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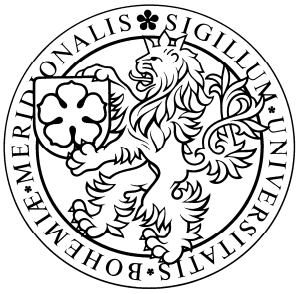

The linkage between denitrification activity, N gas emissions, and the size of the denitrifier community in pasture soils

Ph.D. Thesis

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Annotation

The linkage between denitrification activity, N gas emissions, and the size of the denitrifier community in soils of an upland pasture was investigated. Special emphasis was placed on soil pH as a regulating factor, the spatial distribution of denitrification, and the degree of cattle impact. The thesis has been based on field and laboratory measurements using both conventional and modern methods of soil ecology.

Declaration [in Czech]

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České Budějovice, 18.4.2011 .....

Mgr. Jiří Čuhel

List of papers and author's contribution

The thesis is based on the following papers (listed chronologically):

I. Chroňáková, A., Radl, V., **Čuhel, J.**, Šimek, M., Elhottová, D., Engel, M., Schloter, M., 2009. Overwintering management on upland pasture causes shifts in an abundance of denitrifying microbial communities, their activity and N_2O-reducing ability. Soil Biology & Biochemistry 41, 1132-1138 (IF = 2.978).
Jiří Čuhel participated in gas flux measurement, soil sampling, sample preparations, DNA extraction, quantification of denitrification genes, and revision of the manuscript, and was responsible for measurement of denitrification activity, mineral N content, and statistical analysis.

II. Philippot, L., **Čuhel, J.**, Saby, N.P.A., Chèneby, D., Chroňáková, A., Bru, D., Arrouays D., Martin-Laurent, F., Šimek, M., 2009. Mapping field-scale spatial patterns of size and activity of the denitrifier community. Environmental Microbiology 11, 1518-1526 (IF = 4.909).
Jiří Čuhel participated in soil sampling, sample preparations, DNA extraction, quantification of denitrification genes, and revision of the manuscript, and was responsible for measurement of denitrification activity.

III. Philippot, L., Bru, D., Saby, N.P.A., **Čuhel, J.**, Arrouays, D., Šimek, M., Hallin, S., 2009. Spatial patterns of bacterial taxa in nature reflect ecological traits of deep branches of the 16S rRNA bacterial tree. Environmental Microbiology 11, 3096-3104 (IF = 4.909).
Jiří Čuhel participated in soil sampling, sample preparations, DNA extraction, and revision of the manuscript.

IV. **Čuhel, J.**, Šimek, M., Laughlin, R.J., Bru, D., Chèneby, D., Watson, C.J., Philippot, L., 2010. Insights into the effect of soil pH on N_2O and N_2 emissions and denitrifier community size and activity. Applied and Environmental Microbiology 76, 1870-1878 (IF = 3.686).
Jiří Čuhel was responsible for management of experimental plots, gas flux measurements in the field, soil sampling, sample preparations, DNA extraction, quantification of denitrification genes, measurement of denitrification activity, data assembly, statistical analysis, and writing the manuscript.

V. **Čuhel, J.**, Šimek, M., 2011. Proximal and distal control by pH of denitrification rate in a pasture soil. Agriculture, Ecosystems & Environment 141, 230-233 (IF = 3.130).
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GENERAL INTRODUCTION AND AIMS

Nitrous oxide emissions from pasture soils

After carbon dioxide (CO_2) and methane (CH_4), nitrous oxide (N_2O) is the most potent greenhouse gas contributing to global warming (Conrad, 1996). N_2O, whose emissions into the atmosphere are controlled under the Kyoto Protocol of the United Nations Framework Convention on Climate Change, has a global warming potential about 320 times greater than that of CO_2 and has a lifetime of approximately 120 years (Nakicenovic and Swart, 2000). Moreover, N_2O contributes to the destruction of the stratospheric ozone layer (Crutzen and Ehhalt, 1977) and has become the most dominant ozone-depleting substance (Ravishankara et al., 2009). After further reactions in the atmosphere, N_2O can also produce photochemical smog and acid rain (Vitousek et al., 1997). Its atmospheric abundance before industrialization was 270 ppb, and in 2005 the concentration reached 319.2 ppb (World Meteorological Organization, 2006), exceeding the concentration of preindustrial times by 18%.

Although important sources of N_2O emissions include fossil fuel combustion, biomass burning, waste dumps, industrial and chemical production (e.g., nylon production), soils are responsible for more than two-thirds of global N_2O emissions because of the many microbial transformations of nitrogen compounds in the soil environment (Conrad, 1996; Schimel and Holland, 1998). Agricultural soils have been identified as the major source of N_2O, accounting for about 35% of the global annual emission (Kroeze et al., 1999). Velthof et al. (1996) showed that grasslands are particularly important sources of N_2O emissions (the quantity of N_2O released per unit of fertilizer is generally greater for grasslands than for arable cropland) and also suggested that N_2O emissions are greater in grazed grassland (pastures) than in mowed (ungrazed) grassland. Hynšt et al. (2007) summarized several possible reasons for the large N_2O emissions from pastures: (1) pasture soils are rich in organic carbon, which originates from plant roots, litter, and animal excreta; (2) pasture soils are compacted because of animal trampling, especially under moist or wet conditions, leading to anaerobiosis; (3) animal excreta (dung and urine) deposition in grassland soil results in a patchy distribution of nitrogen and carbon and, consequently, enhanced biogenic transformations of the nutrients; and (4) grazing and defoliation in grasslands reduce nitrogen uptake by plants, leading to the temporary accumulation of mineral forms of nitrogen in the soil.

In 2002, agricultural land occupied about 50 x 10^6 km^2 globally (Smith et al., 2007). Most of this area was under pasture (69%), and cropland occupied 28%. During the last four decades, agricultural land gained almost 5 x 10^6 km^2 from other land uses, a change driven largely by increasing demands for food by a growing population. Every year during this period, an average of 6 x 10^4 km^2 of forestland and 7 x 10^4 km^2 of other land were converted to agriculture, with most of this conversion occurring in the developing world. This trend is also projected to continue into the future (Smith et al., 2007).

Biological emissions of N_2O from soils are largely controlled by two microbial processes: nitrification and denitrification (Firestone et al., 1980; Conrad, 1996). N_2O is a by-product of the first step of nitrification, whereas it is an intermediate or end product in denitrification. Denitrification is the dominant process of N_2O emission from grazed pastures (e.g., de Klein and van Logtestijn, 1994; Saggar et al., 2004; Luo et al., 2008). Because of the enlarging global area of grasslands and their impact on environment through N_2O emissions, it is necessary to identify possible mitigation strategies to lower N_2O emissions from these ecosystems. Such mitigation, however, would be impossible without further in-depth research on the processes involved in N_2O production in pasture soils, with special emphasis on denitrification. The required research would include not only studies on the environmental controllers of denitrification rate and N_2O fluxes, but studies also on the interactions among these controllers and on their possible impact on the community of denitrifying microorganisms.

Denitrification and denitrifiers

Denitrification represents sequential dissimilative reduction of nitrate (NO_3^-) via nitrite (NO_2^-) to gaseous nitric oxide (NO), nitrous oxide (N_2O), and finally molecular dinitrogen (N_2) via four enzymatic complexes. Denitrification is the last step in the global N cycle by which fixed N is returned back to the atmosphere. Although denitrifying organisms normally respire and metabolize aerobically when oxygen is present, a lower partial pressure of oxygen causes them to use oxidized forms of nitrogen as alternative terminal acceptors of electrons in their respiration chain. Thus, denitrification allows denitrifiers to grow and gain energy under anaerobic conditions.

