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Effect of pH and dissolved organic matter on the abundance of *nirK* and *nirS* denitrifiers in spruce forest soil

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Abstract Acid N depositions in the Bohemian Forest during the second half of the last century caused enormous soil acidification which led to the leaching of essential nutrients including nitrates. We investigated the effect of dissolved organic matter (DOM) and pH on the abundance of 16S RDNA, *nirK* and *nirS* gene copies in four spruce forest sites. Soil samples for molecular based quantification (qPCR) were taken from the organic litter and humus layers. The amounts of dissolved organic carbon (DOC) and dissolved nitrogen (DN) were much lower in highly acidified soils. We found a strong correlation between *nirK* denitrifiers and the amount of available P ($r = 0.83$, $p < 0.001$), which suggested a higher nutrient sensitivity of this group of denitrifying bacteria. Additionally, we found that correlations between the amount of *nirK* denitrifiers and DOC and pH are exponentional showing two important threshold values, being 4.8 mol kg^{-1} and 5, respectively. The amount of *nirK* denitrifiers rapidly decreased below these values. The amount of *nirK* and *nirS* denitrifiers was higher in the organic litter horizon than the organic humus horizon at all sampling sites.

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Introduction

Spruce forest soils in Central Europe were exposed to high nitrogen (N) depositions during the second half of the last century. Atmospheric N deposition is the major anthropogenic source of N in terrestrial ecosystems of the United States, Asia and Europe (Galloway et al. 2003; Galloway and Cowling 2002; Waldrop and Zak 2006). Because of N limitation in temperate forests, additional N input can stimulate plant growth and increase aboveground carbon (C) storage (Nadelhoffer et al. 1999; Magill et al. 1997; Waldrop and Zak 2006). However, high N depositions can decrease soil pH, which leads to reduction of the soil acid neutralizing capacity and leaching of a plant's essential cations (Ca^{2+}, Mg^{2+} and K^{+}) (Dauer et al. 2007; Tomlinson 2003; Pawłowski 1997) and NO_3^- from the forest soils (Kopácek et al. 2002a, b; Šantrůčková et al. 2006). One of the reasons for higher NO_3^- leaching could be the reduction in the amount of denitrifying bacteria in the acid forest soils. However, direct evidence is missing.

Denitrification is the main anaerobic biotic process leading to loses of fixed N as well as removal of excess soluble nitrate (NO_3^-) and nitrite (NO_2^-)

from the soil environment. The second step in denitrification, the reduction of NO_2^- to nitric oxide (NO), distinguishes "true" denitrifiers from other NO_3^--respiring bacteria. This reaction is catalyzed by two different types of nitrite reductases, either a cytochrome cd_1 encoded by the *nirS* gene (*nirS* denitrifiers) or a Cu-containing enzyme encoded by the *nirK* gene (*nirK* denitrifiers). *NirS* denitrifiers are located mostly in the rhizosphere, while *nirK* denitrifiers are more abundant in bulk soil (Henry et al. 2004; Tiedje 1988). In this step of the denitrification process, gaseous NO can escape from the environment. Therefore, a change in the amount of *nirK* and *nirS* denitrifiers can regulate NO_3^- and NO_2^- leaching from acid forest soils.

Henry et al. (2004) found that the amount of *nirK* denitrifiers can range from 10^4 to 10^6 gene copies per gram of soil. This high difference was explained by different amounts of soil organic carbon (Kandeler et al. 2006); higher amounts of organic carbon were positively correlated with *nirK* denitrifiers but not *nirS* denitrifiers. The *nirK* denitrifiers are more taxonomically diverse than *nirS* denitrifiers, however, the latter are more abundant. They represent approximately 0.4% of total soil bacteria while the *nirK* less than 0.2% (Kandeler et al. 2006). It seems that the more taxonomically diverse group of *nirK* denitrifiers is more sensitive to environmental changes and nutrient availability than the *nirS* denitrifiers. In highly acidified spruce forest soils, the low pH affects many biological and physical processes. The most important factor is the amount of dissolved organic matter (DOM), which rapidly decreases in low pH soils (Greenland 1971; David et al. 1989). A lower amount of DOM leads to lower nutrient availability for soil bacteria and decreases their numbers. Recently, it was shown that low pH negatively affected the activity of a pure culture of *Pseudomonas mandelii* (*nirS* denitrifier) (Saleh-Lakha et al. 2009). These authors found that the expression of the *nirS* gene was significantly lower when the pH of the medium dropped below 5. However, data from in situ experiments are still missing.

The objectives of this work were to:

(i) evaluate the effect of DOM and pH on the abundance of *nirK* and *nirS* denitrifiers;
(ii) compare the amount of *nirK* and *nirS* denitrifiers in different soil horizons.

