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Ph.D. Thesis

Diversity of the soil microbial community and its functional aspects in man-influenced environments

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Annotation

Diversity of the soil microbial community and its functional aspects were investigated in man-influenced environments, such as colliery spoil heaps in post mining sites and upland pasture used for outdoor cattle husbandry. The study was based on the cultivation of bacteria and streptomycetes as well as culture-independent approaches. Cultivated bacteria and streptomycetes were characterized by phenotypic and genotypic means. The culture-independent approaches were based on an analysis of environmental DNA in terms of both qualitative and quantitative parameters.

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Declaration

I declare here that I worked out this thesis on my own only with the use of the cited literature and other cited sources. I declare that in accordance with the Czech legal code § 47b law No. 111/198 in valid version I consent to the publication of my dissertation in an edition made by removing marked

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Prohlašuji, že jsem svoji disertační práci vypracovala samostatně pouze s použitím pramenů a literatury uvedených v seznamu citované literatury. Prohlašuji, že v souladu s § 47b zákona č. 111/1998 Sb. v platném znění souhlasím se zvěřejněním své disertační práce, a to v úpravě vzniklé vypuštěním vyznačených částí archivovaných Přírodovědeckou fakultou elektronickou cestou ve veřejně přístupné části databáze STAG provozované Jihočeskou univerzitou v Českých Budějovicích na jejích internetových stránkách.

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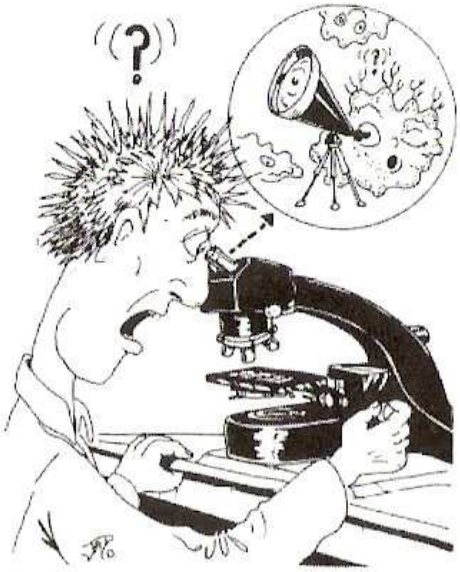

List of papers

The thesis is based on following papers and manuscripts, which are referred to in the text by Roman numerals and Latin letters.

Ia Elhottová D., Krištůfek V., Frouz J., Nováková A., **Chroňáková A.**: Screening for microbial markers in Miocene sediment exposed during open-cast coal mining. *Antonie van Leeuwenhoek International Journal of General and Molecular Microbiology* 89: 459- 463 (2006)

Ib Krištůfek V., Elhottová D., **Chroňáková A.**, Dostálková I., Picek T., Kalčík J.: Growth strategy of heterotrophic bacterial population along successional sequence on spoil of brown coal colliery substrate. *Folia Microbiologica* 50: 427- 435 (2005)

Ic **Chroňáková A.**, Halbritter A., Krištůfek V., Biró B.: Analysis of bacterial isolates and community DNA from four different succession plots in post-mining area. *In: **A. Chroňáková**, V. Krištůfek, D. Elhottová, S. Malý (Eds.): Present methods for investigation of microbial community biodiversity in soils and substrates. Institute of Soil Biology AS CR, České Budějovice, ISBN 80-86525-03-1*, pp. 93-96 (2004)

Id **Chroňáková A.,** Krištůfek V., Tichý M., Elhottová D.: Biodiversity, antibiotic production and resistance of recent and ancient streptomycetes isolated from Miocene lacustrine sediment and a successional sequence of post-mining sites. *Microbiological Research* (manuscript submitted)

IIa **Chroňáková A.**, Radl V., Čuhel J., Šimek, M., Elhottová D., Engel M., Schloter M.: Overwintering management on upland pasture causes shift in the abundance of denitrifying microbial communities, the activity and N_2O producing ability. *Soil Biology & Biochemistry* – manuscript *in press* (2009)

IIb Philippot L., Čuhel J., Saby N.P.A., Chèneby D., **Chroňáková A.**, Bru D., Arrouays D., Martin-Laurent F., Šimek M.: Mapping field-scale spatial distribution patterns of size and activity of the denitrifier community. *Environmental Microbiology* - manuscript *in press* (2009)

IIc Radl V., Gattinger A., **Chroňáková A.**, Němcová A., Čuhel J., Šimek M., Munch J.C., Schloter M., Elhottová D.: Effects of cattle husbandry on abundance and activity of methanogenic Archaea in upland soils. *The ISME J* 1: 443-452 (2007)

III van Elsas J.D., Hill P., **Chroňáková A.,** Grekova M., Topalova Y., Elhottová D., Krištůfek V.: Survival of genetically marked *Escherichia coli* O157:H7 in soil as affected by soil microbial community shift. *The ISME J* 1: 204-214 (2007)

IV **Chroňáková A.,** Horák A., Elhottová D., Krištůfek V.: Diverse Archaeal community of a bat guano pile in Domica Cave (Slovak Karst, Slovakia). *Folia Microbiologica* - manuscript *in press* (2009)

Contents

Souhrn

Tato disertační práce byla zaměřena na studium diversity mikrobiálních společenstev v sedimentech a půdách ovlivněných lidskou činností (extensivní povrchovou těžbou hnědého uhlí a přezimováním skotu na pastvině), přičemž byly aplikovány moderní molekulárně biologické přístupy. Tyto přístupy byly aplikovány jak na kultivovatelnou část, tak i na komplexní mikrobiální společenstva, což umožnilo studovat diversitu na různých úrovních složitosti. Zároveň byly použity přístupy k zhodnocení kvalitativní i kvantitativní složky struktury mikrobiálního společenstva, což umožňuje např. pochopit vztahy mezi složením funkčních skupin mikroorganizmů a dynamikou dusíku v ekosystému a naopak, pro studium toku dusíku je důležité znát kvantitativní zastoupení funkčního mikrobiálního společenstva. Půdní mikrobiální společenstvo zabezpečuje mnoho různých funkcí včetně koloběhu hlavních biogenních prvků, uhlíku a dusíku. Cílem studia bylo propojit informace o struktuře mikrobiálního společenstva s jeho funkcí použitím polyfazického přístupu k řešení problematiky.

Extenzivní povrchová těžební činnost významně narušuje vzhled krajiny a zároveň funkce ekosystému. Preferovanou funkcí z pohledu člověka se stává těžební činnost a vedlejším produktem této činnosti je navršení významného množství hlušiny na tzv. výsypkách. V sokolovské hnědouhelné pánvi jsou tyto hlušiny tvořeny převážně jíly, sedimenty třetihorního jezera, které tvoří nadložní vrstvy hnědého uhlí (Kříbek *et al.*, 1998). Cílem prezentované práce bylo zjistit, jestli existuje životaschopné mikrobiální společenstvo v těchto sedimentech a jak přispívá k jejich kolonizaci a spontánní sukcesi mikrobiálního společenstva po vytěžení a deposici na výsypkách. Ke studiu postupného vývoje sukcesních stadií byly vybrány 4 plochy spontánně vyvýjejících se výsypek, které se lišily stářím (0 - 44 let) a představují stadia kolonizovaná různou hustotou a typem dominantní rostliny: od stadia bez vegetace (iniciální, 1 – 2 roky), stadium s dominantními travami (zejména *Calamagrostis epigeios*; rané, 11 – 12 let), stadium s hustým porostem křovin vrb a olší (zejména *Salix caprea*; střední, 21 – 22 let) až po stadium lesního porostu (*Betula* spp. a *Populus tremuloides;* pozdní, 43 – 44 let). Pozdní stadium je jako jediné charakterizováno přítomností již vyvinutého půdního profilu, ve kterém lze rozeznat fermentační a humusovou vrstvu a vrstvu minerální a také aktivitu žížal. Hlavním cílem práce bylo porovnat a charakterizovat mikrobiální společenstva jednotlivých sukcesních stadií, a to ve svrchní (0-5 cm) a minerální (10-15 cm) vrstvě. Očekávali jsme, že změny v půdních charakteristikách sukcesního gradientu budou posléze doprovázeny změnami mikrobiální biomasy, aktivitou a diversitou půdního mikrobiálního společenstva, hlavně jeho heterotrofní bakteriální složky. Výsledky jednotlivých studií potvrdily, že miocenní sediment obsahuje stopy biologických markerů živých mikroorganizmů, a to fosfolipidy mastných kyselin, které mají rychlý obrat a obecně jsou vnímány jako markery živé mikrobiální biomasy. Struktura zastoupení jednotlivých mastných kyselin ukázala na dominanci heterotrofních bakterií, hlavně aktinomycet a saprofytických mikroskopických hub ve fosilním mikrobiálním společenstvu. Fosilní mikrobiální společenstvo může významně přispívat k usnadnění kolonizace čerstvě vytěžených sedimentů. V sukcesní řadě vyvíjející se na tomto substrátě roste mikrobiální biomasa s časem a s převahou gram pozitivních bakterií ve všech sukcesních stadiích. Mezi kultivovatelnými heterotrofními bakteriemi lze pozorovat trend vývoje společenstva od lépe kultivovatelných (vyšší poměr C:T, poměr kultivovaných k celkovým bakteriím) a r-stratégů (rostoucích na miskách do 3 dnů) k obtížněji kultivovatelným (nižší poměr C:T) a K-stratégům (objevují se na kultivačních miskách mezi 4 - 7 dnem). Zároveň bylo v další studii zjištěno, že Miocenní sediment obsahuje kultivovatelné streptomycety tvořící dvě fylogeneticky oddělené skupiny, z nichž jedna se v dalších sukcesních stadiích již neobjevuje, a druhá skupina pravděpodobně přetrvává a přežívá vytěžení a expozici na zemský povrch. Během sukcesního vývoje jednak rostla biomasa bakterií a streptomycet, množství organického uhlíku, a také podíl steptomycet v kultivovatelné složce bakterií a konečně genetická diversita kultivovatelných streptomycet. Zároveň bylo zjištěno, že existuje vysoký genetický potenciál a také zajímavé fenotypové charakteristiky (produkce antibiotik a

rezistence k nim) kmenů streptomycet izolovaných jak z miocenního sedimentu, tak ze sukcesní řady na sokolovských hnědouhelných výsypkách. Genetické vyhledávání založené na detekci genu pro 5-aminolevulinát synthasu (*hemA-asuA;* Petříček et al., 2006*)* v genomu streptomycet vyústil v objevení 13 nových potenciálních producentů antibiotik manumycinového typu izolovaných právě ze studovaných ploch sokolovských výsypek (výsledky nejsou součástí prezentované disertační práce).

Také různé zemědělské aktivity člověka narušují původní stabilní ekosystém a mění zcela jeho funkci, například podhorská pastvina sloužící jako zimoviště skotu. K studijnímu účelu bylo vybráno zimoviště na farmě v Borové v jižních Čechách o rozloze ca 4 ha (48°52′ severní z.v., 14°13′ východní z.d.). Zimoviště je zvláštní ekosystém, který podléhá sezónnímu koloběhu využívání: od podzimu (obvykle konec října) do jara (začátek května) je tato pastvina intenzivně využívaná pro ca 80 kusů dobytka, které svou činností vytvářejí tzv. gradient zatížení. V letním období zůstává zimoviště bez dobytka a půda regeneruje. Nejvíce ovlivněná část zimoviště se nachází v blízkosti stájí, kde mají zvířata volný přístup ke krmení a vodě, zatímco nejméně ovlivněná část se nachází na nejvzdálenějším konci plochy. Zvířata ovlivňují charakter půdy nejenom nerovnoměrnou deposicí velkého množství exkrementů, ale také pastvou a pošlapáváním. Hlavním cílem práce bylo porovnat mikrobiální přeměny dusíku v půdě zimoviště ovlivněné silnou, střední a žádnou pastevní činností skotu. Práce vycházela z předpokladu, že způsob obhospodařování ovlivní zvýšený vstup organického dusíku a uhlíku do půdy, vlhkost, pH, množství minerálních forem dusíku v půdě, mikrobiální biomasu a relativní zastoupení denitrifikačních bakterií. V důsledku toho se změní aktivita mikrobiálního společenstva, zejména pak denitrifikace, emise plynných forem dusíku z půdy a jejich vzájemný poměr (N_2O:N_2). Výsledky potvrdily předpoklad, že přezimování skotu významně změnilo měřené půdní charakteristiky. Půdní fyzikálně-chemické vlastnosti, stejně jako biologické vlastnosti, byly výrazně ovlivněné způsobem hospodaření, a to hlavně na jaře, kdy se na místech ovlivněných pastvou nacházely velké deposice exkrementů zvířat. Tyto změny byly propojeny s nárůstem mikrobiální biomasy v místech, kde byla vyšší dostupnost organického uhlíku a minerálních forem dusíku a také se zvýšeným výskytem anaerobních mikroprostředí. Narušující efekt sešlapávání půdy zvířaty a deposice exkrementů byl charakterizován zničením původní vegetace a změnou půdní struktury. Funkční změny odlišily půdy ovlivněné pastvou skotu od kontroly zvýšením relativního zastoupení metanogenů a denitrifkačních bakterií a také zvýšenou metanogenezí a denitrifikační aktivitou. Vliv pasených zvířat vyústil v posun v možném fungování ekosystému, jak jasně indikují změny v poměru plynných produktů denitrifikace (N_2O:N_2) a nerovnováhou mezi produkcí a spotřebou metanu vzhledem k 10-letému hospodaření na studované pastvině.