Denitrification is the only biological process of N_2O consumption (Conrad, 1996); however, the denitrification process usually produces more N_2O than it consumes because the denitrification sequence is usually incomplete, i.e., not all of the NO_3^- is reduced all the way to N_2, resulting in two main denitrification products: N_2O and N_2. Denitrification rate and the ratio

of N_2O and N_2 are affected by a number of environmental factors. Tate (1995) summarized that the most important regulators of the denitrification rate and the stochiometry of its products are: the composition and availability of organic matter as a source of energy, the availability of NO_3^-, the partial pressure of oxygen, temperature, and soil pH. All these parameters are affected in pasture soils by the activities of grazing animals. Because N_2O is an intermediate in the denitrification pathway, both the amount of N_2O produced and the ratio of the two products (N_2O and N_2) are important in understanding and predicting N_2O fluxes from soils. Without data on N_2 formation, it is difficult to study the influence of environmental parameters on denitrification activity or to find any linkage between the structure and function of the denitrifying community.

Despite the ecological importance of denitrification for environment, there are very few reports about N_2 emissions (Groffman et al., 2006), likely due to methodological limitations. Quantitative analysis of N_2 is much more difficult than quantitative analysis of N_2O. The acetylene (C_2H_2) blockage method (Balderson et al., 1976; Yoshinari and Knowles, 1976) has been extensively used to quantify both denitrification products. C_2H_2 inhibits reduction of N_2O to N_2. Thus, the amount of N_2O produced with addition of C_2H_2 represents the overall production of N_2O and N_2, and the production of N_2 is then calculated as the difference between N_2O production with and without added C_2H_2. This method has been applied in laboratory experiments under controlled conditions (e.g., Simarmata et al., 1993; Dendooven and Anderson, 1994) and also in the field (e.g., Ryden and Dawson, 1982; Ryden et al., 1987) but has some disadvantages and limitations. First, C_2H_2 also inhibits nitrification (Klemedtsson et al., 1988) and fermentation (Flather and Beauchamp, 1992), and has other side effects on soil processes. Second, the C_2H_2 blockage is generally not suitable for *in situ* measurements. In contrast to the C_2H_2 blockage method, the addition of a labeled denitrification substrate (e.g., $K^{15}NO_3$) to soil and subsequent analysis of the isotopic composition of N_2O and N_2 by mass spectroscopy offers a useful tool to quantify emissions of both N_2O and N_2 *in situ* (Stevens and Laughlin, 1998; Stevens et al., 1998).

Denitrifiers are primarily heterotrophic microorganisms and are characterized by two principal traits: (1) their growth yield is proportional to the amount of N oxide present, and (2) they are able to reduce NO_2^- into N_2O or N_2 (Tiedje, 1988; Mahne and Tiedje, 1995). The ability to denitrify is present in many prokaryotic families such as Thermoproteaceae, Cytophagaceae, Corynebacteriaceae, Streptomycineae, Bacillaceae, Rhodospirillaceae, Rhodobacteraceae, Rhizobiaceae, Burkholderiaceae, Nitrosomonadaceae, Neisseriaceae, and Pseudomonaceae (Philippot and Germon, 2005). Even if only 0.1–5% of the soil bacteria are

denitrifiers (Chèneby et al., 2000), the diversity of the denitrifying community in soil is huge (Philippot and Germon, 2005).

Despite the broad taxonomic and phylogenetic diversity of denitrifiers, the enzymes in the denitrification pathway (reductases) are highly conserved among phylogenetically distant denitrifiers, and the reductases are encoded by similar functional genes (Zumft, 1997). Denitrifying bacteria can express two types of NO_3^- reductase, Nar and Nap NO_3^- reductase, which primarily differ in their location in the cell. Nar NO_3^- reductase is associated with the cytoplasmic membrane, and its functional subunit is encoded by the gene *narG*. Nap NO_3^- reductase is located in the cell periplasm, and its functional subunit is encoded by the gene *napA*. Similarly, two types of NO_2^- reductase have been identified in denitrifiers: copper (Cu) NO_2^- reductase is encoded by the gene *nirK*, and cytochrome cd_1 NO_2^- reductase is encoded by the gene *nirS* (Zumft, 1997). Both types are functionally equivalent but are not present in the same bacteria. The reduction of NO to N_2O is catalyzed by NO reductase, which consists of two functional subunits encoded by the genes *norC* and *norB* (Philippot, 2002). The last step in denitrification is catalyzed by N_2O reductase, which is encoded by the gene *nosZ*. However, denitrifiers may possess a truncated denitrification pathway as revealed by the complete genome sequencing of *Agrobacterium tumefaciens* C58, which lacks the *nosZ* gene and is unable to reduce nitrous oxide (Wood et al., 2001). In theory, it is not essential that each denitrifying organism is capable of performing all steps in denitrification because the different steps in the pathway are relatively independent and can be performed by different components of the soil community; the entire denitrification process has been described as of modular organization of the different steps (Zumft, 1997).

Formerly, most studies on denitrification and N_2O emissions assumed that the process depended on environmental parameters rather than on the structure of the denitrifying community. Holtan-Harwig et al. (2000) and Cavigelli and Robertoson (2000), however, indicated that differences in the structure of the denitrifying community in soil can be of great importance for regulating denitrification activity and N emissions. Recently, the linkage between function and ecology of denitrifiers has become an active research area concerning the microbiology of nitrogen cycling, and the possible impact of the abundance or composition of the denitrifier community on the denitrification rate and the ratio of N_2O and N_2 has been extensively debated (Rich and Myrold 2004; Enwall et al., 2005; Philippot and Hallin, 2005; Ma et al., 2008; Miller et al., 2008, 2009; Hallin et al., 2009). Philippot (2006) pointed out that studying the diversity of a functional community is of limited value for understanding N fluxes if the abundance of this functional community is unknown; Philippot emphasized the necessity to quantify denitrifiers.

Quantitative PCR (qPCR) of functional denitrification genes is a useful molecular technique for quantifying denitrifiers; in contrast, quantification based on the ribosomal gene is unrealistic for denitrifiers because the denitrification trait is present in a broad variety of bacterial species. Moreover, qPCR allows quantification of denitrifiers capable of performing a single step in the denitrification pathway, whereas approaches based on cultivation only allow quantification of denitrifiers that are capable of performing several steps (Philippot, 2002). With qPCR of functional denitrification genes, the sum of *nirK* and *nirS* genes can be used as an estimate of the total number of denitrifiers, because each denitrifier can have only one type of NO_2^- reductase (Philippot, 2002) and the presence of NO_2^- reductase is the main criterion for identifying a bacterium as a denitrifier (Mahne and Tiedje, 1995). Further, the sum of *nirK* and *nirS* genes can be proportional to the measured denitrification activity. Similarly, the ratio of gene abundances *nosZ*/(*nirK*+*nirS*) can be used as an indicator of the proportion of denitrifiers having the genetic capacity to perform the last step of denitrification (reduction of N_2O). However, the real importance of denitrifier abundance for denitrification activity and N_2O/N_2 ratio remains unclear.

