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Regulation potential of earthworms as related to diversity and functioning of soil microbial community

Ph.D. Thesis

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Annotation

Earthworm-microbial interactions with emphasis on the passage effects of *Eisenia* spp. on microbial community were investigated. The study was focused on earthworm potential to regulate functional microbiota in cattle-impacted soils. Microbial communities were studied through a combination of polar lipid analyses, molecular, and culturing methods.

Declaration [in Czech]

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Anna Koubová

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List of papers and author's contribution

The thesis is based on the following papers:

I. **Koubová, A.**, Chroňáková, A., Pižl, V., Sánchez-Monedero, M.A., Elhottová, D., 2015. The effects of earthworms *Eisenia* spp. on microbial community are habitat dependent. European Journal of Soil Biology 68, 42−55 (IF = 1.719).
Anna Koubová was responsible for sample preparation, bacterial cultivations, PLFA procedure, DNA isolation, PCR-DGGE procedure, data evaluation, and writing of the manuscript.

II. **Koubová, A.**, Knapp, B.A., Insam, H., Pižl, V., Elhottová, D., 2010. The effect of passage through the gut of earthworms (Annelida: Lumbricidae) on the diversity of archaea and bacteria. Acta Societatis Zoologicae Bohemicae, 74, 69−74.
Anna Koubová was responsible for sampling, sample preparation, DNA isolation, PCR-DGGE procedure, data evaluation, and writing of the manuscript.

III. Elhottová, D., **Koubová, A.**, Šimek, M., Cajthaml, T., Jirout, J., Esperschuetz, J., Schloter, M., Gattinger, A., 2012. Changes in soil microbial communities as affected by intensive cattle husbandry. Applied Soil Ecology 58, 56−65 (IF = 2.106).
Anna Koubová participated on soil sampling, sample preparation, PLEL procedure, and revision of the manuscript.

IV. Bannert, A., Bogen, C., Esperschütz, J., **Koubová, A.**, Buegger, F., Fischer, D., Radl, V., Fuß, R., Chroňáková, A., Elhottová, D., Šimek, M., Schloter, M., 2012. Anaerobic oxidation of methane in grassland soils used for cattle husbandry. Biogeosciences, 9, 3891−3899 (IF = 3.754).
Anna Koubová participated on PLFA procedure, data evaluation, and revision of the manuscript.

V. **Koubová, A.**, Goberna, M., Šimek, M., Chroňáková, A., Pižl, V., Insam, H., Elhottová, D., 2012. Effects of the earthworm *Eisenia andrei* on methanogens in a cattle-impacted soil: A microcosm study. European Journal of Soil Biology 48, 32−40 (IF = 1.838).
Anna Koubová was responsible for establishment and maintaining of the experiment, sample preparation, PLFA procedure, DNA isolation, microarray analyses, real-time PCR preparation, data evaluation, and writing of the manuscript.

Contents

1 GENERAL INTRODUCTION

1.1 Earthworm-microbial interactions

Soil represents a complex system that provides recycling dead organic material and mineralizing nutrients to receive new energy for plants and other biota anchored in soil (Karaca, 2011). Decomposition processes in soil are dominated by microorganisms via various metabolic pathways and enzymatic activities for breakdown of organic matter (OM). Microbial activity and community structure are highly influenced by diverse populations of soil dwelling fauna. Earthworms, members of soil macrofauna, are often considered key 'ecosystem engineers' (Lavelle, 1997), whereby they have high potential to regulate soil as an environment for other organisms by controlling availability of resources. Moreover, earthworms occur in relatively high densities, e.g., 150–300 ind. m^{-2} with a biomass of 55–100 g m^{-2} in arable soil (Curry et al., 2002) and up to 1000 ind. m^{-2} with a biomass up to 1 kg m^{-2} in cattle grazed pastures with cow dung saturation (Butt et al., 1993), therefore they unavoidably interact with other soil biota in various ways. Soil microbiota come into direct interactions with earthworms during soil passage through the earthworm gut (Egert et al., 2004; Savin et al., 2004). This interaction includes not only predator-prey relationships, but also brings various benefits for microbial proliferation (Sampedro et al., 2006; Marhan et al., 2007; Sampedro and Whalen, 2007). Earthworm-microbial interactions also have indirect effects, such as competition for resources and habitat formation. Earthworm burrowing activity regulates soil porosity and hence aeration and hydration status and forms soil structure (Binet et al., 1998; Frouz, 2002; Frouz et al., 2006). Excrements and mucus secretion change carbon substrate availability to microorganisms and involve biogeochemical cycles of soil trace elements (Scheu, 1990; Borken et al., 2000; Horn et al., 2003; Ihssen et al., 2003).

1.2 Passage effects on microbial community

Earthworms come directly into contact with microorganisms via the passage of material through the gut (ingestion, digestion, and assimilation of OM). Some members of the microbiota are activated during passage through the gut and proliferate, whereas others remain unaffected, or are digested in the intestinal tract (Pedersen and Hendriksen, 1993; Monroy et al., 2008). The selective effects of earthworm gut passage on microbes may be caused by competition between ingested and endosymbiotic microbes that reside in the gut (Brown and Mitchell,

1981) or by the selective defence mechanism of coelomic fluid against microbial pathogens (Beschin et al., 1998).

Several researchers have studied microbial changes during passage through the earthworm gut by means of various quantitative and qualitative microbial assays. Quantitative changes of prokaryotic microorganisms during gut passage were observed using epifluorescence microscopy (Pedersen and Hendriksen, 1993; Krištůfek et al., 1995; Singleton et al., 2003) and with the help of counts of culturable bacteria. In all studied epigeic earthworms (inhabiting and feeding directly on microorganisms and litter in the upper organic horizon of soil; Curry and Schmidt, 2007), gut passage increased direct bacterial numbers in earthworm gut and excrements ($1x10^8$–10^9 cells g^{-1} dw) compared to bulk soil ($1x10^5$–10^6 cells g^{-1} dw; Pedersen a Hendriksen, 1993; Singleton et al., 2003). The numbers of culturable bacteria had a decreasing tendency during the passage (Karsten and Drake, 1995) or were unaffected in comparison to soil (Byzov et al., 2007). Sampedro and Whalen (2007) showed that the total microbial biomass (expressed as phospholipid fatty acid concentration - PLFA) and biomarkers of aerobic bacteria, microeukaryotes and fungi increased 300 times in the gut of anecic *Lumbricus terrestris* compared to bulk soil (anecic species live in deeper zones of mineral soils, but feed at the soil surface and drag litter material into vertical burrows; Curry and Schmidt, 2007). In contrast, Marhan et al. (2007) did not find differences between total amounts of PLFA in soil and gut of the same species. Edwards and Fletcher (1998) revealed that microbial biomass increased up to 1000 times during passage, whereas it decreased to the previous level after excretion of the casts.

The quality of the microbial community associated with the earthworm gut was previously studied mostly using an ester-linked PLFA biomarker method. Gut and cast conditions are suitable for an increase of biomass of micromycetes, eukaryotes and Gram positive as well as Gram negative bacteria (Clapperton et al., 2001; Sampedro et al., 2006; Marhan et al., 2007; Sampedro and Whalen, 2007). Singleton et al. (2003) studied microbial diversity in gut and excrements of *Lumbricus rubellus* using a 16S rRNA analysis. In gut contents, *Acidobacteria*, *Actinobacteria*, *Firmicutes*, *β-* and *γ-Proteobacteria* dominated while excrements were inhabited by bacteria including the phyla *Actinobacteria*, *Rubrobacteria*, *Cytophagales*, *Firmicutes,* and *α-Proteobacteria.* Similar findings were shown by Byzov et al. (2007) in the gut microbial community in the case of an endogeic *Aporrectodea caliginosa* (receiving the nutrients from humified OM in more stable soil mineral layers; Curry and Schmidt, 2007).

Compared to common soil, earthworm intestinal tract is a microenvironment with higher carbon, nitrogen and water contents and a distinct oxygen deficiency (Horn et al., 2003). Many anaerobic or facultatively anaerobic bacteria (e.g.,

Clostridium, *Aeromonas*, *Bacillus*, *Shewanella*, *Propionibacterium*, *Staphylococcus*, *Paenibacillus,* and *Photobacterium*) as well as archaea, are abundant in the guts of earthworms (Toyota and Kimura, 2000; Ihssen et al., 2003; Shin et al., 2004; Knapp et al., 2008; Hong et al., 2011). The conditions of the gut are suitable for the activation of dormant or inactive microbial forms that might be present in soil (Ihssen et al., 2003). The culturable part of anaerobes has been estimated in *L. rubellus* and *Octolasion lacteum* (Karsten and Drake, 1995). Furlong et al. (2002) found that archaeal clones analysed in *L. rubellus* were different between soil and casts.

1.3 Earthworm indirect impact on microbial community

Earthworms indirectly regulate microbial communities by burrowing activity which enables mixing of soil mineral and organic compounds, aeration and improvement of hydration of soil due to macropores (Springett 1983; Kooistra, 1991). On the other hand, earthworms have an indirect impact on microbiota by compaction of soil at burrow walls mechanically and by production of body secretions (Kizilkaya et al., 2011). They add 80-120% water to the ingested soil and 5-38% of the dry weight of soil as mucus, a readily assimilable substrate that they mix in the anterior part of the digestive tract. Excrements and burrows contain mucus formed by intestinal fluids with higher C and N contents and lower C/N ratios (Scheu, 1991; Schmidt et al., 1999). For that reason, the microbial population and their activities in the earthworm excrements and burrow walls are usually higher than that in the surrounding soil (Tiunov and Scheu, 1999). The addition of mucus to the soil triggers a ‘priming effect’ on soil organic carbon, whereby microbial activity is greatly enhanced (Fontaine et al., 2003). Despite scientifically documented and putative beneficial effects of earthworms on soil structure, nutrient dynamics and plant growth, some aspects of earthworm activities are considered as undesirable. Earthworm activity indirectly increases losses of soil nitrogen through leaching and microbial denitrification, and increases loss of soil carbon through enhancement microbial respiration (Lavelle et al., 1998).

1.4 Earthworm influence on functional microbiota

Earthworms may ingest large amounts of soil and litter, transit them through the intestine, and hence participate to biological soil bioturbation processes and become major regulators of litter and OM dynamics in the ecosystem (Lavelle, 1997) Epigeic earthworms are often used in organic waste management for their

potential to eliminate contaminants including deleterious microorganisms from organic waste (Héry et al., 2008; Hickman and Reid, 2008; Vivas et al., 2009). Bacteria and fungi are considered important dietary sources for earthworms, and therefore, the digestion of the organic wastes by these oligochaetes would decrease total bacteria and fungi abundance in worm-worked material, known as vermicompost (Castillo et al., 2013). Sen et al. (2008) observed that total bacterial abundance remained constant during the vermicomposting of industrial sugar wastes as evaluated by real-time PCR. On the other hand, Pramanik and Chung (2011) found increases in the total numbers of bacteria and fungi in vermicomposts inoculated by *Eisenia fetida* and *E. eugeniae*. Nevertheless, vermicomposting influences microbial activity and community structure (Huang et al., 2014).

Various compounds with biogeochemical and climatic relevance are released from soils into the atmosphere. The most important so called greenhouse gases contributing to global warming are carbon dioxide, methane, and nitrous oxide (Conrad, 1996). Agricultural soils with intensive livestock management can be strongly affected by organic wastes and participate in greenhouse gas emissions. Pasture sites used for cattle overwintering are highly influenced by the animal organic wastes. Compared to ordinary pastures, which are used for grazing during summer, a much higher number of animals are present on a small area for a relatively long period during winter time. This causes a reduction in soil aeration through soil compaction (Menneer et al., 2005). The presence of animals is accompanied by a high input of organic material through excrements, which consequently stimulates microbial metabolism, increases oxygen demand and supports the occurrence of anoxic microsites in the soil (Šimek et al., 2006). From this reason, Radl et al. (2007) demonstrated high abundance of methanogens in soils as well as high methane production rates during winter. Cattle excrements are often a main source of alien microorganisms and can induce shifts in the functional and structural composition of microbial communities (Peacock et al., 2001; Griffiths et al., 1998). In particular, microbial communities of such soils have been previously characterized by their high potential to metabolize large nitrogen inputs over short periods (Brůček et al., 2009; Hynšt et al., 2007), by increased abundance of the key greenhouse gases producers, i.e., methanogenic archaea (Radl et al., 2007) and the denitrifiers (Chroňáková et al., 2009), as well as an abundance shift of the main soil bacterial and fungal taxa (Philippot et al., 2009; Jirout et al., 2011). Nevertheless, in-situ methane oxidation rates in those soils were high. It was therefore suggested that methanotrophic microorganisms could be responsible for the consumption of methane. However, no distinctive activity of aerobic methanotrophs could be proved leading to the presumption that anaerobic oxidation of methane (AOM) might play an important role in cattle-impacted soil.

To date, the potential reduction of methanogens by earthworms in organic wastes and soils has been unknown. Although, considerable amounts of methane are produced in the intestines of many soil invertebrates, including millipedes and larvae of tropical scarabaeid beetles (Egert et al., 2003; Lemke et al., 2003; Šustr et al., 2014), only a few earthworm specimens of *Eudrilus eugenie* and *Perionyx excavatus* have been found to emit methane (Depkat-Jacob et al., 2012; Schulz et al., 2015). Representatives of *Eisenia* spp., due to their high tolerance to the environment conditions (ability to live and reproduce in manure or herbivore dung) and no documented methane release, could be suitable model organisms for a bioremediation experiment with cattle-impacted soils typified by high methane emission.

2 AIMS OF THE THESIS

The general aim of the thesis was to determine the influence of earthworms *Eisenia* spp. on complex microbial communities in soil and compost substrates. The first part of the thesis deals with the direct effects of earthworm passage on complex microbial community including anaerobes and archaea. The microbial communities were analysed and compared in samples coming from three different earthworm habitats with various nutrient demands. The second part focuses on the potential of *Eisenia andrei* to regulate microbial communities of agricultural soils affected by outdoor cattle husbandry. We evaluated specific changes in the soil microbial community with emphasis on anaerobes, archaea, and methanogens, originating from cattle activities. We focused on the microbial consortium involved in methane oxidation under anoxic conditions. Finally, we tested the effects of the earthworm *E. andrei* on methanogenic diversity and abundance of total anaerobes in cattle-impacted soils.