Materials and methods

Study site description

Soil was collected from four different sampling sites in October 2008. Two sites are located in the Bohemian Forest in South Bohemia in the watersheds of two glacial lakes (Plešné, PL and Čertovo, CT) and two sites are located in the Jizera Mountains (Černá hora (CH) and Sněžné věžičky (SV)). In each site, four random spots in three replicates were chosen. Soil was collected from the organic litter layer (0–2 cm) and organic humus layer (5–15 cm).

PL and CT are situated at 48°47′ and 49°10′ N, and 13°52′ and 13°11′ E, respectively, at altitudes from 1030 to 1090 m a.s.l. Both sites were affected by the high acid N depositions of the last century. Recently, both sites were subjected to large disturbances caused by bark beetle infestions. Watershed of PL is covered with a 160 year-old Norway spruce (*Picea abies*) forest with small areas of ash. The bedrock is composed of granites. ČL watershed is covered with 90–150 year-old Norway spruce forests, with sparse white fir (*Abies alba*) and European beech (*Fagus sylvatica*); the bedrock is predominantly composed of mica-schist (muscovite gneiss) with quartzite intrusions. The understorey of both watersheds is dominated by hair grass (*Avenella flexuosa*), reedgrass (*Calamagrostis villosa*), blueberry (*Vaccinium myrtillus*), and lady fern (*Athyrium alpestre*, Svoboda et al. 2006). Soil types are mostly cambisols, podzols and litosols on steep slopes in both watersheds. Basic physico-chemical and biochemical properties of the soils are described by Kopáček et al. (2002a, b) and Veselý (1994).

CH and SV are situated at 50°49′ and 50°50′ N, and 15°11′ and 15°13′ E, respectively, at altitudes from 1055 to 1085 m a.s.l. Both sites are covered with 90–150 year-old Norway spruce forests (*P. abies*). The bedrock is composed of granites. Soil types are mostly podzols. The understorey of both sites is dominated by hair grass (*A. flexuosa*), reedgrass (*C. villosa*), and blueberry (*V. myrtillus*).

Soil chemical analyses

Total C and N (C_{TOT} and N_{TOT}, respectively) and oxalate extractable P (P_{OX}) were analyzed in air-dried, finely ground soil. C_{TOT} and N_{TOT} were

measured using an elemental analyzer (NC Thermo-Quest, Germany). Dissolved organic carbon (DOC) and dissolved nitrogen (DN) were extracted step by step in both cold water (CW) and hot water (HW): water:soil, 10:1, v/w, 30 min at 20°C and 16 h at 80°C, respectively. DOC and DN in the CW and HW extracts were determined on a TOC/TN analyzer (SKALAR FORMACS HT). P_{OX} was determined by extraction of 0.5 g of litter with 50 ml of acid ammonium oxalate solution (0.2 M $H_2C_2O_4$ + 0.2 M $(NH_4)_2$ C_2O_4 at pH 3) according to Cappo et al. (1987), but with a three-step instead of continuous extraction (Kopáček et al. 2002a). P_{OX} is supposed to characterize the biological bioavailability of P in soil (Koopmans et al. 2004; Pote et al. 1996; Schwertmann 1964; van der Zee et al. 1987). Active pH was determined in the CW extracts.

Extraction of DNA

Three replicates of each soil sample (0.25 g) were taken for DNA extraction. For the isolation of genomic DNA from soil, the Power Soil DNA Isolation kit (MoBio Laboratories Inc. Carlsbad, CA, USA) was used according to manufacturer's instructions with some modification. A Mini Bead-Beater (BioSpec Products, Inc; speed of 6 m s^{-1} for 45 s) was used for better disruption of cell walls. DNA was stored in 1.5 ml Eppendorf microtubes in a freezer (−20°C) until the next analyses.

