

Field-Scale Pattern of Denitrifying Microorganisms and N₂O Emission Rates Indicate a High Potential for Complete Denitrification in an Agriculturally Used Organic Soil

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Abstract More than 50% of all anthropogenic N₂O emissions come from the soil. Drained Histosols that are used for agricultural purposes are particularly potent sources of denitrification due to higher stocks of organic matter and fertiliser application. However, conditions that favour denitrification can vary considerably across a field and change significantly throughout the year. Spatial and temporal denitrifier dynamics were assessed in a drained, intensely managed Histosol by focusing on the genetic nitrite and N₂O reduction potential derived from the abundance of *nirK*, *nirS* and *nosZ* genes. These data were correlated with soil properties at two different points in time in 2013. N₂O emissions were measured every 2 weeks over three vegetation periods (2012–2014). Very low N₂O emission rates were measured throughout the entire period of investigation in accordance with the geostatistical data that revealed an abundance of microbes carrying the N₂O reductase gene *nosZ*. This, along with neutral soil pH values,

is indicative of high microbial denitrification potential. While the distribution of the microbial communities was strongly influenced by total organic carbon and nitrogen pools in March, the spatial distribution pattern was not related to the distribution of soil properties in October, when higher nutrient availability was observed. Different nitrite reducer groups prevailed in spring and autumn. While *nirS*, followed by *nosZ* and *nirK*, was most abundant in March, the latter was the dominant nitrite reductase in October.

Keywords Denitrification · Organic soil · Geostatistic · *nirK* · *nirS* · *nosZ* · Histosol

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Nitrous oxide (N₂O) is a potent greenhouse gas. More than 50% of all anthropogenic N₂O is emitted from agricultural soil [1]. Histosols that are intensively farmed release large amounts of N₂O. Drainage, cultivation and fertilisation enhance the mineralisation of organic N in soil organic matter (SOC) and its subsequent denitrification into N gases. In Europe, up to 17% of N₂O emitted from agricultural soils can be assigned to organic soils, i.e. soils with a total organic carbon (TOC) content of >12% [2]. A decrease in N₂O emissions can be achieved by reducing the denitrification rates either in general or by minimising the N₂O/N₂ ratio during denitrification. Both processes depend on the presence of oxygen, total nitrogen content (N_t), TOC content and soil pH [3]. Numerous studies have observed that the spatial distribution of denitrifying microbes in soils varies considerably and that the denitrifier community's spatial patterns of size and activity correlate strongly with environmental factors [4, 5]. This might be due to (i) the niche partitioning of microbes containing copper (encoded by *nirK*) or cytochrome cd₁ (encoded by *nirS*) nitrite reductases, (ii) the fact that one third of all denitrifying microbes use truncated denitrification pathways

Table 1 Primer and thermal profiles used for real-time PCR quantification of the genes *nirS*, *nirK* and *nosZ*. Primers were purchased from Metabion, Germany and dimethylsulfoxide (DMSO) from Sigma, Germany. Per-reaction primer and DMSO amounts are given in microliters

Target gene	Source of standard	Thermal profile	No. of cycles	Primer source	Primer (10 µM)	DMSO
<i>nirS</i>	<i>Pseudomonas stutzeri</i>	95 °C-15 s/57 °C-30s/72 °C-30s	40	cd3aF, R3cd [28, 29]	0.5	0.625
<i>nirK</i>	<i>Azospirillum irakense</i>	95 °C-15 s/63 °C-30s/72 °C-30s	5 ^a	nirK876, nirK5R	0.5	0.625
		95 °C-15 s/58 °C-30s/72 °C-30s	40	[30, 31]		
<i>nosZ</i>	<i>Pseudomonas fluorescenz</i>	95 °C-30s/65 °C-30s/72 °C-30s	5 ^a	nosZ2F, nosZ2R [32]	0.5	-
		95 °C-15 s/60 °C-15 s/72 °C-30s	40			