The specific objectives of the thesis were:

- To determine the microbial changes accompanying the passage of the substrate through the intestine of *E. fetida* and *E. andrei* and to test whether the specific microbial changes are dependent on different earthworm habitats.

- To characterise the complex microbial community in pasture soil with different degrees of cattle disturbance and to assess the microbial input from cattle excrements to soil.

- To reveal whether microorganisms in cattle-impacted soils oxidise methane under anaerobic conditions and whether ^{13}C incorporated in microbial biomass comes from labelled methane.

- To test the potential of earthworms (*E. andrei*) to regulate soil anaerobes with emphasis on the diversity and abundance of methanogenic archaea in cattle-impacted soils.

3 SAMPLING SITES AND METHODS

3.1 Experimental earthworms

The epigeic earthworms *Eisenia fetida* and *E. andrei* were chosen as experimental model species. Although earthworms of this genus have been the focus of many research studies, their interactions with microorganisms are still poorly understood and data concerning gut microbiota are fragmented. *Eisenia fetida* (Savigny, 1826) and *E. andrei* Bouché (1972) are closely related sibling species with similar anatomy and morphology (Albani, et al., 2003). Both species live in the fresh litter layer of forest soil, in litter mounds, and synantropically in manure heaps, herbivore dung and compost piles. In Central Europe, *E. andrei* commonly inhabits anthropogenic cultures, and *E. fetida* lives in unique forest populations (Pižl, 2002). Both species are used for the management of organic wastes through vermicomposting and vermiremediation processes (Vivas et al., 2009; Suthar et al., 2014). Moreover, they are often used in ecotoxicology (Renoux et al., 2000; Hickman and Reid, 2008; Contreras-Ramos et al., 2009), physiological studies (Velando et al., 2008), and as model organisms in immunology to understand the defence mechanisms of soil invertebrates against microbial pathogens (Beschin et al., 1998; Bilej et al., 2000; Bilej et al., 2001; Procházková et al., 2006; Dvořák et al., 2013).

Specimens of *E. fetida* (**Papers I and II**) were collected from moist soil of a mixed forest brook rich in leaf litter (Hluboká nad Vltavou, Czech Republic) in 2007, 2008 and 2009. *Eisenia andrei* earthworms (**Papers I, II, and V**) were collected from a compost pile with plant remains (České Budějovice, Czech Republic) in 2007 and 2009; the earthworms invaded the compost spontaneously from a neighbouring compost pile. The specimens of a second *E. andrei* population used in **Paper I** were collected from a large-scale vermiculture plant consisting of straw, cattle manure and other organic agricultural wastes (Mikulčice, Czech Republic) in 2007 and 2008; the substrate was inoculated using commercially acquired animals.

3.2 Overwintering cattle pasture

The research published in **Papers III–V** of this thesis was realised on an experimental cattle overwintering area at an organic farm in Borová near Český Krumlov in South Bohemia (details in Šimek et al., 2006). The farm has used the overwintering area, which was occupied by about 90 cows from the end of October till the beginning of May, periodically each year since 1995. Soils under long-term

investigation were categorized into sections according to the level of visible cattle impact (severe, moderate, and no impact). The most affected section was situated near the barn (SI = severe impact). The soil surface and plant cover at this site were totally destroyed by the end of winter season. A less impacted section in the middle of the gradient (MI = moderate impact) was characterized by partly destroyed vegetation. The third section at the opposite side of the overwintering area was used as a control (NI or CO = no impact, control site) because cattle impact was minimal and the soil and original vegetation were unaffected. At each sampling point (number of sampling points and samples differed in individual experiments), random single samples of soil from the top 0–20 cm were collected directly after cattle abandoned the overwintering area in spring (**Papers III - V**) and right before the gathering of cattle for overwintering at beginning of fall (**Paper III**). Two incubation experiments were established to simulate i) soil anaerobic conditions induced by high input of excrements and cattle trampling during winter, and ii) soil regeneration during summer with potential bioturbation activity of added earthworms *E. andrei*. The details of experimental procedure are described in 'Materials and methods' of **Papers IV and V**.

3.3 Methods

A complex approach combining phospholipid biomarker analyses, molecular and culturing methods was used for the purposes of this thesis. This approach may help to better understand microbial processes in the studied soils and in the earthworm gut and excrements. Below-mentioned methods applied in the thesis were used to estimate total viable microbial biomass and complex community structure, bacterial community structure and diversity, archaeal diversity, and relative distribution of functional microbial groups in microbial communities with emphasis on anaerobes and their archaeal part of methanogens. Table 1 shows an overview of the individual methods for detection of anaerobes applied in the thesis.

A PLFA analysis was used to quantify total microbial biomass and microbial community composition and to detect the microorganisms involved in anaerobic methane oxidation. A simple PLFA analysis evaluates only ester-linked (EL) fatty acids. An extended PLFA method includes both EL-PLFA indicative of aerobes, and non-ester-linked (NEL) PLFA interpreted as anaerobes, and allows a complex and detailed comparison of microbial community structure (Zelles, 1999).

In the thesis we used the following technique to determine PLFA. The phospholipid fraction was subjected to mild alkaline methanolysis to liberate EL-PLFA, which were separated from the unsaponifiable lipids by the solid-phase extraction. The NEL-PLFA were obtained through the acidic hydrolysis and methylation of the unsaponifiable fraction. The EL-PLFA and NEL-PLFA profile

Table 1
Methodological approaches for detection of anaerobes including archaea applied in particular papers of the thesis. Abbreviations: CFU – colony forming units, NEL-UNSFA – unsubstituted non-ester linked phospholipid fatty acids, SIP-PLFA – stable isotope probing in combination with phospholipid fatty acid analysis, PLEL – phospholipid ether lipid analysis, ip – isoprenoid, DGGE – denaturing gradient gel electrophoresis, qPCR – quantitative polymerase chain reaction, *mcrA* – methyl-coenzyme M reductase gene, *Msar* 16S rRNA – *Methanosarcina* genus specific sequences.

Method		**Targeted anaerobes**	**Paper**
Plate cultivation	CFU, growth strategy, species identification	Facultative anaerobes	I
Polar lipid analysis	NEL-UNSFA	Strict anaerobes	I, III, IV, V
	SIP-PLFA	Anoxic methane oxidisers	IV
	PLEL	Archaea	III
	PLEL (ip20:1)	Methanogens	III
Molecular analysis	DGGE	Archaea	I, II
	microarray AnaeroChip	Methanogens	V
	qPCR *mcrA*	Methanogens	V
	qPCR *Msar16S rRNA*	*Methanosarcina* spp.	V

was identified by gas chromatography with flame ionisation detector (GC, FID) followed by mass spectrometry (GS/MS).

Stable isotope probing has been reported as a method to study key bacterial populations involved in methane-dependent denitrification under aerobic conditions (Osaka et al., 2008). The SIP-PLFA analysis focuses on the detection of PLFA with incorporated ^{13}C (coming from labelled $^{13}CH_4$). In this thesis, an extended PLFA method was used to separate phospholipids into individual fractions according to Zelles (1999). Fatty acid methylesters were detected via GC/MS, while the isotopic composition of fatty acids was detected after combustion in the isotope-ratio mass spectrometry (GC/MS-C-IRMS).

The PLEL analysis is a biomarker method based on the determination of isoprenoid hydrocarbons. This method enables the quantification of microbial biomass of archaea and identification of archaeal etherlipids including isoprenoids typical for methanogens (Gattinger et al., 2003). In principal, the phospholipid fraction after the formation of ether core lipids is subjected to the cleavage of ether bonds with hydroiodic acid and reductive dehalogenation with Zn in glacial acetic acid. The resulting isoprenoid hydrocarbons are determined via GC/MS.

The DGGE is a molecular technique that was used to analyse archaeal and bacterial community structures (Muyzer et al., 1993; Heuer et al., 1997; Coolen et al., 2004).

To screen the methanogenic diversity we used the AnaeroChip - a phylogenetic microarray targeting the 16S rRNA gene of most lineages of methanogens (Franke-Whittle et al., 2009a). Soil DNA was PCR-amplified, digested, hybridised with the arrays, and fluorescently scanned at wavelengths of 543 nm and 633 nm. The signal-to noise ratio (SNR) was calculated, and all the positive probes (SNR ≥ 2) were included in the evaluations.

Quantitative PCR was used to evaluate *mcrA* gene and 16S rRNA gene abundances in environmental samples. The *mcrA* gene is a characteristic functional marker coding for the α-subunit of methyl-coenzyme M reductase, a key enzyme of methanogenesis (Luton et al., 2002). The abundances of *Methanosarcina* were quantified using genus specific primers and qPCR assay targeting 16S rRNA sequences (Zhang et al., 2006; Franke-Whittle et al., 2009b).

The dilution plate cultivation technique was used to estimate the number of CFU. The development of CFU during a one-week cultivation period on specific medium was expressed as a colony forming curve and used for a bacterial growth strategy evaluation (Krištůfek et al., 2005). The identification of culturable bacteria was performed by the MIS Sherlock System (TSBA 6, MIDI Inc., USA).

Evaluations of microbial activities, in particular measurements of CO_2 and CH_4 production rates, were done using GC with a thermal conductivity detector.

4 RESULTS AND GENERAL DISCUSSION

Paper I deals with the direct effects of earthworm passage on a complex microbial community ubiquitous in the earthworm intestine and excrements. Three earthworm populations (one belonging to *Eisenia fetida* and two to *E. andrei*) were collected from different habitats: forest soil, compost, and vermiculture, respectively. Passage increased total microbial biomass in the gut and faeces of all three studied *Eisenia* populations. Compared to soil, we observed a significant increase in biomass of anaerobes in earthworm gut and faeces, suggesting the fact that the intestinal tract is a microenvironment with a higher carbon, nitrogen and water content and distinct oxygen deficiency (Horn et al., 2003; 2006). Many anaerobic or facultatively anaerobic bacteria as well as archaea have been reported as abundant in the guts of earthworms (Hong et al., 2011; Knapp et al., 2008; Shin et al., 2004; Toyota and Kimura, 2000). Counts of culturable bacteria and their identification showed that the gut environment, mainly of compost earthworms, activated spore-forming bacteria (e.g., *Bacillus* and *Paenibacillus*). Similarly, Fisher et al. (1997) revealed that the passage by *L. terrestris* enhanced the germination of *B. megaterium* spores. Bacterial DGGE fingerprints did not confirm the differences among substrate, faeces and gut content of *E. andrei* inhabiting vermiculture, but showed a specific bacterial profile in gut and faeces different from forest soil. Nevertheless, archaeal DGGE patterns were strictly divergent in different stages of passage in all studied earthworms. Bacteria were characterised by a higher diversity compared to archaea, and the differences might correspond to the changes in dominant representatives; therefore, the total bacterial community might have responded less sensitively to environmental changes than archaea. Earthworms feeding in various habitats showed different effects on passaged microbial community composition. Passage affected the intestinal microbiome of indigenous *E. fetida* in more aspects than in related *E. andrei*: the results indicated greater changes in archaeal community structure and higher biomass of anaerobes in passaged soil. Compost and vermiculture *E. andrei* are evolutionarily adapted to a substantially higher quantity of microbes and their PLFA profile was well balanced between gut and faeces (Dvořák et al., 2013). On the other hand, the intestine of indigenous *E. fetida* selected and activated anaerobes that predominated in faeces. Distinct antimicrobial defence mechanisms could play a significant role among studied earthworm species (Procházková et al., 2006).

Paper II describes passage effects of the earthworms *E. fetida* and *E. andrei* on archaeal and bacterial diversity. Archaeal diversity of different *Lumbricus* species has been previously characterised in the digestive tracts (Furlong et al., 2002; Knapp et al., 2008), whereas archaea associated with *E. fetida* were investigated only in vermicompost substrates (Vivas et al., 2009). Our results

showed that the numbers of DGGE bands, corresponding to archaeal and bacterial richness, increased after passage through the guts of *E. andrei*, but not *E. fetida*. Nevertheless, the passage in both earthworm species had a significant effect on the archaeal diversity whereas the bacterial diversity was affected only by *E. andrei*. The effects of earthworms corresponded to previous studies on earthworm-bacterial interactions. Karsten and Drake (1997), Singleton et al. (2003) and Egert et al. (2004) showed that passage through the guts of earthworms had a substantial effect on bacterial diversity. Digestive fluid may select bacterial species and enable proliferation of more resistant anaerobes rather than facultative anaerobic bacteria and fungi (Byzov et al., 2007).

Paper III characterises in detail the microbial communities in pasture soils with different degrees of cattle disturbance caused by overwintering management. Moreover, in this work we optimised extended PLFA analysis and applied it on non-soil samples such as cow excrements. The results showed that the microbial biomass of severely impacted soil increased up to four times compared to controls and a new microbial community was derived from cattle intestinal microorganisms, typical by increased content of archaeal PLEL and by new fatty acids indicative of bacterial and fungal faecal anaerobes. Archaeal ether lipids were enriched by methanogen markers in excrements and soils under cattle impact. Gattinger at al. (2007) showed that repeated long-term application of organic manure increases archaeal biomass, which could be related to methanogen *Methanoculleus* and *Methanosarcina* species. Cattle-impacted soils were enriched by unsubstituted non-ester-linked fatty acids typical of anaerobic bacterial genera ubiquitous in cattle faeces and bacteria tolerant to high pH and anaerobic conditions. The relative abundance of anaerobes was positively correlated with the concentration of the fraction of soil organic matter (OM) containing aromatic compounds and with soil water content. The differences between spring and fall sampling periods were evident only at sites with a moderate degree of cattle disturbance. Compared to the severely-impacted site, the moderately-impacted site was characterised by higher activity of the microbial community and higher OM transformation at the end of the vegetation season. Our study documented that the structure of the microbial community in soils under repeated cattle impact was completely changed and particularly enriched by anaerobes including methanogens.

