibit N_2O reductase activity, thus results only from the NIR abundances. Therefore, we did not test the relation between *nos*Z gene abundance and DEA. General linear model obtained from data of all eight soils (*nos*Z abundance = date + OM + NO_3^-) indicated that almost half of the variation in *nos*Z gene abundance was explained by temporal variation (44.70%; Table 1), which was also significant for both models of the clayey (65.60%; *nos*Z abundance in clayey soils = date) and sandy (54.70%; *nos*Z abundance in sandy soils = date + location) soils. The physico-chemical variables that explained part of the variance of the *nos*Z gene abundance in the overall data set were OM (13.40%) and nitrate (5.70%). When separating by soil type, none of the physico-chemical parameters explain changes in *nos*Z abundance (Table 1). When we removed soils V and S, we observed that the effect of OM was no longer significant (*nos*Z abundance = date + NO_3^-) for all data, and that all soil parameters except location were able to explain variations in *nos*Z abundance in the clayey soils (*nos*Z abundance in clayey soils = pH + NO_3^-+OM + date), but none of them explained the variation in the sandy ones. However Date and Location were close to be significant in the analysis (Table S4; Fig. S3). In this case, date was the variable that consistently explained the majority of the variation in the *nos*Z abundance in almost all models.

Regarding the abundance of *nir*S gene, the community size varied from 1×10^3 to 2.6×10^6 gene copies g^{-1} dw when considering the overall

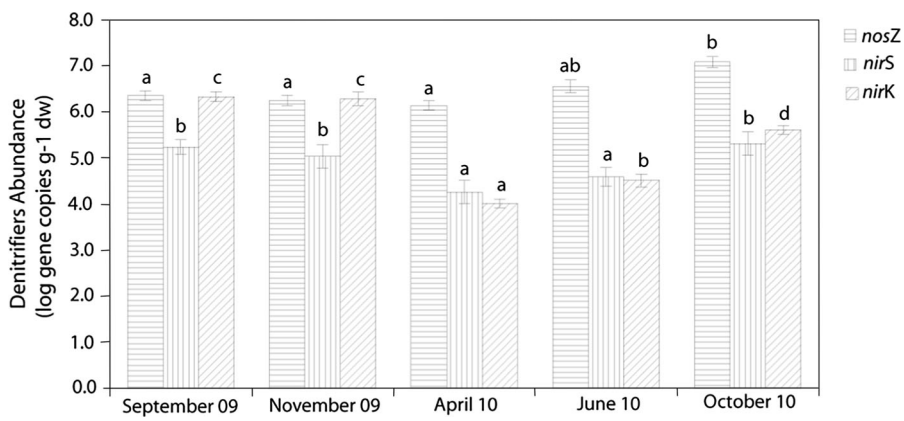

Fig. 2 Real-time quantification of *nos*Z, *nir*S and *nir*K denitrifiers across the sampling period in potato fields. Average between sandy and clay soils and standard errors are shown for each sampling date

data (Fig. 2). Analyses across the sampling period disclosed that the *nir*S gene were less abundant during the beginning of the growing season (5.8×10^4 gene copies g^{-1} dw in April and 6.8×10^4 gene copies g^{-1} dw in June of 2010) and more abundant close to the end of agricultural season (3.1×10^5 gene copies g^{-1} dw in September 2009, 3.5×10^5 gene copies g^{-1} dw in November 2009 and 5×10^5 gene copies g^{-1} dw in October 2010). Classifying the soils by texture revealed that there were not significant differences between clayey (9.5×10^5 gene copies g^{-1} dw) and sandy soils (5.6×10^5 gene copies g^{-1} dw). General linear model considering the overall dataset showed that *nir*S abundance was mostly explained by soil location (28.80%), date (20.86%), soil type (18.90%) and pH (16.60%; *nir*S abundance = location + date + type of soil + pH; Table 1). After removing soils V and S we observed that all variables, except nitrate, had an effect on *nir*S gene abundance (Table S4; *nir*S abundance = location + date + type of soil + pH + OM).