as they lack the nitrous oxide reductase enzyme (encoded by *nosZ*), (iii) the time of sampling as the availability of alternative electron acceptors and donors can vary considerably throughout the year [6] or (iv) the interaction of different environmental factors [3]. However, most of these studies were performed in soils with a carbon content of less than 5%. Thus, it is still not known which are the major drivers of denitrification in soils with a high TOC content and whether a spatial correlation pattern exists. By targeting the *nirK*, *nirS* and *nosZ* genes, the current study compares spatio-temporal distribution of denitrifying microbes in an agricultural soil with high organic matter content at the beginning (March) and end (October) of the vegetation period. Abundance data from the individual functional groups were correlated to soil properties that have an effect on denitrification processes (TOC, N_t, NO₃⁻, NH₄⁺, pH and water content). Moreover, the N₂O fluxes were monitored over three consecutive years (2012–2014). The findings suggest that the high TOC content (i) leads to the emission of high amounts of N₂O and that (ii) the availability of nitrate drives the spatial pattern of denitrifying microbes.

N₂O fluxes were measured every 2 weeks from January 2012 until December 2014 using closed chambers

(3 × 0.5625 m²). At each sampling date, four gas samples were taken from each chamber at 20-min intervals. N₂O was analysed and calculated as described by Flessa et al. [7]. The soil samples used for the geostatistical analyses were taken from the upper 5 cm of a Histosol used for agricultural purposes in the Upper Rhine Valley (Germany) (49° 10' 12' N, 8° 28' 6' E) based on an orthogonal grid (50 × 60 m). Field properties and grid design are summarised in Table S1 and Fig. S1. All soil samples (106 collected in March and 106 in October) were stored at -80 and -6 °C and used for the analysis of denitrifiers and soil properties. The TOC and N_t contents were measured with the Elemental Analyser “Euro EA” (Eurovector, Italy). NH₄⁺ and NO₃⁻ were extracted with 0.01 M CaCl₂ from bulk soil with an extracting agent:soil ratio of 4:1 and analysed using the FIAstar 5000 continuous flow analyser (FOSS, Denmark). Nucleic acids were extracted using the phenol-chloroform based extraction protocol described by Töwe et al. [8] and stored at -80 °C until further use. The number of genes involved in nitrite and N₂O reduction was determined using real-time PCR (qPCR) [9] (Table 1). Pre-experiments indicated an inhibition-free amplification when DNA extracts were diluted 1:8 (March) or 1:16 (October, data not shown). The amplification efficiencies were

Table 2 Median, mean values, standard deviation and coefficient of variance of all parameters analysed (*n* = 106) as well as their range values and sill-to-nugget ratios (referred to as Cs/(Co + Cs)) derived from geostatistical analyses. Missing values indicate the absence of spatial correlation