qPCR assay

Quantitative PCR was performed with an ABI Step One (Applied Biosystems) by using SYBR green as the detection system in a reaction mixture of 20 µl containing 0.5 µM (each) primer; 10 µl of Power SYBR®Green PCR master mix, including AmpliTaq Gold® DNA Polymerase, Power SYBR®Green PCR buffer, deoxynucleoside triphosphate mix with dUTP, SYBR green I, ROX, and 5 mM $MgCl_2$ (Power SYBR®Green PCR Master Mix; Applied Biosystems, USA); and 2 µl of tempalte DNA corresponding to 10 ng of total DNA. Bovine serum albumin (BSA, 500 ng/reaction; Fermentas, Italy) and dimethylsulfoxide (DMSO, 12.5 µmol/reaction) were used to enhance PCR efficiency. 16S rDNA gene copy numbers were determined using the eubacterial primers 341f (5-CCT ACG GGA GGC AGC AG-3) and 515r (5-ATT CCG CGG CTG GCA-3) as described by Henry et al. (2004). The conditions for 16S rDNA real-time PCRs were 600 s at 95°C for enzyme activation (based on manufacturer recommendation) and 30 cycles of 15 s at 95°C, 30 s at 60°C and 30 s at 72°C for the denaturation, annealing and extension steps, respectively (López-Gutiérrez et al. 2004). The primers nirK876 (5′-ATY GGC GGV CAY GGC GA-3′) and nirK1040 (5′-GCC TCG ATC AGR TTR TGG TT-3′) were used for quantification of *nirK* denitrifiers (Henry et al. 2006). For the quantification of *nirS* denitrifiers, the primers nirSCd3aF (5′-AACGY-SAAGGARACSGG-3′) and nirSR3 cd (5′-GASTTCGGRTGSGTCTTSAYGAA-3′) were used as described by Kandeler et al. (2006). The conditions for *nirK* and *nirS* real-time PCRs were described elsewhere (Henry et al. 2006; Kandeler et al. 2006). Fluorescence was measured after each extension step. Melting curve and agarose electrophoresis (1.5% w/v, 110 V, 45 min) was performed for quality verification of the PCR product after each qPCR. Thermal cycling, fluorescent data collection, and data analysis were carried out with ABI Step One. Two independent qPCRs were performed for each gene and soil replicate. Standard curves were obtained with serial tenfold plasmid dilutions of a known amount of plasmid DNA containing a fragment of the 16S rDNA, *nirK* and *nirS* genes, respectively.

The extracted DNA from the soil samples was also tested for the inhibitory effects of co-extracted substances (i.e. humic and fulvic acids) by determining the 16S rDNA, *nirK* and *nirS* gene copy numbers in tenfold and 100-fold dilutions of the soil DNA extract. In addition, standard plasmid DNA was quantified with and without the addition of environmental DNA, and the obtained values were included into the calculation of 16S rDNA, *nirK* and *nirS* gene copy numbers as correction factors. Also DNA extraction efficiency (ng DNA per g soil) was included into the calculation.

Using the SYBR green assay and degenerate primers for the *nirK* and *nirS* genes, it was possible to discriminate against the primer dimer fluorescence by acquiring data at a temperature of 80°C, which is above the melting point of these by-products (Henry et al. 2006). This temperature was verified by melting curve analysis (data not shown). The gene copies in the no template controls (NTCs) were 0 and less than ten copies for *nirK* and *nirS*, respectively. Less than

ten copies per assay were observed for the 16S rDNA gene. According to threshold cycles (C_T) of standards and the NTC values, a detection limit of approximately 10–100 gene copies per assay was achieved for *nirK*, *nirS* and 16S rDNA quantification, which corresponds to 10^2–10^3 gene copies per gram of dry soil.

Statistical evaluation

Basic statistical analyses were performed using Statistica 8.0 (StatSoft). One-way ANOVA (soil layer as independent variable), followed by the Tukey HSD test was used for testing the differences between soil quality characteristics of the four sampling sites. One-way ANOVA (site as independent variable), followed by the Tukey HSD test was used for testing the differences between the amount of *nirK*, *nirS* and 16S rDNA gene copies of the four sampling sites. Linear regression was used to test the correlation of DOC, DN, DOC_{CW}, DN_{CW}, and pH with the 16S rDNA, *nirK* and *nirS* gene copy numbers. Correlation coefficients (r) with p values were evaluated.

For pH and DOC, an exponential relationship was compared ($R^2_{PH} = 0.802$, $R^2_{DOC} = 0.756$), because it fitted better the experimental data values and explained more variability than the linear relationship ($R^2_{pH} = 0.728$, $R^2_{DOC} = 0.742$).

Results

Amount of DOM in different soil horizons and sampling sites

DOC and P_{OX} were the only compounds significantly lower in the organic humus horizons than organic litter horizons in all four sites. Amounts of C_{TOT} and N_{TOT}, as well as the amount of DN, were also lower in organic humus horizons than in organic litter horizon, but insignificantly (Table 1). pH values were not statistically different between the soil horizons in most cases, with only the organic litter horizon at CH being significantly more alkaline than the organic humus horizon from the same site (Table 1). However, pH was significantly lower at PL and CT than SV and

Table 1 Spatial variation of chemical properties of organic litter and organic humus layers in the Bohemian Forest (PL, Plešné Lake catchment; CT, Čertovo lake catchment), and The Jizera Mountains (SV, Sněžné věžičky catchment; CH, Černá hora catchment)