Summary

This Ph.D. thesis focused on the study of soil microbial community diversity in man-influenced environments: an extensive open cast brown coal mining and overwintering of cattle on upland soil, where modern molecular biology tools have been applied. Cultivation-dependent as well as culture-independent studies of complex microbial communities were studied using this approach, which enabled the study of diversity on different levels of organization. At the same time, qualitative and quantitative components of microbial community structure have been assessed, which made it possible to understand, i.e., the relationship between structure of functional microbial guilds and the dynamic of nitrogen in the ecosystem and vice versa. A soil microbial community supports plenty of diverse functions, including carbon and nitrogen cycling of main biogenic elements; as well as ecosystem stability, productivity and resilience towards stress and disturbance.

Extensive open cast mining significantly disturbs the landscape and simultaneously ecosystem functions. Man-preferred function started with mining activity and the excavation of a notable amount of overburden on spoil heaps occurred as a side effect. In the Sokolov mining area (Podkrušnohorská Dump), these heaps are formed mainly by the clays of Miocene lacustrine sediments, which lay as overburden on the brown coal layer (Kříbek *et al.,* 1998). The aim of the study was to discover a viable microbial community in these sediments and to find out whether it contributes to the colonization of the substrates and spontaneous succession after excavation and deposition on the heaps. Four sites undergoing spontaneous succession were chosen to reflect successional stages, which differed by the age of deposition (0 – 44 y. scale). These stages are differentially vegetated: the initial stage without vegetation (0 – 2 y. old), early stage with dominant annual grasses (mainly *Calamagrostis epigeios*, 10 – 12 y. old), middle stage with shrub vegetation (*Salix caprea*, 20 – 22 y. old) and the late stage with forest vegetation (*Betulla* spp. and *Populus tremuloides*, 42 – 44 y. old). The late stage only is characterized by the occurrence of a developed soil profile where it is possible to recognize fermentation and humus layers, as well as a mineral layer and activity of earthworms. The main aim of the study was to compare microbial communities among different stages of spontaneous succession in both top (0-5 cm) and mineral (10-15 cm) layers and characterize them. We expected that changes in soil properties along a succession gradient would be followed by changes in microbial biomass activity and diversity of soil microbial community, with the major emphasis on heterotrophic bacteria. Our results showed that the Miocene lacustrine sediment consists of a viable microbial biomass as observed by Phospholipid Fatty Acid Analysis (PLFA). The structure and composition of individual fatty acids showed the dominance of heterotrophic bacteria, mainly actinobacteria and saprotrophic fungi in the fossil microbial community. A fossil microbial community can significantly contribute to and facilitate the colonization of freshly excavated sediments. Microbial biomass increased with age along a studied successional gradient and was dominated by Gram positive species at all stages. The trend in replacement of r-strategists and high proportion of cultivable bacteria (cultivable to total bacteria ratio, C:T ratio) by K-strategists and low C:T ratio can be recognized among heterotrophic bacteria. Additionally, it was observed that cultivable streptomycetes isolated from the Miocene sediment are part of two distinct phylogenetic clusters, one of which did not occur in the subsequent successional stages, while the other one represents species surviving the excavation and deposition on the Earth's land surface. These species with high probability contribute to the development of the streptomycete community on colliery spoil heaps. The increase in bacterial and actinomycetes biomass, the amount of organic C and proportion of actinomycetes in cultivable bacteria with the increasing age of succession were observed in this study. The genetic diversity of Streptomycetes based on 16S rDNA-ITS (16S rRNA gene and 16S – 23S Intergenic Transcribed Spacer) RFLP profiles (Restriction Fragment Length Polymorphisms) increased until the middle stage, where the richness of OTUs (Operational Taxonomic Units) was the highest, both in top and mineral layers. The replacement of pioneer species by late successional species was observed using RFLP and phylogenetic approaches. Moreover, the high genetic potential of streptomycetes isolated from Miocene sediment and successional gradient on colliery spoil heaps was observed. The screening for detection of gene coding for 5-aminolevulinate synthase (*hemA-asuA*; Petříček *et al.*, 2006) in the genome of streptomycetes revealed in retrieval of 13 new putative producers of manumycin-type antibiotics (the results are not included in this thesis).

The various agricultural activities of man disturb the original stable ecosystem and change its function too; i.e., upland grassland used for outdoor cattle husbandry. The overwintering area was selected at Borová Farm in South Bohemia, which was approximately 4 ha large (latitude 48°52′ N, longitude 14°13′ E). The overwintering area is a special ecosystem which conducts seasonal cycling of usage: from autumn (usually the end of October) to spring (the begining of May) is intensively used for cattle (ca 80 cows), which create a gradient of impact on the grassland soil. In the summer, the overwintering area remains intact without cattle impact and the soil as well as vegetation regenerates. The most impacted site is near the stables where animals have free access to feeding and water, whereas the least

impacted site is the farthest away. Animals affect the soil by the uneven deposition of large amounts of excrement, but also by grazing and treading. The aim of the study was to compare microbial transformation of carbon and nitrogen in soil affected by different degrees of animal activity. It was expected that a change in management influences the input of organic C and N into soil, soil moisture, pH, nitrate and ammonium concentration, microbial biomass and a relative proportion of denitrifying bacteria and methanogenic Archaea. As a result, the pattern and rate of microbial activity would change, in particular denitrification and methanogenesis, emissions of N gases and methane from the soil and a relative proportion of denitrification products (N_2O:N_2). Our results showed that a cattle overwintering on grassland soils changes the soil quality significantly. Soil physico-chemical as well as biological properties are highly influenced by this management technique, especially in spring when intensive decay of dung and urine occurred at the sites. These changes were coupled with microbial biomass increases at sites with a higher availability of organic carbon and mineral nitrogen as well as increased anaerobic microsites. The disturbing effect of animal treading and excrement deposition was detected also visually and was characterized by the damage of the original vegetation and changes in soil structural properties. Functional changes were differentiated by increases in abundance of both methanogenes and denitrifiers in soils under animal impact and also by increased methanogenesis and denitrification activity. The impact of cattle resulted in a shift of potential ecosystem functioning, as clearly indicated by changes in the denitrification product ratio (N_2O:N_2) and imbalance between methanogenesis and methanotrophy due to the overwintering management practiced for 10 years.

Outline of the thesis

Chapter 1 contains a review of soil microbial community diversity in general, streptomycetes, denitrifiers and methanogens in particular, and its relationship to ecosystem functioning. The disturbing effect in man-influenced environments is summarized and two study ecosystems are described. Finally, the scopes and the aims of the thesis are formulated.

In ***Chapter 2*** the known methods and approaches developed to study soil microbial diversity are summarized and possible applications are indicated. A literature overview and list of recent molecular biology methods used are given.

Chapter 3 is concerned with the most important results of the thesis, which are summarized and analyzed, and concluding remarks are clarified.

Chapter 4 brings experimental results arranged as a List of Papers cited in a literature overview and analysis chapter. The List of Papers are separated into two groups, each of them concerning a single studied man-influenced environment: *i)* Spontaneous succession on colliery spoil heaps in Sokolov (South Bohemia) and, *ii)* Cattle overwintering area in Borová farm (West Bohemia). Additional two papers are included (Paper III and IV), where the application of some methods is described.

Chapter 1 – General introduction

Man-influenced ecosystems and landscape changes

One of the oldest human activities pertaining to changes of ecosystem management and nature's landscape is agriculture. Agriculture started independently in several areas of the world 10,000 years ago. Since then, human-induced changes to soil – "metapedogenesis" (Yaloon and Yaloon, 1966), or "anthropedogenesis" (Richter 2007) began to occur on different levels. Domesticated nature, including plants, animals and landscapes (Kareiva *et al.*, 2007) encompasses about 50 % of Earth's land surface now. This area has been converted to grazed land and cultivated crops mainly from forests and half of the world's forests have been lost (Millenium Ecosystem Assessment, 2005). The domesticated landscape includes broad human activities, such as cities, industrial zones, mining activities, promotion of commerce (road construction, enhanced trade), reduced risk (fire suppression, flood control, predator removal, coastal engineering), and in the end maximized productivity, such as, increased food and animal production and fisheries yield. All of them bring some impacts and tradeoffs for the natural ecosystem. For example, the selection of certain ecosystem attributes, such as, increased animal production led to a seven-fold global increase in pastures (1700–1990, Kareiva *et al.,* 2007).

The major consequences of changes in landscape management for increased food and animal production include disturbed nitrogen cycle, damaged riparian zones, overuse of antibiotics and 30 % of global loss of grassland. The younger, but not less important man-disturbing activity is mining. Open-cast mining is primarily accompanied by negative impacts on the environment with destruction of surface soil and removal of overburden, which is simultaneously collected on the Earth's land surface. For example, the acreage of almost 9,300 ha has been disturbed in the study area (Sokolov mining district, West Bohemia, Sokolovská uhelná, a.s., Frouz *et al.*, 2007) since the beginning of open-cast mining in the early 1950s. In turn, land reclamation is carried out in the district of Sokolov to amend the negative effects of mining activities and a new landscape has been created for human recreation, agriculture and forestry. All of these processes affecting landscape (land-use and land-cover) changes involve also soil change (Yaloon, 2007). These mechanisms can lead to changing (increasing or decreasing) diversity and activity of the soil microbial community.

Note: The accessory side effect of enhanced microbial transformations in soil is an increased abundance of greenhouse gases, which has been to a significant extent of anthropogenic origin since the 19th century (Prather and Enhalt, 2001). The major greenhouse gases of anthropogenic origin are carbon dioxide (CO_2), methane (CH_4), nitrous oxide (N_2O) and halocarbons, which are released into the atmosphere by the burning of fossil fuel and biomass, as well as other fallout of industry and agriculture. Methane and nitrous oxide are produced mainly by microorganisms that are stimulated by human activities, such as the increased use of fertilizer, cattle production, soil water management and waste management. Human activity thus indirectly causes global climate change, i.e., human activity affects microbial communities, which, in turn, affects the composition of the atmosphere by gas emissions. Therefore, global climate change will not be understood without proper knowledge of what the relevant microorganisms do and how they react (Conrad, 2007).

Colliery spoil heaps after brown coal mining (Papers Ia, Ib, Ic and Id) and the area used for outdoor cattle husbandry (Papers IIa, IIb and IIc) were chosen as examples of man-influenced ecosystems in this Ph.D. thesis. Both of them creates some benefits and at the same time some impacts and tradeoffs.

The impact of these managements on the soil microbial community's diversity and function is studied and analyzed.

Diversity of soil microbial communities with respect to ecosystem functioning

Diversity is the criterion of genetic complexity of the microbial community as described by two components: number of species (*richness, R*) and relative abundance (*evenness, E*). In the same way, it can be assumed that the amount and distribution of information is directly applicable to the total genetic diversity or complexity in a community. The genetic complexity or the size of microbial community genomes assessed by re-association of community DNA equals the size of 6,000 – 10,000 *E. coli* genomes in unperturbed organic soils and 350 – 1,500 genomes in arable or heavy-metal contaminated soils (Torsvik and Øvreås, 2002). By contrast, the genomic complexity recovered by culturing methods was less than 40 genomes. The total genomic complexity, assessed, for example, by an analysis of total community DNA, provides the information about the overall (potential) taxonomic and functional variability at the community level and therefore can reflect the real situation more accurately than cultivation-dependent methods. The significance of biodiversity arose in the mid-1900s, when MacArthur and Hutchinson (for a review, see Morris *et al.*, 2002) claimed that biodiversity is a measure of important ecological processes such as resource partitioning, competition, succession and community stability. In the 1960s, microbiologists began to investigate the impact of biodiversity on the function and structure of microbial communities (Hariston *et al.,* 1968; Swift, 1974). However, the field of microbial biodiversity has grown markedly since the Diversitas initiative in 1991, together with the development of techniques for characterizing diversity, in particular at the molecular level. These techniques can be applied to both culturable and nonculturable microorganisms (Rondon *et al.,* 2000). A summary of molecular biology methods applied to assess microbial diversity is presented in Chapter 2.

Functional diversity is an aspect of the overall microbial diversity in soil, and encompasses a range of activities. The relationship between microbial diversity and function in the soil is largely unknown, but biodiversity has been assumed to influence ecosystem stability, productivity and resilience towards stress and disturbance (Torsvik and Øvreås, 2002). From this point of view it can be assumed that the higher diversity the higher or improved functioning of the soil microbial community. But this is a questionable point indeed. Inside the complex soil microbial community there are several functional guilds, which are redundant or unique, non-reduntant. This is congruent with the characters of entire microbial communities as they can reflect the impact of different kinds of disturbance: they can act as resistant, sensitive or resilient communities with functional redundancy (for a review see Allison and Martiny, 2008). There are conflicting theories on the importance of species biodiversity in relation to resilience since it has been proposed that functional diversity is more important than species diversity (Tilman *et al.,* 1997). Finally, these manifestations may play important roles in entire ecosystem functioning. With complex functions characterized, for example, by protecting the soil microbial community against pathogen invasion, diversity plays an important and significant role (van Elsas *et al.*, 2007 – Paper III). The higher the diversity of the microbial community, a lower rate of survival of genetically modified *E. coli* O157:H7 strain T in the soil occurs. The authors further concluded that soil microbial diversity is a key factor that controls the extent to which bacterial invaders become established and thrive.