Separating the data according to soil type revealed that pH explained *nir*S abundance in both clayey (42.30%) and sandy (54.70%) soils, when all eight soils were considered (*nir*S abundance in clay soils = location + pH; *nir*S abundance in sandy soils = date + pH). However, when we removed soils V and S, the effect of pH was no longer significant in the clayey soils (*nir*S abundance in clay soils = date + location + OM), however, it was even stronger in the sandy soils (56.68%; Table S4; *nir*S abundance in sandy soils = date + pH + NO_3^-). In general the pH, site location and Date showed an influence over *nir*S abundance. Additionally we found no correlation between *nir*S gene abundance and DEA (Table S5), either considering the eight soils or the six soils (without V and S).

The total copy numbers of *nir*K gene varied from 1.7×10^3 to 1.6×10^7 gene copies g^{-1} dw. As in the case of *nir*S, variations in the abundance of *nir*K gene were found across the sampling period (Fig. 2). The *nir*K gene abundance was significantly lower in April 2010 (1.5×10^5 gene copies g^{-1} dw), increasing gradually to the end of agricultural season. The *nir*K abundance in June 2010 (5.4×10^4 gene copies g^{-1} dw) increased significantly towards October of the same year (5.4×10^5 gene copies g^{-1} dw). Moreover, maximum abundances for this gene were found in September and November of the previous year (3.5×10^6 and 3.3×10^6 gene copies g^{-1} dw, respectively). Grouping of the soils according to their texture revealed that there were not significant differences in the size of *nir*K community between clayey and sandy soils. General linear model considering the entire dataset indicated that *nir*K abundance was largely explained by temporal variation (78.47%; Table 1; *nir*K abundance = date + location + NO_3^-), and this was higher in the sandy (49.70%; *nir*K abundance in sandy soils = date + location + pH + NO_3^-) than in the clayey soils (39.40%; *nir*K abundance in clay soils = date + location + OM + NO_3^-). The same trend was observed when considering only six soils (*nir*K abundance = date + type of soil + OM + pH + NO_3^-) with a larger percentage of variation explained by date for both clayey (43.85%; *nir*K abundance in sandy soils = date + pH + NO_3) and sandy soils (66.17%; Table S4; n*ir*K abundance in clayey soils = date + OM + pH + NO_3^-). Based on the overall dataset, the only physicochemical parameter that explained changes in *nir*K gene abundance was nitrate (2.93%; Table 1). Moreover, in the clayey soils *nir*K gene abundance was explained by nitrate (9.50%) and OM (8.50%), whereas in the sandy soils it was soil pH (17.10%) and nitrate (3.30%; Table 1). When we removed the data from soils V and S from the analysis we found that these soils were masking the effect of OM (20.25%) and pH (1.73%), especially in the clayey soils (Table S4). Similarly to the results observed for *nir*S, the abundance of *nir*K communities was not correlated with DEA (Tables S5, S6).

Discussion

The aim of this work was to evaluate the temporal variations in denitrifying activities and in the abundances of the different subsets of denitrifiers, i.e. the *nir*S-, *nir*K- and *nos*Z- harboring communities, in agricultural soils. Moreover, we were interested in determining which soil parameters, i.e. soil type, pH, NO_3^- and/or OM would have a stronger influence over the size and activity of these communities. Because two of the soils (V and S) presented soil properties (OM and NO_3^-) out of the mean that could bias our classification of sandy and clay, we performed all the analyses twice, one with all eight soils and one with six soils.