The results described in **Paper IV** revealed that ^{13}C labelled methane was consumed under anaerobic experimental conditions in samples with soil from the SI site. The consumption of methane was accompanied by production of carbon dioxide containing the label to an increasing extent. Furthermore, the ^{13}C label was also found to be incorporated into microbial biomass carbon for SI soil while non-impacted control soil lacked a comparable incorporation. The analysis of SIP-PLFA showed that respective microorganisms incorporated ^{13}C from labelled methane in their phospholipid membranes. This provided the opportunity to analyse ^{13}C-

marked phospholipid fatty acids. Highest relative and absolute label incorporation was detected in PLFA 16:1ω7, indicating that organisms harbouring this fatty acid are highly active in degrading methane under anaerobic conditions. These lipids were also found as most ^{13}C-enriched fatty acids by Raghoebarsing et al. (2006) after addition of $^{13}CH_4$ to an enrichment culture coupling denitrification of nitrate to anaerobic oxidation of methane. This might be an indication of anaerobic oxidation of methane by relatives of "*Candidatus* Methylomirabilis oxyfera" (Wu et al., 2011) in the investigated grassland soil under the conditions of the incubation experiment. Stable isotope experiments, using ^{13}C-labelled methane, allowed us to separate methane production and consumption processes occurring simultaneously. The percentage of CH_4-C incorporated into CO_2 was significantly increased in anaerobically incubated SI soil compared to control soil. Decreasing amounts of nitrate and increasing amounts of ammonium indicate absence of nitrification and prevalence of denitrification or other anaerobic processes during incubation. Results from the present study give clear evidence for an anaerobic activity of methane-oxidizing microorganisms in pasture soil.

Paper V focuses on the potential of earthworms to regulate anaerobes, especially methanogens, in soils sampled in a severely cattle-impacted site from the overwintering area. Phylogenetic microarray analysis completed in detail the findings about methanogens in pasture soils (Radl et al., 2007), and provided a first view into earthworm-methanogenic interactions on the basis of a laboratory incubation experiment. The phylogenetic DNA microarray showed that severely impacted soils were characterised by high methanogenic diversity with predominance of *Methanosarcina* and other methanogenic members of Euryarchaeota. The inoculation of cattle-impacted soils with earthworms did not significantly reduce the total numbers of positive methanogenic probes, but DNA microarray and qPCR analysis confirmed a significant decrease in *Methanosarcina* abundance and a decrease below detectable levels (SNR $\geq$ 2) of three other genera of methanogens. Total anaerobes (indicated by NEL-PLFA) decreased significantly in worm-worked soils after 4 months of incubation. They could have been reduced not only due to bioturbation, but also due to the digestion of microorganisms during the passage of soil through the earthworm intestinal tract. At the level of soil-earthworm microcosms we did not observe a decrease of potential methane production rate. This corresponds to a non-significant decrease of total methanogens analysed as *mcrA* gene abundance. Uncultured methanogens (approximately 41% of the clones reported by Radl et al., 2007) that were not detected by AnaeroChip, may have contributed to the methanogenic activity in worm-worked soil. The earthworm-mediated incorporation of fresh OM into soil in the form of polysaccharide mucus may have promoted the 'priming effect' (Fontaine et al., 2003). Substantial earthworm activity in a small soil volume could cause large local increases of labile compounds and result in diverse

aerobic/anaerobic activities and fluctuating oxic and anoxic microsites. The results of this study indicated that *E. andrei* is a member of the soil macrofauna with high potential to regulate the abundance of anaerobes and most common methanogen *Methanosarcina* spp. in soils strongly disturbed by cattle.

5 CONCLUSIONS

The thesis elucidated the interactions between earthworms and microbial comunities in their natural habitats. The work brought new knowledge about the relationships between earthworms and soil anaerobes including methanogens. The passage of soil or compost through the gut of *Eisenia* spp. substantially changed microbial community structure, increased total viable microbial biomass, and reduced the richness of functional microbial groups in gut and faeces. The influence of *Eisenia* spp. on the associated microorganisms depended on the earthworm feeding habitat. The passage indicated greater changes in archaeal than bacterial community structure and higher abundance of anaerobes in samples from forest soil than compost and vermiculture substrates.

The study of soil microbial communities under the impact of cattle husbandry showed a specific community profile derived from ruminant microbiota with significant enrichment of methanogenic archaea. Cattle-impacted soils were enriched by unsubstituted non-ester-linked fatty acids typical for anaerobic bacterial genera and bacteria tolerant to high pH and anaerobic conditions.

Specific incubation experiment showed the oxidation of methane under anoxic conditions in cattle-impacted soil. The consumption of $^{13}CH_4$ labelled methane was accompanied by the production of carbon dioxide containing the labelled ^{13}C. Carbon was also found to be incorporated into microbial biomass. Decreasing amounts of nitrate and increasing amounts of ammonium indicate absence of nitrification and prevalence of denitrification. This experiment showed that nitrate and nitrite were utilised as electron acceptors during anaerobic methane oxidation in upland pasture soils.

Another laboratory microcosm experiment documented that *E. andrei* had the potential to regulate functional microbiota in cattle-impacted soils with high anoxic microenvironments related to animal activities. Earthworms reduced the abundance of total anaerobes and the concentration of the most common methanogen *Methanosarcina* spp. in these soils.

The thesis brought a new knowledge about the importance of earthworms *Eisenia* spp. in relationship with soil microbiota for the control of flux of greenhouse gases at the ecosystem level.

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RESEARCH ARTICLES

Paper I

Koubová, A., Chroňáková, A., Pižl, V., Sánchez-Monedero, M.A., Elhottová, D., 2015. The effects of earthworms Eisenia spp. on microbial community are habitat dependent. European Journal of Soil Biology 68, 42−55.

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Original article

The effects of earthworms *Eisenia* spp. on microbial community are habitat dependent

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ABSTRACT

The effects of earthworms *Eisenia* spp. on microorganisms of three different habitat soil, compost, and vermiculture were studied. Microbial communities of gut and fresh faeces of earthworms and substrates, the worms were collected from, were analysed. Microbial biomass and composition of the total microbial community were examined using phospholipid fatty acid (PLFA) biomarkers. Archaeal and bacterial communities were studied by polymerase chain reaction-denaturing gradient gel electrophoresis (DGGE). The culturing methods were used for assessment of counts, species richness and growth strategy of bacteria.

In comparison with the substrates, the viable microbial biomass and the group of non-ester-linked PLFAs indicative of anaerobes were higher in both the gut and faeces of all earthworm populations. The prokaryotic community evaluated using DGGE revealed that archaeal community structure in the gut and faeces of earthworms from populations differed from that in substrates, whereas the passage through the gut had less influence on the bacterial community structure, particularly in compost and vermiculture.

The counts of culturable bacteria increased due to gut passage only in forest and vermiculture populations. The fast-growing bacteria increased due to gut passage only in forest soil population. Actinobacteria (*Arthrobacter*, *Microbacterium*, *Lechevalieria* and *Nesterenkonia*) and Firmicutes (*Bacillus* and *Paenibacillus*) were generally favoured in substrates and their species richness decreased with gut passage, whereas Gammaproteobacteria (*Aeromonas*, *Enterobacter*, *Pseudomonas* and *Salmonella*) dominated in gut contents. The impact of earthworm activity on the microbial community was higher in nutrient-poor forest soil than in nutrient-rich compost and vermiculture substrates.

1. Introduction

Earthworms are remarkable drivers of decomposition of dead organic matter and bioturbation processes in soil; they increase mineralisation rate of organic matter due to the enhancement of microbial activity [1] and modify the physical structure of soil. Earthworms come into interactions with microorganisms not only during their direct ingestion but also affect microbial life indirectly by forming and altering their habitat [2]. Their casts enrich the microbial nutrient status [3]. Passage of feeding material through the earthworm gut leads to a different bacterial composition of gut and faeces compared to that of the original soil or compost [4–6]. The shift in microbial biomass, structure and species diversity of the intestinal microbial community has been previously studied in different earthworm ecological groups (anecic, endogeic and epigeic), however the data are fragmented. Sampedro et al. [6] and Sampedro and Whalen [7] revealed that the total microbial biomass (expressed as PLFA concentration) and biomarkers of aerobic bacteria, microeukaryotes and fungi increased in gut of anecic *Lumbricus terrestris* compared to bulk soil. In contrast, Marhan et al. [8] did not found the differences between total amount of PLFA in soil and gut of the same species. Counts of culturable bacteria isolated

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from gut of epigeic *Eisenia fetida* increased compared to soil and were found three fold higher than those from anecic (*L. terrestris*) and endogeic earthworms (*Aporrectodea caliginosa*) that remained constant [9,10]. In contrast to other earthworm species, relatively little is known about the prokaryotic microorganisms (archaea and bacteria) associated with the intestinal tract of epigeic *E. fetida* and *Eisenia andrei* [11–14] and only few studies include both species [11,15–17]. To date archaea have been studied as associated with the intestine of *Eudrilus eugeniae* [18] and *Lumbricus rubellus* [19] and culturable anaerobes have been estimated in *L. rubellus* and *Octolasion lacteum* [9].

Thakuria et al. [20] found that earthworm ecological group is the strongest factor for the composition of the gut-wall associated bacteria. Little is known about the influence of earthworm habitat on microbiota within the same ecological group on the microbial changes in earthworm gut [8,21]. To study the effects of different feeding habitats on microbiota associated with the intestine of earthworms we tested three earthworm populations living in three specific habitats: an indigenous soil population of *E. fetida* and a compost and a vermiculture population of *E. andrei*, representing the same ecological group.

The epigeic earthworms *E. fetida* (Savigny, 1826) and *E. andrei* Bouché (1972) are closely related sibling species with similar anatomy and morphology [22]. Both species can be properly differentiated at the molecular level [16]. In nature *E. fetida* and *E. andrei* lives in fresh litter layer of forest soil, in litter mounds and synantropicaly in manure heaps, herbivore dungs and compost pile. In Central Europe *E. andrei* commonly inhabits antropogenic cultures, although *E. fetida* lives in unique forest populations [23]. Both species are used for the management of organic wastes as vermicomposting [24], in ecotoxicology [25–27], in physiology [28], and as model organisms in immunology to understand the defense mechanisms of soil invertebrates again microbial pathogens [29–32].

The aim of this study was to determine (i) the microbial changes occurring during the passage of the substrate through the intestine of *Eisenia* spp; (ii) the specific microbial changes dependent on three different habitats (forest soil, compost, and vermiculture). To achieve these aims, microbial characteristics were determined in substrates, and in earthworm guts and faeces. The concomitant earthworm microbiota was assessed using a combination of biomarkers, molecular and culturing methods, to obtain information concerning the total microbial biomass, community structure, bacterial and archaeal composition, culturable bacteria-counts, -growth strategy, and -species richness.

2. Material and methods

2.1. Earthworm and sample collection

The study is based on two to three independent observations for each *Eisenia* population over three years. *E. fetida* earthworms were collected from moist soil of a mixed forest brook rich in leaf litter (mixed forest, Hluboká nad Vltavou, Czech Republic) in 2007, 2008 and 2009. *E. andrei* specimens were collected from a compost pile with plant remains (České Budějovice, Czech Republic) in 2007 and 2009; the earthworms invaded the compost spontaneously from a neighbour compost pile. The specimens of a second population of *E. andrei* were collected from a large-scale vermiculture plant consisting of straw, cattle manure and other organic agricultural wastes (Mikulčice, Czech Republic) in 2007 and 2008; the substrate was inoculated using commercially acquired animals. Earthworm species were identified on the base of species-specific mitochondrial gene for cytochrome c oxidase subunit I [16]. Three composite soil samples, each consisting of three randomly collected partial samples, were taken from a bottom of the brook in a depth 0–10 cm. Similarly, three composite samples, each of three partial samples, were collected from compost pile/vermiculture bed in a depth 0–20 cm and stored at 4 °C. Microbial characteristics of substrates (SUB) were compared with those in the gut content (GT) and faeces (FS) of earthworms. Six earthworms per one sample considered for gut content analysis were used and the samples were acquired according to Pižl and Nováková [33]. To obtain one sample of fresh faeces, nine to twelve worms were placed on moist sterile filter paper over the night and fresh excrement was collected into a sterile tube. Samples were immediately performed to the chemical analyses or stored in microtubes at minus 18 °C for molecular analyses.

2.2. Soil, compost and vermiculture properties

Basic chemical and biochemical characteristics of soil (SO), compost (CO), and vermiculture (VE) are summarized in Table 1. The SO differed significantly in most of properties in comparison to CO and VE. The soil pH was weakly acidic in contrast to neutral pH

Table 1
Chemical and biochemical parameters of forest soil, compost, and vermiculture substrates. Values are mean ± standard error. Chemical parameters were assessed in three replicates (n = 3); carbon- and humic-substances and enzymatic activity were analysed in two replicates (n = 2).