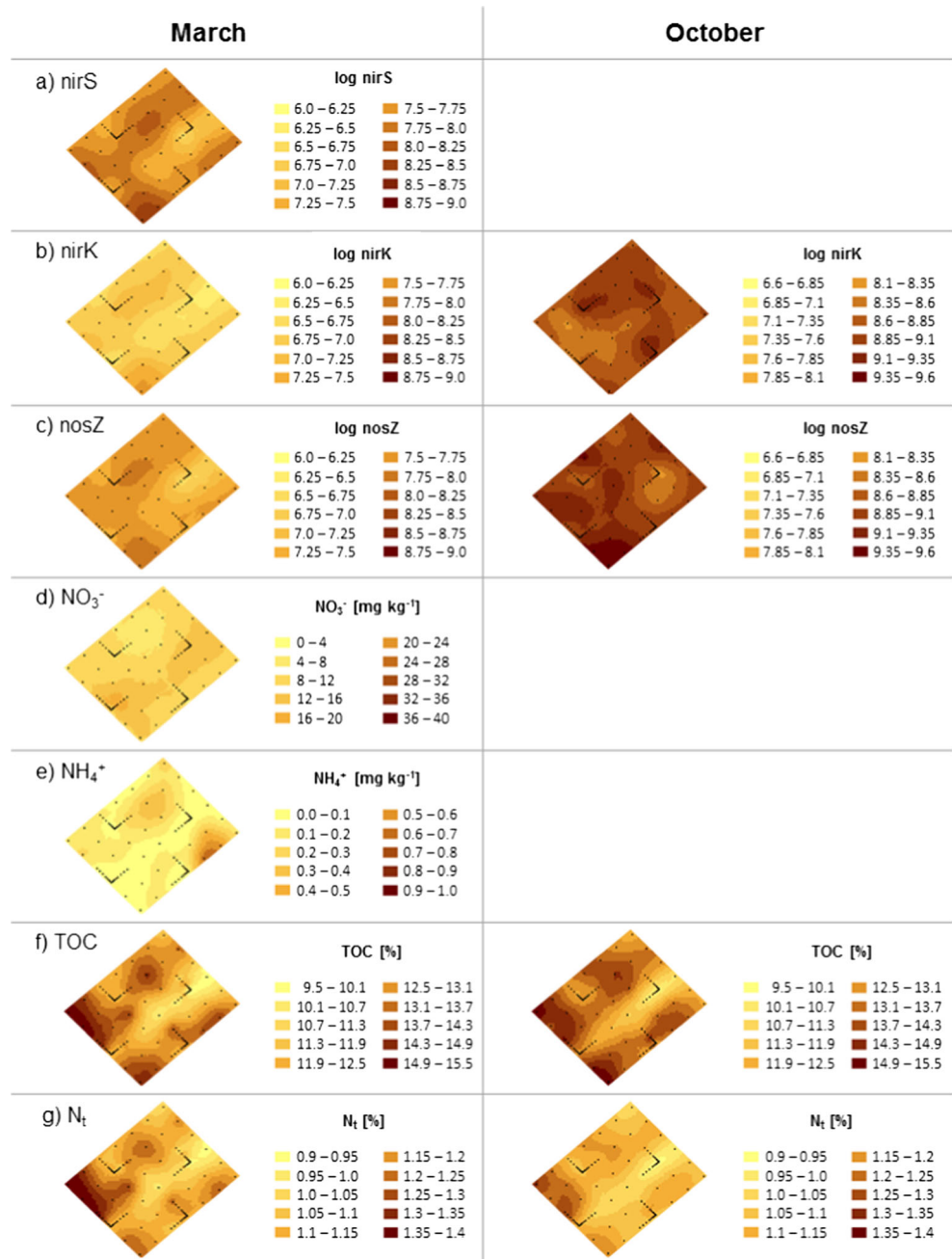
	pH	TOC mg g ⁻¹	N mg g ⁻¹	C/N	NO ₃ mg kg ⁻¹	NH ₄ mg kg ⁻¹	DNA µg g ⁻¹	<i>nirK</i> gene copies g ⁻¹ soil	<i>nirS</i> gene copies g ⁻¹ soil	<i>nosZ</i>	<i>nirK</i> + <i>nirS</i>
March											
Median	7.6	125.2	11.2	11.0	11.8	0.02	9.3	6.9 × 10 ⁶	4.0 × 10 ⁷	3.4 × 10 ⁷	5.0 × 10 ⁷
Mean	7.6	124.2	11.3	11.0	11.6	0.09	10.8	9.5 × 10 ⁶	6.0 × 10 ⁷	4.0 × 10 ⁷	7.0 × 10 ⁷
SD	0.1	12.9	1.0	0.4	5.5	0.15	7.3	9.1 × 10 ⁶	6.5 × 10 ⁷	3.0 × 10 ⁷	7.2 × 10 ⁷
Range (m)	16.7	23.9	19.3	25.7	35.9	21.9	22.7	13.1	21.2	17.0	20.9
Cs/(Co + Cs) (%)	52.5	95.9	100	84.7	38.5	70.3	64.5	85.0	96.0	84.7	95.5
October											
Median	7	129	11	11.7	8.8	1.6	56.7	1.1 × 10 ⁹	2.1 × 10 ⁸	7.7 × 10 ⁸	1.4 × 10 ⁹
Mean	7	127.3	10.9	11.7	20.3	3.4	58.5	1.2 × 10 ⁹	2.6 × 10 ⁸	1.2 × 10 ⁹	1.4 × 10 ⁹
SD	0.2	13.5	0.9	0.5	26.3	5.6	25.9	8.6 × 10 ⁸	2.2 × 10 ⁸	1.3 × 10 ⁹	9.5 × 10 ⁸
Range (m)	22.8	22.3	18.0	26.2			32.1	20.9		21.3	26.6
Cs/(Co + Cs) (%)	64.1	90.7	83.9	67.1			31.4	46.0		55.0	32.0