In turn, decomposition is also a complex process characterized by partitioning, and several microbes of different taxonomical distribution are involved during degradation succession (Begon *et al.*, 1990).

Diverse species colonize the substrate or disappear from it, depending upon the kind of sources consumed and whether others are generated. It is assumed that many compounds can be degraded by a wide variety of microorganisms, i.e., there is considerable functional redundancy, but recalcitrant compounds are degraded by more specialized organisms requiring specific enzymes. Some of them (i.e., degradation of chitin or organopollutants) require the concerted action of several different functional groups for degradation (Prosser, 2007). Hence, in this particular case, the overall microbial diversity does not matter, in contrast to the presence and activity of certain species or, putting it another way, functional diversity is more important.

Spontaneous microbial succession and diversity of the microbial community on excavated substrates and post-mining sites

Human activity including mining, transfer, agriculture and many similar pursuits has its negative accessory impacts on environmental quality, defensibility, function and development of ecosystems. These can be both direct and indirect. Among direct impacts we can easily expect the change of landscape shape after extensive surface mining of brown coal in the Sokolov area. There can be recognized the excavation of huge amounts of tertiary sediment from a depth of 200 m and even deeper on the Earth's land surface. The spoil heaps, used as experimental plots in our studies, have been formed mainly by the clays of Cypriss formation (Kříbek *et al.*, 1998) and they represent a very different environment from that colonized by recent microflora. The name is derived from the frequency of water crustacean – the ostracoderm *Cypris angusta.* These rock clays comprise the main part of the overburden, and therefore also the substrate of dump bodies, where it is possible, thanks to their composition and content of organic components, to realize certain kinds of biological reclamation directly, without the resorting to resoiling. The spontaneous succession undergone on these colliery spoil heaps and development of soil *de novo* occurs without reclamation hits. Contrastingly to other common substrates, where spontaneous succession has been ongoing and has been intensely studied, this spoil material doesn't contain significant amounts of recent organic carbon, but only the fossil one (Kříbek *et al.*, 1998; Frouz and Nováková, 2005). This factor together with encouraging soil reaction (pH ~ 7.8) facilitates this process. The roles of an entire microbial community of heaped substrate, which can act as *inoculum* or facilitator of colonization as well as a contribution of heterotrophic prokaryotes, remain unknown. The successional theory predicts that opportunistic (pioneer) species with a high investment of energy in reproduction and an extensive niche width would be replaced by equilibrium (late successional) species with a relatively higher investment of energy in maintenance and narrower niche width as communities develop. Little is known about the development of microbial communities during spontaneous succession, however, Sigler and Zeyer (2002) proposed that microbial populations increased in number and activity during the primary succession on substrates after glaciers receded. Garland *et al.* (2001) signalized also that the ratio of culturable to total bacterial counts (C:T) can change significantly during soil community development and that a shift in resource allocation from growth to maintenance occurs with time. Kandeler *et al.* (2006) studied the development of the denitrifying bacterial community during the primary succession of the receding glacier and showed increased bacterial densities (16S rRNA copy numbers) with progressing soil development. Their results signalized that the amount of organic substances can drive the abundance of bacteria, NirK and NosZ denitrifiers, but NirS and NarG denitrifiers showed a different development. Several studies have been carried out concerning the development of communities of algae (Lukešová, 2001), microfungi, and macrofauna (Frouz *et al.*, 2002) in the studied area. The development of the heterotrophic streptomycete community as a significant part of the microbial community has not yet been studied in detail. As streptomycetes are key players in decomposition,

signaling (production of antibiotics), putative spread of antibiotic resistance, humification, promotion of plant growth and protecting plants against pathogens, we focused on their community development on spoil heaps.

Streptomycetes, their diversity, functional traits and roles in the environment

Streptomycetes represent one of the most abundant groups of bacteria cultivated from soils and sediments and it is assumed that soil and compost serve them as primary reservoirs. From the taxonomic point of view, they belong to the order *Actinomycetales* (domain: Bacteria, phylum: Actinobacteria, class: Actinobacteria, order: Actinomycetales, family: Streptomycetaceae; Sedláček, 2007), consisting of Gram-positive chemoorganotrophic bacteria with an oxidative type of metabolism. They utilize a broad scale of carbon and nitrogen compounds served as energy sources. They are characterized by high GC content in the genome (> 55 %) and filamentous type of growth. *Streptomyces* is a type genus of this heterologous group of bacteria and consists of more than 500 species. Their genome size is two times larger in comparison to common bacteria, i.e., *S. coelicolor* has a genome size of 8.7 Mb (ca two times larger than *Escherichia coli*). This encourages streptomycetes since they can possess various enzymatic apparatuses and, a feature that in general is incident only to eukaryots, *viz.,* cell differentiation (Chater, 1989). Their cells differentiate during the growth on solid media and create two types of long filamentous cells. Vegetative hyphes (the average of the filament is 0.5 – 2.0 μm) produce large-scale branched mycelium, which rarely fragments. In the conditions of nutrient limitation they create serial mycelium with the chains of arthrospores (Holt *et al.,* 1994). Spores are very specialized cells intended for the survival of unfavorable environmental conditions or for spreading to more adequate conditions in comparison with that of the mother colony. This feature together with the ability to metabolize and/or survive under anaerobic conditions proves streptomycetes are a pioneer of life in conditions generally thought to be inhospitable.

Actinomycetes are responsible mainly for decomposition of soil organic matter and their products enrich available nutrients for other microflora or plants. At the same time, they are involved in the process of humus formation. Some species (i.e., *Frankia*) conduct nitrogen fixation as a consequence of symbiosis and therefore facilitate the nitrogen input to the ecosystem and colonization of poor soils or sediment by symbiotic and other plants (i.e., g. *Alnus*). Moreover, they are involved in other nitrogen transformation processes, either only in mineralization (i.e., proteolytic and chitinolytic activities), or in denitrification and codenitrification and consequent N_2O production. They are able to use mineral nitrogen forms of nitrates or nitrites instead of oxygen as an electron acceptor in reductive reactions to gain energy similarly to denitrifying bacteria (Albrecht *et al.*, 1997; Shoun *et al.*, 1998; Kumon *et al.*, 2002). Findings of Kumon e*t al.* (2002) indicate that denitrification takes place also under aerobic conditions, which is strictly repressed in denitrifying bacteria, and that the entire process is more similar to co-denitrification known in fungi.

Note: Actinomycetes and streptomycetes are also of major biotechnological interest, because they are the most important producers of natural bioactive compounds (Lazzarini *et al.,* 2000; Hopwood, 2007). Secondary metabolism begins when nutrient limitation occurs and when incubated in laboratory batch cultures. Therefore, it has been proposed that secondary metabolism can be an adaptive mechanism to environmental stress and streptomycetes can assure growth at the expense of microorganisms suppressed by the antibiotic activity. Another ecological role of antibiotics has been postulated by Challis and Hopwood (2003). These authors stressed that antibiotics can play a role as signal molecules in a real environment and can modulate the communication between microbes (similar to quorum sensing). Nevertheless, antibiotic biosynthetic clusters of

streptomycetes and improvements of strains have generated major interest today, as the need for new bioactive compounds is crucial, mainly due to the increase of antibiotic resistance pathogens amongst human beings and animals (MRSA, VRSA - Methicilin or Vancomycin resistant *Staphylococcus aureus*, TB – *Mycobacterium tuberculi*, etc.; Levy, 2002).

The disturbing effect of extensive cattle grazing on soil microbial communities

Agricultural lands occupy 37 % of the Earth's land surface and intensive agriculture accounts for 52 % and 84 % of global anthropogenic methane and 84 % of nitrous oxide emissions (Smith *et al.,* 2008). The major sources of N_2O production in soil are denitrification and nitrification and mitigation of gaseous products simultaneously lead to a loss of nitrogen from ecosystems. Therefore, it is crucial to identify the factors controlling the formation of CH_4 and N_2O by microorganisms in soil, particularly the known processes of consumption of both green house gases (CH_4 oxidation and N_2O reduction). Upland grasslands in low-input farming systems are usually well aerated and therefore, it is assumed to be a sink for methane (Hütsch *et al.,* 1994; Conrad, 2007) and only weak sources for nitrous oxide (Mosier *et al.,* 1991). Management changes, such as cattle grazing and overwintering, may lead to a significant shift in microbial community structure and functioning (Clayton *et al.,* 1994). For the most part nitrogen transformation can be highly affected as a huge amount of nitrogen inputs to the pasture soil in the form of animal urine and excrement. Additionally, a significant amount of organic carbon enters the soil and therefore may enhance microbial transformation under changed physico-chemical conditions. The effect of animal treading was also studied by Meenner *et al.* (2005). These authors described significant changes in denitrification rates in the soil depending on the intensity level of treading. These assumptions were confirmed by studies of Šimek *et al.* (2006) and Hynšt *et al.* (2007) on the same overwintering area (Borová farm in South Bohemia).

Note: Soil under animal impact showed enhanced rates of microbial transformation, especially denitrification, and three main factors steering microbial processes have been determined (Hynšt *et al.*, 2007):

- dung and urea, the main organic input, are enriched in these soils leading to extraordinary amounts of organic carbon and nitrogen;
- compaction of soil by animal traffic and other changes reduce soil aeration;
- grazing, trampling and defoliation result in reduced plant N uptake.

Decreased soil aeration facilitates anaerobic processes, such as denitrification and methanogenesis. For example, it has been shown that methane emissions can significantly increase in usually well-aerated prairie soil after the snowmelts and precipitation (Wang and Bettany, 1995). The research focused on soil microorganisms responsible for methane and N_2O production is essential for understanding the processes as well as finding the solution for decreasing emissions of both gases indisturbed ecosystem.

However, the consumption of N_2O occurs as a last step of denitrification under anaerobic conditions; the mechanisms controlling the last step of this pathway and environmental factors facilitating it are not yet fully understood. One assumption is the presence and expression of gene coding for nitrous oxide synthase (*nosZ*). Some denitrifying bacteria can lack this gene in the operon as revealed by the complete genome sequencing of *Agrobacterium tumefaciens* C58, and thus be unable to reduce nitrous oxide (Henry *et al.*, 2006). Hence, the quantifying of *nosZ* gene abundance can be crucial for the estimation of N_2O emissions. The other key factor is the activation of expression of *nosZ* gene under

certain environmental conditions, such as soil reaction (pH). This factor strongly influences nitrification (Morkved *et al.,* 2007) and denitrification activity (Šimek and Cooper, 2002) and affects their NO product (NO, N_2O) ratios. It also influences microbial community structure, and with high probability also the activation of *nosZ* gene expression. The other factors controlling denitrification are the organic carbon, aeration status and the concentration of mineral nitrogen in the soil. They often act in reciprocal correlation and therefore, it is difficult to know if one of them is crucial.

On the other hand, there are two ways methane can be removed from the environment and decrease the CH_4 emissions from the soil: i) aerobic oxidation by a specialized group of bacteria and ii) anaerobically by specialized archaea (Christoserdova *et al.,* 2005). Thus, the balance between methanogenesis and two processes of methane oxidation influences the actual CH_4 fluxes. Aerobic methane oxidation seems to be ubiquitous in upland pasture soil (Le Mer and Roger, 2001), but little is known about the occurrence of anaerobic methane oxidation in these soils even though it has been detected in rice paddy and wetland soils and marine deep subsurfaces. There is a presumption, that reverse methanogenesis plays a role in the methane oxidation process and, therefore, can occur in soils, where methane is simultaneously produced. Pasture soils usually act as a sink for methane, where methanotrophic consortia consume almost all methane produced in the soil or from the nearby atmosphere. Environmental conditions affect the composition of the methanogenic communities. Therefore, changes of soil physico-chemical properties as a result of intensive cattle overwintering may cause changes of the methanogenic community structure.

Denitrification, methanogenesis and microbial guilds involved in these processes

The denitrification process is supplied by a wide variety of microorganisms that range from archaea to Gram-positive bacteria and to fungi (Tiedje, 1988). This stepwise reaction has been intensely studied largely in denitrification bacteria belonging to α-, β- or γ- *Proteobacteria*. The denitrification pathway consists of several reduction steps where nitrate, nitrite, nitric oxide and nitrous oxide play a role as electron acceptors. Three steps, nitrate, nitrite and nitric oxide reduction, are carried out by two functional guilds each bearing different enzymes in biochemical and genetic bases: Nap denitrifiers with periplasmic nitrate reductase and Nar denitrifiers act with membrane bound nitrate reductase. Both types of enzymes can be present in one strain (Philippot *et al.,* 2007). The reduction of soluble NO_2^- to gaseous nitric oxide (NO), the key step in the denitrification pathway, can be catalyzed by two evolutionary unrelated enzymes that are different in terms of structure and prosthetic metals: a copper (NirK) and cytochrome cd_1 (NirS) nitrite reductase. In contrast to nitrate reductases, bacteria carry either copper or cd1 nitrite reductase but the two enzymes are functionally equivalent (Glockner *et al.,* 1993). Reduction of NO to nitrous oxide is also catalyzed by two types of enzymes: one NO reductase receives the electrones from cytochrome *c* (cNor) and one from the quinol pool (qNor). The last step of the denitrification pathway, the reduction of nitrous oxide to molecular nitrogen, is performed by multicopper homodimeric N_2O reductase (nosZ), which is located in the periplasm in Gram-negative bacteria (Zumft, 1997).