Site	Soil horizon	Selected soil chemical characteristics							
		C_{TOT} (mol kg^{-1})	N_{TOT} (mol kg^{-1})	DOC (mol kg^{-1})	DOC_{CW} (mmol kg^{-1})	DN (mol kg^{-1})	DN_{CW} (mmol kg^{-1})	P_{OX} (mmol kg^{-1})	pH (H_2O)
PL	Organic litter	38.58^{B} (1.72)	1.37^{BC} (0.21)	2.02^{B} (0.85)	124.0^{A} (7.3)	0.19^{A} (0.01)	5.3^{AB} (0.3)	4.95^{B} (0.79)	3.59^{A} (0.15)
	Organic humus	25.61^{A} (7.14)	1.08^{A} (0.14)	1.26^{A} (0.01)	121.8^{A} (10.0)	0.21^{A} (0.04)	5.2^{B} (0.0)	3.30^{A} (0.34)	3.45^{A} (0.13)
CT	Organic litter	40.53^{C} (2.46)	1.58^{D} (0.02)	3.24^{C} (0.87)	138.2^{B} (12.6)	0.22^{A} (0.04)	5.8^{C} (0.2)	5.61^{C} (0.03)	3.67^{A} (0.14)
	Organic humus	28.09^{AB} (8.57)	0.98^{A} (0.35)	2.47^{B} (0.36)	125.7^{AB} (15.4)	0.20^{A} (0.02)	5.4^{AB} (0.5)	3.38^{A} (0.75)	3.48^{A} (0.19)
SV	Organic litter	33.22^{AB} (19.4)	1.47^{BC} (0.34)	7.04^{E} (0.07)	440.0^{E} (49.1)	0.41^{D} (0.01)	5.6^{BC} (1.0)	9.70^{C} (3.99)	5.75^{B} (0.07)
	Organic humus	33.68^{AB} (9.8)	1.49^{C} (0.09)	5.45^{D} (0.12)	249.3^{D} (3.8)	0.31^{C} (0.01)	4.2^{A} (0.1)	7.78^{C} (3.48)	5.58^{B} (0.20)
CH	Organic litter	29.94^{AB} (3.6)	1.54^{D} (0.05)	7.20^{E} (0.71)	513.3^{F} (14.9)	0.44^{D} (0.09)	9.0^{D} (0.1)	11.71^{D} (2.88)	6.05^{C} (0.15)
	Organic humus	30.98^{AB} (8.8)	1.44^{BC} (0.27)	5.12^{D} (0.02)	184.5^{C} (28.2)	0.27^{B} (0.01)	4.2^{AB} (0.8)	5.81^{BC} (2.43)	5.28^{B} (0.14)

Different letters show significant differences between values within columns ($p < 0.05$)

Mean values of total carbon (C_{TOT}), total nitrogen (N_{TOT}), total dissolved organic carbon (DOC), dissolved organic carbon in cold water extract (DOC_{CW}), total dissolved nitrogen (DN), dissolved nitrogen in cold water extract (DN_{CW}) and pH in water extract ($n = 4$) are given with the standard deviation in brackets ($n = 4$)

CH. Comparing the highly acidic soils (PL and CT) with the more alkaline soils (SV and CH) we can see also significantly higher amounts of DOM and P_{OX} at SV and CH soils than at PL and CT soils (Table 1).

Amounts of 16S rDNA, *nirK* and *nirS* gene copy numbers in soil horizons

The total number of 16S rDNA gene copy numbers in the organic litter horizon was significantly higher at SV and CH than PL and CT (Fig. 1). A similar pattern was observed for the *nirK* denitrifiers. On the contrary, *nirS* denitrifiers showed the opposite trend. However, the number of *nirS* denitrifiers was two orders lower than the number of *nirK* denitrifiers (Fig. 1).

The total number of bacteria at the organic humus horizon was significantly lower than the organic litter layer and significantly higher at SV and CH (Fig. 1). The amount of *nirS* denitrifiers was again two orders lower and was on the detection limit of the qPCR methodology.