Parameters	Substrates		
	Forest soil	Compost	Vermiculture
pH ($CaCl_2$)	5.7 ± 0.1	7.4 ± 0.1	6.9 ± 0.1
Cox (%)	2.1 ± 0.4	34.5 ± 6.9	65.8 ± 13.8
Ntot (%)	0.2 ± 0.0	2.6 ± 0.5	2.6 ± 0.5
C/N	9.7	13.5	25.0
P (g kg^{-1} dw)	0.06 ± 0.01	2.96 ± 0.59	4.37 ± 0.87
K (g kg^{-1}dw)	0.06 ± 0.01	7.11 ± 1.42	17.58 ± 3.52
Mg (g kg^{-1}dw)	0.22 ± 0.03	1.90 ± 0.29	4.69 ± 0.70
Ca (g kg^{-1}dw)	1.86 ± 0.37	9.36 ± 1.87	9.40 ± 1.88
Water-soluble carbon (WSC) (%C)	0.08 ± 0.06 a	0.33 ± 0.14 a	0.52 ± 0.17 a
Water-soluble carbohydrates (%C)	0.00 a	0.02 ± 0.00 b	0.04 ± 0.00 c
Extractable C_{tot} (%C)	1.50 ± 0.26 a	4.06 ± 1.33 a	4.33 ± 0.58 a
Humic acids (%C)	1.15 ± 0.27 a	3.56 ± 1.33 a	3.37 ± 0.59 a
Fulvic acids (%C)	0.35 ± 0.03 a	0.50 ± 0.05 ab	0.96 ± 0.15 b
Enzymatic activity			
β-glucosidase (μg p-nitrofenol g^{-1} h^{-1})	165.7 ± 39.5 a	167.8 ± 1.2 a	284.1 ± 1.9 a
Protease (μg consumed tyrosine g^{-1} h^{-1})	43.1 ± 3.6 a	67.8 ± 1.4 b	100.7 ± 0.2 c
Urease (mg N–NH_4^+ g^{-1} h^{-1})	0.95 ± 0.12 a	4.58 ± 0.10 b	4.66 ± 0.85 b

Means in a row followed by variable lowercase letters are significantly different at $P \leq 0.05$. Differences between means were evaluated by one-way ANOVA followed by Tukey's post hoc test.

in CO and VE. The content of nitrogen (N_{tot}) and other nutrient elements were lower in SO than in CO/VE about one or even two orders, respectively. Similarly there were measured lower organic carbon (C_{org}), water soluble carbohydrates and fulvic acids in SO compared to CO/VE substrates. Nutrient and carbon poor soil habitat was also characterized by lower enzymatic activity, especially urease and protease. The VE was in most of parameters (C_{org}, P, K, Mg, protease) the richest habitat characterized by more than twice higher C/N ratio compared to CO and SO.

The pH was determined in 1:5 (v:v) soil/extract (0.01 M $CaCl_2$ extraction solution; [34]). Organic carbon was determined by dry combustion and total nitrogen was determined by Kjeldahl digestion [35]. Trace elements P, K, Mg, and Ca were analysed using flame atomic absorption spectroscopy (FAAS). Water-soluble and extractable carbon and humic substances were analysed according to modified methods described by Sanchéz-Monedero et al. [36]. Protease activity was measured by determining tyrosine consumed after incubating the samples (1 g dw) with sodiumcaseinate (2%) for 2 h at 50 °C. Optical density was measured at 700 nm after Folin-Ciocalteu addition as a reagent to develop colour [37]. Beta-glucosidase activity was determined as p-nitrofenol released after incubating the samples with p-nitrofenil-β-glucoside for 1 h at 37 °C. Optical density was measured at 400 nm after developing the colour with 0.5 M $CaCl_2$ [38]. Urease activity was assessed by measuring the $N{-}NH_4^+$ content after incubating the samples with urea solution (0.2 M) for 2 h at 37 °C and ammonium extraction with 2 M KCl and 0.015 M HCl in a spectrophotometer at 660 nm [39].

2.3. Fatty acid analysis

A simple PLFA analysis was used to quantify total microbial biomass and microbial community composition [40] in all samples during three years-observation. Simple PLFA analysis evaluates ester-linked (EL-) PLFA without later fractionation of methylesters. An extended PLFA method includes EL-PLFA indicative of aerobes, and non-ester-linked (NEL-) PLFA interpreted as anaerobes. NEL-PLFA group represents only minor part of soil microbiome PLFA profile (e.g., 21% of total PLFA in fertilised soil [41]). Extended PLFA procedure [42] was used for complex comparison of all samples in a single experiment (2008). The extended PLFA analysis was repeated in all sample types of *E. fetida* also in year 2009 to verify high concentration of NEL-PLFA in faeces.

Fatty acid abbreviations (used in Fig. 1) according to Zelles [41] denote the total number of carbon atoms followed by the number of double bonds and their position from the aliphatic end (ω). The prefixes i- and a-refer to the terminal iso- and anteiso-branched fatty acid chain, respectively. A number followed by Me-indicates the position of the methyl group from the carboxyl end of the molecule (i.e. mid-branching), and the prefix cy-represents cyclopropyl branching. The number preceding OH refers to the position of the hydroxyl group of a hydroxy-fatty acid, and DMA refers to aldehyde dimethylacetals. The individual PLFAs were classified according to their composition and specific predominance in microbial taxa into groups (Table 2).

2.4. PCR-DGGE analysis of the archaeal and bacterial community structure

DNA was extracted from 0.12 g earthworm gut content and faeces and 0.5 g soil, compost and vermiculture substrates according to Griffiths et al. [56]. The sample was placed in a Bio-101 Multimix 2 Matrix Tube (Qbiogene, Germany) and 0.5 ml CTAB extraction buffer and 0.5 ml phenol-chloroform-isoamylalcohol (25:24:1, v:v:v) were added. After the homogenisation step (40 s, 16,000 *g*) and centrifugation (5 min, 16,000 *g*), the aqueous phase was transferred and phenol was removed by mixing with an equal volume of chloroform-isoamylalcohol (24:1, v:v). The centrifugation step was repeated and total nucleic acid was extracted using two volumes of 30% polyethylene glycol-1.6 M NaCl on ice for 2 h. Subsequently, the centrifugation step was repeated (10 min) and the supernatant was removed, DNA was air-dried and washed with 0.6 ml ice-cold 70% ethanol (centrifugation for 10 min) and again air-dried. Extracted DNA was quantified with a spectrophotometer (Genesys 6, Thermo Spectronic, USA) and subjected to electrophoresis in a 1% (w:v) agarose gel using 1 × TAE buffer and ethidium bromide staining [57].

PCR amplification of the target DNA was performed in a PCR thermocycler (T 3000 Thermocycler, Biometra, Germany) using different bacterial and archaeal primer sets. Each PCR mixture contained 50 ng extracted DNA, 0.2 μM each primer, 0.625 U Dream Taq DNA Polymerase, 1 × DNA polymerase buffer, 5 μg Bovine serum albumin (BSA), 0.2 mM dNTP-Mix and 3.5 mM $MgCl_2$ in a final volume of 50 μl (all MBI Fermentas, Lithuania).

Bacterial 16S rRNA genes were amplified using the universal 16S

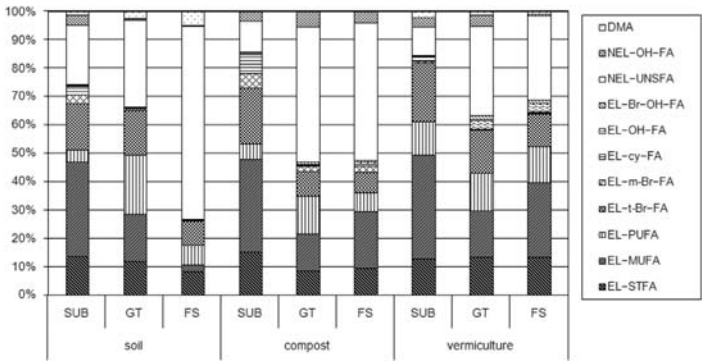

Fig. 1. Relative distribution of phospholipid fatty acid (PLFA) functional groups in the PLFA profile evaluated in substrates (SUB), gut contents (GT), and faeces (FS) of three different earthworm populations. The individual PLFAs were classified according to their source and structure into fatty acid groups: ester-linked (EL-) saturated (EL-STFA), monounsaturated (EL-MUFA), polyunsaturated (EL-PUFA), terminally-branched (EL-t-Br-FA), mid-branched (EL-m-Br-FA), cyclopropyl-branched (EL-cy-FA), hydroxy-substituted (EL-OH-FA), and branched hydroxy-substituted (EL-Br-OH-FA) fatty acids. The non-ester-linked fatty acids (NEL-PLFA) included the group of unsubstituted (NEL-UNSFA), hydroxy-substituted fatty acids (NEL-OH-FA), and aldehyde dimethylacetals (DMA).

Table 2
Phosholipid fatty acid (PLFA) functional groups used in this study and their interpretation. The abbreviations of FA groups were used according to Zelles [41].

Abbreviation	Indicator	Interpretation	Reference
EL-PLFA	ester-linked fatty acid	aerobic microorganisms	[41,43]
NEL-PLFA	non-ester-linked fatty acids	anaerobic and extremophilic microorganisms	[41,43]
EL-STFA	saturated EL-PLFA	all microorganisms	[44]
EL-MUFA	monounsaturated EL-PLFA	aerobic eukaryotes and prokaryotes	[45]
EL-PUFA	polyunsaturated EL-PLFA	microeukaryotes, rare in bacteria	[44,46,47]
EL-t-Br-FA	terminally branched EL-PLFA	Gram-positive bacteria	[48,49]
EL-m-Br-FA	mid-branched saturated EL-PLFA	actinomycetes	[50,51]
EL-cy-FA	cyclopropyl-branched saturated EL-PLFA	Gram-negative bacteria	[41,43]
EL-OH-FA	hydroxy-substituted saturated EL-PLFA with straight chain	Gram-negative bacteria, fungi	[45,49]
EL-Br-OH-FA	hydroxy-substituted saturated EL-PLFA with branched chain	Gram-negative bacteria, mainly Bacteroidetes	[45]
NEL-UNSFA	unsubstituted NEL-PLFA	anaerobes	[45,52]
NEL-OH-FA	hydroxy-substituted NEL-PLFA	facultative anaerobes (*Sphingomonas*, Bacteroides, *Flavobacterium*, *Candida*)	[45,52–54]
DMA	aldehyde dimethylacetals	plasmalogen-containing strict anaerobes	[52,55]

rRNA primer set 984FGC (5′- [gc] GCACGGGGGGAACGCGAAGAACCTTAC − 3′) and 1378R (5′- CGGTGTGTACAAGGCCCGGGAACG − 3′) [58]. The PCR included an initial 4 min denaturation at 95 °C and was followed by 10 thermal cycles of 1 min at 94 °C, 1 min at 60 °C and 2 min at 72 °C followed by 25 thermal cycles of 1 min at 94 °C, 1 min at 55 °C and 2 min at 72 °C. Amplification was completed with a final extension step at 72 °C for 15 min.

The archaea 16S rRNA genes were amplified according to Coolen et al. [59] using primers Parch519F (5′- CAGCMGCCGCGGTAA − 3′) [60] and Arch915RGC (5′- [gc] GTGCTCCCCCGCCAATTCCT − 3′) [61]. The PCR started with an initial denaturation step at 96 °C for 5 min, followed by 35 thermal cycles of 30 s at 94 °C, 40 s at 57 °C, and 40 s at 72 °C and a final extension step at 72 °C for 10 min. PCR products were visualised by electrophoresis in 1% (w:v) agarose gels and ethidium bromide staining (10 mg ml^{-1}).

The DGGE was performed with the Ingeny PhorU2 system (Ingeny International BV, The Netherlands) according to Chroňáková et al. [62] using some modifications: for bacterial as well as archaeal DGGE, 30 μl PCR product was loaded onto 6% (w:v) polyacrylamide gels with a denaturing gradient of 45%–60% and 45%–75% for bacteria and archaea, respectively, and was run at 100 V at 60 °C in 1 × TAE-buffer (pH 7.4) for 16 h. After electrophoresis, the gels were stained with SYBR Green. A comprehensive comparison of all samples was performed in a single experiment, building on the preliminary experiment; the partial results are summarized in methodological work by Koubová et al. [63].

2.5. Counts and identification of culturable bacteria

The dilution plate cultivation technique was used to estimate the number of colony forming units (CFU). The development of CFU during a one-week cultivation period on BBL Tripticase™ Soy Broth Agar medium (Becton Dickinson, USA) at 28 °C was expressed as a colony forming curve and used for a bacterial growth strategy evaluation [64]. The identification of culturable bacteria was performed by the MIS Sherlock System (TSBA 6, MIDI Inc., USA) after previous preparation of the samples according to the manufacturer's protocol.

Table 3
Viable microbial biomass (EL-PLFA) evaluated in substrates (SUB), gut contents (GT), and faeces (FS) of three different *Eisenia* populations: soil (SO), compost (CO) and vermiculture (VE); (mean ± standard error; n = 6).

	EL-PLFA		
	[nmol PLFA g^{-1} dw]		
	SO	CO	VE
SUB	29.6 ± 11.8 aA	73.1 ± 43.7 aA	48.5 ± 15.6 aA
GT	1216.0 ± 286.8 bA	1335.3 ± 267.9 bA	2095.9 ± 426.0 bA
FS	1148.7 ± 578.2 abA	656.3 ± 95.8 aA	787.0 ± 71.4 aA

Means followed by variable lowercase letters in a column are significantly different at $P \leq 0.05$; variable uppercase letters in a row show significantly different PLFA mean values within earthworms. Differences between means were evaluated by One-way ANOVA followed by Tukey's post hoc test.

2.6. Statistical analysis

The analysis of variance (one-way ANOVA) and Tukey's post-hoc test were used to detect the differences between the abundance values of microbial biomass at $P < 0.05$ within samples in consequence of the gut passage in a single earthworm population. Kruskal–Wallis test ($P < 0.05$) and multiple comparisons of mean ranks for all groups were used to find the significant differences between CFU numbers since data were not normally distributed (STATISTICA 7). The differences between important microbial taxonomic groups were evaluated based on the relative abundance of specific biomarkers or fatty acid groups within the PLFA profile using one-way ANOVA and Tukey's test. Principal component analysis (PCA) was applied to visualise the sample distribution within the PLFA profile, log [mol% + 1] values were used (Canoco for Windows 4.5, Centre for Biometry Wageningen, The Netherlands). The ordination plot was created with CanoDraw for Windows 4.5 [65]. One-way ANOVA and Tukey's post-hoc test of PC sample scores were used to depict the effect of passage and the earthworm population on the FA distribution.

DGGE banding patterns were normalised and analysed using the GelCompar II software package, version 4.0 (Applied Maths, Ghent, Belgium). Calculation of the pair-wise similarities was based on the Dice correlation coefficient and an unweighed pair-group method using arithmetic averages (UPGMA). The PCA of DGGE patterns was used to explain the variability of the samples, and PCA sample scores were tested using one-way ANOVA ($P < 0.05$) and Tukey's post-hoc test to reveal the differences between DGGE banding patterns.