Spatial distribution of soil bacteria and denitrifiers

Although microbial processes exhibit substantial spatial variability at the field scale (Parkin, 1993), it is not clear whether the microbial communities that mediate these processes also exhibit spatial distribution patterns. This is also true for denitrifiers because while many studies have dealt with the spatial variability of the denitrification process (Parkin, 1993), the spatial variability of the corresponding functional community has largely been ignored. Although microorganisms are key players in ecosystem functioning, investigations into the spatial distribution of microbial communities have only recently been reported (Martiny et al., 2006). Most previous studies investigating the spatial distribution of soil bacteria have focused either on the total bacterial community or on particular species (Green et al., 2008). However, this taxon-centered perspective is unlikely to detect the possible linkage between the composition and function of bacterial communities, because a broad range of functional variation may occur among similar organisms while a unique function may be present among a broad variety of bacterial species. Therefore, Green et al. (2008) highlighted the use functional trait-based approaches for studying the spatial distribution of microorganisms. Analysis of functional guilds (communities of populations that share certain traits) could bridge the gap between the ecology of microbial communities and ecosystem functioning. Denitrifiers have

been recently considered as a model guild of global concern in functional ecology (Philippot and Hallin, 2005), and so spatial analysis of that community is now needed. Spatial patterns of denitrifying communities at the field or landscape scales deserve special attention because the patterns could be linked with land use; understanding the patterns could lead to better management strategies for lowering N_2O emissions from soils.

From the taxon-centered point of view, the detection of spatial patterns of taxon-specific groups of bacteria largely depends on the choice of taxonomic level used to define the operational unit (Levin, 1992; Ramette and Tiedje, 2007). At which level of taxonomic organization can spatial patterns of distribution be observed remains one of the unanswered and central questions in microbial ecology. Spatial patterns of bacteria are more likely to be observed at low taxonomic levels (strain or species), because there is a higher probability that organisms will share similar responses to environmental gradients if they belong to the same strain or species than if they belong to, for example, the same genus or family. However, Fierer et al. (2007) have recently suggested that certain bacterial phyla could be differentiated into *r*- and *K*-selected ecological categories, which provides a basis for the hypothesis that ecological traits and therefore patterns of spatial distribution differ among bacterial phyla.

Effect of pH on denitrification

pH is a master soil variable because it affects all soil properties, whether chemical, physical, or biological (Brady and Weil, 1999). This is also true for denitrification, and pH is one of the most important factors influencing both denitrification rate and ratio of the two main denitrification products, N_2O and N_2 (Šimek and Cooper, 2002). In general, denitrification activity increases with increasing pH (until the pH optimum), while the $N_2O/(N_2O+N_2)$ ratio decreases with increasing pH (Tate, 1995). Under acidic pH, the activity of N_2O reductase is lowered, and the synthesis of new N_2O reductases is inhibited, resulting in increased accumulation of N_2O. This means that at low pH, the major denitrification product is often N_2O. In soils with higher pH values, the activity and synthesis of N_2O reductase is supported and therefore more N_2 is produced. This relationship has been characterized in laboratory experiments (e.g., Šimek et al., 2002; Čuhel and Šimek, 2011), but it is unclear whether the same relationships exist in the field because of methodological limitations for *in situ* measurement of N_2 emissions (Groffman et al., 2006). However, a better understanding of the effect of pH on the regulation of field fluxes of both denitrification products is essential if liming is to be used to increase soil pH and thereby lower N_2O emissions from pasture soils.

Fierer and Jackson (2006) have recently shown that the structure of bacterial communities in soil is not random but is significantly driven by pH. They used a ribosomal DNA-fingerprinting method to quantitatively compare the composition and diversity of bacterial communities in soils from across North and South America. Surprisingly, bacterial diversity was not related to those variables that typically predict the diversity of plants and animals. The diversity and richness of soil bacterial communities differed by ecosystem type, and these differences could largely be explained by soil pH. These findings were further expanded and verified by Lauber et al. (2009), who used the bar-coded pyrosequencing technique to examine the structure and diversity of bacterial communities in a similar set of soils; these authors found that the influence of soil pH on bacterial community composition is evident at even relatively coarse levels of taxonomic resolution. Rousk et al. (2010) compared data from Lauber et al. (2009) with their own results of bacterial community structure across a 180-m pH gradient (pH 4.0–8.3) that was achieved by long-term liming of an arable soil and showed that there was as much variability in bacterial communities across the 180-m distance (Rousk et al., 2010) as across soils sampled in a wide range of biomes in North and South America (Lauber et al., 2009). This suggests the dominance of pH in establishing the structure of bacterial communities. Because of the broad taxonomical diversity of denitrifiers and because denitrification represents only a facultative trait of denitrifiers, soil pH is likely to also be important in structuring denitrifying communities, which has been already shown in several studies (Parkin et al., 1985; Enwall et al., 2005; Philippot et al., 2007). This raises the possibility that soil pH influences the denitrification rate, the ratio of the denitrification products, and subsequent N gas emissions not only directly through the kinetics of denitrification reductases but also indirectly through its impact on composition and abundance of the denitrifier community. The denitrifier community, in turn, acts as a transducer through which the direct effect of pH on denitrification activity and N_2O emissions is realized (Wallenstein et al., 2006). However, the relative significance of direct and indirect controls by pH on denitrification remains unclear.

In-depth research on the effect of pH on denitrification is limited by the high buffering capacity of most soils, which makes difficult to achieve fast and long-lasting changes of soil pH; it follows that experimental control of soil pH is difficult. Therefore, studies on the influence of pH on denitrification and other activities of soil microorganisms commonly use either a set of soils differing in pH (e.g., Bremner and Shaw, 1958; Drury et al., 1991; Bååth and Anderson, 2003) or soils whose pH was modified gradually over time (e.g., Adams and Adams, 1983; Aciego Pietri and Brookes, 2008; Nicol et al., 2008) by application of lime or other compounds. In both cases, however, possible linkages between pH and biological

processes or variables might not represent direct causal relationships because variables other than pH may differ among different soils and because the effect of pH may be indirect. Moreover, pH manipulation can take years and require repeated lime applications.

Aims of the thesis

The overall aim of this Ph.D. thesis was to determine whether production of N_2O and N_2 via denitrification, subsequent emissions of these gases into the atmosphere, size of the denitrifying community, and environmental factors are linked to each other in pasture soils. The specific objectives of the thesis were:

1. To describe the shifts in the abundance of functional denitrification genes, denitrification activity, and N_2O emissions in a cattle overwintering area as a result of different cattle-induced impacts on soil physico-chemical parameters.
2. To explore spatial patterns of size and activity of the denitrifying community in a grassland field subjected to different cattle grazing regimes, and to investigate whether the pattern of denitrifier abundance was related to those of denitrifying activity, the ratio of the denitrification products (N_2O and N_2), and environmental regulators of denitrification.
3. To investigate the effect of changes in soil pH on *in situ* N_2O and N_2 emissions, potential denitrification activity, and the size of the microbial community possessing the different denitrification genes, and to detect possible relationships among these characteristics.
4. To determine how soil pH influences the denitrification rate and the relative production of N_2O both directly (through kinetics of denitrification reductases) and indirectly (through changes in the structure of denitrifier community).

To meet these objectives, a set of field and laboratory experiments and measurements were conducted using a unique methodological approach that combined the traditional acetylene blockage method, ^{15}N-labeling techniques (to provide information on *in situ* emissions of both N_2O and N_2), molecular techniques (to quantify denitrifiers), and geostatistics (to characterize and map spatial patterns).