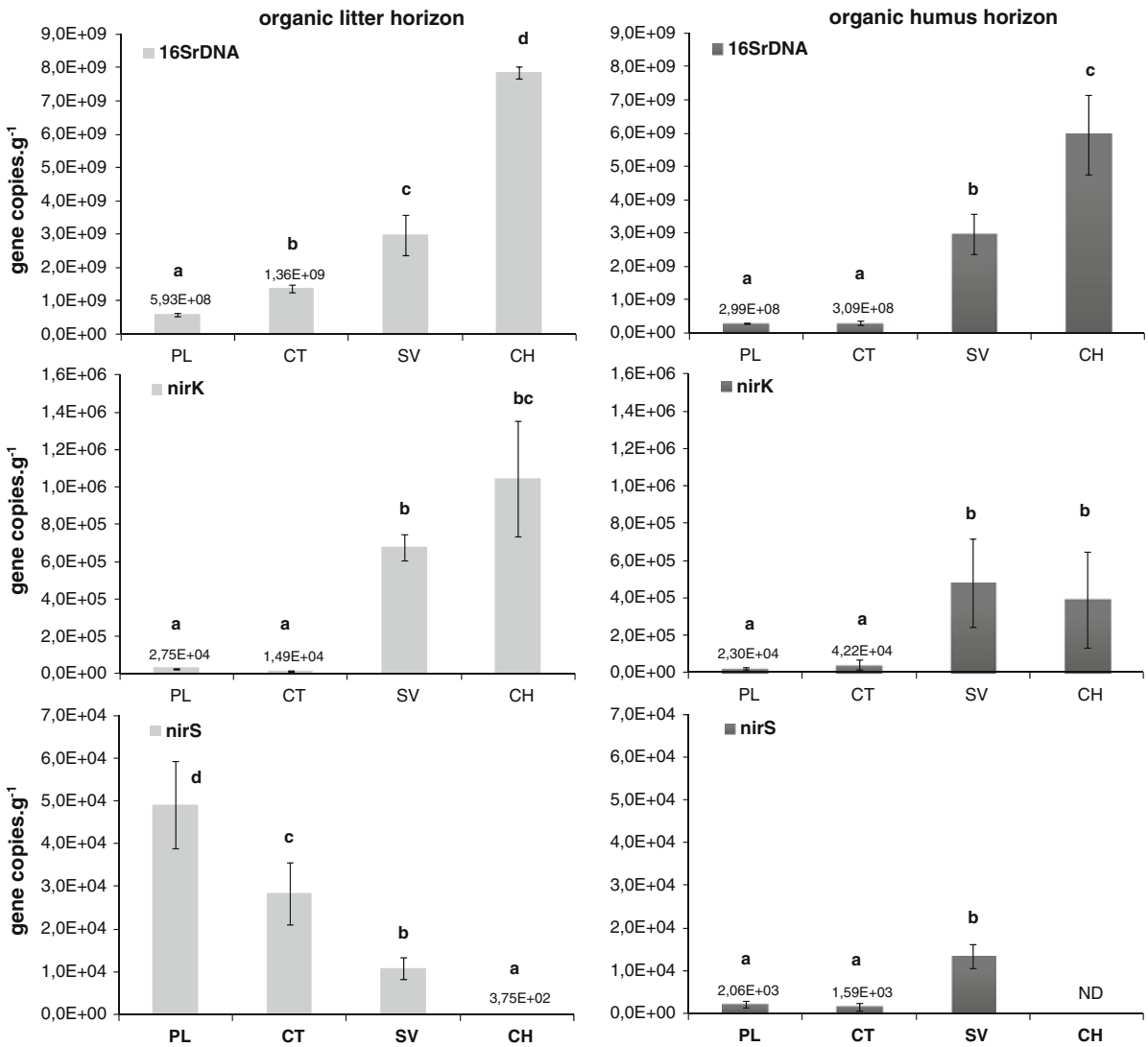

Fig. 1 Total amount of bacteria (16SrDNA gene copy numbers), and *nirK* and *nirS* denitrifiers in organic litter and organic humus horizons at PL, CT, SV and CH. *ND* not detected. *Different letters* show significant differences between sites ($n = 4$, $p < 0.05$)

Correlations between DOM, P_{OX}, pH and the 16S rDNA, *nirK* and *nirS* gene copy numbers

DOC and DN were highly positively correlated (Table 2). Also the amount of P_{OX} was highly positively correlated with pH suggesting lower P bioavailability in the PL and CT soils. A similar relationship was found between P_{OX} and DOC, and DOC and DN, showing a very close cross connection between pH and DOM.

There were also high positive correlations between DOC, DN, P_{OX} and pH, and the total amount of 16S rDNA and *nirK* gene copy numbers (Table 3). The *nirS* denitrifiers showed a positive correlation with only C_{TOT}. Interestingly, there were high positive correlations between DOC, DN and P_{OX}, and the *nirK*/*nirS* gene ratio, showing a shift between the relative amount of these two groups depending on nutrient availability in soil (Table 3).