3. Results

3.1. Microbial biomass and PLFA microbial community profile

Viable microbial biomass, examined as EL-PLFA by simple PLFA analysis, increased significantly by about one-to two-fold in earthworm gut compared to substrates for all studied habitats (Table 3, Table A.1). The EL-PLFA measured in faeces had an intermediate response compared to that in substrates and guts; the

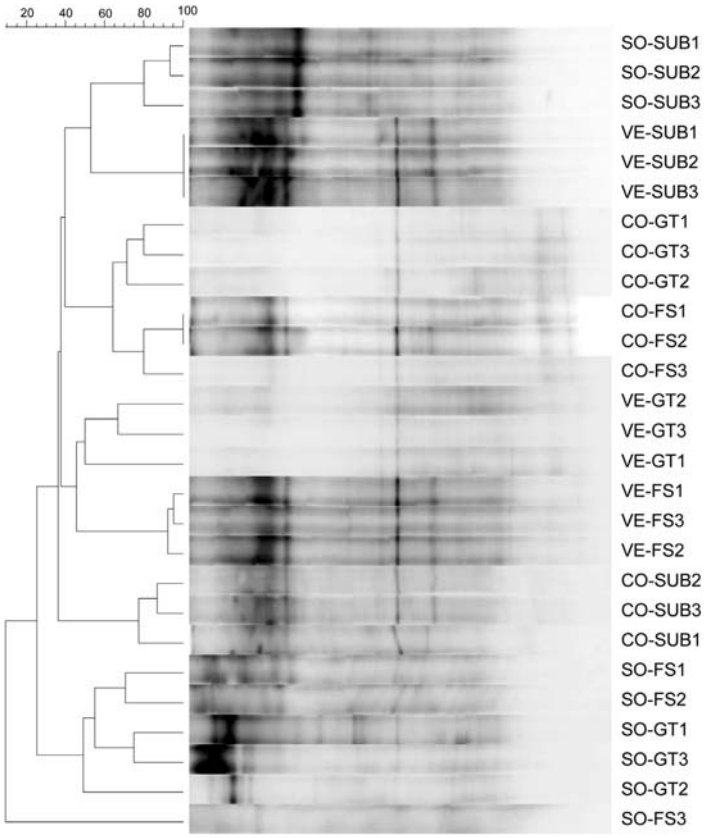

Fig. 2. Dendrogram of archaeal DGGE fingerprints based on 16S rDNA extracted from substrates (SUB), gut content (GT) and faeces (FS) of forest (SO), compost (CO), and vermiculture (VE) earthworm populations. Profiles were clustered by Dice correlation and an unweighted pair-group method using arithmetic averages (UPGMA).

values were not significantly different. The extended PLFA results confirmed above described trend of quantitative microbial differences caused by passage of the substrates (Table A.2) and in more details explained qualitative composition (Fig. 1, Fig. A.1). It showed that substrates of all habitats had well-balanced proportions of individual PLFA functional groups. Passage significantly affected several groups. (i) The total NEL-PLFA relative abundance significantly increased and accounted for 73%, 53%, and 31% of the total microbial profile in faeces of soil, compost, and vermiculture earthworms, respectively. In detail, the DMA (strict anaerobes) relative abundance increased significantly in gut and faeces of soil earthworm at the expense of NEL-OH-FA (facultative anaerobes). (ii) The EL-PUFA accounted for a relatively low molar percentage in soil and in compost substrate (4% and 6%, respectively) but increased significantly in gut of soil and compost earthworms (to 21% and 13%, respectively) (Fig. 1). The EL-PUFA represented 13% in all vermiculture samples. (iii) In contrast, the individual EL-PLFA groups (MUFA, t-Br-FA, cy-FA), that accounted for a relatively high molar percentage of the PLFA profile in substrates, were suppressed in gut contents and faeces of all earthworm populations. (iv) EL-OH-FA and EL-Br-OH-FA increased in gut and faeces after the passage of vermiculture (Fig. 1). The multivariate comparison (PCA) performed with the PLFA community profiles extracted two axes explaining 80.6% of the total variance (Fig. A.1). All the substrates were discriminated in a cluster around the negative pole of the PC1 axis which explained 64.5% of the total variance. All the GT samples and CO-FS samples were distributed along the negative pole of the PC2 axis which explained 16.1% of the variance. These samples were characterised by anaerobic markers (NEL-UNSFA, 30–55% of the PLFA profile), by the fungal marker 18:2ω6,9 (Fig. A.1), and evenly balanced groups of EL-t-Br-FA, EL-PUFA, EL-MUFA, and EL-STFA (Fig. 1). Soil-earthworm-faeces samples (SO-FS) created one separate cluster, where NEL-UNSFAs and DMAs extremely enriched the PLFA profile (Fig. 1, Fig. A.1). The PC1 axis separated the faeces of the vermiculture population (VE-FS) from that of both soil and compost populations. The VE-FS samples were clustered together with all samples of substrate. The PLFA profile of VE-FS was well-balanced and the relative content of EL-PLFA was significantly higher compared to the other FS samples (Fig. 1). One-way ANOVA of the PC sample scores demonstrated a significant effect ($P < 0.05$) of passage on the PLFA microbial community profile in all earthworm populations. The post-hoc test showed a different microbial PLFA composition of FS samples compared to both SUB and GT samples, whereas no differences were found between SUB and GT samples in all three populations.

3.2. Archaeal and bacterial community structure

Cluster analysis of archaeal DGGE patterns revealed that passage shifted the archaeal community structure in GT and FS compared to

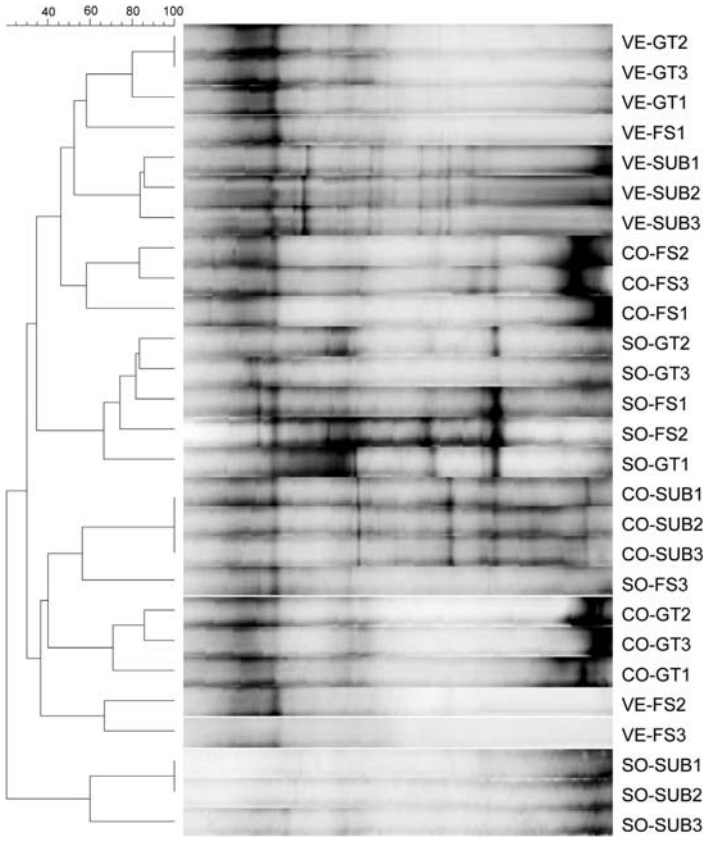

Fig. 3. Dendrogram of bacterial DGGE fingerprints based on 16S rDNA extracted from substrates (SUB), gut content (GT) and faeces (FS) of forest (SO), compost (CO), and vermiculture (VE) earthworm populations. Profiles were clustered by Dice correlation and an unweighted pair-group method using arithmetic averages (UPGMA).

SUB in all three earthworm populations (Fig. 2). This analysis of fingerprints also depicted the lowest similarity among substrate, gut, and faeces primarily in *E. fetida* inhabiting forest soil. PCA of DGGE patterns extracted two PC axes explaining 35.0% of the total variability (Fig. A.2a). One-way ANOVA of PC1 sample scores confirmed significant differences ($P < 0.001$) among SUB, GT, and FS archaeal DGGE profiles of all *Eisenia* populations.

For bacterial banding patterns, passage showed a lower influence on the bacterial community structure (Fig. 3). Bacterial community of soil (SO-SUB) was significantly different from that in GT and FS samples. Compost and vermiculture substrate communities were more similar to those of GT and FS samples than those in the archaeal fingerprint. Principal component analysis of DGGE patterns extracted two PC axes explaining 36.0% of the total variability (Fig. A.2b). No evident separation of the samples was created along the first two PC axes. One-way ANOVA of PC1 sample scores revealed differences among SUB, GT, and FS bacterial DGGE banding patterns of the soil population ($P < 0.001$). A significant effect of passage on the bacterial community was also found for the compost population ($P < 0.01$), although banding patterns were only different in FS samples. Gut passage through the vermiculture population had no significant impact on the bacterial DGGE fingerprint. The effect of passage on bacterial composition decreased along the gradient in the direction from nutrient-poor forest soil to the richest vermiculture habitat.

Table 4
Colony forming units (CFU) of cultivable bacteria in substrates (SUB), gut contents (GT), and faeces (FS) of three different *Eisenia* populations: soil (SO), compost (CO) and vermiculture (VE); (mean ± standard error; n = 6).

	Counts of cultivable bacteria [CFU g^{-1} dw]		
	SO	CO	VE
SUB	$5.6 \pm 1.2 \times 10^{6}$ aA	$2.7 \pm 0.8 \times 10^{10}$ aB	$5.8 \pm 0.8 \times 10^{9}$ aB
GT	$2.8 \pm 0.1 \times 10^{7}$ bA	$5.2 \pm 0.6 \times 10^{9}$ aB	$1.0 \pm 0.3 \times 10^{10}$ abB
FS	$1.7 \pm 0.1 \times 10^{7}$ abA	$3.7 \pm 0.5 \times 10^{9}$ aB	$1.2 \pm 0.1 \times 10^{10}$ bC

Means followed by variable lowercase letters in a column are significantly different at $P \leq 0.05$; variable uppercase letters in a row show significantly different CFU mean values within earthworms. Differences between means were evaluated by Kruskal–Wallis test followed by multiple comparisons of mean ranks for all groups.

3.3. Culturable bacteria-counts, growth strategy, and species richness

The average counts of culturable bacteria were two to three orders higher in the GT and FS of compost and vermiculture earthworms compared to the GT and FS of soil earthworms and three to four orders higher in compost and vermiculture substrates

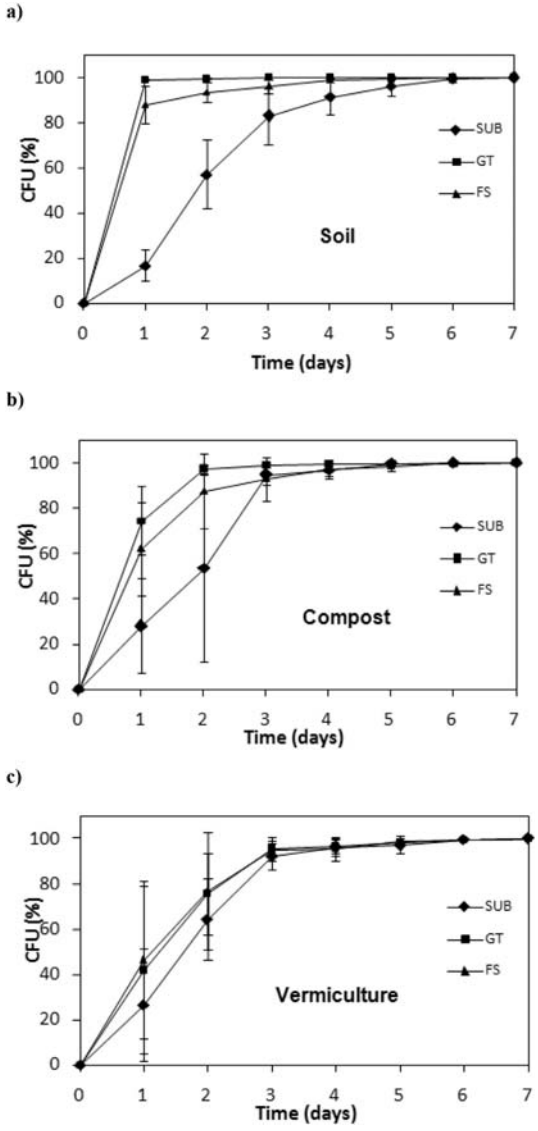

Fig. 4. Growth strategy of culturable bacteria isolated from substrates (SUB), gut contents (GT) and faeces (FS) of soil (a), compost (b), and vermiculture (c) earthworm populations.

compared to soil substrate. Earthworm passage significantly increased the counts of culturable bacteria in the GT of the soil earthworm population and in the FS of the vermiculture population, no differences were found after compost passage (Table 4). Soil earthworm also accelerated the bacterial growth rate in passed material (Fig. 4a) in contrast to compost and vermiculture earthworms (Fig. 4b; Fig. 4c).

The richness of culturable bacterial species decreased during passage in all earthworm populations (Fig. 5, Table A.3, Table A.4). Bacterial species identification revealed the following differences

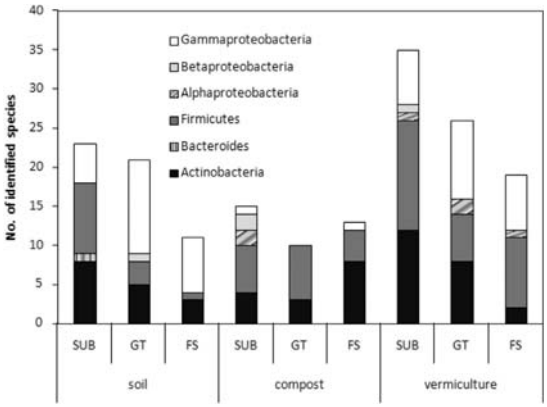

Fig. 5. Species richness of culturable bacteria and distribution in taxonomic groups.

between samples derived from the three earthworm populations: whereas soil compost and vermiculture substrates favoured by Actinobacteria (e.g., *Arthrobacter globiformis*, *Microbacterium* sp., *Nesterenkonia halobia*) and Firmicutes (e.g., *Bacillus* sp., *Paenibacillus* sp.), in the gut content and faeces of soil earthworms dominated Gammaproteobacteria (e.g., *Aeromonas* sp., *Pseudomonas* sp., *Salmonella typhimurium*). Gut content of compost population inhabited almost Firmicutes (e.g., *Bacillus* sp., *Paenibacillus* sp.) and in faeces dominated Actinobacteria (e.g., *Microbacterium* sp., *Micrococcus luteus*, *Oerskovia turbata*, *Rhodococcus rhodochrous*). Whereas, Gammaproteobacteria dominated in gut content of vermiculture earthworm, Firmicutes increased in faeces.