Methanogenesis and methanogenic soil microbial community

The production and consumption of methane together with fixation and respiration of carbon dioxide are involved in the global carbon cycle. The production and consumption of methane is brought about

by different processes, and also by different microorganisms. Microbial production of methane is strictly linked to anaerobic conditions and the activity of methanogenic archaea, which all belong to the *Euryarchaeota* based on 16S rDNA phylogeny. The key enzyme in methane production is methyl coenzyme M reductase (Mcr), which is ubiquitous and unique to methanogens. The *mcrA* gene encoding for a subunit of the Mcr, seems to be monophyletic, because its evolution is more or less congruent with that of the 16S rRNA gene (Lueders *et al.*, 2001). Hence, methanogenic communities can be analyzed by targeting either a unique functional gene (*mcrA*) or the 16S rRNA gene sequences that are characteristic for methanogen clusters. Communities of methanogenic archaea occur in all environments where biogenic methane is produced. They inhabit mainly deep oceanic sediments, hydrothermal springs, and are rarely found in terrestrial environments, such as rice paddy soils, wetlands and permafrost. Moreover, they are known to inhabit termites' hindgut (Lee *et al.,* 1987) and cattle rumen (Tatsuoka *et al.,* 2004). A common characteristic of all methanogenic environments is the lack of oxygen and other oxidants such as nitrate, ferric ion or sulphate, so that CH_4 and CO_2 become the end products of the degradation of organic matter (Conrad, 1996). A complex community of fermenting microorganisms degrades all organic compounds equally to acetate, H_2 and CO_2. These are the substrates from which CH_4 is subsequently produced.

Aims and research questions

The general aim of the Ph.D. thesis is to describe soil microbial community diversity and change in their activity and functioning in man-influenced environments. To study this, two man-influenced ecosystems were selected: spoil heaps in post-mining sites and upland pasture under outdoor cattle husbandry.

The particular objectives of the thesis are as follows:

To master techniques of various DNA and RNA extractions from soil and sediments as well as DNA/RNA-based methods for assessment of diversity of soil microbial communities, test and prepare them for use in the laboratory (All papers).

To describe spontaneous succession of the heterotrophic bacterial community with emphasis on soil streptomycetes and to compare four different stages of ongoing succession on colliery spoil heaps (Papers Ib, Ic, Id).

To address the question whether Miocene lacustrine sediment used as original material for heaping excavated from a hanging wall situated at a depth of 200-m can bring viable microbial biomass to the surface and thus facilitate the succession process (Papers Ia, Id).

To characterize the shift in abundance and activity of denitrifying as well as methanogenic microbial consortia in upland pasture soil as a result of changed soil physico-chemical properties due to animal impact (Papers IIa, IIc).

To correlate the shift in microbial community structure with its potential activity and green house gases emission from the soil (Papers IIa, IIb, IIc).

To address the question whether the spatial distribution pattern of denitrifiers in pasture soil is random, or it is dependent on soil physico-chemical properties (Paper IIb).

Chapter 2 - Methods used to assess diversity of microbial community and its functional aspects

Diversity of microbial communities in soils and approaches used for its assessment

Diversity of prokaryotic microbes exceeds the diversity of eukaryots in soil ecosystems. One gram of soil may host more than 10 billion microbes of thousands of species (Øvreås and Torsvik, 1998). Based on the fact, that only 0.1 – 1 % of microbes visible under light microscopy can be cultivated and characterized, soil ecosystems still remain undiscovered. Microbial diversity describes complexity and variability on the various levels of biological organization. It compiles genetic variability inside the taxons (species), number (*richness, R*) and relative abundance (*evenness, E*) of taxons or functional guilds in the community. Last two components, evenness and richness, can be used to calculate the diversity index, such as Simpson's (*D*) or Shannon's (*H'*) indices (Begon *et al.,* 1990). Overview of various diversity indices and calculations formulae are given in Youssef and Elshahed (2009). An alternate way to interpret data about distribution and abundance of taxa is to calculate rank-abundance diagrams and rarefaction curve analysis. Methods used to measure microbial diversity in soil can be categorized into two groups (adapted from Kirk *et al.,* 2004):

- biochemically-based or phenotype-based methods, including Plate counts, Community level physiological profiling (CLPP), and Fatty acid methyl ester analysis (FAME)
- molecular-based techniques (which are summarized in Table 1) will be of further interest.

Taxonomy studies and fingerprinting techniques

Bacterial taxonomy is grounded on the polyphasic approach which in principle unites all genotyping, phenotyping, and phylogenetic information about taxa (Vandamme *et al.,* 1996). Modern microbial ecology uses rRNA gene sequences (rRNA) for phylogenetic studies and applies various analyses of amplified DNA, a product of polymerase chain reaction (PCR). It is a universal, ubiquitous, highly conservative gene bearing simultaneously information about the evolution of an organism, which is signified by changes in nucleotide sequence (Woese 1987; Woese *et al.,* 1990). These sequences have been used to map the phylogenetic affiliations of all living organisms, especially bacteria (Pace *et al.,* 1986), because the bacterial species concept is built up on 16S rDNA relatedness and DNA-DNA homology (Staley, 2006).

Note: Bacterial polyphasic taxonomy consists of genotyping information derived from nucleic acids (DNA, and RNA) present in the cell, phenotypic information derived from proteins and their functions (electrophoretic analysis of total cell proteins and enzyme patterns), different chemotaxonomic markers (cellular fatty acids, quinones, mycolic acids, polar lipids, polyamines, cell wall compounds and exopolysaccharides) and a wide range of other expressed features (morphology, physiology, enzymology and serology).
The species is the basic unit of bacterial taxonomy and is defined as a group of strains, including the type strain, sharing 70 % or greater DNA-DNA relatedness with 5 °C or less ΔT_m (T_m as the melting temperature of the hybrid as determined by stepwise denaturation; ΔT_m is the difference in T_m in degrees Celsius between the homologous and heterologous hybrids formed under standard conditions) (Vandamme *et al.,* 1996).

Abbreviations:

AFLP - Amplified Fragment Length Polymorphism
ARDRA - Amplified Ribosomal DNA Restriction Analysis
cPCR – competitive PCR
DGGE / TGGE – Denaturing / Temperature Gradient Gel Electrophoresis
FISH – Fluorescence *in situ* hybridization
ITS – Intergenic Transcribed Spacer
LH-PCR – Length Heterogeneity PCR
MPN-PCR – Most Probable Numbers-PCR
qPCR (quantitative PCR)
RAPD - Random Amplification of Polymorphic DNA
rep-PCR - repetitive DNA elements amplification (box-PCR – boxA1 elements)
RFLP / t-RFLP – Restriction Fragment Length Polymorphism / Terminal RFLP
RISA / ARISA – Ribosomal Intergenic Spacer Analysis / Automated RISA
SSCP – Single Strand Conformational Polymorphism
SSU rRNA – Small Subunit rRNA (16S in Prokaryota, 18S in Eukaryota)

Conservative regions of the rRNA gene serve the space for universal primers or probe application targeting; for example, domains and in turn, variable regions are suitable for taxon-specific primer or probe design. A number of methods with high resolving power have been developed for characterization of microbial communities that include both cultured and uncultured microorganisms (Table 1.). They can be differentiated according to the obtained information into two classes: i) quantitative, and ii) qualitative, with respect to some overlapping, i.e., qPCR (comprising of real-time PCR, MPN-PCR, cPCR) and FISH, which can serve both. The relative proportion of present taxa can be estimated also using the T-RFLP and PCR-clone-sequence approaches and they have been used for describing microbial community structure.

A number of DNA-based methods has been used to address taxonomy and diversity of the bacterial population on the culture dependent level. As there is no official definition of a bacterial species (Colwell *et al.,* 1995) and this problem is associated with measuring microbial diversity, microbial ecologists introduced the phylogenetic species concept or genomic-phylogenetic species concept (GPSC; Staley, 2006) with phylotypes, ecovars or geovars instead of species. The major reason to facilitate usage of these definitions and terms is that the number of species of bacteria and archaea is surprisingly small (ca 5,000) considering their early evolution, genetic diversity and residence in all environments (Staley, 2006). Several methods to discriminate bacterial environmental isolates have been applied to microbial ecology, such as rep-PCR, box-PCR, RAPD, AFLP, ARDRA, RFLP, which used either genomic DNA or 16S rDNA polymorphisms (for reference see Table 1). The environmental strains can be compared to known bacterial strains from the culture collections or in the collection from given ecosystems, and subsequent sequencing of unique ribotypes or fingerprints can be accompanied. Box-PCR fingerprinting was applied to distinguish the number of bacterial isolates and to evaluate the shift in the community during spontaneous succession (Paper Ic). 16S rDNA-ITS RFLP (ARDRA including ITS sequences) was used to group streptomycete isolates and was complemented with the sequencing of 16S rDNA and phylogeny (Paper Id). RFLP is usually used to analyse clone libraries and it has been performed in two studies (Papers IIc and IV).

Fingerprinting techniques were developed to overcome the problem of time-consuming and an expensive PCR-clone-sequencing approach. In ecological studies, a tremendous number of replicates is needed, and usually the high number of samples are analysed for studying spatial or temporal distribution changes and response to perturbation or others. Fingerprinting techniques in principle can accurately address the research questions with less time and money, but one should consider some of the limitations of what they can bring, which is common for all PCR based methods, *viz.,* the PCR bias. It comprises the overestimation of dominant species at the expence of minor contributors, caused by an exponential growth of amplicons produced during PCR and preferent amplification of domiants by *Taq* DNA polymerase. In these methods, DNA or RNA is extracted from the environmental sample and purified. Target DNA (16S, 18S or ITS) is amplified using universal or specific primers and the resulting products are separated in different ways. The way of separation can be agarose or acrylamide

gels (ARDRA, RFLP, SSCP, RISA), which can be either of a denaturing chemical or temperature gradient (DGGE, TGGE), or the separation process can be automatized (ARISA, T-RFLP) using capillary sequencer instruments. A summary of methods and review of their application has been presented several times (Head *et al.,* 1998; Wellington et *al.,* 2003; Kirk *et al.,* 2004). We applied ARDRA analysis of bacterial and actinomycetal 16S rDNA genes to compare stages of spontaneous succession on colliery spoil heaps (Paper Ic). A DGGE analysis of 16S rDNA amplicons was applied to determine the degree of diversity alteration in soil microcosms as affected by chloroform fumigation (Paper III). Subsequent gel analyses should be performed to analyze results of gel fingerprinting methods and we used the Juke-Cantor method to calculate distance matrix and the Unweighted Pair Group Method with Arithmatic Means (UPGMA) (Sneath and Sokal, 1973) to construct dendrograms using Quantity One 1D software (Bio-Rad Laboratories, USA) and Gel Compar II (Applied Maths, Belgium).

Table 1. The overview of DNA based techniques used to assess diversity of soil microbial communities at different levels. Adapted from Kirk *et al.* (2004). The citation of the first work introducing the method to microbial ecology studies is given as a reference. The list of links to our work in presented thesis, in which techniques were used, is given in the right column.

Techniques	**Reference**	**Used in the thesis**
quantitative analyses		
real-time PCR	Hermansson and Lindgren (2001)	Paper IIa, IIb, IIc
cPCR	Hallier-Soulier *et al.* (1996)	-
MPN-PCR	Mäntynen *et al.* (1997)	-
FISH, CARD-FISH, FISH-MAR	DeLong (1989)	Paper IV
qualitative analyses		
G+C content (%)	Nusslein and Tiedje (1999)	-
nucleic acids reassociation and hybridization	Torsvik *et al.* (1990)	-
DNA microarrays and DNA hybridization	Gushin *et al.* (1997)	-
rep-PCR (including box-PCR, RAPD, etc.)	Versalovic *et al.* (1994)	Paper Ic
DGGE and TGGE	Muyzer et al. (1993)	Paper III
SSCP	Lee *et al.* (1996)	-
ARDRA or RFLP	Massol-Deya *et al.* (1995)	Paper Ic, Id
t-RFLP	Liu *et al.* (1997)	-
RISA or ARISA	Fisher and Triplett (1999)	-
LH-PCR	Mills *et al.* (2007)	-
PCR-clone-sequence + rarefaction analysis	Hongoh *et al.* (2003)	Paper Id, IIc, IV
metagenomics	Rondon *et al.* (2000)	-
SSU rRNA hypervariable tag sequencing	Huse *et al.* (2008)	-

Most of these techniques are based on analyses of ribosomal RNA genes (rDNA), but other carefully chosen taxonomic marker genes (i.e., *gyrB*, *rpoD*) can be used, especially with the multilocus sequence alignment (MLSA) approach (Wellington *et al.,* 2003). They have uncovered part of the microbial diversity in soil yielding sequences from many novel phylogenetic lineages which have not been discovered using a culture-dependent strategy. However, these analyses provide extensive information

about the taxa present in an environment, although they provide little insight into the functional role of each phylogenetic group.