Closer investigation of the correlations between the number of *nirK* denitrifiers and DOC and pH revealed that these correlations had a more likely exponential relationship than linear (Fig. 2). This suggests that there should be some critical point below/above which the number of *nirK* denitrifiers rapidly decreases/increases. For DOC and pH, the critical point was approximately 4.8 mol kg^{-1} and 5, respectively. The number of *nirK* denitrifiers rapidly decreased below these thresholds. On the contrary, DN and P_{OX} showed linear correlations with the number of *nirK* denitrifiers (Fig. 2).

Table 2 Correlation coefficients (*r*) of selected soil quality characteristics (C_{TOT}, N_{TOT}, DOC, DN, P_{OX}, and pH (H_2O)) from the organic litter and organic humus soil layers ($n = 16$)

	C_{TOT}	N_{TOT}	DOC	DN	P_{OX}	pH (H_2O)
C_{TOT}	–	0.78***	ns	ns	ns	ns
N_{TOT}		–	0.55*	ns	ns	ns
DOC			–	0.90***	0.77***	0.93***
DN				–	0.85***	0.84***
P_{OX}					–	0.72**
pH (H_2O)						–

Correlation coefficients are given and their significances are marked by asterisks as follows: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

ns not significant

Discussion

Quantification of 16S rDNA, *nirK* and *nirS* genes in soil environment

Quantitative real-time PCR of 16S rDNA and denitrification genes encoding NO_2^- reductase was used to study the ecology of denitrifiers in acidified spruce forests. 16S rDNA copy number ranged from 2.9×10^8 to 7.8×10^9 copies er gram of soil. These numbers are one order of magnitude lower than reported by Kandeler et al. (2006) suggesting a lower amount 16S rDNA gene copies in our experimental sites. The absolute number of 16S rDNA gene copies cannot be accurately compared to the total number of bacteria because the numbers of 16S rDNA gene

Table 3 Correlation coefficients (*r*) of selected soil quality characteristics (C_{TOT}, N_{TOT}, DOC, DOC_{CW}, DN, DN_{CW}, P_{OX}, and pH (H_2O)) versus the 16SrDNA, *nirK*, and *nirS* gene copy numbers and *nirK*/16SrDNA, *nirS*/16SrDNA, and *nirK*/*nirS* ratios

	16SrDNA	*nirK* denitrifiers	*nirS* denitrifiers	*nirK*/16SrDNA	*nirS*/16SrDNA	*nirK*/*nirS*
C_{TOT}	ns	ns	0.66**	ns	ns	ns
N_{TOT}	ns	ns	ns	ns	ns	ns
DOC	0.93***	0.86***	ns	ns	−0.51*	0.53*
DOC_{CW}	0.88***	0.90***	ns	0.50*	ns	0.66**
DN	0.87***	0.94***	ns	0.59*	−0.53*	0.69**
DN_{CW}	ns	0.60**	ns	ns	ns	0.80***
P_{OX}	0.67**	0.83***	ns	0.51*	ns	0.69**
pH (H_2O)	0.93***	0.85***	ns	ns	−0.54*	0.50*

Correlation coefficients are given and their significances are marked by asterisks as follows: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