4. Discussion

4.1. Different feeding habitats

Various feeding habitats resulted in different passage effects of indigenous soil earthworm and related compost and vermiculture earthworms. Earthworm gut passage had a higher impact on the microbial community of forest soil than on that of compost and vermiculture. The difference in chemical and biochemical parameters, especially in the organic C, water soluble carbohydrates and soil organic matter appears as an important factor in various earthworm responses in different habitats [66]. Distinct NEL-PLFA concentrations in faeces of three earthworm populations could be explained by different antimicrobial defense mechanisms among earthworm species from various habitats [16]. Compost and vermiculture earthworms are evolutionary adapted to substantially higher quantity of microbes and their PLFA profile was well balanced between gut and faeces. In the other hand, the intestine of soil *E. fetida* selected and activated anaerobes that predominated in faeces.

The lower numbers of cultivable bacteria (CFU) in soil population corresponded to the nutrient demands in forest soil habitat. High amount of organic carbon in compost and vermiculture forms these substrates more suitable for growth of microorganisms. Our estimates of CFU in substrates were in agreement with those of previous measurement of Dvořák et al. [16] where culturable bacterial numbers in soil and compost inhabited by Eisenia worms reached 10^5 and 10^8 CFU g^{-1} dry matter, respectively. According to previous studies, the counts of bacteria isolated from the gut in compost earthworm *E. fetida* ranged from 10^6 to 10^9 CFU g^{-1} dry matter [14,67,68], whereas the counts of culturable bacteria in the

gut contents of other earthworm species from different ecological groups (anecic *Lumbricus terrestris*, epigeic *L. rubellus*, and endogeic *Aporrectodea caliginosa* and *Octolasion lacteum*), reached only 10^5–10^6 CFU g^{-1} dry matter [9,10]. The acceleration of the bacterial growth rate derived from gut and faeces of *E. fetida* correlates with the relatively low CFU observed in forest soil. It is clear that the nutrient-rich vermiculture substrate contained a large community of fast-growing bacteria, a strategy that was preferred after entering into an intestinal environment (Fig. 4). The richer the habitat in nutrients and organic carbon was, the lower the influence of earthworms to stimulate intestinal bacterial growth.

4.2. Passage effects

Previous studies showed that earthworm gut increases or does not change the abundance of microbial biomass [6–8]. Our results demonstrate that passage increased total microbial biomass as indicated by a one-to two-fold increase in the EL-PLFA molar concentration in the gut and faeces of three *Eisenia* populations. In accordance with our study, Sampedro and Whalen [7] found about 300 times greater total PLFA concentration in the gut of *L. terrestris* than in bulk soil. Similarly, they found significant changes in microbial-derived PLFA profiles of soil and gut and described that the passage significantly increased the concentration of biomarkers indicative for aerobic bacteria, microeukaryotes and fungi. The passed material through the earthworm gut may differ between species and this difference might result in various increments of gut microbial biomass. For instance, some species such anecic *L. terrestris* feeds mainly upon intact organic wastes for nutrition, whereas other species such epigeic *E. fetida* prefer organic matter in an advanced stage of decomposition. Endogeic species e. g. *Allolobofora caliginosa* even extract the nutrients from finely fragmented organic matter mixed with soil [69]. Moreover, we showed the significant increase of NEL-PLFA concentration in earthworm gut and faeces. The increased concentration of the NEL-UNSFA group in earthworm gut and faeces is mainly attributable to the presence of anaerobes [52]. Compared to soil, the intestinal tract is a microenvironment with a higher carbon, nitrogen and water content and distinct oxygen deficiency [70]. Many anaerobic or facultatively anaerobic bacteria (e.g., *Clostridium, Aeromonas*, *Bacillus*, *Shewanella, Propionibacterium, Staphylococcus, Paenibacillus* and *Photobacterium*) as well as archaea, are abundant in the guts of earthworms [13,14,71–73]. The conditions of the gut are suitable for the activation of dormant or inactive microbial forms that might be present in soil [70]. In this study, we obtained these results by culturable bacteria counts and identification. We revealed that the gut environment, mainly of compost earthworms, activated spore-forming bacteria (*Bacillus licheniformis, Bacillus megaterium*, *Bacillus mycoides, Bacillus pumilus, Bacillus subtilis, Paneibacillus macerans,* etc.). The same findings were described previously by Fischer et al. [74], who demonstrated that passage by *L. terrestris* enhanced the germination of *B. megaterium* spores. It is evident for soil and vermiculture earthworm populations that the richness of the Gammaproteobacteria increased in the gut contents. The Gram-negative facultative anaerobic bacteria, *Aeromonas*, together with *Pseudomonas*, which are able to grow in anaerobic conditions, have been previously identified by culture-dependent and independent approaches as typical genera in the intestine of *E. fetida* [12,13]. So called 'sleeping beauty paradox', the contradiction between the short generation time of microorganisms and their slow turnover [75], underlines the fact that earthworm gut passage might stimulate dormant microbiota.

Although culturable bacterial phyla differed in vermiculture gut contents and faeces, the PCR-DGGE analysis did not confirmed the differences in bacterial molecular fingerprints. Furlong et al. [19] revealed in *L. rubellus*, that soil and cast bacterial isolates were different but represented the same phylogenetic groups. It should be taken into account that facultative anaerobes were found to be as abundant in an anaerobic gut environment and these were not identified by cultivation [71], thus the differences between culturable aerobic heterotrophic bacterial communities were not obvious in the resulting bacterial molecular profiles. Bacteria were distinguished by a higher diversity compared to archaea, and the differences that we observed might correspond to the changes in dominant representatives, therefore, the total bacterial community might have reacted less sensitively to environmental changes than archaea, as was reported in previous study by Koubová et al. [63]. The archaeal DGGE patterns were strictly divergent in different stages of passage. Furlong et al. [19] found that RFLP patterns analysed in *L. rubellus* were different between soil and casts archaeal clones. The differences in archaeal profiles might be influenced by typical soil and compost methanogenic and non-methanogenic Euryarchaeota as well as NH_4-oxidising Thaumarchaeota [76]. Nevertheless, many uncultured representatives of these two groups exist, whose functional properties are unknown. Koubová et al. [42] showed an earthworm-mediated decrease in *Methanosarcina* sp. abundance and methanogenic diversity changes in soil altered by *E. andrei*; nevertheless, the quantity of total methanogens was stable. A recent study by Depkat-Jakob et al. [18] demonstrated that methanogenic taxa *Methanosarcinaceae*, *Methanobacteriaceae*, and *Methanomicrobiaceae* might be associated with the emission of methane by *Eudrilus eugeniae*. Methanogenic and ammonium-oxidizing archaea might be the dominant archaeal groups attending composting processes [77], however, the participation of individual archaeal taxa on earthworm ingestion processes remains unresolved. Microbial species selection and reduction during earthworm gut passage could play a key role in biological soil amendments like bioturbation and vermicomposting.

5. Conclusions

In summary, we have shown that the passage of consumed material through the gut of *Eisenia* spp. increased total viable microbial biomass, changed the microbial structure and reduced the richness of the microbial communities in gut and faeces. We have revealed that the influence of *Eisenia* spp. on associate microorganisms depends on the earthworm feeding habitat. Passage affected the intestinal microbiome of indigenous soil earthworm in more aspects than related compost and vermiculture populations: the results indicated greater changes in archaeal community structure, higher abundance of anaerobes in passed forest soil. The richer the habitat in nutrients and organic carbon was, the lower the influence of earthworm gut passage on bacterial growth rate has been found. This work brought original reveals about archaeal community and total anaerobes accompanying intestinal tract of *Eisenia* spp. Further molecular screening of the microbial community might help the understanding of the microbial functions associated with *Eisenia* spp. earthworms.

Acknowledgements

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Appendices

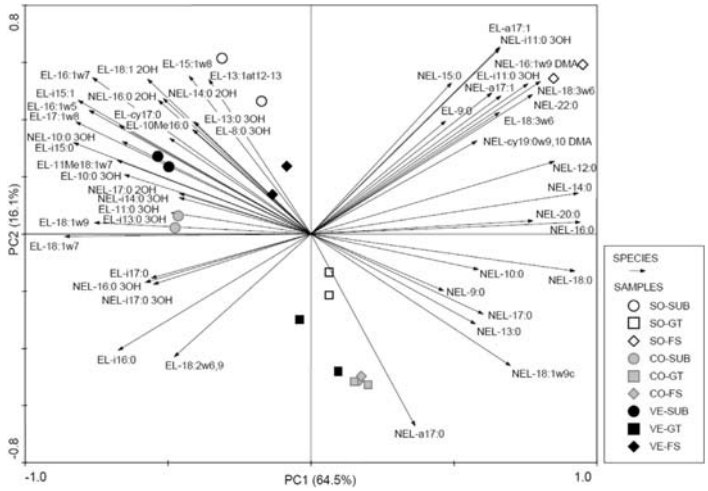

Fig. A.1. Ordination plot of sample distribution after principal component analysis of the complex PLFA profile. Only PLFAs that fitted with PC1 and PC2 by more than 40% are projected in the figure. The symbols represent: soil (SO), compost (CO), vermiculture (VE), substrate (SUB), earthworm gut contents (GT) and earthworm faeces (FS). The arrows demonstrate EL- and NEL-PLFAs.

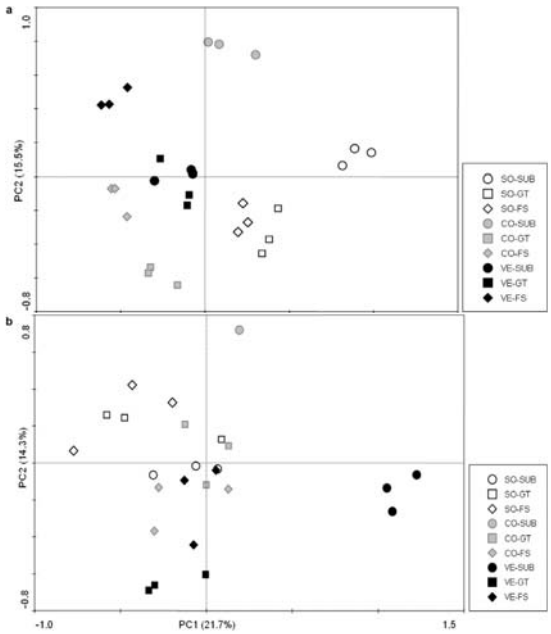

Fig. A.2. Principal component analysis of DGGE banding patterns for archaea (a), and bacteria (b). The symbols represent: soil (SO), compost (CO), vermiculture (VE), substrate (SUB), earthworm gut contents (GT), and earthworm faeces (FS). Values on the axes indicate the percentage variability in the DGGE banding patterns.

Table A.1
PLFA microbial functional groups [nmol PLFA g^{-1} dw] and substrate availability to microbes evaluated in substrates (SUB), gut contents (GT), and faeces (FS) of three different *Eisenia* populations: soil (SO), compost (CO) and vermiculture (VE); (mean ± standard error; n = 6). Specific PLFA biomarkers were used to estimate the aerobic bacteria (i15:0; a15:0; 15:0; 16:1ω7c; i17:0; cy17:0; cy19:0), actinomycetes (10Me18:0; 10Me17:0; 10Me16:0), fungi (18:2ω6; 18:1ω9), and aerobic microeukaryotes (20:4ω6; 20:2ω6; 18:3ω6; 18:2ω6). The substrate availability to microbes was estimated as the ratio EL-MUFA/EL-STFA.

Microbial parameters	Samples analysed								
	SO			CO			VE		
	SUB	GT	FS	SUB	GT	FS	SUB	GT	FS
Aerobic[a] bacteria	12.7 ± 5.3 a	226.4 ± 82.2 a	149.6 ± 100.2 a	25.0 ± 16.1 a	296.3 ± 86.5 b	69.1 ± 38.7 a	25.0 ± 8.8 a	460.2 ± 92.1a	226.0 ± 24.5 a
Actinomycetes[b]	1.1 ± 0.3a	23.2 ± 20.5 a	54.8 ± 38.0 a	4.1 ± 2.6 a	24.5 ± 15.6 a	14.9 ± 9.7 a	0.8 ± 0.3 a	0.0 ± 0.0 a	0.3 ± 0.3 a
Aerobic[c] microeukaryotes	2.3 ± 1.2 a	180.3 ± 91.5 a	252.8 ± 127.1 a	4.7 ± 2.8 a	181.3 ± 120.8 a	43.9 ± 27.9 a	7.1 ± 3.5 a	614.5 ± 162.3 b	93.1 ± 19.3 a
Fungi[a]	3.0 ± 1.6 a	181.5 ± 58.8 b	30.1 ± 22.9 a	10.6 ± 0.9 a	157.1 ± 34.4 b	85.1 ± 5.8 ab	4.1 ± 2.0 a	232.3 ± 61.7 b	44.8 ± 5.3 a
Substrate[d] availability	0.8 ± 0.3 a	0.4 ± 0.2 a	0.7 ± 0.2 a	0.6 ± 0.2 a	0.3 ± 0.2 a	0.5 ± 0.3 a	1.1 ± 0.2 a	0.5 ± 0.0 b	0.7 ± 0.2 ab

Means in a row followed by variable lowercase letters are significantly different at $P \leq 0.05$ within samples in a single earthworm population. Differences within samples in consequence of the gut passage in a single earthworm population were evaluated by one-way ANOVA followed by Tukey's post hoc test.