DESCRIPTION OF SAMPLING SITES

All experiments, measurements, and soil sampling described in this thesis were conducted at an upland pasture of the Borová Farm near Český Krumlov in South Bohemia, Czech Republic (latitude 48°52′N; longitude 14°13′E; altitude 630 m). The farm is situated in the Protected Landscape Area Blanský Les and is specialized for beef cattle husbandry and hay production under an organic farming regime; the thesis research, however, was focused only on the parts of the pasture more or less impacted by cattle. The mean annual precipitation in the area is 650 mm, and the annual average temperature is 7 °C (data from meteorological station located 7 km from the pasture). The soil at the site is classified as Haplic Phaeozem (arenic; World Reference Base system) containing 60 to 80% sand, 14 to 32% silt, and 6 to 14% clay (USDA classification system). The plant cover of the pasture is a mixture of grasses, clovers, and other dicotyledonous plants.

The first study (**Paper I**) was performed at an overwintering area within the pasture. This so-called winter pasture occupied 4.04 ha and had been used since 1995 for overwintering of ca. 90 cows. In this husbandry system, the cattle herd is present at the winter pasture each year from October/November to April/May. Because the overwintering area is adjacent to a cow barn with open access to the pasture, the cows use the overwintering area very unevenly. Soon after the cattle arrive in autumn, a gradient of cattle impact becomes apparent on the winter pasture. Along the gradient, three sites differing in cattle impact were identified. Site SI represents a severely impacted part of the pasture near the cow barn. At the end of the overwintering period in spring, site SI is characterized by destroyed plant cover, degraded soil structure, and an oversupply of excrements. The site with moderate impact, MI, is ca. 100 m from the cow barn; cattle are irregularly present at site MI. Site NI represents a control with no or slight cattle impact. A more detailed description of the sites is provided by Šimek et al. (2006a) and Hynšt et al. (2007).

Another experimental site was selected within the pasture area for studying spatial distribution of the size and activity of the denitrifier community (**Paper II**) and spatial distribution of the abundance of selected bacterial taxa (**Paper III**). Since 1998, different grazing regimes had been applied to the site, which resulted in three adjacent areas or plots that differed in the following manner in spring 2007 (Fig. 1): (i) plot covered with original vegetation, under very low impact of cattle (estimated average density of two to four heads per ha); (ii) plot lacking vegetation because of high impact of cattle for about 6 months preceding soil sampling (estimated average density of up to 80 heads per ha); and (iii) plot under medium

impact of cattle (estimated average density of 20–30 heads per ha). A total of 60 soil samples (20 per plot) were collected from the 39.6 by 14.4 m grid (the combined area of the three plots) with 3.6 m between sampling points (Fig. 1).

The third experimental site was also at the Borová Farm and concerned the artificial manipulation of soil pH (**Papers IV and V**). The experimental field (12×18 m) was divided into 24 plots (3×3 m each), 12 of which were subjected to one of the following three pH manipulations: (i) four random plots were amended with a KOH solution to increase the pH, (ii) four random plots were amended with an H_2SO_4 solution to decrease the pH, and (iii) four random plots were amended with water (control plots with a natural pH) (Fig. 2). The KOH and H_2SO_4 solutions were applied three times before the soil was sampled or N gas emissions were measured. The KOH and H_2SO_4 solutions were applied uniformly to the whole surface of each plot with a sprinkling can, and the same volume of water was applied to the control plots (for details see **Paper IV**).

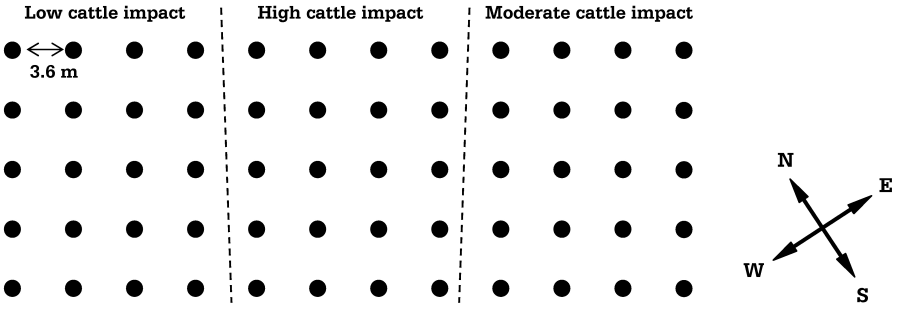

Fig. 1. Distribution of 60 sampling points for the geostatistical research.

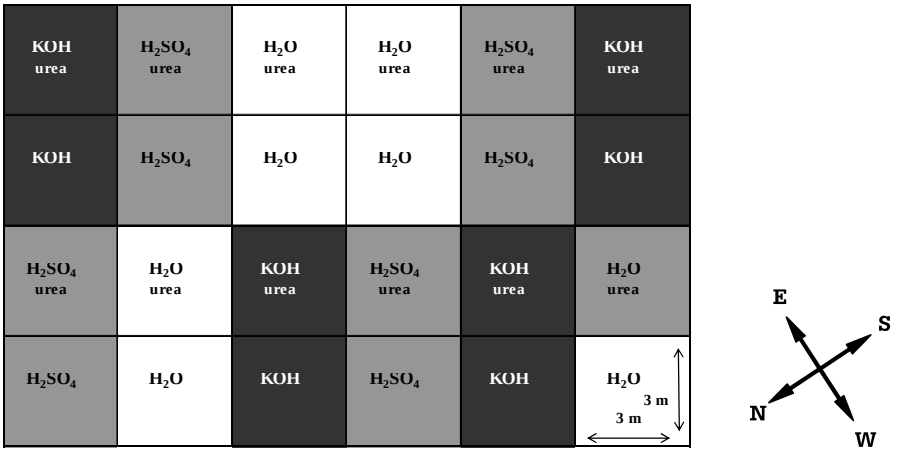

Fig. 2. Distribution of experimental plots with different pH treatments. The plots with application of urea were not used in the thesis.

RESULTS AND GENERAL DISCUSSION

This thesis research began with a study of the function of the denitrifying community in relation to its abundance at a cattle overwintering area. Previous studies at the same area had shown that different levels of cattle impact had led to the differences in denitrification activity, physico-chemical properties including pH (Šimek et al., 2006a), field N gas emissions (Šimek et al., 2006b; Hynšt et al., 2007), composition of bacterial communities (Elhottová and Šimek, 2002), and abundance and activity of methanogenic Archaea (Radl et al., 2007). We used qPCR to quantify three functional denitrification genes (*nirK*, *nirS*, and *nosZ*) at the three sites that differed in degree of cattle impact; samples were collected in spring (before cattle left the overwintering area) and in autumn (before cattle returned to the overwintering area). The results were further related to soil physico-chemical properties (especially carbon and nitrogen content) as well as potential denitrification rates and actual N_2O emissions *in situ* (**Paper I**).

The degree of cattle impact was positively correlated with total N, organic C, pH, and moisture in the pasture soils. These changes in soil physico-chemical properties also influenced soil microbial processes (Šimek et al., 2006a), including denitrification in that the activity of denitrifying enzymes (DEA) followed the increasing degree of cattle impact: DEA was highest in the severely impacted soil (SI) and lowest in the NI soil (which had no or slight cattle impact). Similar results were obtained by Meneer et al. (2005), who described a significant increase of denitrification rates in soil depending on the intensity level of animal treading. In contrast to DEA, *in situ* N_2O emissions were highest at the moderately impacted site (MI) and lower N_2O emissions at the SI site indicated that most gaseous loss of N was in the form of N_2 from soil SI. This inference is supported by Šimek et al. (2006b), who used the ^{15}N tracer method to determine emissions of both N_2O and N_2 from the same sites. One of the reasons for the reduced N_2O emissions from the SI site could be higher soil compaction (due to the animal impact), which would reduce N_2O diffusion rates and thereby prolong the residence time of N_2O in the soil profile and increase the probability of complete denitrification (all the way to N_2) (Weier et al., 1993). Another explanation for the low N_2O emissions from the SI site could be the site's significantly higher soil pH, which is known to reduce relative N_2O production (Šimek and Cooper, 2002); the latter inference is supported by the higher N_2O-reducing ability of the SI soil during the DEA assay. Nevertheless, the discrepancy between the results of DEA and N_2O emissions highlights the necessity of determining field emissions of both N_2O and N_2.

