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Overwintering management on upland pasture causes shifts in an abundance of denitrifying microbial communities, their activity and N_2O-reducing ability

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ABSTRACT

Pasture soils used for cattle overwintering may represent significant sources of N_2O emissions from soils. Therefore, the long-term effect of cattle overwintering on the abundance and activity of a denitrifying community was explored. The study was performed at a cattle overwintering area in South Bohemia (Czech Republic), where three sites differing in the degree of animal impact were selected: severely impacted (SI) and moderately impacted (MI), as well as a control site with no impact (NI). N_2O flux measurement and soil sampling were performed in spring and fall of 2005. The activity was measured in terms of potential denitrification activity. Bacterial *nirK*, *nirS* and *nosZ* genes were used as functional markers of the denitrifying communities; abundance was analyzed using a real-time PCR assay. Surprisingly, *in situ* N_2O emissions were the highest in spring at MI and significantly differed from those at SI and NI, while in autumn, rates of emissions generally decreased. In contrast potential denitrification rates were highest at SI, followed by MI, and the lowest at NI. An overall significant shift in N_2O/N_2 molar ratio was shown in cattle impacted sites. The highest abundance of all genes measured at both sampling times was found at site SI, whereas at site MI increased numbers were observed only in spring. Our results indicate a strong influence of cattle on the abundance as well as the activity of microbes involved in denitrification.

1. Introduction

The use of grasslands as permanent pastures has become increasingly popular in the European Union since the early 1990s. Typical pasture management is characterized by low-input agriculture and ecological farming including regional overwintering of animals on pasture sites. Obviously, this type of management is favorable to the animals as it improves their health status. However, the consequences of this management technique on nutrient cycling, including nitrogen transformations, in soil are not well understood. As the overwintering areas are close to the stables and mostly restricted in size, the impact of the animals on the soil is assumed to be larger than in the summer season, when animals use a larger area for grazing.

Several studies concerning nitrous oxide emissions and nitrogen losses from the system during the last few years have been carried out in the cattle overwintering area located in South Bohemia, Czech Republic (Šimek et al., 2006a, b; Hynšt et al., 2007a, b). Three main factors steering microbial processes have been identified in those areas: (i) dung and urea, the main organic inputs, are enriched in these soils leading to extraordinary amounts of organic carbon and nitrogen; (ii) compaction of soil by animal traffic and other changes reduce soil aeration; and (iii) grazing, trampling and defoliation result in reduced plant N uptake. Not surprisingly, these factors also lead to significant changes in soil microbial community structure and functioning. An increase in abundance and activity of methanogenic Archaea, that are strictly anaerobic, was detected in soils used as overwintering pasture (Radl et al., 2007) and indicates the increased occurrence of anaerobic micro-sites as a result of cattle grazing. Moreover it implies that the microbial community is sensitive to this kind of disturbance (cattle overwintering activity), because not only community structure was affected, but also the functioning (for a review see Allison and Martiny, 2008).

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In this study we investigated the influence of cattle overwintering on denitrification and the corresponding denitrifier community structure. We postulate that a low oxygen status combined with high concentrations of carbon and nitrogen in soils of highly cattle impacted areas increases the abundance of genes involved in denitrification compared to non-impacted sites, thus resulting in higher potential as well as actual denitrification rates. Furthermore we hypothesize that increased abundances of denitrifiers will also be observed after cattle have left the overwintering area, indicating the sensitivity of denitrifiers for the disturbance of the soil ecosystem.

To review these hypotheses we applied qPCR (Philippot, 2006; Sharma et al., 2007) to quantify three functional genes, which catalyze two key steps in the denitrification process at three sites with a different degree of cattle impact in spring (before cattle have left the overwintering area) and in autumn (before cattle have returned to the overwintering area). We studied the abundance of genes coding for enzymes of the denitrification pathway responsible for the nitrite reduction (*nirK* and *nirS*; Braker et al., 1998; Heylen et al., 2006) and the nitrous oxide reduction (*nosZ*; Thröback et al., 2004). The obtained data were related to soil parameters (especially carbon and nitrogen content) as well as potential denitrification activities and actual N_2O fluxes.

2. Materials and methods

2.1. Study area

For this study an experimental site close to Český Krumlov in South Bohemia (latitude 48°52′N, longitude 14°13′E) 170 km south of Prague was chosen; a more detailed description is provided by Šimek et al. (2006a). Briefly, the overall size of the field is approximately 4 ha, and it has been used for overwintering approximately 90 cows since 1994/95. The animals are normally on the site from late October until the end of April. Soon after the animals arrived, a gradient of animal impact from the most impacted areas near the animal barn to much less impacted areas in the middle and almost unaffected areas at the opposite side of the overwintering area became apparent. Along this gradient three sites were identified, differing in the presumed rate of animal impact. These included a severely impacted site (SI) with totally destroyed plant cover and surface soil in spring, at the end of the overwintering period; a site with moderate impact (MI) where effects of trampling and disturbance of the vegetation were still visible; and a control site (NI) with very slight or no impact, as judged from soil and vegetation (see also Hynšt et al., 2007b for more details).