The PCR-clone-sequence approach has been widely used to assess diversity of bacteria or archaea as well as functional marker genes. Various experiences obtained showed that this approach can provide a limited amount of information when a complex microbial community is studied. The number of clones which should be sequenced and analysed to estimate a relevant bacterial community, i.e., in agricultural soil, where thousands of genomes are expected, is enormous and should be tested using rarefaction analysis. This approach can provide useful information about community composition in the environment, where a lesser number of species is expected, or when the functional gene marker is used rather than 16S rDNA. PCR-clone-sequencing was successfully used in studying the archaeal community in the bat guano heap using 16S rDNA as a marker, where very acidic pH indicated a lowered diversity of species (Paper IV). In addition this approach was used to identify the methanogenic population using *mcrA* gene as a marker in soil severely impacted by cattle and to infer phylogenetic relatedness from translated aminoacid sequences with rumen-borne and soil-borne methanogenes (Paper IIc). The rarefaction curve analysis was performed in both studies to estimate the number of clones to be sequenced without losing the information.

Methods used to quantify DNA markers in the environment

Quantitative PCR methods (real-time PCR, cPCR, MPN-PCR) are widely used to quantify marker genes in environmental samples and dominated usage of FISH techniques in studies carried out in terrestrial environments. Another alternative of the culture-independent approach to quantify genes is Southern hybridization, but it only allows comparison with the relative abundance of genetic markers between samples and cannot be used to estimate cell numbers. The detailed review of advantages and limitations of qPCR- based approaches was given by Smith and Osborn (2009), and quantitation of functional genes from prokaryotes in soil by PCR was summarized by Sharma *et al.* (2007). The authors stressed that obtaining the information about the gene pool and possibilities for its *in situ* induction is required to control turnover processes and fluxes in soils and other environments. The choice of method for quantitative PCR is highly dependent on the characteristics of individual experiments and whether relative or absolute quantification is required. The call to quantify denitrifying microorganisms for understanding nitrogen fluxes was proposed by Philippot (2006). In our studies we used the real-time PCR method to quantify marker genes for bacteria (16S rDNA), denitrifiers (*narG, napA, nirS, nirK, nosZ*) and methanogenes (*mcrA*) in soil under a different impact of cattle overwintering (Papers IIa, IIb, IIc).

Novel methods linking phylogenetic groups to their activities and function

Metagenomic is a valuable tool for recognizing which phylogenetical groups can be linked to a specific function regarding the presence of a functional marker. In essence, DNA isolated from an environment is archived in the form of bacteriophage lambda, cosmid, fosmid or bacterial artificial chromosome (BAC) library (Wellington *et al.,* 2003). Applying this approach, many novel bacterial lineages of uncultured microorganisms were described (Rappe and Giovannoni, 2003), and moreover, linked to function (i.e., the soil crenarchaeotic clone 54d9 possessed a putative gene for ammonia monooxygenase with high similarity to its bacterial counterpart; Treusch *et al.*, 2005). A powerful

approach to metagenomics analysis is to identify clones that express a function in an appropriate host, the heterologous expression (Handelsman, 2004). Metagenomics analysis provides some functional information through genomic sequence and expression traits, but other methods are required to link specific functions with the group responsible for them. Recently, the presence of gene coding for an enzyme (or its catabolic subunit) active in a specific metabolic pathway is used for targeting functional microbial guilds and evaluation of their diversity. The usage of functional markers overcomes the problem for studying diversity of functional guilds, which are of polyphyletic origin, and therefore the monitoring of diversity and abundance was impossible using the SSU rDNA approach. Studying diversity of functional microbial guilds together with activity measurements offer us the option of monitoring changes in an ecosystem functioning as a whole, because microorganisms take a major role in most soil transformations. Today, the biggest challenge of microbial ecology is to find a link between structural and functional biodiversity. Measures of microbial patterns (fingerprinting) and taxonomic variability have been coupled with an analysis of functional genes and activity measurements. Such investigations aim to reveal and understand the relationship between structural and functional diversity in soil microbial ecosystems. This approach was used to study denitrifying and methanogenic microbial communities in presented papers IIa, IIb and IIc. The concomitant qualitative and comparative analyses of expressed rRNA genes and genes for key enzymes in relation to environmental factors can be used to obtain information about the phylogeny and ecology of functional bacterial or archaeal groups responsible for processes like denitrification, nitrification, methanogenesis and methane oxidation.

The major advance in linking functional activity to community structure came with the development of a stable isotope probing (SIP), which relies on the labeling of DNA with ^{13}C, resulting in the separation of heavier labeled DNA during density gradient centrifugation (Wellington *et al.*, 2003). The labeled DNA can then be analyzed for functional and taxonomic marker genes. Radajewski *et al.* (2000) pioneered this approach for the study of methylotrophs in soil. SIP can be also combined with extracted labeled RNA analysis as introduced by Manefield *et al.* (2002), which is proposed to be a more responsive biomarker as its turnover in much higher than that of DNA in active cells. The other possibility is to combine a metagenomic approach with SIP to extend this general concept and link metabolic activity to structure, as was explored by Radajewski *et al.* (2003).

In the last few years, DNA-DNA hybridization based techniques such as DNA or RNA (rRNA or mRNA) microarrays have begun to be employed widely in microbial ecology. This tool can be valuable in ecological studies since a single array can contain thousands of DNA sequences (Cho and Tiedje, 2001). The arising usage of these techniques goes together with the improvement of methodologies, especially with an extensive growth in the number of sequence data in the public databases and more accurate designing of probes targeting genes of interest. This microarray can contain specific target genes to provide functional diversity information (Kirk *et al.*, 2004).

Active versus dormant communities

It is unlikely that dead cells will contribute significantly to community DNA as they show a reduced residence time in soil, but some may be protected from degradation by adsorption to clays, as with small amounts of free DNA. The rapid developments in reverse transcription-PCR (RT-PCR) and improvement in RT enzymes has provided opportunities for an evaluation of active communities inferred from analysis of rRNA and mRNA (for review see Wellington *et al.*, 2003).

The direct detection of active cells *in situ* is provided by FISH techniques and their further improvements, such as CARD-FISH and FISH-MAR combining FISH with microautoradiography (FISH-MAR) (Rogers et al., 2007). These techniques are widely used in aquatic microbial ecology studies, and less in soil environment, as soil represents considerable difficulties for FISH analysis because of background fluorescence and low metabolic activity of soil communities. In our studies, a CARD-FISH technique was used to estimate the relative proportion of archaea in an entire prokaryotic community in bat guano heap (Paper IV).

Chapter 3 – Results and general discussion

Man-influenced environments, in general, provide an opportunity to study the human impact on the Earth's global ecosystem and at the same time the mechanisms of putative revitalization of disturbed landscape or soil. The methodological approach - DNA tools for assessing microbial community diversity - were learned and mastered in the frame of broad collaboration with laboratories abroad as well as with home laboratories. Nowadays, these techniques are routinely used in our laboratory to address research questions of ongoing projects.

Development of the soil microbial community during spontaneous succession on colliery spoil heaps

The processes of spontaneous succession and development of soil *de novo* can show the means of natural revitalization processes. It is an example of prosperous natural restoration. The results of studies summarized here show that the Miocene lacustrine sediment consists of viable bacterial and fungal biomass as assessed by PLFA (Paper Ia), and also by isolation of viable streptomycete strains, characterized by the polyphasic approach (Paper Id). The structure of FA components showed the dominance of heterotrophic bacteria and actinobacteria as well as saprotrophic fungi in the fossil microbial community (Paper Ia). Spontaneous succession on colliery spoil heaps is characterized by increased microbial biomass with increasing age of development, which is dominated by Gram positive bacteria all over the stages. The increase of microbial biomass along successional transect has been described frequently (Ohtonen *et al.,* 1999; Frouz and Nováková, 2005; Baldrian *et al.,* 2008). The proportion of Gram positive species (G^+/G^-) decreased with the age of succession in both the culture dependent and independent survey. The shift in the bacterial community was accompanied with soil organic matter availability as a result of the development of plant roots (Paper Ib), being attracted by the rhizosphere, where mainly G^- dominate bacterial community according to culture isolation techniques (Paul and Clark, 1989). This is congruent with findings characterizing the development of a soil microbial community in the barren soil of a glacier forefield (Ohtonen *et al.,* 1999). The heterotrophic bacterial community changed the community structure during the spontaneous succession. The shift from prevalence of *r*-strategists and easier to cultivate bacteria (higher C:T ratio, *cultivable to total bacteria ratio*) to *K*-strategists and harder to cultivate bacteria (lower C:T ratio), which is congruent with results of Sigler and Zeyer (2002) and Garland *et al.* (2001). This can be explained by the changes in organic matter nature (from fresh to recalcitrant organic matter), free niche diminishing and the increase of microbial density and growth of nutrient competition (Paper Ib). Bacterial biomass, G^+/G^- ratio and *r-K* continuum showed more pronounced differences among

successional stages in the surface layer than in the mineral one. The development of microbial communities in a mineral layer was slower compared with the surface layer, being accelerated by earthworm-mediated mixing of soil layers and changed soil organic carbon availability after ca 25 y. The significant positive effect of soil fauna on translocation of organic matter into the mineral layer was described by Frouz and Nováková (2005). The community development was studied also by a culture-independent approach. Applying ARDRA we found that development of bacterial and actinomycetes communities' showed the biggest changes between 11 – 22 y. after succession begun (Paper Ic). This is in agreement with the assumption of Baldrian *et al.* (2008), who suggested that rate of nutrient accumulation and development of soil under primary succession is largely regulated by the vegetation succession. The switch from grassland to a forest-dominated ecosystem that occurs between 11- and 21- y. old sites can be of great importance in our sites, since these ecosystems differ in soil properties and annual dead plant biomass production.

In a later study, the development of the soil streptomycete community was described in more detail. The presence and survival of heterotrophic bacteria, including streptomycetes, in Miocene lacustrine sediment was expected according to our previous findings (Paper Ia). In Paper Id we report the isolation of viable streptomycetes (19 strains) from Miocene sediment in Cypris formation (17- to 19-million-year-old, see Kříbek *et al.,* 1998). Heterotrophic bacteria seems to be ubiquitous in deep subsurface sediments, as they were isolated from various sediments such as deep sediments in the Siberian permafrost (Mindlin *et al.,* 2008) and deep terrestrial sediments (Fliermans and Balkwill, 1989). These findings are supported by the prevalence of aerobic or facultative anaerobic heterotrophic bacteria in ancient or deep subsurface samples with oxidative rather that fermentative metabolism (Fliermans and Balkwill, 1989; Vreeland *et al.,* 2000; Mindlin *et al.,* 2008). Remarkably, some strains originally present in the Miocene sediment can survive the changes brought about by excavation and may contribute to the colonization of the bare substrate. These strains were related to *Streptomyces microflavus, S. spororaveus* and *S. flavofuscus*. Some species disappeared and new colonizers replaced them and these non-colonizing strains were related to *S. gougerotii, S. champavatii* and *S. aureus* in our study (Paper Id). We proposed that Miocene-borne (an ancient) streptomycetes together with other microbial counterparts probably facilitated the colonization and contributed to soil microbial community development on colliery spoil heaps. Streptomycete abundance (CFU counts) increased along with their relative proportion in total bacterial CFUs during spontaneous succession. Genetic diversity of streptomycetes was the highest after 21 y. of development in both the surface and mineral layer and then decreased, maybe due to changes in plant cover and K-selection indication, as a result of prevalence of recalcitrant substrates and higher microbial densities. The shift in the streptomycete community composition was shown by the replacement of pioneer species (*S. microflavus* and *S. flavofuscus*) and by the late succession species (*S. phaeochromogenes, S. exfoliates, S. prunicolor, S. californicus, S. spinicoumarensis, S. tauricus*), followed by some *Kitasatospora* and *Amycolatopsis* species and changes at the functional level. Phenotypic properties of isolated streptomycete strains indicated the presence of genetic markers for antibiotic production and resistance in pristine environments and showed equal frequency of these determinants in all studied habitats. This is not in agreement with the studies Davelos *et al.* (2004) conducted in prairie soil, where ATB resistance determinants predominated ATB producing. The occurence and putative roles of ATBs and ATB resistance determinants in pristine environments are under wide-ranging discussion and their major role can be placed on the level of microbial communication in the natural environment as suggested by Challis and Hopwood (2003).

This study contributed in a major way to establish the Culture Collection of Actinomycetes at České Budějovice (CCACB). The CCACB was founded at the Biology Centre AS CR, v. v. i. – Institute of

Soil Biology in 2007 and serves as a depository for cultures of soil actinomycetes (www.actinomycetes.cz).

The impact of cattle overwintering on denitrifying and methanogenic microbial communities with respect to their activities and functioning

Human activities, mainly intensive agriculture, disturb original stable ecosystems and change its functioning, for example, when upland pasture began to be used as overwintering area. Overwintering pasture is the accessory result of increasing animal production demands for man. The impact of increased deposition of dung and urine in small areas together with trampling and treading of cattle resulted to significant changes in soil structure, its physico-chemical properties and a subsequent shift in microbial community structure. The activity of cattle (grazing, treading, dung and urine deposition, defoliation) resulted in increased levels of organic C (C_{org}), total N, ammonium, pH and soil moisture along the gradient from no impact (NI) above moderate impact (MI) to severe impact (SI) (Paper IIa, IIc). Similar observations have already been described by several authors (Menneer *et al.,* 2005; Šimek *et al.,* 2006; Hynšt *et al.,* 2007). Higher soil moisture caused by C_{org} accumulation and intensive soil compaction lead primarily to creation of more anoxic microsites. Additionally, defoliation resulted in limited plant N uptake and creation of rhizosphere-free sites. Denitrifying and methanogenic communities were also stimulated to grow and their activities were enhanced with the increasing impact of animals. As a result of these changes, emissions of green house gases (nitrous oxide and methane) or potential for them increased significantly (Papers IIa, IIc).