[a] Frostegård and Bååth (1996) [78].
[b] Kroppenstedt (1985) [50].
[c] Erwin (1973) [79].
[d] Bossio and Scow (1998) [80].

Table A.2
Abundance PLFA functional groups [nmol PLFA g^{-1} dw] in the PLFA profile evaluated in substrates (SUB), gut contents (GT), and faeces (FS) of three different *Eisenia* populations: soil (SO), compost (CO) and vermiculture (VE); (mean ± standard error; n = 2). Measurements were obtained from detailed extended PLFA analysis from observations over one year.

PLFA functional groups	Samples analysed								
	SO			CO			VE		
	SUB	GT	FS	SUB	GT	FS	SUB	GT	FS
EL-STFA	2.9 ± 0.3 a	193.5 ± 46.3 b	87.0 ± 16.6 ab	37.1 ± 2.5 a	350.0 ± 26.8 b	180.3 ± 0.8 c	13.7 ± 1.2 a	198.5 ± 42.0 a	119.4 ± 36.0 a
EL-MUFA	7.2 ± 1.5 a	272.4 ± 15.7 b	26.9 ± 5.7 a	78.4 ± 3.5 a	521.7 ± 157.5 a	386.8 ± 57.5 a	39.0 ± 2.5 a	246.7 ± 94.2 a	234.3 ± 42.1 a
EL-PUFA	0.9 ± 0.2 a	331.2 ± 8.8 b	75.8 ± 37.8 a	13.9 ± 1.7 a	544.1 ± 146.4 b	131.8 ± 10.6 ab	12.9 ± 1.8 a	193.6 ± 63.7 a	116.7 ± 36.5 a
EL-t-Br-FA	3.6 ± 0.5 a	257.8 ± 41.2 b	92.8 ± 10.5 a	47.4 ± 2.5 a	350.6 ± 69.4 b	129.8 ± 14.3 a	22.1 ± 1.5 a	230.9 ± 69.5 b	104.3 ± 4.6 a
EL-m-Br-FA	0.7 ± 0.1 a	8.4 ± 6.4 a	2.5 ± 2.5 a	12.4 ± 0.3 a	73.5 ± 5.4 b	44.6 ± 9.0 ab	0.5 ± 0.0 a	0.0 ± 0.0 a	0.7 ± 0.8 a
EL-cy-FA	0.7 ± 0.1 a	6.0 ± 4.3 a	0.0 ± 0.0 a	16.7 ± 0.6 a	17.5 ± 3.0 a	11.4 ± 1.7 a	1.8 ± 0.2 a	2.5 ± 0.2 a	3.4 ± 2.1 a
EL-OH-FA	0.1 ± 0.0 a	5.3 ± 0.8 b	0.7 ± 0.7 a	1.6 ± 0.3 a	10.9 ± 3.1 ab	15.1 ± 1.2 b	0.1 ± 0.1 a	50.3 ± 18.2 a	25.8 ± 9.5 a
EL-Br-OH-FA	0.0 ± 0.0 a	1.7 ± 0.2 b	1.6 ± 0.4 b	0.7 ± 0.0 a	28.4 ± 0.0 b	12.7 ± 1.5 c	0.3 ± 0.0 a	23.6 ± 2.4 b	11.2 ± 0.3 c
EL-$PLFA_{tot}$	**16.2 ± 2.9 a**	**1076.3 ± 124.8 b**	**287.4 ± 61.6 a**	**210.6 ± 11.3 a**	**1901.1 ± 419.3 b**	**917.6 ± 96.2 ab**	**91.0 ± 7.2 a**	**946.0 ± 163.6 b**	**616.7 ± 128.1 ab**
NEL-UNSFA	4.6 ± 0.2 a	499.4 ± 64.4 ab	736.6 ± 140.7 b	26.1 ± 0.5 a	1945.2 ± 412.3 b	933.2 ± 137.0 ab	11.0 ± 2.5 a	471.1 ± 69.8 b	268.6 ± 77.6 ab
NEL-OH-FA	0.8 ± 0.0 a	6.1 ± 1.5 b	4.0 ± 0.2 ab	7.9 ± 0.2 a	213.8 ± 100.3 a	76.0 ± 13.7 a	3.6 ± 0.7 a	61.0 ± 26.6 a	5.8 ± 0.2 a
DMA	0.2 ± 0.0 a	38.5 ± 3.0 b	50.9 ± 27.1 b	0.0 ± 0.0 a	0.0 ± 0.0 a	0.0 ± 0.0 a	2.0 ± 0.5 a	14.0 ± 5.2 a	6.5 ± 6.5 a
NEL-$PLFA_{tot}$	**5.6 ± 0.2 a**	**543.9 ± 65.8 ab**	**791.5 ± 167.9 b**	**34.0 ± 0.7 a**	**2159.0 ± 512.6 b**	**1009.2 ± 150.8 ab**	**16.6 ± 2.7 a**	**546.1 ± 101.6 b**	**280.9 ± 83.9 ab**
$PLFA_{tot}$	**21.8 ± 2.7 a**	**1620.2 ± 190.6 b**	**1078.9 ± 106.4 ab**	**244.6 ± 10.6 a**	**4060.0 ± 931.9 b**	**1926.7 ± 246.9 ab**	**107.6 ± 4.6 a**	**1492.1 ± 265.2 b**	**897.6 ± 212.0 ab**

Means in a row followed by variable lowercase letters are significantly different at $P \leq 0.05$ within samples in consequence of the gut passage in a single earthworm population. Differences between means were evaluated by one-way ANOVA followed by Tukey's post hoc test.

Table A.3
Cultivable species of Actinobacteria and Firmicutes isolated from substrates (SUB), gut contents (GT), and faeces (FS) in three different *Eisenia* populations: soil (SO), compost (CO) and vermiculture (VE).

Phyla and species	Soil			Compost			Vermiculture		
	SUB	GT	FS	SUB	GT	FS	SUB	GT	FS
Actinobacteria									
Arthrobacter aurescens								+	
Arthrobacter globiformis	+				+		+		
Arthrobacter oxydans			+						
Brevibacterium lyticum									+
Cellulomonas fimi	+								
Cellulomonas flavigena				+					
Cellulomonas gelida							+		
Cellulosimicrobium cellulans							+	+	+
Corynebacterium xerosis						+			
Curtobacterium flaccumfaciens				+	+	+			
Dactylosporangium fulvum	+								
Kocuria rhizophila								+	
Kocuria rosea		+	+	+					
Kocuria varians			+						
Lechevalieria flava				+			+		
Microbacterium barkeri		+						+	
Microbacterium esteraromaticum	+								
Microbacterium flavescens						+			
Microbacterium chocolatum							+	+	
Microbacterium lacticum							+		
Microbacterium laevaniformans						+			
Microbacterium liquefaciens								+	
Microbacterium trichothecenolyticum							+		
Microbispora diastatica							+		
Micrococcus luteus						+	+		
Micrococcus lylae								+	
Nesterenkonia halobia	+						+	+	
Nocardiopsis dassonvillei dassonvillei	+								
Oerskovia turbata	+					+			
Rhodococcus erythropolis		+					+		
Rhodococcus fascians		+							
Rhodococcus rhodochrous		+				+			
Streptomyces biverticillatus							+		
Streptoverticilium reticulum	+				+				
Species richness	8	5	3	4	3	7	12	8	2
Firmicutes									
Aneurinibacillus migulanus				+			+		
Bacillus atrophaeus				+			+		+
Bacillus cereus	+	+	+					+	
Bacillus clausii				+			+		
Bacillus coagulans							+		
Bacillus lentus							+		
Bacillus licheniformis					+	+	+	+	+
Bacillus megaterium		+			+		+		
Bacillus mycoides	+	+			+				
Bacillus pumilus	+			+	+	+	+		
Bacillus sphaericus	+						+		+
Bacillus subtilis				+	+	+	+		
Bacillus thuringiensis kurstakii									+
Bacillus viscosus	+						+		
Brevibacillus reuszeri								+	+
Carnobacterium piscicola	+								
Listeria innocua							+		
Paenibacillus lautus							+		
Paenibacillus macerans	+				+			+	+
Paenibacillus polymyxa	+			+		+	+	+	+
Staphylococcus cohnii cohnii									+
Staphylococcus sciuri					+				
Staphylococcus simulans									+
Virgibacillus pantothenticus	+								
Species richness	9	3	1	6	7	4	14	5	9

Table A.4
Cultivable species of Proteobacteria and Bacteroides isolated from substrates (SUB), Cultivable species of Proteobacteria and Bacteroides isolated from substrates (SUB), gut contents (GT), and faeces (FS) in three different Eisenia populations: soil (SO), compost (CO) and vermiculture (VE).

Phyla and species	Soil			Compost			Vermiculture		
	SUB	GT	FS	SUB	GT	FS	SUB	GT	FS
α-Proteobacteria									
Brevundimonas diminuta				+					
Brevundimonas vesicularis								+	
Hyphomonas hirschiana								+	
Rhizobium radiobacter							+		+
Sphingomonas paucimobilis				+					
Species richness	0	0	0	2	0	0	1	2	1
β-Proteobacteria									
Aquaspirillum autotrophicum		+							
Delftia acidovorans				+					
Duganella zoogloeiodes							+		
Hydrogenophaga pseudoflava				+					
Species richness	0	1	0	2	0	0	1	0	0
γ-Proteobacteria									
Aeromonas ichthiosmia/hydrophila		+	+					+	+
Aeromonas jandaei			+						
Aeromonas salmonicida achromogenes		+					+		
Aeromonas salmonicida-masoucida	+	+	+						
Aeromonas veronii		+							+
Enterobacter intermedius			+					+	
Chryseomonas luteola								+	
Lysobacter antibioticus				+			+	+	+
Lysobacter sp.						+			
Pantoea agglomerans (Enterobacter)								+	
Photobacterium angustum	+								
Pseudomonas agarici	+								
Pseudomonas alcaligenes							+		
Pseudomonas fluorescens/mandelii	+	+	+						
Pseudomonas fluorescens/taetrolens		+							
Pseudomonas chlororaphis	+								
Pseudomonas putida		+							
Pseudomonas putida/vancouverensis		+					+		
Pseudomonas syringae-syringae		+							
Pseudomonas syringae-tabaci		+							
Pseudomonas syringae-tomato		+							
Pseudoxanthomonas broegbernensis							+	+	
Pseudoxanthomonas sp.								+	
Raoultella terrigena									+
Salmonella typhimurium		+						+	+
Serratia plymuthica								+	
Shewanella putrefaciens									+
Shewanella putrefaciens/algae							+		+
Stenotrophomonas acidaminiphila							+	+	
Vibrio fischeri			+						
Yersinia aldovae			+						
Species richness	5	12	7	1	0	1	7	10	7
Bacteroides									
Chryseobacterium balustinum	+								
Species richness	1	0	0	0	0	0	0	0	0

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Paper II

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The effect of passage through the gut of earthworms (Annelida: Lumbricidae) on the diversity of archaea and bacteria

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Abstract. The changes that occurred in the diversity of microbes in soil and compost during its passage through the gut of earthworms were evaluated in two epigeic earthworm species *Eisenia fetida* (Savigny, 1826) and *E. andrei* (Bouché, 1972), which inhabit forest soil and garden organic compost, respectively. A modified extraction protocol was used to extract DNA from forest soil, compost and fresh earthworm faeces. DNA was amplified in a polymerase chain reaction (PCR). The products were analyzed using denaturing gradient gel electrophoresis (DGGE) fingerprinting. Analysis of archaeal and bacterial DGGE fingerprints revealed that the diversity in both microbial domains increased after passage through the guts of *Eisenia andrei*, but not *E. fetida*. Nevertheless, cluster and correspondence analyses revealed that the passage of soil and compost through the guts of *Eisenia fetida* and *E. andrei*, respectively, had a significant effect on the composition of the archaeal community. Shifts in the composition of the compost bacterial community were more apparent after passage through the guts *Eisenia andrei* than of *E. fetida*. This study is the first on the changes in the composition of the archaeal community in substrates that occur during their passage through the guts of two species of *Eisenia*.

Key words. Soil zoology, soil microbiology, microbial diversity, Archaea, Bacteria, *Eisenia fetida*, *Eisenia andrei*, Lumbricidae, Annelida, gut passage, PCR-DGGE, molecular fingerprinting.

INTRODUCTION

Passage of soil through the guts of earthworms has positive or negative effects on biomass and activity of microorganisms depending on the species of earthworm, type of microbe and soil conditions (Clapperton et al. 2001, McLean et al. 2006, Marhan et al. 2007, Aira et al. 2008). During passage of soil through the gut of earthworms the microbial diversity is modified (Egert et al. 2004, Byzov et al. 2007, Vivas et al. 2009). Ingested microorganisms may survive or are even activated by passage through the gut of earthworms (Hendriksen 1990). On the other hand many species are sensitive to specific gut conditions and as a consequence do not survive the digestion process (Khomyakov et al. 2007).

Epigeic earthworms of the genus *Eisenia* are often used in vermicomposting (Adi & Noor 2009, Vivas et al. 2009), bioremediation of contaminated soils (Hickman & Reid 2008, Contreras–Ramos et al. 2009) and as model organisms in immunology (Bilej et al. 2001, Procházková et al. 2006). There are few studies on the diversity of microflora associated with *Eisenia* earthworms. Microbial community fingerprinting, based on 16S rRNA, is one of the most popular methods. It was recently used to reveal differences in microfloral diversity during passage through the guts of the

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anecic earthworm *Lumbricus terrestris* (Egert et al. 2004) and epigeic *L. rubellus* (Singleton et al. 2003, Knapp et al. 2008). Vivas et al. (2009) used polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) to show that the bacterial structural and functional diversity in vermicompost produced by *E. fetida* is greater than in ordinary compost. The optimized DGGE method (Muyzer et al. 1993) was used to determine the diversity of microbes in both soil and compost after it has passed through the guts of two species of *Eisenia*. The main aim of this study was to determine the bacterial and archaeal diversity in the material ingested *E. fetida* and *E. andrei* before and after it passes through the intestinal tracts of worms inhabiting different environments and feeding on different substrates. The objective is to enhance the understanding of the interactions between earthworms and microorganisms, in particular its functional significance.