The abundance of the genes encoding NO_2^- reductase (*nirK* and *nirS*) was congruent with animal impact on soils, and positively correlated with DEA. In spring, these genes were most

abundant at the SI site and least abundant at the NI site. The gene abundances also decreased in autumn, which is related to the reduced values of DEA. On the other hand, the size of the community possessing the gene *nosZ* was not changed as significantly as nirS and nirK community: the *nosZ* abundance was relatively stable under different levels of cattle impact and also during the season. The ratio *nirK*/(*nirS*+*nirK*) was higher at the NI site than at the SI or MI sites for both sampling periods, which might be due to preferential niche differentiation between NirK and NirS denitrifiers (Oakley et al., 2007; Smith and Ogram, 2008). Jones and Hallin (2010) hypothesized that NO_3^- concentration was driving the community assembly process among *nirS* denitrifiers in their study whereas *nirK* denitrifiers may be responding to a different environmental parameter, e.g., soil Cu content as indicated by Enwall et al. (2010). In summary, **Paper I** demonstrated that it is possible to find a linkage between the abundance and activity of the denitrifier community in pasture soils differing in cattle impact.

The next part of the thesis (**Paper II**) investigated the spatial distribution of the size and activity of the denitrifier community. This research used geostatistical modelling in a grassland field with three areas that had experienced different cattle grazing regimes (Fig. 1). Geostatistics is used to quantify spatial variation; with geostatistics, researchers can estimate values in non-sampled areas and produce detailed interpolation maps of specific parameters by kriging (Krige, 1951). The size of the denitrifier community was determined with quantification of the genes *narG*, *napA*, *nirK*, *nirS*, and *nosZ*.

We observed a nonrandom distribution pattern of the gene abundances with a spatial autocorrelation range of 6–16 m. Given this autocorrelation range, the distribution of denitrifier abundance could be modelled at the field scale. The kriged maps of the genes *narG*, *napA*, *nirK*, and *nosZ* revealed a gradient in their distribution between the north and south areas of the field. The spatial distribution of the 16S rRNA gene also followed this gradient, which confirms that the denitrification trait is not a strong factor controlling abundance of denitrifying community and that the sizes of both the total bacterial community and of the denitrifier community are controlled by the same factors. Sizes of the total bacterial community and of the denitrifier community were not affected by the presence of cattle, and none of the soil properties were significant predictors of the 16S rRNA, *narG*, *napA*, *nirK*, and *nosZ* gene distributions. This indicates that factors that were not taken into account in our study (e.g., topography) were driving the abundance of denitrifiers and total bacteria. The exception is the distribution of *nirS* gene abundance, which exhibited a completely different pattern from that of the other genes; *nirS* abundance was highest in the central area of the field and was positively correlated with several soil properties such as NO_3^- and NH_4^+ concentrations, pH, and soil

moisture. This result is consistent with Hallin et al. (2009), who reported that among the denitrification genes, only the abundance of *nirS* was correlated with soil properties. Interestingly, the *nosZ*/*narG* and *nosZ*/16S rRNA ratios, which represent the proportion of bacteria genetically capable to perform the last step in the denitrification cascade compared with those genetically capable to perform the first step in the cascade or to total bacteria, respectively, were higher in the central area of the field. These results indicate that the proportion of bacteria able to reduce N_2O is not constant and can be affected by environmental gradients. As was found in **Paper I**, analysis of the distribution of the *nirS*/*nirK* ratio revealed significantly lower ratios in the area of the field more impacted by cattle, which indicates again that NirK and NirS denitrifiers prefer different niches.

In contrast to the abundance of most denitrification genes, the spatial distributions of DEA and relative N_2O production were strongly affected by the presence of cattle. Significant correlations between DEA and NO_3^- and NH_4^+ concentrations, pH, soil moisture, and organic C indicated that the spatial distribution of some soil properties, as altered by cattle activity, imposed significant control on denitrification. We found that the distribution patterns of denitrifier abundance and activity were correlated when the gene *nirS* was used as a molecular marker. These results can be directly compared to Enwall et al. (2010), who used geostatistical modelling to map spatial patterns of the activity, size, and structure of denitrifiers on a 44-ha farm (thus, at a scale compatible with land management). Enwall et al. found that the rate of potential denitrification was more closely related to the spatial pattern for *nirS* gene abundance than for *nirK* gene abundance. Moreover, the spatial pattern of denitrification activity in their study was reflected in the maps of the *nirS* community structure but not in the maps of the *nirK* community structure (as determined with terminal restriction fragment length polymorphism). Denitrifiers with the *nirS* gene are clearly of special importance in determining denitrification rate.

We found that relative N_2O production was higher in the low impacted areas, perhaps due to the lower soil pH compared with the more impacted areas (Šimek and Cooper, 2002); accordingly, we documented a significant negative correlation between the $N_2O/(N_2+N_2O)$ ratio and pH. Moreover, the relative abundance of bacteria with the *nosZ* gene encoding N_2O reductase in the total bacterial community was a strong predictor of relative N_2O production, indicating that the proportion of bacteria able to reduce N_2O would be crucial in determining the nature of the denitrification end product, i.e., N_2 or N_2O. Thus, we provided evidence for a relationship between bacterial community composition based on the relative abundance of denitrifiers in the total bacterial community and ecosystem processes. More generally, the presented geostatistical approach allows integrated mapping of microbial communities, and

hence can facilitate our understanding of relationships between the ecology of microbial communities and microbial processes along environmental gradients.

Paper III does not fully meet the objectives and scope of this thesis, because it is not focused on denitrification. Nevertheless, because we used the same soil samples and DNA extracts as in **Paper II** and because we used similar methods (qPCR and geostatistical analysis) to describe and map the spatial patterns of genes, we believe that **Paper III** should be included in the thesis. In **Paper III**, we investigated the spatial distribution of the abundance of bacterial groups at high taxonomical levels (phylum or class): *Acidobacteria*, *α-Proteobacteria*, *Actinobacteria*, *Bacteroidetes*, *β-Proteobacteria*, *Gemmatimonadetes*, *Firmicutes*, *Verrucomicrobia*, and the total bacterial community. Recent consultation of 32 16S rRNA gene clone libraries had revealed these to be the predominant bacterial taxa in soil (Janssen, 2006). The distributions of the relative abundance of most taxa (except *Firmicutes*) displayed strong spatial patterns at the field scale and could be predicted at the field scale. Moreover, the interpolated maps of the relative abundance of the taxa revealed differences between the northwest, central, and southeast areas of the field, which mainly reflected the degree of cattle impact. Comparison of the interpolated maps also revealed that some of the targeted taxa displayed different or even contrasting spatial patterns, which indicates that they respond differently to spatially structured environmental factors. This indicates that, within a given environment, members of a bacterial clade defined at high taxonomic levels shared specific ecological characteristics. While researchers still question whether the branching pattern of the tree of life corresponds to anything in nature (Doolittle and Bapteste, 2007), our results provide evidence that the 16S rRNA gene tree divisions are not only based on evolutionary theory but also are mirrored in the nature.