ns not significant

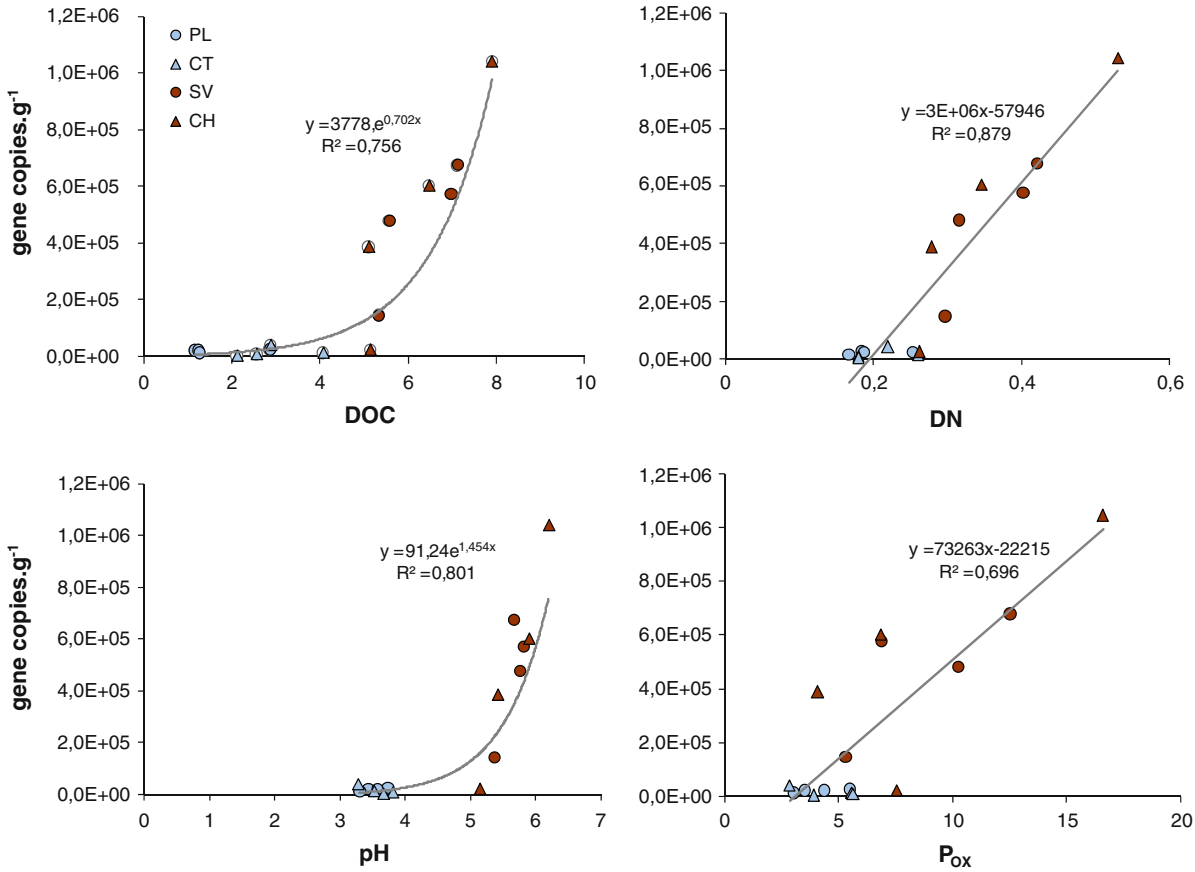

Fig. 2 Correlations of *nirK* denitrifiers with DOC, DN, pH and P_{OX}. Data are summarized for both soil horizons and sampling sites

copies per bacterial genome range from 1 to 13 copies (Chéneby et al. 2000; Fogel et al. 1999). On the contrary, *nirK* and *nirS* genes are present in only one copy per genome but also they cannot represent the absolute numbers of *nirK* and *nirS* denitrifiers because of the limitation of PCR based methods, i.e. primer specificity (Heylen et al. 2006). Our results showed that the number of *niK* and *nirS* gene copies in forest soil is higher in the organic litter horizon (i.e. from 0 to 2 cm below ground) suggesting higher denitrification potential in higher soil horizons (Fig. 1). These results confirmed the previous study of Clément et al. (2002) who found a rapid decrease of denitrification rate with increasing soil depth.

Soil samples in our sites were taken during October 2008, which could have additional impact on denitrification. Soils are wetter at this time of year, there is a higher input of plant litter into the soil horizons, and decomposition produces higher amounts of DOM than in drier summer periods. Together with higher decomposition goes higher O_2 consumption. This creates almost ideal conditions for denitrifiers. Clément et al. (2002) found that the seasonal variation in denitrification in the upper soil horizon is different in grassland and forest ecosystems. In grasslands, the maximum denitrification rate was reached during the autumn, while during the summer period in forests. Therefore, a vegetation shift with different DOM input can alter denitrification processes (Cabrita and Brotas 2000; van Kessel et al. 1993) and the amount and composition of *nirK* and *nirS* denitrifiers (Wertz et al. 2009). The contribution of grasses (i.e. *Calamgrostis villosa* and *A. flexuosa*) recently increased in PL and CT due to forest dieback. Therefore, the maximum denitrification rate and the amount of nirK and nirS denitrifiers can be closer to grassland ecosystems (i.e. in autumn).

Amount of DOM in acid forest soils

The amount of DOM in soil pore water plays a crucial role in the availability of nutrients for soil microorganisms. When the amount of DOM is low it becomes limiting for soil microorganisms leading to starvation and reduction of biochemical processes. The major factor influencing the amount of DOM is pH (Quails and Raines 1992; Greenland 1971; David et al. 1989). Soils at PL and CT are highly acidic, which leads to significantly lower amounts of DOC, DN and available P (P_{OX}) than at SV and CH (Table 1). Close relationships between these DOM fractions and pH were confirmed in our soils (Table 2). Three major mechanisms were proposed to explain this relation: (i) formation of insoluble compounds and subsequent flocculation of organic molecules, (ii) adsorption of organic molecules on clay material through cation bridging and (iii) complexation of organic molecules with polyvalent cations (Greenland 1971). All three processes seem to be operating. However, complexation of OM with polyvalent cations should be the most important process in our soils. In PL and CT, acidification was interconnected with higher mobility of toxic Al^{3+} forms, which can precipitate DOM making it unavailable for biological processes (Kana and Kopácek 2006; Northup et al. 1998; Tomlinson 2003). Schindler et al. (1992) described the negative effect of Al^{3+} on OM solubility in acidified lakes. These authors found that DOC may also precipitate with $Fe(OH)_3$ at pH <4, which leads to an additional decrease of DOC amount. Moreover, high concentrations of Al^{3+} can damage bacterial membranes leading to higher bacterial mortality (Yaganza et al. 2004), which corresponds with our results of a one order magnitude decrease in 16S rDNA gene copies at PL and CT (Fig. 1). All of these effects could explain the exponential relationship between the number of *nirK* denitrifiers and DOC, and the rapid reduction of *nirK* denitrifiers at pH <4 at these sites.