calculated using the equation $\text{Eff} = [10^{(1/\text{slope})} - 1]$: *nirS*: 80–92%, *nirK*: 86–88% and *nosZ*: 73–83%. All standard curves were linear ($r^2 > 0.99$) over 6 orders of magnitude. For data interpretation, correlation analyses were calculated using Spearman's rank correlation coefficients (Sigma Plot 11.0, Systat Software Inc., USA). The spatial behaviour of TOC, N_t , NH_4^+ , NO_3^- , *nirS*, *nirK* and *nosZ* data was analysed with the R software version 3.0.2 in combination with the gstat software package [10–13]. *nirS*, *nirK* and *nosZ* abundance data were log transformed for geostatistical analyses. Omnidirectional experimental variograms were calculated and a spherical model was fitted to each experimental

variogram. Exponential models were tested when no spherical model could be fitted. If no model could be fitted, either the parameter under investigation was homogeneously distributed or the spatial distribution was independent of the scale chosen and thus could not be visualised using kriged maps. In order to display the spatial distribution of selected soil properties, thematic maps were created with an ordinary kriging procedure (Arc Map 10.0, ESRI® 2010, Germany) using the variogram parameters obtained with gstat.

The mean annual N_2O emissions were very low ($5.4 \mu\text{g N m}^{-2} \text{h}^{-1}$) throughout the entire investigation period from 2012 to 2014. Episodic peak emissions only occurred

Fig. 1 Kriged maps showing the spatial distribution of **a** a nitrite reductase (cytochrome cd_1) encoded by the *nirS* gene and **b** a Cu-containing enzyme encoded by the *nirK* gene, **c** a N_2O reductase (*nosZ* gene, gene copy numbers were related to g soil), **d** NO_3^- , **e** NH_4^+ , **f** TOC and **g** N_t in March and October 2013. Kriging could only be performed when the parameters reflected a spatial correlation (see Table 2)



directly after fertilisation (up to $800 \mu\text{g N m}^{-2} \text{h}^{-1}$) (Fig. S2). This observation is in contrast to data obtained by other studies [14]. Geostatistical and correlation analyses were carried out in 2013 to gain a deeper understanding of why only low amounts of N_2O were emitted. qPCR data revealed that the abundance of nitrite- and N_2O -reducing microbes was quite similar (Table 2). This observation, too, is in contrast to other studies, which found that the N_2O reductase gene *nosZ* was less abundant than the nitrite reductase genes [5, 15]. This suggests that microbes in the soil under investigation are able to perform the entire denitrification pathway, which in turn explains the lower N_2O emissions observed. This assumption is substantiated by a soil pH of 7.6 in March and of 7 in October. These pH values are in the optimal pH range for denitrification and favour the production of N_2 over N_2O . The latter is more prominent under acidic conditions [3]. Moreover, drained organic soils are usually characterised by high TOC content and high water-holding capacity, thus providing optimal conditions for denitrification throughout the year [16]. Most samples taken from the soil under investigation had a water content of between 45 and 60% (w/w) (data not shown) and a TOC content of above 12% and were thus characterised by favourable denitrification conditions.

Besides the observation of low N_2O emissions, the spatial and correlation analyses differed considerably between the

samples taken in March and October. In March, the abundance of denitrifiers was highly correlated ($R^2 > 0.8$, $p < 0.001$) and indicated a spatial coexistence of microbes harbouring *nirK* and *nirS* (Fig. 1, Table 3). This is in contrast to other geostatistical studies that observed niche partitioning between the two groups [4, 5, 17]. It can therefore be assumed that in drained organic soils the overall high denitrification capacity surpasses the niche preferences of the two nitrite reducer groups. Although the *nirS*- and *nirK*-type nitrite reducers formed the same spatial pattern, *nirS*-type nitrite reducers were one order of magnitude more abundant, something that has also been reported for constructed wetlands [18, 19]. Moreover, the abundance of nitrite- and N_2O -reducing microbes strongly correlated with TOC and N_t concentrations. Low range values and very high sill-to-nugget ratios further suggest a high spatial dependency of the microbes. The labile nitrogen compounds NO_3^- and NH_4^+ showed opposite trends. While NH_4^+ correlated positively with the abundance of denitrifying microbes, NO_3^- correlated negatively, even though NO_3^- is the direct denitrification substrate and regarded as a major driver of the denitrification processes. However, it must be taken into account that the NO_3^- pool is very dynamic and to a large extent affected by the NO_3^- uptake by plants and leaching to groundwater. Furthermore, the role of NO_3^- ammonification in this type of soil is still poorly understood.