The results described in **Papers I** and **II** indicated that soil pH could greatly affect denitrification activity (especially the ratio of the products N_2O and N_2) and the abundance of denitrifiers in the studied pasture ecosystem. This agrees with previous findings that pH is one of the most important factors influencing denitrification (Šimek and Cooper, 2002) and that pH affects the structure of denitrifier communities (Parkin et al., 1985; Enwall et al., 2005). Therefore, we decided to conduct a field experiment that included the relatively rapid adjustment of soil pH (Fig. 2), where the impact of pH on the denitrification process could be explored more deeply.

The field experiment was conducted using replicated plots in which the soil pH was modified by addition of either acid or hydroxide to obtain soil that was acidic, pH natural, or

alkaline. A ^{15}N-tracer method was used to provide information on N_2O and N_2 emissions. In addition to measuring DEA, we measured the size of the denitrifier community by qPCR of the denitrification genes (**Paper IV**). Because the application of the acid and hydroxide to the soil also affected soil properties other than pH, the correlations between pH and N fluxes or denitrifying community size observed in our work might not represent direct causal relationships, as confounding effects cannot be ruled out.

Analysis of the denitrification process in the soils with different pH treatments in **Paper IV** revealed a significant decrease in N gas production with decreasing soil pH for both cumulative N fluxes *in situ* and DEA, which agrees with previous findings of lower denitrification rates in acidic soil (Šimek et al., 2002; Enwall et al., 2005). In contrast, we did not find any differences in N_2O emissions between the pH treatments for either the cumulative *in situ* N_2O emissions or potential N_2O production. This result was quite strange because higher N_2O emissions in acidic soils have been reported in several studies (Mkhabela et al., 2006; Weslien et al., 2009). Calculation of the $N_2O/(N_2O+N_2)$ ratio showed a decreasing molar ratio with increasing soil pH, which agrees with previous studies (Šimek and Cooper, 2002). Surprisingly, the $N_2O/(N_2O+N_2)$ ratio in the field was significantly correlated with the $N_2O/(N_2O+N_2)$ ratio calculated from the DEA assay under laboratory conditions. Moreover, the total N fluxes *in situ* were significantly correlated with potential denitrification (DEA). This is an important finding indicating that determination of the $N_2O/(N_2O+N_2)$ ratio under laboratory conditions can reflect treatment differences in the field. Overall, our results confirm the role of soil pH *in situ* in determining the nature of the denitrification end products and process rates. We emphasize, however, that many variables in our experimental field study, such as pH and NO_3^-, were correlated with each other.

The abundance of the denitrification genes was not correlated with total N fluxes *in situ*, and only the abundance of the *nirS* gene was correlated with DEA. This positive correlation between *nirS* gene abundance and DEA in **Paper IV**, which was also found in **Papers I** and **II**, suggests a link between nirS denitrifiers and denitrification rate. Further, we found a positive correlation between the relative abundance of the gene *nirS* and soil pH, which indicates that soil pH influences denitrification also through a pH-driven change in the structure of the denitrifier community. In contrast to the results described in **Paper II**, which suggested that the proportion of bacteria able to reduce N_2O could be of importance in determining the nature of the denitrification end products, we did not find a negative correlation between the proportion of denitrifiers possessing the *nosZ* gene and the $N_2O/(N_2O+N_2)$ ratio. This indicates that, not surprisingly, the routes for N_2O production are numerous and that the relative importance of

denitrification enzyme regulation, denitrifier community composition, and the proportion of denitrifiers lacking the gene for an N_2O reductase remains to be experimentally demonstrated.

As noted earlier, soil pH can influence denitrification both directly through effects on the kinetics of denitrification reductases and indirectly through changes in the denitrifier community. This agrees with the concept of proximal and distal control of denitrification, as proposed by Wallenstein et al. (2006). In the previous study (**Paper IV**), we observed a relationship between soil pH, the abundance of denitrifiers possessing the *nirS* gene, and denitrifying activity (DEA). It was unclear, however, whether soil pH influenced denitrification directly or indirectly; thus, it was also unclear whether there is a causal relationship between the size and activity of the denitrifying community in soils differing in pH. Consequently, we explored how soil pH influences the denitrification rate and $N_2O/(N_2O+N_2)$ ratio both directly and indirectly in **Paper V**. We subjected the soils from the field pH manipulation experiment to further pH adjustment just before DEA measurements to determine the effect of short-term changes in pH.

Our results clearly indicated that DEA (overall N_2O and N_2 production) was more affected by the relatively long-term pH management in the field, which led to the changes in the abundance of denitrifiers possessing the *nirS* gene, than by short-term changes in pH. It was evident that the denitrification rate was controlled by pH indirectly, i.e., denitrification was more affected by the size and composition of the denitrifying community than by the current or direct pH effect. On the other hand, the ratio of the denitrification products (N_2O and N_2) was affected by the current pH value of the soil slurries during measurement rather than by the long-term pH value. Thus, the balance between N_2O production and reduction was controlled exclusively by the direct pH effect. These findings partly refute the results from **Paper II**, where we found a correlation between the relative N_2O production and the proportion of bacteria genetically capable of performing N_2O reduction relative to the total bacteria. We suspect that there probably was no causal relationship between the relative N_2O production and the relative abundance of N_2O-reducing bacteria in **Paper II**, because the $N_2O/(N_2O+N_2)$ ratio was also negatively correlated with soil pH.

The finding that the ratio of denitrification products is directly controlled by pH agrees with Liu et al. (2010), who recently reported that the dependency of the ratio on soil pH is a post-transcriptional phenomenon because the relative N_2O production from soils differing in pH in their study did not correspond to the relative abundances or to the relative transcription rates of the denitrification genes *nosZ* and *nirS*. Liu et al. suggested that pH affects the translation, protein assembly, or the activity of N_2O reductase. We demonstrated only the direct influence

of pH on the activity of N_2O reductase because during the DEA measurement induction of new denitrification reductases does not occur (Smith and Tiedje, 1979). Similarly, Chèneby et al. (1998) indicated that the presence of the *nosZ* gene is a poor predictor of the ability of bacteria to reduce N_2O to N_2 among denitrifying isolates. In contrast, Philippot et al. (2011) inoculated three different agricultural soils with serial dilutions of the denitrifying strain lacking the *nosZ* gene so as to modify the proportion of denitrifiers having the genetic capability for N_2O reduction; in general, they provided evidence that the inability of denitrifiers to synthesize N_2O reductase can affect the ratio of the denitrification products, which indicated that the extent of N_2O reduction can have a genetic basis. N_2O production in their study, however, increased only when the size of the inoculated population was in the same range as the indigenous community having the *nosZ* gene, which may not occur under field conditions.

These discrepancies in results from different studies may be explained by the fact that detection of denitrifying genes in soil does not necessarily indicate that the organisms with those genes are actively denitrifying at the time of measurement. In other words, gene abundance is not likely to be completely correlated with potential denitrification rate or ratio of the denitrification end products because the genetic pool only partly contributes to the activity at a given time (Enwall et al., 2010). Moreover, the development of primers for the denitrification genes is an ongoing process. Therefore, the targeting of the denitrifying community by approaches based on DNA remains an imprecise tool for determining the linkage between the ecology and function of denitrifiers. To better determine the relationship between community structure and function, we require the improvement and utilization of methods based on targeting mRNA or proteins of the denitrification enzymes for better detection of active denitrifiers in soil, as underlined by Philippot and Hallin (2005).