Denitrification activity (DEA) increased considerably with the degree of animal impact, which is corresponds with previous findings (Menneer *et al.,* 2005; Šimek *et al.,* 2006). However, the highest N_2O emissions were measured at the MI site and a discrepancy between potential denitrification activity and actual N_2O fluxes at the SI site was observed (Paper IIa). We assumed that reduced N_2O emission rates at site SI might be the result of high compaction of the soil due to animal impact, resulting in lower diffusion rates of N_2O and consequently longer residence times of N_2O in the soil, which increases the probability for a complete reduction to N_2. Studied soils under the differing impact of cattle differed in N_2O reducing ability, which can be explained by differences in oxygen availability, quality and amount of C and N sources, soil pH and presence of plant cover (rhizosphere-related species). Additionally, nitrification and emissions of its gaseous products can be affected (Hatzenpichler *et al.,* 2008) and contribute different ways to overall N_2O emissions. The indication of pH modulation of denitrification pathway in studied soils is consistent with studies of Firestone *et al.* (1980), Nägele and Conrad (1990) and Šimek and Cooper (2002). The abundance of denitrifier's markers (*NirS* and *NirK*) followed microbial biomass and increased along with animal impact and positively correlated with DEA and DEA-N_2: in the spring, their abundance was the highest at SI and the lowest at NI and was related to reduced DEA rates in autumn. In contrast, *nosZ* abundance was not changed as significantly as *nirK* and *nirS*, indicating a relative stability of this community under cattle impact and also during the season. The changes in the denitrifying community affected by cattle overwintering were reflected by the ratio *nirS* : *nirK* denitrifiers, which was the highest in the severely impacted site and indicated also niche preference of both groups of Nir denitrifiers. Our results as well as those of Chèneby *et al.* (1998) indicated that *nosZ* abundance is not a good predictor of the ability of bacteria to reduce N_2O and thus, is not a good predictor of N_2O emissions. In summary, our results (Paper IIa) from spring sampling showed good agreement with the study of Swerts *et al.* (1996), who found that the molar ratio of gaseous denitrification products is strongly influenced by the balance between the NO_3^- concentrations, available organic C as well as enzyme status.

The significant shift from pasture soil acting as a sink for methane to the soil with significant emissions of methane was described (Paper IIc). Cattle dung has been proposed to serve as an *inoculum* for the rumen-borne methanogenic community and together with changed soil properties might lead to facilitation of soil-born methanogenic consortia and their activity. This hypothesis was proven to be a higher abundance (real-time PCR and archaeal etherlipids – PLEL analysis) and activity (CH_4 fluxes) of methanogenes was observed in a severely impacted site. Cattle impacted sites emitted a significant amount of methane and reduced activity of methane oxidizers was observed. It can be explained by the inhibition of methane oxidation by a high concentration of ammonia (originating from cattle urine), nitrogen limitation in contrast, or oxygen limitation (increased anaerobiosis). In any case, methane oxidation by low-affinity methane oxidizers or anaerobic methane oxidation may play an important role at the SI site. Additionally, a shift in microbial community structure reflected by the presence of rumen-borne as well as soil-borne methanogens (*Methanosarcinaceae*) was detected in SI soil using a phylogenetic analysis of molecular marker for methanogenic archaea (*mcrA* gene) (Paper IIc). A similar phenomenon was also described for arable soils receiving high amounts of cattle manure using PLEL analysis (Gattinger *et al.,* 2007). Finally, it can be concluded that after a period of at least six months with no cattle impact, the formerly severely impacted site maintained its methane production potential, whereas the methane production potential under moderate impact returned to background values.

The spatial distribution patterns of denitrifying bacteria were mapped in a grassland field divided into three areas based on the intensity of cattle impact using a geostatistic approach (Paper IIb). Geostatistics is a tool used to quantify spatial variation and predict values of non-sampled locations (Krige, 1951). Kriged maps revealed a gradient in the distribution of the 16S rRNA, *napA, nirK* and *nosZ* genes, between southeast and northwest areas of the field. The *narG* map also showed a similar trend but the gradient was weaker. These similarities between the spatial distributions of denitrification and 16S rRNA genes confirm that the denitrification trait is not a strong factor in controlling denitrifier abundance and that the abundance of both total bacteria and of the denitrifier community is controlled by the same factors (Paper IIb). Accordingly, it has been proposed that most of the denitrifiers in nature exist because they are effective aerobic competitors (Tiedje, 1988). However, the distribution of *nirS* gene abundance exhibited a completely different pattern and was positively correlated to several soil properties such as nitrate and ammonia concentrations, pH and soil moisture. Additionally, all these soil properties were affected by the presence of cattle. A NirS denitrifying community is proposed to be most affected by environmental changes and therefore can reflect the change of environmental conditions more pronouncedly (Philippot, personal communication). Other genetic markers – *narG*, *napA*, *nirK* and *nosZ* showed that the rest of denitrifying community is quite stable in the environment. Moreover, it was reported that the abundance of nitrous oxide reductase (coded by *nosZ* gene) is not a good predictor of N_2O emissions, because not the presence but the actual regulation of transcription is the major player in this process.

To conclude, the results observed and presented in this Ph.D. thesis contributed to a better understanding of microbial colonization of Miocene sediments and ongoing spontaneous succession after their exposition on the Earth's land surface. At the same time, the second part of the study described the interference of soil microbial community structure and function after cattle overwintering had begun on upland pasture. An integral part of the thesis was to introduce almost all molecular biology methods into the workplace of the Institute of Soil Biology (BC AS CR, v. v. i.), which contributed to the task handling of the presented work as well as the issues of ongoing projects, on which the student worked during her Ph.D. study.

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Paper Ia

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Screening for microbial markers in Miocene sediment exposed during open-cast mining

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Key words: Fatty acids, Microfungi, Mining, Miocene sediment, Subsurface bacteria

Abstract

Viable microorganisms were found in Miocene lacustrine clays of the cypris formation excavated from 200-m below the surface as spoil during open-cast brown coal mining (Sokolov Brown Coal Basin, North-Western Bohemia, Czech Republic). Both saprotrophic microfungi of the genera *Penicillium, Verticillium, Cladosporium* and *Aspergillus* as well as heterotrophic bacteria were isolated from an intact sediment cores. Heterotrophic bacteria were classified by the MIS Sherlock System as representatives of genera *Nocardiopsis, Arthrobacter, Micrococcus, Kocuria, Rothia, Clavibacter, Bacillus, Paenibacillus, Brevibacillus, Microbacterium, Acinetobacter* and *Pseudomonas.* A bacterium found among the strains had an atypical fatty acids profile enriched by branched fatty acids and polyunsaturated fatty acid (18:3ω6) and gave no MIS Sherlock match. Phospholipid fatty acids analyss indicates a relatively high (535 pmol g^{-1}) but inhomogenously distributed viable microbial biomass. Fatty acids analyses of non-fractioned lipids (representing viable, storage and dead biomass; 8390 pmol g^{-1}) detected rich and homogenous profiles with fungal, bacterial and actinmycetal markers but no protozoan and algal fatty acids markers.

Abstrakt

Byly nalezeny živé mikroorganismy v jílech miocenního jezera cyprisové formace, které byly vytěžené z hloubky 200 m jako odpad po povrchové těžbě hnědého uhlí (Sokolovská uhelná pánev, severozápadní Čechy, Česká republika). Saprotrofní mikromycéty z rodů *Penicillium, Verticillium, Cladosporium* a *Aspergillus* společně s heterotrofními bakteriemi byly izolovány z neporušeného jádra sedimentu. Heterotrofní bakterie byly identifikovány pomocí systému MIS Sherlock a byly určeny jako zástupci rodů: *Nocardiopsis, Arthrobacter, Micrococcus, Kocuria, Rothia, Clavibacter, Bacillus, Paenibacillus, Brevibacillus, Microbacterium, Acinetobacter* a *Pseudomonas.* Byla nalezena bakterie, která měla neobvyklé zastoupení mastných kyselin a nebyla identifikována pomocí MIS Sherlock metody, přičemž profil mastných kyselin této bakterie byl obohacen o větvené mastné kyseliny a polynenasycené mastné kyseliny (18:3ω6). Analýza fosfolipidů mastných kyselin poukazuje na relativně vysoký (535 pmol g^{-1}), ale nehomogenně rozložený obsah živé mikrobiální biomasy. Analýza mastných kyselin celkových lipidů (reprezentujících živou, zásobní a mrtvou biomasu; 8390 pmol g^{-1}) odhalila bohaté a rovnoměrně rozložené profily houbových, bakteriálních a aktinomycetových markerů, a naopak nepotvrdila přítomnost markerů protozoí a řas.

Nasledující pasáž o rozsahu 4 stran obsahuje utajované skutečnosti a je obsažena pouze v archivovaném originále disertační práce uloženém na Přírodovědecké fakultě Jihočeské univerziy v Českých Budějovicích.

Contribution: 10 %

Paper Ib

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Growth Strategy of Heterotrophic Bacterial Population Along Successional Sequence on Spoil of Brown Coal Colliery Substrate

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Abstract

The bacterial population of brown coal colliery spoil (Sokolov coal mining district, Czechia) was characterized by measuring viable bacterial biomass, the culturable to total cell ratio (C:T), colony-forming curve (CFC) analysis and species and/or biotypes diversity. Bacterial representatives that differed in colony-forming growth (fast and/or slow growers) were used for growth-strategy investigation of heterotrophic bacteria. Spoil substrates from the surface (0-50 mm) and the mineral (100-150 mm) layers were sampled on 4 sites undergoing spontaneous succession corresponding to 1, 11, 21 and 43 years after deposition (initial, early, mid and late stages). The bacterial biomass of the surface layer increased during the initial and early stages with a maximum at mid stage and stabilised in the late stage while mineral layer biomass increased throughout the succession. The maxima of C:T ratios were at the early stage, minima at the late stage. Depending on the succession stage the C:T ratio was 1.5-2 times higher in the mineral than the surface layer of soil. An increase in the fraction of nonculturable bacteria was associated with the late succession stage. CFC analysis of the surface layer during a 3-d incubation revealed that the early-succession substrate contained more (75 %) rapidly colonizing bacteria (opportunists, r-strategists) than successively older substrates. The culturable bacterial community of the mineral layer maintained a high genera and species richness of fast growers along the succession line in contrast to the surface layer community, where there was a maximum in the abundance of fast growers in the early stage. There was a balanced distribution of Gram-positive and Gram-negative representatives of fast growers in both layers. A markedly lower abundance of slow growers was observed in the mineral in contrast to the surface layer. Gram-positive species dominated the slow growers in the surface as well as in the mineral layers. The growth strategy of the heterotrophic bacterial population along four successional stages on spoil of brown coal colliery substrate in the surface layer displayed a trend indicative of a r-K continuum in contrast to the mineral layer, where an r-strategy persisted.

Abstrakt

Populace bakterií hnědouhelných výsypek (Sokolovská uhelná pánev, Čechy) byla charakterizována pomocí měření živé bakteriální biomasy, poměru kultivovatelných k celkovým bakteriím (C:T), analýzou křivek jednotek tvořících kolonie (CFC) a druhovou a/nebo biotypovou diverzitou. Představitelé bakterií, kteří se lišily v rychlosti růstu (rychle a/nebo pomalu rostoucí kolonie), byly použity pro studium růstové strategie heterotrofních bakterií. Výsypkový substrát z povrchové (0-50 mm) a minerální (10-150 mm) vrstvy byl odebrán na 4 místech, kde dochází k spontánní sukcesi, a které odpovídají stáří 1, 11, 21 a 43 let po navršení (iniciální, rané, střední a pozdní stadia). Bakteriální biomasa povrchové vrstvy rostla v průběhu iniciálního a raného stadia, dosáhla maxima ve středním stadiu a ustálila se v pozdním stadium, zatímco biomasa minerální vrstvy rostla po celou dobu sukcese. Maxima poměru C:T byly v raném stadiu, minima v pozdním stadiu. V závislosti na sukcesním stadiu byl poměr C:T 1.5–2 krát vyšší v minerální vrstvě než ve vrstvě povrchové. Nárůst podílu

nekultivovatelných bakterií je spojován s pozdním sukcesním stadiem. CFC analýza povrchové vrstvy během 3-denní inkubace odhalila, že raný sukcesní substrát obsahuje více (75 %) rychle rostoucích bakterií (oportunistických, r-stratégů) než sukcesně starší stadia. Kultivovatelná část bakteriálního společenstva minerální vrstvy si podržela vysokou druhovou i rodovou bohatost oportunistických bakterií po celou sukcesní řadu, na rozdíl od společenstva povrchové vrstvy, kde největší množství oportunistických bakterií bylo zaznamenáno v raném stadium. Rozložení Gram pozitivních a Gram negativních zástupců oportunistických bakterií bylo vyrovnané. Významně menší abundance pomalu rostoucích bakterií byla zaznamenána v minerální vrstvě oproti vrstvě povrchové. Gram pozitivní druhy převažovaly v populaci pomalu rostoucích bakterií jak v povrchové, tak i v minerální vrstvě. Růstová strategie heterotrofních bakterií v povrchové vrstvě substrátů podél 4 sukcesních stadií na hnědouhelných výsypkách naznačovala r-K kontinuum, naopak u bakterií z minerální vrstvy přetrvávala r-strategie.