Correlation between the amount of denitrifiers and available DOM

As mentioned above, PL and CT were strongly affected by a bark beetle infestation, which has led to an enormous spruce forest dieback and high amount of spruce needles which have accumulated in the organic litter horizon. The relatively slow decomposition of spruce needles (Bárta et al. 2010) produces a high amount of organic acids, which leads to secondary acidification of PL and CT (Šantrůčková et al. 2004, 2006, 2007).

Therefore, in these extremely acidic soils, both Al^{3+} and Fe^{3+} ions co-precipitate organic matter which leads to an enormous nutrient limitation for soil microorganisms. The *nirK* denitrifiers seem to be more sensitive to these nutrient changes than *nirS* denitrifiers. These results are in agreement with Kandeler et al. (2006). However, the *nirK* denitrifiers are still more abundant than *nirS* denitrifiers in our sites. They are more abundant in the bulk soil where selective pressure and nutrient limitation are higher than in the rhizosphere where the *nirS* denitrifiers are preferred (Henry et al. 2004). The *nirS* denitrifiers have one advantage over the *nirK* denitrifier which is that plants exhude compounds which stimulate the overall growth of bacteria in the rhizosphere (Rösch et al. 2002; Livsey and Barklund 1992). Thus, the lower amount of DOM in bulk soil does not have so dramatic effect on shift of *nirS* gene abundance. Recently, some studies showed that the *nirK* gene may be more preferable for horizontal gene transfer (HRT) than the *nirS* gene. The *nirK* gene was found in highly taxonomically diverse bacterial groups. The gene was "spread" among the same habitat and does not correlate with 16S rDNA phylogeny as the *nirS* does (Heylen et al. 2006). At PL and CT, the lower amount of DOM could therefore decrease the rate of HRT between soil bacteria making them less effective in reducing higher amounts of NO_3^- and NO_2^-. These can than leach from the forest ecosystem.

In our previous work we found that P availability (P_{OX}) plays an important role in litter decomposition at PL and CT and is more important than the availability of C and N. Also the composition of bacterial and fungal communities strongly correlated with the amount of P_{OX}. The overall biodiversity and enzymatic activity of decomposing microorganisms were much higher in litter with higher amount of P_{OX} (i.e. grasses) than in litter which contained low amount of P_{OX} (i.e. spruce needles) (Bárta et al. 2010). In our current study, we found that also the amount of *nirK* denitrifiers strongly depends on P_{OX} (Table 3). Litter decomposition and nutrient availability in the upper soil horizons are closely connected. Lower microbial biodiversity on spruce

needles can lead to lower decomposition and lower amount of released nutrients into the upper soil horizons (Sinsabaugh et al. 2002, 2005). Additionally, lower ligninolytic enzymatic activity leaves a higher amount of recalcitrant polyphenolics which are not available to most soil bacteria including denitrifiers (Snajdr et al. 2008). Therefore, it seems that this is an additional process to those mentioned above, which leads to the reduction of denitrification potential in acidified spruce forests.

The *nirK*/*nirS* ratio

The amount of *nirS* denitrifiers did not show any correlation with DOM, however, there was a high positive correlation between DOM and the *nirK/nirS* ratio. A higher amount of DOM shifts the ratio to the *nirK* denitrifiers while in lower amounts of DOM the ratio decreases with a higher relative contribution of *nirS* denitrifiers. These results are in agreement with Winder and Levy-Booth (2009). These authors studied the effect of clear-cutting in Douglas-fir stands and found a similar reduction in the amount of denitrifiers.

In conclusion, DOM appears to strongly affect the amount of denitrifying bacteria, especially the *nirK* denitrifiers, which are highly sensitive to the amount of DOM and soil pH. This can reduce denitrification rate in the organic litter and organic humus horizons, which can lead to increased NO_3^- and NO_2^- leaching from highly acidic spruce forest soils.

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