In conclusion, the results obtained in this Ph.D. thesis have increased the understanding of the microbial control and regulation of denitrification in and of the N gas emission from pasture soils. We detected shifts in both the abundance and activity of the denitrifying community as the result of increased cattle impact or artificial changes in soil pH. Most importantly, we found a linkage between the size of the denitrifier community, the denitrification rate, and the proportion of the denitrification end products. We also determined that relative N_2O production under laboratory conditions can reflect treatment differences in the field. Moreover, the information obtained by various manipulations of soil pH provided insights into the possible ways in which soil pH influences denitrification activity and the ratio of N_2O and N_2. More generally, the results have increased our understanding of relationships between the ecology of microbial communities and microbial processes along environmental

gradients. It is crucial in future studies to continue to bridge the gap between studies of denitrifier ecology and of N fluxes for a comprehensive understanding of the role of denitrifier community ecology in determining not only total denitrification rates but also the nature of the denitrification end products.

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RESEARCH ARTICLES

Paper I

Chroňáková, A., Radl, V., **Čuhel, J.**, Šimek, M., Elhottová, D., Engel, M., Schloter, M., 2009. Overwintering management on upland pasture causes shifts in an abundance of denitrifying microbial communities, their activity and N_2O-reducing ability. Soil Biology & Biochemistry 41, 1132-1138.

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.

Abstrakt
Pastevní půdy využívané pro přezimování dobytka mohou představovat významný zdroj emisí N_2O. Proto jsme studovali, jak přezimování skotu na pastvině dlouhodobě ovlivňuje abundanci a aktivitu denitrifikačního společenstva. Tato práce byla provedena na zimovišti skotu v jižních Čechách (Česká republika), kde byly vybrány 3 plochy s různou zátěží pasoucími se zvířaty: plocha silně zastižená (SI), středně zastižená (MI) a kontrolní bez vlivu zvířat (NI). Stanovení emisí N_2O spolu s odběrem půdních vzorků proběhlo na jaře a na podzim roku 2005. Byla stanovena potenciální denitrifikační aktivita. Bakteriální geny *nirK*, *nirS* a *nosZ* byly použity jako funkční markery denitrifikačního společenstva; jejich abundance byla stanovena s využitím metody PCR v reálném čase. Emise N_2O *in situ* byly překvapivě nejvyšší na jaře na ploše MI a lišily se od emisí na plochách SI a NI, zatímco na podzim byly emise N_2O obecně nižší. Na druhou stranu byla potenciální denitrifikační aktivita nejvyšší v půdě SI, poté v půdě MI, a nejnižší v půdě NI. Byl pozorován celkově významný posun v molárním poměru N_2O/N_2 v půdách se zátěží zvířat. Nejvyšší abundance všech stanovených genů byla zaznamenána v půdě SI na jaře i na podzim, zatímco v půdě MI byly zvýšené hodnoty abundancí jenom na jaře. Naše výsledky naznačují silný vliv skotu na abundanci a aktivitu denitrifikačních mikroorganismů.

Publikace vyšla tiskem v časopise Soil Biology & Biochemistry.
Podíl studenta na publikaci: 40%.

Paper II

Philippot, L., **Čuhel, J.**, Saby, N.P.A., Chèneby, D., Chroňáková, A., Bru, D., Arrouays, D., Martin-Laurent, F., Šimek, M., 2009. Mapping field-scale spatial patterns of size and activity of the denitrifier community. Environmental Microbiology 11, 1518-1526.

Abstract
There is ample evidence that microbial processes can exhibit large variations in activity on a field scale. However, very little is known about the spatial distribution of the microbial communities mediating these processes. Here we used geostatistical modelling to explore spatial patterns of size and activity of the denitrifying community, a functional guild involved in N-cycling, in a grassland field subjected to different cattle grazing regimes. We observed a non-random distribution pattern of the size of the denitrifier community estimated by quantification of the denitrification genes copy numbers with a macro-scale spatial dependence (6–16 m) and mapped the distribution of this functional guild in the field. The spatial patterns of soil properties, which were strongly affected by presence of cattle, imposed significant control on potential denitrification activity, potential N_2O production and relative abundance of some denitrification genes but not on the size of the denitrifier community. Absolute abundance of most denitrification genes was not correlated with the distribution patterns of potential denitrification activity or potential N_2O production. However, the relative abundance of bacteria possessing the *nosZ* gene encoding the N_2O reductase in the total bacterial community was a strong predictor of the $N_2O/(N_2 + N_2O)$ ratio, which provides evidence for a relationship between bacterial community composition based on the relative abundance of denitrifiers in the total bacterial community and ecosystem processes. More generally, the presented geostatistical approach allows integrated mapping of microbial communities, and hence can facilitate our understanding of relationships between the ecology of microbial communities and microbial processes along environmental gradients.

Abstrakt
Rychlost mikrobiálních procesů je v polním měřítku velmi variabilní. Na druhou stranu máme k dispozici velmi málo informací o prostorovém uspořádání mikrobiálních společenstev, které jsou za tyto procesy zodpovědné. V této práci jsme použili geostatistické modelování, abychom v pastevní půdě s různým režimem pastvy skotu stanovili prostorového uspořádání velikosti a aktivity denitrifikačního společenstva – funkční skupiny zapojené do cyklu N. Pozorovali jsme nenáhodné prostorové uspořádání velikosti společenstva denitrifikátorů stanovené kvantifikací denitrifikačních genů. Prostorové uspořádání půdních parametrů, které bylo značně ovlivněno přítomností dobytka, statisticky průkazně ovlivnilo rychlost denitrifikační aktivity, potenciální produkci N_2O a relativní abundanci některých denitrifikační genů, ovšem neovlivnilo velikost denitrifikačního společenstva. Absolutní hodnoty abundance většiny denitrifikačních genů nekorelovaly s prostorovým uspořádáním denitrifikační aktivity nebo relativní produkce N_2O. Nicméně relativní abundance bakterií mající gen *nosZ* pro N_2O reduktasu z celého bakteriálního společenstva byla silným prediktorem poměru $N_2O/(N_2+N_2O)$, což poskytuje důkaz o vztahu mezi složením bakteriálního společenstva založeném na relativní abundanci denitrifikátorů z celého bakteriálního společenstva a ekosystémovými procesy. Popsaný geostatistický přístup umožňuje integrované mapování mikrobiálních společenstev a tak lepší porozumění vztahům mezi ekologií mikrobiálních společenstev a mikrobiálních procesů podél environmentálních gradientů.

Následující pasáž o rozsahu 9 stran obsahuje skutečnosti chráněné autorskými právy a je obsažena pouze v archivovaném originále disertační práce uloženém na Přírodovědecké fakultě Jihočeské univerzity v Českých Budějovicích.

Publikace vyšla tiskem v časopise Environmental Microbiology.

Podíl studenta na publikaci: 60%.

Paper III

Philippot, L., Bru, D., Saby, N.P.A., **Čuhel, J.**, Arrouays, D., Šimek M., Hallin, S., 2009. Spatial patterns of bacterial taxa in nature reflect ecological traits of deep branches of the 16S rRNA bacterial tree. Environmental Microbiology 11, 3096-3104.

Abstract
Whether bacteria display spatial patterns of distribution and at which level of taxonomic organization such patterns can be observed are central questions in microbial ecology. Here we investigated how the total and relative abundances of eight bacterial taxa at the phylum or class level were spatially distributed in a pasture by using quantitative PCR and geostatistical modelling. The distributions of the relative abundance of most taxa varied by a factor of 2.5–6.5 and displayed strong spatial patterns at the field scale. These spatial patterns were taxonspecific and correlated to soil properties, which indicates that members of a bacterial clade defined at high taxonomical levels shared specific ecological traits in the pasture. Ecologically meaningful assemblages of bacteria at the phylum or class level in the environment provides evidence that deep branching patterns of the 16S rRNA bacterial tree are actually mirrored in nature.