Table 3 Spearman correlations between soil parameters and gene abundances. Numbers that were significant are bolded

	NH_4^+	DNA	<i>nirK</i>	<i>nirS</i>	<i>nosZ</i>	<i>nirK</i> + <i>nirS</i>	pH	N_t	TOC	C/N
March										
NO_3^-	-0.717***	-0.438***	-0.422***	-0.434***	-0.434***	-0.433***	-0.147	-0.225*	-0.280**	-0.338***
NH_4^+			0.396***	0.398***	0.418***	0.399***	0.125	0.296**	0.344***	0.341***
DNA			0.909***	0.822***	0.854***	0.852***	-0.091	0.442***	0.517***	0.545***
<i>nirK</i>				0.795***	0.849***	0.839***	-0.076	0.391***	0.487***	0.565***
<i>nirS</i>					0.919***	0.993***	-0.002	0.490***	0.541***	0.511***
<i>nosZ</i>						0.932***	-0.072	0.532***	0.586***	0.536***
<i>nirK</i> + <i>S</i>							-0.031	0.478***	0.532***	0.517***
pH								-0.066	-0.007	0.062
N_t									0.941***	0.398***
TOC										0.610***
October										
NO_3^-	-0.431***	0.163	-0.152	-0.031	0.180	-0.140	0.079	0.155	0.269**	0.311**
NH_4^+		-0.128	0.168	0.113	0.122	0.162	-0.047	0.162	0.206*	0.176
DNA			-0.005	0.266**	0.279**	0.062	0.016	0.199*	0.213*	0.096
<i>nirK</i>				0.363***	-0.039	0.978***	0.046	0.027	-0.031	0.085
<i>nirS</i>					0.273**	0.523***	-0.114	0.083	0.100	0.214*
<i>nosZ</i>						0.015	-0.063	0.357***	0.491***	0.520***
<i>nirK</i> + <i>S</i>							0.025	0.045	-0.007	0.118
pH								-0.023	0.000	0.024
N_t									0.873***	0.250**
TOC										0.608***

All labile soil parameters were higher in October. In addition, the microbial gene abundance pattern also differed between April and October. These results are in line with many other seasonally resolved studies [20–22] and reflect the presence of more favourable growth conditions (e.g. higher temperatures, availability of easily decomposable substrates due to fertilisation, root exudates, retention of crop residues and root biomass) during the field management season. However, while the number of microbes with the *nirS*-type nitrite reductase gene only doubled during the growing season, the number of microbes with the *nirK*-type nitrite reductase gene increased from 9.6×10^6 to as many as 1.2×10^9 copies g^{-1} dwt and, thus, out-competed their *nirS* counterparts (Table 2). Although a clear niche separation of the two groups as suggested by other studies [9, 17, 23] was not observed, the high abundance of *nirK*-type microbes can be attributed to higher than normal nutrient availability. This is in line with other studies that have found a sensitive response by *nirK*-type microbes to environmental changes [24] and their preference for habitats with easily degradable nutrients [17, 25–27]. This observation was further underlined by the fact that the correlation of *nirK* and the slowly degradable carbon and nitrogen pools (TOC or N_t) decreased from an R^2 of 0.49 and 0.39 ($p < 0.001$) in March, respectively, to R^2 of <0.03 in October.

In conclusion, our data indicate that the high denitrification potential of this drained organic soil prevents the distinct niche separation of microbes harbouring either *nirK*- and *nirS*-type nitrite reductase genes and favours microbes that possess *nosZ* genes, thus leading to the emission of N_2 rather than N_2O . Future studies will need to clarify whether these results are site specific or whether they change in the presence of different crops, pH values or management practices. Our study was based on genes that provide information on the genetic potential and gene expression of microbial communities and that reflect long-term changes. The analysis of transcripts may provide a key to understanding the complexity of microbial activity and variability of $\text{N}_2/\text{N}_2\text{O}$ fluxes. It can be expected that the spatial and temporal variability $\text{N}_2/\text{N}_2\text{O}$ fluxes will also require adapting sampling strategies to varying conditions.

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