Environmental conditions change throughout the year and rates of denitrification are expected to change in consequence. Temperature is one of the major factors controlling denitrification in field samples (Dorland and Beauchamp 1991). Our results showed no significant differences in DEA across our sampling period, although a tendency to increase in DEA was detected in the summer. In fact, the temporal variation in DEA was very low (about 9.0% using the whole dataset). Nevertheless, the values for DEA found here provide an indication of the potential denitrification rates in the respective soils, being of the same order of magnitude in previously reported rates in agricultural fields (D'Haene et al. 2003; Attard et al. 2011). Previous studies that used the same method to measure DEA have reported seasonal changes in denitrification rates with a peak in the mid-summer (Strauss et al. 2006; Wallenstein et al. 2006; McGill et al. 2010). However, the low variation in DEA through time in our sampling sites could be due to the sensitivity of the method used to assess denitrification, which provides optimal denitrifying conditions and therefore might lead to more constant DEA over time than in situ measurements (Groffman et al. 1999; Morse et al. 2012).

Our data suggest that DEA was not a good indicator for N_2O flux patterns across land uses, but in situ N_2O fluxes measurements are necessary to corroborate this hypothesis. Despite the small variations detected, our data showed that, considering all eight soils, the most important factor explaining the variability observed in denitrification across all locations was soil type. However, when we removed the two soils V and S, we found that this observation did not hold true, and the effect of pH appeared as the main driver of denitrification rates, as we had previously hypothesized and it was previously reported (Firestone and Davidson 1989; Knowles 1982; Van den Heuvel et al. 2011). Therefore, when defining such classification, care must be taken, especially when dealing with soils that vary enormously in terms of physico-chemical characteristics. This does not mean that general patterns cannot be recovered from a large region data, but there are sites with especial characteristics that can mask the patterns.

Indeed, soil pH has been considered a master variable that influence N transformations (Mørkved et al. 2007; Baggs et al. 2010), and an important control on denitrification through enzyme sensitivity (Firestone and Davidson 1989; Van den Heuvel et al. 2011). A pH between 7.0 and 8.0 has been suggested as optimum for denitrification (Knowles 1982). In particular, it has been suggested that denitrification could be reduced in soils with acid pH due to a decrease in organic carbon and mineral nitrogen that are available for denitrifying populations under this condition, rather than to direct toxicity over the denitrifying reductases (Šimek and Cooper 2002). Organic carbon availability is one of the most important factors affecting denitrification activity in soils (Although soil type did not significantly influence denitrification in the model without soils V and S, we did observe higher rates of DEA in clayey than in sandy soils. These results were expected as, providing that electron receptors and donors are not limiting, fine textured and structured soils provide favorable conditions for denitrification as their smaller pores are more prone to water saturation (D'Haene et al. 2003; Gu and Riley 2010).

The level of temporal fluctuations in the abundance of the denitrifier communities varied considerably depending on the targeted gene, although the population sizes of denitrifying bacteria, quantified by qPCR targeting the genes *nos*Z, *nir*S and *nir*K were in agreement with the ranges observed for other agricultural soils (Henry et al. 2004, 2008; Chroňáková et al. 2009). As we previously hypothesized, the *nos*Z and *nir*K harboring communities showed the highest temporal variability, as around 50 and 60% of the differences in *nos*Z and *nir*K abundance, respectively, could be explained by sampling time, whereas the *nir*S gene varied only about 20%. The *nir* gene types are functionally and physiologically equivalent (Glockner et al. 1993), but studies indicate that they might not be ecologically comparable (Jones and Hallin 2010; Attard et al. 2011), possibly having different habitat preferences. The ecologically distinct role of *nir*K and *nir*S communities has been also observed in cropping systems where changes in denitrification were related to the abundance of *nir*K rather than *nir*S-harboring bacteria (Attard et al. 2011). In addition to the differences in temporal variation, we also observed differential response to physical–chemical parameters among *nir* types. For instance, the abundance of *nir*S-harboring communities was strongly affected by pH, whereas *nir*K abundance was slightly correlated with nitrate. The lack of effect of nitrate content on *nir*S gene abundance was also reported for glacier foreland (Kandeler et al. 2006) and farmland (Enwall et al.