Abstrakt
Zda bakterie vykazují prostorové uspořádání a na jaké taxonomické úrovni lze toto uspořádání pozorovat, jsou klíčové otázky mikrobiální ekologie. V této práci jsme za využití metod kvantitativní PCR a geostatistického modelování studovali, jak jsou celkové a relativní abundance osmi bakteriálních taxonů na úrovni kmene či třídy prostorově uspořádány v pastevní půdě. Distribuce relativních abundancí většiny taxonů kolísalo s faktorem 2,5-6,5 a v polním měřítku projevovala silný prostorový vzorec. Prostorové uspořádání relativních abundancí většiny taxonů bylo pro daný taxon specifické a korelovalo s půdními parametry, což naznačuje, že se zástupci vyšších taxonomických skupin na pastvině vyznačovali podobnými ekologickými charakteristikami.

Publikace vyšla tiskem v časopise Environmental Microbiology.
Podíl studenta na publikaci: 20%.

Paper IV

Čuhel, J., Šimek, M., Laughlin, R.J., Bru, D., Chèneby, D., Watson, C.J., Philippot, L., 2010. Insights into the effect of soil pH on N_2O and N_2 emissions and denitrifier community size and activity. Applied and Environmental Microbiology 76, 1870-1878.

Abstract
The objective of this study was to investigate how changes in soil pH affect the N_2O and N_2 emissions, denitrification activity, and size of a denitrifier community. We established a field experiment, situated in a grassland area, which consisted of three treatments which were repeatedly amended with a KOH solution (alkaline soil), an H_2SO_4 solution (acidic soil), or water (natural pH soil) over 10 months. At the site, we determined field N_2O and N_2 emissions using the ^{15}N gas flux method and collected soil samples for the measurement of potential denitrification activity and quantification of the size of the denitrifying community by quantitative PCR of the *narG*, *napA*, *nirS*, *nirK*, and *nosZ* denitrification genes. Overall, our results indicate that soil pH is of importance in determining the nature of denitrification end products. Thus, we found that the $N_2O/(N_2O + N_2)$ ratio increased with decreasing pH due to changes in the total denitrification activity, while no changes in N_2O production were observed. Denitrification activity and N_2O emissions measured under laboratory conditions were correlated with N fluxes *in situ* and therefore reflected treatment differences in the field. The size of the denitrifying community was uncoupled from *in situ* N fluxes, but potential denitrification was correlated with the count of NirS denitrifiers. Significant relationships were observed between *nirS*, *napA*, and *narG* gene copy numbers and the $N_2O/(N_2O + N_2)$ ratio, which are difficult to explain. However, this highlights the need for further studies combining analysis of denitrifier ecology and quantification of denitrification end products for a comprehensive understanding of the regulation of N fluxes by denitrification.

Abstrakt
Cílem této práce bylo zjistit, jak změny půdního pH ovlivňují emise N_2O a N_2, denitrifikační aktivitu a velikost denitrifikačního společenstva. V pastevní oblasti byl založen polní experiment skládající se ze tří variant, které byly po dobu 10 měsíců opakovaně ošetřovány přídavky roztoku KOH (zásaditá půda), roztoku H_2SO_4 (kyselá půda) nebo vodou (půda s přirozeným pH). Na této pokusné ploše byly stanoveny emise N_2O a N_2 s použitím metody toku plynů značených ^{15}N a byly odebrány půdní vzorky pro měření potenciální denitrifikační aktivity a pro stanovení velikosti denitrifikačního společenstva pomocí kvantitativní PCR denitrifikačních genů *narG*, *napA*, *nirS*, *nirK* a *nosZ*. Naše výsledky celkově naznačují, že půdní pH je významné při stanovení poměru koncových produktů denitrifikace. Zjistili jsme, že poměr $N_2O/(N_2O+N_2)$ vzrůstá se zvyšujícím se pH kvůli změnám v celkové denitrifikační aktivitě, zatímco nebyly pozorovány změny v produkci N_2O. Denitrifikační aktivita a emise N_2O měřené za laboratorních podmínek korelovaly s toky N *in situ*, a proto odrážely rozdíly v polních variantách. Velikost denitrifikačního společenstva nebyla spojena s toky N *in situ*, avšak potenciální denitrifikace byla korelována s počtem denitrifikátorů majících gen *nirS*. Byly pozorovány průkazné vztahy mezi počtem kopií genů *nirS*, *napA* a *narG* a poměrem $N_2O/(N_2O+N_2)$, což je těžké objasnit. Tento fakt nicméně upozorňuje na potřebu dalších studií kombinujících analýzu ekologie denitrifikátorů a kvantifikaci koncových produktů denitrifikace pro ucelené pochopení regulace emisí N denitrifikací.

Publikace vyšla tiskem v časopise Applied and Environmental Microbiology.

Podíl studenta na publikaci: 70%.

Paper V

Čuhel, J., Šimek, M., 2011. Proximal and distal control by pH of denitrification rate in a pasture soil. Agriculture, Ecosystems & Environment 141, 230-233.

Abstract
Soil pH can influence denitrification both proximally and distally. Proximal control by pH involves direct changes in denitrification reductase activity while distal control by pH involves changes in the denitrifier community, which is a key component affecting the denitrification rate. The current study separated the proximal and distal control by pH of the denitrification rate and of the relative proportion of two denitrification gas products (N_2O and N_2). The potential denitrifying enzyme activity (DEA) was measured in the presence or absence of acetylene in three pasture soils differing in pH management in the field. The pH of these soils was further manipulated just before DEA measurement to determine the effect of short-term changes in pH. DEA was driven by the pH management in the field rather than by current pH resulting from short-term changes in pH. However, the $N_2O/(N_2O+N_2)$ ratio was driven by the effects of the current pH value on the kinetics of N_2O production and reduction. The data suggest that even if the pH-induced changes in the structure of denitrifying community can control the absolute denitrification rate (distal control by pH), the community does not influence the proportion of denitrification products, which is regulated solely by the proximal control by pH.

Abstrakt
Půdní pH může ovlivňovat denitrifikaci jak přímo tak nepřímo. Přímý vliv pH spočívá v okamžitém ovlivnění aktivity denitrifikačních reduktas, zatímco nepřímý vliv pH spočívá ve změnách denitrifikačního společenstva, které je klíčovou součástí kontroly denitrifikace. Tato práce oddělila přímý vliv pH na rychlost denitrifikace a na relativní produkci denitrifikačních produktů (N_2O a N_2) od vlivu nepřímého. Potenciální aktivita denitrifikačních enzymů (DEA) byla stanovena s acetylenem a bez acetylenu ve třech pastevních půdách lišících se v polní manipulaci pH. pH těchto půd bylo ještě dále ovlivňováno těsně před stanovením DEA, abychom mohli zaznamenat vliv krátkodobých změn pH. DEA byla řízena spíše polní manipulací pH než-li okamžitou hodnotou pH vycházející z krátkodobých změn pH. Avšak poměr $N_2O/(N_2O+N_2)$ byl řízen vlivem okamžité hodnoty pH na kinetiku produkce a redukce N_2O. Výsledky naznačují, že i když změny ve struktuře denitrifikačního společenstva indukované půdní reakcí (pH) mohou ovlivňovat absolutní rychlost denitrifikace (nepřímý vliv), tak společenstvo neovlivňuje poměr denitrifikačních produktů, který je regulován pouze přímým vlivem pH.

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