Nasledující pasáž o rozsahu 8 stran obsahuje utajované skutečnosti a je obsažena pouze v archivovaném originále disertační práce uloženém na Přírodovědecké fakultě Jihočeské univerziy v Českých Budějovicích.

Contribution: 10 %

Paper Ic

Proceedings of the 9th Methodological workshop: Present Methods for investigation of microbial community biodiversity in soils and substrates. Chroňáková A., Krištůfek V., Elhottová D., Malý S. (eds). Institute of Soil Biology AS CR, České Budějovice 2004

Analysis of Bacterial Isolates and Community DNA from Four Different Succession Plots in Post-mining Area

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Abstract

Microbial communities of 4 different stages (initial [0 years after heaping of the excavated material], early [10], middle [20] and late [42]) of primary succession process were studied. They were characterized by genotyping methods: ARDRA – Amplified ribosomal DNA restriction analysis and box-PCR - genomic fingerprinting using specific primer for boxA-element. Box-PCR method was chosen for comparison of methodological approaches used for characterization of heterotrophic bacterial communities. ARDRA patterns of eubacterial communities were so complex and there weren't shown any differences among individual communities on different stages of primary succession process. *Actinomycetes* communities were analyzed using two sets of primers: Actinomycetes 1. (243F-1378R, Heuer *et al.* 1997) and Actinomycetes 2. (243F-A3R; Monciardini *et al.* 2002). Fingerprints, which were obtained using both of actinomycetes specific primers, showed differences between communities on the early and later stages of primary succession process. Box-PCR analysis is still in data processing and will be published later.

Abstrakt
Byla studována mikrobiální společenstva 4 rozdílných stadií primární sukcese: iniciální [0 let po navršení substrátu], rané [10], střední [20] a pozdní [42]. Mikrobiální společenstva byla charakterizována pomocí 2 genotypových metod ARDRA (Amplified ribosomal DNA restriction analysis) a box-PCR (genomický fingerprinting pomocí primeru specifického pro boxA element). Metoda box-PCR byla vybrána pro porovnání metodických přístupů vhodných pro charakterizaci heterotrofních společenstev bakterií. ARDRA profily bakteriálních společenstev byly komplexní a neukázaly rozdíly mezi jednotlivými společenstvy různých stadií primární sukcese. Společenstvo aktinomycetů bylo analyzováno pomocí 2 sad primerů: Actinomycetes 1. (243F-1378R, Heuer *et al.* 1997) and Actinomycetes 2. (243F-A3R; Monciardini *et al.* 2002). Fingerprinty, které byly získány pomocí obou sad primerů specifických pro aktinomycety, ukázaly rozdíly ve složení společenstva mezi raným a pozdějšími stadiemi primární sukcese. Analýza box-PCR je právě ve stavu zpracování a bude publikována později.

Nasledující pasáž o rozsahu 3 stran obsahuje utajované skutečnosti a je obsažena pouze v archivovaném originále disertační práce uloženém na Přírodovědecké fakultě Jihočeské univerziy v Českých Budějovicích.

Contribution: 70 %

Paper Id

Microbiological Research – submitted manuscript

Biodiversity, antibiotic production and resistance of recent and ancient streptomycetes isolated from Miocene lacustrine sediment and successional sequence of post mining sites

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Key words: Actinomycetes, Antibiotics, Diversity, Primary succession, Resistance, Subsurface, 16S rDNA-ITS

Abstract
The amount and genetic diversity of soil streptomycetes, a major source of natural bioactive compounds, was investigated on colliery spoil heaps (Sokolov, Czech Republic) using the restriction pattern analysis of 16S-internal transcribed spacer rDNA and 16S sequences. We sampled freshly excavated Miocene sediment and four sites of primary succession (initial, early, middle and late stages, aged 1-44 years) developed on the same sediment. Even in fresh Miocene sediment, active bacteria were present and the relative proportion of actinomycetes among total bacterial isolates and their genetic diversity significantly increased with the age of deposition. The clustering of phylotypes well corresponded to the stage of succession and clusters were dominated by sequences: (i) from the initial and early stages; (ii) from the middle and late stages; (iii) from the Miocene sediment and finally (iv) of relatives of *S. turgidiscabies*, which were spatially dispersed. The replacement of pioneer species by

the late succession species during succession was observed. Plate assays of *Streptomyces* strains revealed 27% antibiotic-producing strains. Screening for nonribosomal peptide synthases and type I polyketide synthases systems showed putative production of biological active compounds in 90% and 55% streptomycetes, respectively. The frequencies of tetracycline, amoxicillin and chloramphenicol resistant streptomycetes were 6%, 9% and 15%, respectively. These findings documented the occurrence of genetic elements coding for antibiotic resistance and production even in pristine environments. Our results demonstrated a key role of ancient streptomycetes for pioneer community development and dynamic changes in their community on freshly excavated substrates during the first 20 years.

Abstrakt

Studovali jsme množství a genetickou diverzitu půdních streptomycetů (hlavních zdrojů přírodních bioaktivních látek) na hnědouhelných výsypkách (Sokolov, Česká republika) pomocí analýzy restrikčních profilů 16S-ITS (internal transcribed spacer/vnitřní přepisovaná intergenová sekvence) rDNA a 16S sekvencí. Vzorkovali jsme čerstvě vytěžený miocenní sediment a 4 místa primární sukcese (iniciální, rané, střední a pozdní stadia, staré 1-44 let), která se vyvíjela na stejném sedimentu. Dokonce i v čerstvém miocenním sedimentu byly přítomné aktivní bakterie, a relativní zastoupení aktinomycet mezi celkovými bakteriálními izoláty a jejich genetická diverzita významně rostly se stářím navážky. Shlukování fylotypů dobře odpovídalo stáří sukcese a u klastrů /skupin/ převládaly sekvence: (i) z iniciálního a středního stadia; (ii) ze středního a pozdního stadia; (iii) z miocénního sedimentu a konečně (iv) sekvence příbuzné *S. turbidiscabies*, které byly prostorově rozptýlené. Bylo zaznamenáno vytlačení pionýrských druhů pozdně sukcesními druhy v průběhu sukcese. Plotnové zkoušky kmenů streptomycetů odhalily přítomnost kmenů s antibiotickou aktivitou (27 %). Vyhledávání genetických markerů pro neribozomální peptid syntázy a polyketid syntázy typu I ukázalo na možnou produkci biologicky aktivních látek u 90%, respektive u 55 %, streptomycetů. Frekvence streptomycetů rezistentních na tetracyklin, amoxicilina chloramfenikol byla 6 %, 9 %, respektive 15 %. Tato zjištění dokumentovala přítomnost genetických elementů kódujících antibiotickou rezistenci a produkci dokonce v původních, neovlivněných prostředích. Naše výsledky ukázaly na klíčovou roli pradávných streptomycetů ve vývoji pionýrského společenstva a dynamické změny v jejich společenstvu na čerstvě vytěžených substrátech během prvních 20 let.

Nasledující pasáž o rozsahu 14 stran obsahuje utajované skutečnosti a je obsažena pouze v archivovaném originále disertační práce uloženém na Přírodovědecké fakultě Jihočeské univerziy v Českých Budějovicích.

Contribution: 60 %

Paper IIa

Soil Biology & Biochemistry (2009) - accepted manuscript

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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***Keywords*:** Nitrous oxide, qPCR, *nirS*, *nirK*, *nosZ*; DEA, grassland soils

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 užívané pro přezimování dobytka mohou reprezentovat významné zdroje emisí N_2O z půd. Z tohoto důvodu byl zkoumán dlouhodobý vliv přezimování skotu na pastvině na abundanci a aktivitu denitrifikačního společenstva. Studie byla provedena na zimovišti v jižních Čechách (Česká republika), kde byla vybrána 3 místa, která se lišila stupněm zátěže zvířat: silně zatížená (SI) a středně zatížená (MI) společně s kontrolní půdou bez vlivu zvířat (NI). Měření toků N_2O a vzorkování půd proběhlo na jaře a na podzim roku 2005. Byla měřena potenciální denitrifikační aktivita. Bakteriální geny *nirK*, *nirS* a *nosZ* byly použity jako funkční markery denitrifikačního společenstva; abundance byla analyzována pomocí real time PCR. Emise NO_2 *in situ* byly překvapivě nejvyšší na MI na jaře a významně se lišily od emisí na SI a NI, kdežto na podzim, míra emisí obecně klesla. Na rozdíl od emisí, potenciální denitrifikační aktivita byla nejvyšší na SI, poté na MI, a nejnižší na NI. Byl prokázán celkový významný posun v molárním poměru N_2O / N_2 na lokalitách se zátěží zvířat. Nejvyšší abundance všech detekovaných genů v obou odběrových časech byla zaznamenána na SI, kdežto na MI byly zvýšené hodnoty naměřeny jenom na jaře. Naše výsledky naznačují silný vliv skotu na abundanci a také aktivitu mikroorganizmů, které se účastní denitrifikace.

Nasledující pasáž o rozsahu 10 stran obsahuje utajované skutečnosti a je obsažena pouze v archivovaném originále disertační práce uloženém na Přírodovědecké fakultě Jihočeské univerziy v Českých Budějovicích.

Contribution: 70 %

Paper IIb

Environemntal Microbiology (2009) - accepted manuscript

Mapping field-scale spatial distribution patterns of size and activity of the denitrifier community

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Summary

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 modeling to explore spatial patterns of abundance 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 nonrandom distribution pattern of denitrifiers with a macro-scale spatial dependence (6 to 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 and potential N_2O production but not on denitrifier abundance. 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

Aktivita mikrobiálních procesů v půdě vykazuje velkou prostorovou variabilitu. Zároveň je málo informací o prostorové distribuci mikrobiálních společenstev, které tyto procesy zprostředkovávají. V naší studii jsme použili geostatistické modelování pro zjištění prostorových vzorců abundance a aktivity denitrifikačního mikrobiálního společenstva, funkční skupiny zapojené do koloběhu dusíku, na pastvině vystavené různé zátěži pastvy skotem. Obdrželi jsme nenáhodnou distribuci denitrifikačních bakterií závislou na prostorové makro-škále (6 - 16 m) a zmapovali jsme distribuci této funkční skupiny v terénu. Prostorové vzorce půdních charakteristik, které byly silně ovlivněné přítomností skotu, významně kontrolovaly potenciální denitrifikační aktivitu a potenciální produkci N_2O, ale nekontrolovaly abundanci denitrifikačních bakterií. Absolutní hodnoty abundancí většiny denitrifikačních genů nebyly v korelaci s distribučními vzorci potenciální denitrifikační aktivity nebo potenciální produkce N_2O. Avšak relativní abundance bakterií nesoucích gen *nosZ*, kódující reduktázu oxidu dusného v celkovém bakteriálním společenstvu, byla silným predikátorem poměru $N_2O/(N_2+N_2O)$, který poskytuje důkaz, že existuje vztah mezi složením bakteriálního společenstva založeném na relativním zastoupení denitrifikačních bakterií v celkové bakteriálním společenstvu a procesy na úrovni ekosystému. Představený geostatistický přístup obecně poskytuje ucelené mapování mikrobiálních společenstev a proto může napomáhat k lepšímu pochopení vztahů mezi ekologií mikrobiálních společenstev a mikrobiálních procesů podél environmentálních gradientů.

Nasledující pasáž o rozsahu 8 stran obsahuje utajované skutečnosti a je obsažena pouze v archivovaném originále disertační práce uloženém na Přírodovědecké fakultě Jihočeské univerziy v Českých Budějovicích.

Contribution: 10 %

Paper IIc

The ISME Journal (2007) 1, 443-452

Effects of cattle husbandry on abundance and activity of methanogenic Archaea in upland soils

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Keywords: Diversity - Methane Emission - Methyl coenzyme M reductase

Abstract

In the present study we tested the hypothesis that animal treading associated with a high input of organic matter would favour methanogenesis in soils used as overwintering pasture. Hence, methane emissions and methanogen populations were examined at sections with different degree of cattle impact in a Farm in South Bohemia, Czech Republic. In spring, methane emission positively corresponded to the gradient of animal impact. Applying phospholipid etherlipid (PLEL) analysis, the highest archaeal biomass was found in section SI (severe impact), followed by MI (moderate impact) and NI (no impact). The same trend was observed for the methanogens as showed by real-time quantitative PCR analyses of methyl coenzyme M reductase (*mcrA*) genes. The detection of mono-unsaturated isoprenoid side chain hydrocarbons (i20:1) indicated the presence of acetoclastic methanogens in the cattle impacted sites. This result was corroborated by the phylogenetic analysis of *mcrA* gene sequences obtained from section SI, which showed that 33% of the analysed clones belonged to the genus *Methanosarcina*. The majority of the sequenced clones (41%) showed close affiliations with uncultured rumen archaeons. This leads to the assumption, that a substantial part of the methanogenic community in plot SI derived from the grazing cattle itself. Compared to the spring sampling, in autumn a significant reduction in archaeal biomass and number of copies of *mcrA* genes was observed mainly for section MI. It can be concluded that after 5 months without cattle impact, the severely impact section maintained its methane production potential, whereas the methane production potential under moderate impact returned to background values.