The soil on the site is a sandy loam originally classified as Cambisol and recently re-classified as Haplic Phaeozem (arenic; WRB system) containing 60–80% sand, 14–32% silt, and 6–14% clay (USDA classification system). The original plant cover was a perennial mixture of grasses, clovers and other dicotyledonous plants, which did not recover fully at sites SI and MI over the cattle-free period (at these sites the original vegetation was replaced with a mixture of fast growing plants including ruderal herbs (*Polygonum* sp., *Plantago major*, *Galinsoga parviflora*), grasses (*Echinochloa cruss-galli*) and red clover (*Trifolium pratense*). No additional fertilization was used at the investigated sites. The mean annual precipitation in the area is 650 mm and the average annual temperature is 7 °C (data from the meteorological station located at 7 km distance from the experimental site). Data about important soil properties of the three sites (including pH, gravimetric water content, C_{org} and N_{tot}) are given in Table 1.

2.2. Gas flux measurements and soil sampling

Field work was done in spring and fall of 2005, at the end of the 2004/2005 overwintering period in May, and in autumn (October) before the new overwintering season began. In each of the three experimental sites nine plots, which were treated as independent replicates, were selected randomly for gas flux measurements and soil sampling. To avoid biased sampling, e.g., due to climatic conditions, all sampling was carried out on the same day; furthermore, sampling of the 27 plots was obtained at random to ensure no systematic error due to time of day of sampling. Nitrous oxide fluxes were determined using medium-sized (basal area 0.076 m^2, volume 15 dm^3) non-vented manual closed chambers (for details see Radl et al., 2007). Gas samples were stored in pre-evacuated 12 ml glass vials, transported to the laboratory and immediately analyzed using HP 5890 gas chromatograph (Hewlett Packard, USA) equipped with a 3 m, 0.318 cm i.d. stainless steel Porapak Q column and an electron capture detector. Bulk soil samples (0–20 cm depth) were collected from each chamber position with a spade after the final gas sampling. The soil was immediately homogenized by sieving through a 5 mm screen sieve. Samples for molecular biology and DEA analyses were stored at −80 °C, respectively, 4–8 °C. Soil mineral N (NH_4^+, NO_3^-) was measured calorimetrically in 1 M KCl extracts using a soil (fresh, field-moist): solution ratio of 40 g: 200 ml (Zbíral et al., 1997). Soil moisture was determined gravimetrically after drying at 105 °C for 24 h; it is expressed as relative mass of water per mass of dry soil.

2.3. Potential enzyme activities

Denitrifying enzyme activity (DEA) was determined using an anaerobic slurry technique similar to the phase I assay of Smith and Tiedje (1979), slightly modified (Šimek and Hopkins, 1999). Soil slurries were made by mixing 25 g of field-moist soil in 120 ml serum bottles with 25 ml of a solution containing 1 mM glucose and 1 mM KNO_3 and incubated in helium. The slurries were shaken on an end-to-end shaker at 25 °C. Thirty and 60 min after the addition of acetylene, 0.5 ml samples of headspace atmosphere were taken with a gas-tight syringe and N_2O was measured on a gas chromatograph (see above). DEA was calculated from the N_2O increase during half hour incubation (60–30 min). For an estimation of both N_2 and N_2O production, parallel soil samples were incubated with and without 10 vol. % acetylene.

2.4. DNA extraction from environmental samples

DNA was extracted from 0.5 g of soil according to Griffiths et al. (2000), including a step of homogenization at 6000 × *g* for 30 s using Pre-cell lysis equipment (Precelles, France). DNA quantity and quality were evaluated using a spectrophotometer (Nanodrop, PeqLab, Germany) and on 1% agarose gels stained with ethidium bromide (Sambrook and Russell, 2001). The optimal dilutions for the obtained DNA mainly related to inhibitory substances has been evaluated in detail (see Radl et al., 2007).

2.5. Quantitative PCR assays of nirK, nirS and nosZ genes

SybrGreenI® based real-time PCR assays were carried out in a volume of 25 μL, containing: 5 μL qPCR ROX-&GO™ Green Master mix (Qbiogene, France), 1.5 μg BSA, 5% DMSO (both Sigma–Aldrich, Steinheim, Germany), 10 pmol of each primer (Thermo Hybaid, Germany) and 100 ng DNA template. Primer pairs used in our study were: Cd3aF–R3bcd (Thröback et al., 2004), nirK1F–nirK5R (Braker et al., 1998) and nosZ2F–nosZ2R (Henry et al., 2006) for *nirS*, *nirK*