2010). This is consistent with the idea that the abundance of denitrifying communities is primarily controlled by other factors rather than nitrate concentration (Mergel et al. 2001; Wallenstein et al. 2006), and together, these results corroborate the fact that *nir*K and *nir*S harboring communities are adapted to different niches in soils (Smith and Ogram 2008; Yoshida et al. 2010).

Overall, our results indicate that *nir*K harboring communities are more responsive to temporal changes than to any soil parameter measured in this study, as temporal variation explained more than two-thirds of their variation. The ecologically distinct role of *nir*K and *nir*S communities has been also observed in cropping systems where changes in denitrification were related to the abundance of *nir*K rather than *nir*S-harboring bacteria (Attard et al. 2011). The *nos*Z gene abundances showed intermediate temporal fluctuations when compared to nitrite-reductase genes, which might reflect the large range of bacterial and archaeal phyla harboring this gene (Green et al. 2010; Jones et al. 2011), thus corroborating our initial hypothesis. (Green et al. 2010; Jones et al. 2011). Our analysis provided evidence that the amount of nitrate had the largest influence in the overall *nos*Z abundance.

The temporal variability observed for the different denitrifying communities in our study did not follow any pattern related to plant development, as previously observed (Dandie et al. 2011) except that all three genes analyzed showed lower abundances on April 2010, which corresponds with the beginning of the growing season. Although it is not possible to pinpoint why abundances dropped from November 2009 to April 2010, we could speculate that the changes the soil went through during winter and early spring, both in terms of temperature and agricultural practices used to prepare the soil, such as ploughing, could potentially affect the abundances of denitrifies. It is interesting to note that this drop in abundances was not observed for total bacterial abundance in the same soil samples (Pereira e Silva et al. 2012), and in fact, total bacterial abundances increased linearly from September 2009 to October 2010. Overall, our results indicate that the abundance of denitrifiers increased towards the end of the growing season, most likely due to higher water saturation and the lack of competition with the plants for nitrate. In addition, *nos*Z gene can be less affected by environmental factors than other genes of the denitrification pathway (Wallenstein et al. 2006; Chroňáková et al. 2009), based on the final model with six soils, thus removing outliers.

Overall, no significant correlations were found between DEA and the community size of denitrifiers, with activity rates being driven mostly by soil environmental parameters, as observed by Attard et al. (2011). Previous studies did not find relationships between denitrifier abundance in soils and N_2O production (Dandie et al. 2008; Miller et al. 2008; Baudoin et al. 2009; Djigal et al. 2010; Henderson et al. 2010; Song et al. 2012; Attard et al. 2011). On the contrary, *nos*Z gene abundance was significantly correlated to DEA in a long-term fertilization experiment (50 years) (Hallin et al. 2009), in a grassland field subjected to different cattle grazing regimes (Philippot et al. 2009) and across Alaskan ecosystem types (Petersen et al. 2012), whereas *nir*S and *nir*K gene abundances have previously been found to correlate to DEA (Chroňáková et al. 2009; Dong et al. 2009; Cuhel et al. 2010). It has been recently suggested (Petersen et al. 2012) that abundances of functional genes are not suitable for predicting small changes in DEA. Indeed the low temporal variation we observed in DEA in the overall dataset might have hampered the correlation. Moreover, we do not discard the possibility of the number of denitrifiers was underestimated, even though we used degenerate primers, which had shown a wide range of affinity for different templates (Liu et al. 2003; Throbäck et al. 2004; Henry et al. 2006). Finally, Our data support the idea that, in certain soil types, the rates of denitrification may be decoupled from the density of the denitrifier communities, being the environmental conditions the major drivers (Dandie et al. 2008; Philippot et al. 2009; Henderson et al. 2010; Attard et al. 2011).

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Conflict of interest The authors declare no conflict of interest.

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