Abstrakt

V této studii jsme testovali hypotézu, zda sešlapování půdy zvířaty, společně s vysokým vstupem organické hmoty, bude podporovat methanogenezi v půdách využívaných jako zimoviště. Proto byly zkoumány emise methanu a populace methanogenů na plochách různě zatížených pobytem zvířat na farmě v jižních Čechách (Česká republika). V jarním období emise methanu pozitivně korelovaly s gradientem zátěže zvířat. Použitím fosfolipid-etherlipid analýzy bylo zjištěno, že nejvyšší biomasa

archeí se nachází na ploše s nejvyšší zátěží (SI), poté na středně zatížené (MI) a nejnižší na kontrolní půdě. Stejný trend byl zaznamenán pro methanogenní archea, jak bylo ukázáno pomocí real-time kvantitativní PCR analýzy genů pro methyl koenzym M reduktázu (*mcrA*). Detekce mononenasycených isoprenoidů v postranních řetězcích uhlovodíků (i20:1) naznačovala přítomnost "acetoclastic" methanogenů na plochách ovlivněných skotem. Tento výsledek byl potvrzen fylogenetickou analýzou *mcrA* sekvencí získaných z plochy SI, který odhalil, že 33 % analyzovaných klonů patří do rodu *Methanosarcina.* Většina analyzovaných klonů (41 %) byla blízce příbuzná se sekvencemi nekultivovatelných archeí bachoru skotu. Tato zjištění vedou k domněnce, že podstatná část metanogenního společenstva půdy SI je odvozena od samotných pasoucích se zvířat. V porovnání s jarním odběrem bylo na podzim zaznamenáno významné snížení biomasy archeí a počtu kopií genu *mcrA* hlavně v půdě MI. Může být shrnuto, že po 5 měsících bez vlivu skotu, si silně ovlivněná půda uchovala potenciál produkovat methan, kdežto potenciál půdy se středním zatížením se vrátil na úroveň kontrolních hodnot.

Nasledující pasáž o rozsahu 9 stran obsahuje utajované skutečnosti a je obsažena pouze v archivovaném originále disertační práce uloženém na Přírodovědecké fakultě Jihočeské univerziy v Českých Budějovicích.

Contribution: 30 %

Paper III

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Survival of genetically-marked Escherichia coli O157:H7 in soil as affected by soil microbial community shifts

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Key words: Soil – fumigation – survival – Escherichia coli O157:H7 – Bacterial community structure – Molecular methods – DGGE – PLFA

Abstract

A loamy sand soil sampled from a species-rich permanent grassland at a long-term experimental site (Wildekamp, Bennekom, The Netherlands) was used to construct soil microcosms in which the microbial community compositions had been modified by fumigation at different intensities (depths). As expected, increasing depth of fumigation was shown to result in progressively increasing effects on the microbiological soil parameters, as determined by cultivation-based as well as cultivation-independent (PCR-DGGE, PLFA) methods. Both at 7 and at 60 days after fumigation, shifts in the bacterial, fungal and protozoan communities were noted, indicating that altered community compositions had emerged following a transition phase. At the level of bacteria culturable on plates, an increase of the prevalence of bacterial r-strategists was noted at 7 days followed by a decline at 60 days, which also hinted at the effectiveness of the fumigation treatments. The survival of a non-toxigenic

Escherichia coli O157:H7 derivative, strain T, was then assessed over 60 days in these microcosms, using detection via colony forming units counts as well as via PCR-DGGE. Both data sets were consistent with each other. Thus, a clear effect of fumigation depth on the survival of the invading strain T was noted, as a progressive increase of depth coincided with a progressively enhanced inoculant survival rate. As fumigation depth was presumably inversely related to community complexity, this was consistent with the hypothesis that soil systems with reduced biological complexity offer enhanced opportunities for invading microbial species to establish and persist. The significance of this finding is discussed in the light of the ongoing discussion about the complexity-invasiveness relationship within microbial communities, in particular regarding the opportunities of pathogens to persist.

Abstrakt

Hlinitopísčitá půda s bohatou druhovou diverzitou, pocházející z trvalé pastviny na experimentálním stanovišti (Wildekamp, Bennekom, Holandsko), byla použita pro vytvoření půdních mikrokosmů, u kterých bylo modifikováno složení mikrobiálního společenstva pomocí fumigace. Podle očekávání, zvyšující se intenzita fumigace měla za následek postupně rostoucí vliv na půdní mikrobiologické parametry, které byly určeny pomocí kultivačních i na kultivaci nezávislých metod (PCR-DGGE, PLFA). Sedm a také 60 dní po fumigaci byly zaznamenány posuny ve skladbě bakteriálního, houbového i protozoálního společenstva, které naznačovaly, že po přechodném období se objevilo změněné společenstvo. Na úrovni bakterií schopných kultivace byla zaznamenána převaha r-stratégů 7 dní po fumigaci, po které následoval pokles (60 dní, respektive), což ukazovalo na efektivitu fumigační zátěže. Přežívání netoxického kmene T, odvozeného od kmene *Escherichia coli* O157:H7, bylo stanoveno po dobu 60 dní v mikrokosmech pomocí detekce kolonie-formujících jednotek a také PCR-DGGE. Oba datasety byli navzájem shodné. Byl tedy zaznamenán jasný vliv intenzity fumigace na přežívání invazivního kmene T, protože postupný nárůst intenzity fumigace se shodoval s postupným zvyšováním schopnosti inokulantu přežívat. Protože intenzita fumigace byla pravděpodobně negativně korelována se složitostí/komplexitou diversity, toto zjištění potvrdilo hypotézu, že půdní systémy se sníženou biologickou složitostí umožňují zvýšené možnosti pro přežití invazivního mikrobiálního druhu. Význam tohoto poznatku je diskutován ve smyslu probíhající diskuze o vztahu mezi invazivitou a složitostí uvnitř mikrobiálních společenstev, zejména s ohledem na možnosti přetrvávání patogenů.

Nasledující pasáž o rozsahu 10 stran obsahuje utajované skutečnosti a je obsažena pouze v archivovaném originále disertační práce uloženém na Přírodovědecké fakultě Jihočeské univerziy v Českých Budějovicích.

Contribution: 20 %

Paper IV

Folia Microbiol. (2009) – accepted manuscript

Diverse Archaeal Community of a Bat Guano Pile in Domica Cave (Slovak Karst, Slovakia)

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Abstract

Although the microbial biodiversity of terrestrial habitats is studied in a great scale, little is known about microbial communities in caves and related habitats. Here we examined the molecular diversity of *Archaea* in a bat guano pile in Cave Domica (Slovakia), temperate cave ecosystem with significant bat colony (about 1,600 individuals). Guano pile was created mainly by an activity of the Mediterranean horseshoe bat (*Rhinolophus euryale*) and provides a source of organic carbon and other nutrients in the oligotrophic subsurface ecosystem. The upper and the basal parts of guano surface were sampled where the latter one had higher pH and higher admixture of limestone bedrock and increased colonization of invertebrates. The relative proportion of *Archaea* determined using CARD-FISH in both parts ranged between 3.5 – 3.9% (the basal and upper part, respectively). The archaeal community of the bat guano pile was dominated by non-thermophilic *Crenarchaeota* (99 % of clones). Phylogenetic analysis of 115 16S rDNA sequences revealed presence of *Crenarchaeota* previously isolated from temperate surface soils (group 1.1b, 62 clones), deep subsurface acid waters (group 1.1a, 52 clones) and *Euryarchaeota* (1 clone). Four of the analyzed sequences were found to have little similarity to those in public databases. The composition of both studied archaeal communities differed, with respect to higher diversity of *Archaea* in the upper part of the bat guano pile. Our study revealed for the first time that a high diversity archaeal population is present in the bat guano deposit and consists of both soil and subsurface - born *Crenarchaeota*.

Abstrakt

Ačkoliv je biodiversita mikroorganismů v terestrických ekosystémech studována ve velké míře, jenom málo je známo o mikrobiálních společenstvech v jeskyních a příbuzných prostředích. V této práci jsme pomocí molekulární analýzy studovali diversitu archeí kupy netopýřího guána v jeskyni Domica (Slovensko), typické temperátní jeskyni s významnou kolonií netopýrů (kolem 1 600 jedinců). Guánová kupa byla vytvořena aktivitou Vrápence jižního (*Rhinolophus euryale*) a slouží jako zdroj organického uhlíku a živin v oligotrofním podpovrchovém ekosystému. Byly vzorkovány horní a bazální části povrchu guánové kupy, přičemž druhá z nich byla charakteristická vyšším pH a větší příměsí vápencového podloží a také zvýšenou kolonizací bezobratlými živočichy. Relativní zastoupení archeí v obou místech určené metodou CARD-FISH se pohybovalo mezi 3.5 až 3.9 % (bazální a horní část, resp.). Ve společenstvu archeí kupy netopýřího guána převládala netermofilní *Crenarchaeota* (99 % klonů). Fylogenetická analýza 115 sekvencí genu 16S rRNA odhalila přítomnost *Crenarchaeota*, která byla dříve isolována z temperátních povrchových půd (skupina 1.1b, 62 klonů) a z hlubinných podpovrchových kyselých vod (skupina 1.1a, 52 klonů), a přítomnost *Euryarchaeota* (1 klon). Čtyři analyzované klony vykazovaly velice nízkou příbuznost se sekvencemi z veřejných databází. Struktura obou studovaných společenstev archeí se lišila, s ohledem na vyšší diversitu archeí v horní části guánové kupy. Naše studie poprvé odhalila, že ložisko netopýřího guána skýtá vysokou diversitu společenstva archeí, které je složeno jak ze zástupců původně půdních, tak podpovrchových *Crenarchaeota*.

Nasledující pasáž o rozsahu 11 stran obsahuje utajované skutečnosti a je obsažena pouze v archivovaném originále disertační práce uloženém na Přírodovědecké fakultě Jihočeské univerziy v Českých Budějovicích.

Contribution: 80 %

Curriculum vitae

Alica Chroňáková (maiden name Pristachová) was born 2 May 1972 in Trenčín, Slovakia (formerly Czechoslovakia). She graduated from the Faculty of Science of Charles University in Prague in 1995. Her master's thesis focused on the expression of human oncoprotein (MN) and putative inducer protein NP (LCMV) in insect cells using bacculovirus expression system and also on a study of post-translation modifications of MN protein in this environment. She was supervised by doc. RNDr. Jitka Forstová, CSc., the chief of the Laboratory of Virology in the Department of Microbiology and Molecular Biology. After completing her MSc. studies she began a happy time on maternity leave with two girls. She returned to work as a technician in the Department of Soil Microbiology and Soil Chemistry of the Institute of Soil Biology AS CR, in České Budějovice in 2002 and began her Ph.D. study at the University of South Bohemia in České Budějovice, Faculty of Science in September 2003 under supervision of Ing. Václav Krištůfek, CSc. During her Ph.D. studies (2003 - 2008) she was employed at the Institute of Soil Biology AS CR as a junior researcher and participated in 14 projects of the Soil Microbiology Group at the same department led by Prof. Miloslav Šimek. She focused on the research of the ecology and biology of soil streptomycetes with regard to screening for secondary metabolites production, methanogenic and the nitrifying Archaea and denitrification bacterial community by means of modern molecular techniques applied in microbial ecology (PCR, ARDRA, DGGE, TGGE, T-RFLP, qPCR, sequencing and Southern hybridization). Nowadays, she is fully employed as a researcher and acts as a curator of the Culture Collection of Actinomycetes České Budějovice of the Biology Centre, v. v. i. – Institute of Soil Biology, České Budějovice, Czech Republic.

I visited following international and home collaborating institutions:

Éötvös Loránd University in Budapest, Hungary, Prof. K. Márialigeti (several short term stages in 2004 and 2005)

Research Institute for Soil and Agricultural Chemistry of the Hungarian Academy of Sciences, Budapest, Hungary, Dr. A. Halbritter (two short term stages in 2004 and 2005)

Institute of Soil Ecology, GSF - German Research Center for Environment and Health, Neuherberg, Germany, Dr. M. Schloter (3 stages one month-long and several short term stages from 2005)

University of Gröningen, Department of Microbial Ecology, Haren, The Netherlands, Prof. J.D. van Elsas (3 weeks in 2006)

INRA - University of Burgundy, Department of Soil and Environmental Microbiology, Dijon Cedex, France, Dr. L. Philippot (1 week in 2007)

University of Florence, Department of Soil Science and Plant Nutrition, Florence, Italy, Prof. P. Nannipieri (2 weeks in 2008)

Institute of Microbiology AS CR, v. v. i., Laboratory of Molecular Biology of Actinomycetes, Prague, Ing. Miroslav Petříček

BC AS CR, v. v. i. – Institute of Parasitology, Laboratory of Molecular Taxonomy, České Budějovice, Doc. Miroslav Oborník

Masaryk University, Department of Experimental Biology, Brno, Doc. Jiří Damborský

International workshopes I have attended:

University of Aveiro, Portugal - International Course on Molecular Methods to Study Complex Microbial Communities, Department of Biology & CESAM (Centre for Environmental and Marine Studies), Dr. I. Henriques, University in Aveiro, Portugal, January 21 – 25, 2008.

John Innes/Rudjer Bošković Summer School on Microbial Secondary Metabolites: Genomes, Signals and Communities, Prof. D. Hopwood and Prof. J. Davies, IUC Dubrovnik, Croatia, August 24 - September 1, 2008.

Workshop on Molecular Evolution, Dr. M.P. Cummings and Dr. S.A. Handley, Český Krumlov, Czech Republic, January 11 – 23, 2009.

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