MATERIAL AND METHODS

Sample collection

Specimens of *Eisenia fetida* and *E. andrei* (Annelida: Lumbricidae) were collected in a mixed forest from moist brook sediment rich in leaf litter, at Hluboká nad Vltavou, Czech Republic, and from garden compost, in České Budějovice, Czech Republic, respectively. Three samples of forest soil (pH_{CaCl2}=5.7±0.1; C_{ox}=2.14±0.43%; N_{tot}=0.22±0.04%) and compost (pH_{CaCl2}=7.4±0.1; C_{ox}=34.5±6.90%; N_{tot}=2.55±0.51%) were randomly collected from the same habitats from which the earthworms were collected. The microorganisms inhabiting soil (SO) and compost (CO) were compared with those present in the faeces (FS) of *E. fetida* (EF) and *E. andrei* (EA), respectively. Surface sterilized living worms were placed in sterile Petri dishes containing damp filter paper and three samples of freshly produced faeces were collected after 16 h. Each sample consisted of the faeces of 12–15 worms. DNA was extracted and subjected to PCR followed by DGGE as described by Knapp et al. (2009) with the modifications detailed below.

DNA extraction

Total DNA was extracted from about 0.1 g of earthworm faeces, 0.3 g forest soil and 0.3 g compost using the PowerSoil DNA Isolation Kit (MoBio Laboratories Inc., USA) according to the manufacturer's protocol. To improve DNA yield but reduce shearing of large fragments the following modifications were used: after an abbreviated bead beating step (5 min), samples were frozen at –80 °C for 1h and thawed in a water bath at 37 °C for 30 min. This freezing-thawing step was repeated once, before continuing with the regular protocol. DNA yield and quality were assessed by 1% (w/v) agarose gel electrophoresis and the concentration of DNA extracts was measured using PicoGreen dsDNA quantitation reagent (Invitrogen, USA) (Juen & Traugott 2005).

PCR amplification of target DNA

Extracted DNA was amplified in a PCR thermocycler (PCR Express, ThermoHybaid) with different primer sets. Each PCR mixture contained 0.5 ng of extracted DNA, 0.2 µM of each primer, 0.625 U BioTherm TM DNA Polymerase (Gene Craft, Germany), 1x DNA polymerase buffer, 2.5 µg Bovine serum albumin (BSA), 0.2 mM dNTP-Mix and 2.5 mM $MgCl_2$ in a final volume of 25 µL.

To amplify total bacterial communities the universal 16S rRNA primer set 63f (CAG GCC TAA CAC ATG CAA GTC) (Marchesi et al. 1998) and 1378r (CGG TGT GTA CAA GGC CCG GGA ACG) (Heuer et al. 1997) were used in the first step. The PCR included an initial 3 min denaturation at 94 °C followed by 30 thermal cycles of 1 min at 94 °C, 1 min at 62 °C and 2 min at 72 °C. Amplification was completed with a final extension step at 72 °C for 10 min. PCR products of the first amplification were used as a template for a second PCR performed using primer pair 984fGC (CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG GAA CGC GAA GAA CCT TAC) (Heuer et al. 1997) and 1378r. The PCR program for these primers differed in an additional annealing step of 30 sec at 56 °C.

Members of the domain archaea were amplified using 16S rRNA primers A934f (AGG AAT TGG CGG GGG AGC A) and 1390rGC (CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GCG GTG TGT GCA A) (Wu et al. 2001). The PCR started with an initial denaturation step at 94 °C for 5 min, followed by 35 thermal cycles of 30 sec at 94 °C, 1 min at 53 °C, and 1 min at 72 °C and a final extension step at 72 °C for 15 min.

After amplification the PCR products were checked by electrophoresis on 1% (w/v) agarose gels stained with ethidium bromide (10 mg ml^{-1}) and quantified using PicoGreen quantitation.

DGGE analysis

Denaturing gradient gel electrophoresis was performed using the Ingeny PhorU2 system (Ingeny International BV, The Netherlands). For bacterial and archaeal DGGE, 2 µL of PCR products (corresponding to 43–160 ng DNA) was loaded on to 8% (w/v) polyacrylamide gels with a denaturing gradient of 40% to 70% (100% denaturant according to 7M urea plus 40% formamide in 1X TAE-buffer) and was run for 16 h at 100 V and a constant temperature of 60 °C in 1x TAE-buffer

Table 1. Numbers of bands per lane of the archaeal and bacterial DGGE profiles of compost (CO), soil (SO) and faeces (FS) of *Eisenia andrei* (EA) and *E. fetida* (EF) (mean±SD; n=3). Values in the columns with differing superscripts are significantly different in the two species of earthworm (Student's t-test for dependent samples; P≤0.05)

	number of bands	
	archaeal profile	bacterial profile
SO	11.0 ± 0.0[a]	10.3 ± 1.5[a]
EF-FS	12.0 ± 0.0[a]	8.7 ± 0.6[a]
CO	14.0 ± 4.6[a]	13.7 ± 1.5[a]
EA-FS	25.0 ± 1.0[b]	20.7 ± 0.6[b]

(pH 7.4). After electrophoresis, the gels were stained with silver nitrate (Sanguinetti et al. 1994) using an automated gel stainer (Amersham Pharmacia Biotech, Germany), photographed and air dried for storage. DGGE banding patterns were normalized and analysed using the GelCompar II software package, version 4.0 (Applied Maths, Ghent, Belgium). Cluster analysis using the algorithm of Ward (Legendre & Legendre 1998) was performed to infer similarities among the different banding patterns and expressed in percentages. Differences in the numbers of banding patterns in the archaeal and bacterial profiles before and after passage through the guts of worms were tested using Student's t-test for dependent samples with a significance level of P≤0.05 (STATISTICA 9). Ordination plots were created using correspondence analysis (Canoco for Windows, software package, version 4.5, Centre for Biometry Wageningen, the Netherlands). Statistically significant differences (P≤0.05) among sample scores were tested using one-way ANOVA and Tukey's test (STATISTICA 9).

RESULTS

The DGGE fingerprints of bacterial and archaeal 16S rRNA amplified fragments in earthworm faeces and substrates revealed complex banding patterns. Inter-sample distances in correspondence analysis plots indicate that the samples are not similar (Fig. 1). Fingerprints showed differences in numbers of bands in the forest and compost microbial DGGE profiles (Table 1). The number of archaeal bands increased significantly after passage through the gut of *E. andrei*. Similarly, the number of bands in the bacterial fingerprints increased after passage through the gut of *E.*

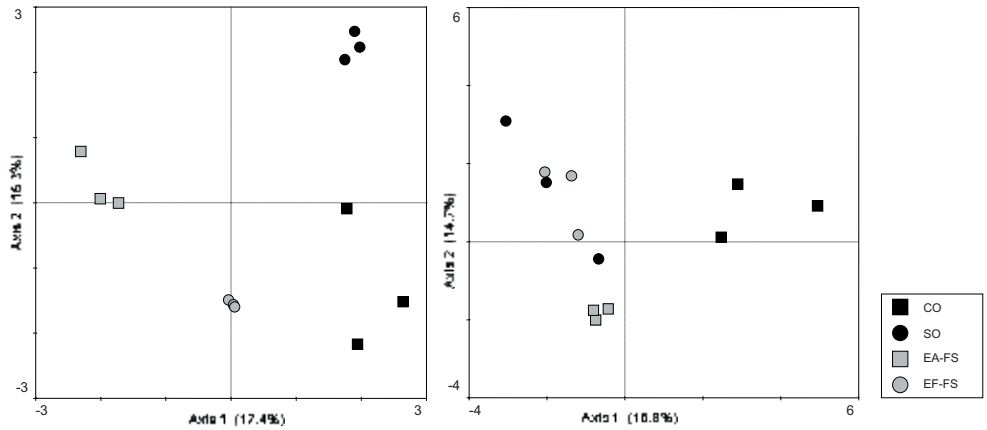

Fig. 1. Correspondence analysis of DGGE patterns for compost (CO), soil (SO) and faeces (FS) of *Eisenia andrei* (EA) and *E. fetida* (EF): left – Archaea; right – Bacteria. Values in the borders indicate the percentage variability in the DGGE banding patterns.

andrei. In contrast, the numbers of archaeal and bacterial bands in the fingerprints of SO and FS of *E. fetida* did not differ.

The cluster analysis (dendrogram not shown) separated the archaeal community in FS of both earthworm species from the original substrate communities. The similarity of the archaeal fingerprints of FS of both earthworms was only 41%. Correspondence analysis of DGGE patterns showed that the archaeal community differed in all the samples other than SO and CO (Fig. 1).

Compost substrate-based bacterial community was completely different from that in *E. andrei* FS with a <30% similarity (dendrogram not shown). Correspondence analysis indicates that these differences are statistically significant (Fig. 1). Bacterial communities in SO and *E. fetida* FS were similar (>56% similarity). Bacterial fingerprint of *E. andrei* FS was even more similar to the SO than to the CO bacterial profile. Correspondence analysis confirmed the results of the cluster analysis: there is no significant difference in the bacterial communities associated with *E. fetida* FS and SO.

DISCUSSION

In this study PCR-DGGE was used for the first time to evaluate the faecal microbial community of *Eisenia fetida* and *E. andrei*. Vivas et al. (2009) recently investigated bacterial communities in *E. fetida* vermicomposts. The archaeal diversity of various *Lumbricus* species is recorded (Furlong et al. 2002, Knapp et al. 2008). This study provides the first infomation on differences in diversity and composition of archeal communities in substrates and faeces of the above two species of *Eisenia*.

During passage of compost through the gut of *E. andrei* the microbial diversity is enhanced and the compositions of compost archaeal and bacterial communities changed. The passage of forest soil through *E. fetida* did not increase the microbial diversity, but did result in changes in the composition of the archaeal community. The bacterial community in faeces was obviously derived from the soil microbial community. *E. fetida* is an epigeic earthworm that typically inhabits and feeds within the litter layer of the soil (Brown et al. 2000). However, forest soil used in this study contained less readily decomposable compounds and had a low C/N ratio. In contrast, the compost contained high amounts of fresh, undecomposed organic matter rich in carbohydrates and with a high C/N ratio. According to Vivas et al. (2009) the nature of the compost may facilitate the differentiation of the bacterial community during its passage through the gut and increase bacterial diversity. Similar results are reported by Egert et al. (2004) for *Lumbricus terrestris*. The faeces that resulted from ingesting soil with added beech litter had a changed bacterial diversity, whereas the mineral soil community was not changed significantly.

Differences in archaeal diversity in soil and faeces are reported by Furlong et al. (2002) for *Lumbricus rubellus*. They sequenced two dissimilar phylogenetic archaeal groups derived from soil and faeces, respectively. In the current study the differences in the archaeal fingerprints of soil, compost and earthworm faeces may be due the changes in oxygen availability during the passage through the gut. More anoxic conditions inside the gut activate ingested microbes and enhance certain species that are unable to grow in soil (Horn et al. 2003). In particular, many archaeal species may be excreted in faecal pellets rich in easily degradable carbon as reported by Lemke et al. (2003) and Kammann et al. (2009).

Several studies indicate that passage through the guts of earthworms has a very big effect on microbial diversity (Karsten & Drake 1997, Singleton et al. 2003, Egert et al. 2004). This supports the hypothesis that the microflora is totally changed during the passage through the gut but the microbial community in the faeces rapidly adapts to soil conditions. Future studies should aim

at revealing the changes in microbial diversity that occurred during the passage through the guts of both *E. fetida* and *E. andrei*.

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Paper III

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Changes in soil microbial communities as affected by intensive cattle husbandry

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ABSTRACT

The present field study documents substantial changes in the soil microbial community (SMC) and organic matter (SOM) in an upland pasture soil resulting from 10 years of "cattle outdoor over-wintering practice". Soils from a long-term investigated pasture area were compared under three different levels of cattle impact (SI – severe, MI – moderate, NI – no impact). Extended polar lipids analysis (PLA) confirmed a qualitatively new microbial community profile and a several-fold increase of the microbial biomass in the impacted soils (SI) compared to the control NI soil. The new SMC was derived from cattle intestine microorganisms, typical by increased content of archaeal phospholipid ether lipids and by new fatty acids indicative for bacterial and fungal fecal anaerobes. A quality of the SI-SOM, evaluated by the relative content of the pyrolytic fragments profile was more similar to the cattle excrements than to the MI and NI soils, and an organic carbon content of the SI soil was not more than three times higher in comparison to the control NI soil. The quality and quantity of the SOM as well as the SMC in both, the most impacted SI and the control NI soils, were stable in contrast to the moderately impacted MI soil. During the growing season, the MI soil lost 75% of the C_{org} and 65% of the soil microbial biomass that had accumulated during winter; its aromatic-rich-SOM showed transformation into SOM, enriched by N, P-organic derivates. This transformation was positively correlated to a significant recovery of the actinobacteria and reduction of anaerobic microorganisms during the vegetation season. Results in this study showed that the stability of the soil microbial changes due to the cattle outdoor over-wintering husbandry depended on the stability of the quantitative and qualitative changes of the SOM.

1. Introduction

Microbial communities drive organic matter transformation in soil. At the same time, the quality and quantity of organic matter (OM) entering the soil greatly affects the structure and function of soil microbial communities (SMC). The application of animal excrements to the soil has traditionally been considered as benefiting plant growth as well as soil microbial activities (Lovell and Jarvis, 1996). The animal excrements, beside indubitable beneficial effect on soil fertility and agriculture ecosystem, however, can also be a source of undesirable nutrient release to the environment (Kurz et al., 2006). It is usually accompanied by the introduction of alien microorganisms to the soil including pathogens and risky genes (Mawdsley et al., 1995; Cools et al., 2001; Blanco et al., 2009), and can induce shifts in the functional and structural composition of the micro-edaphon communities (Peacock et al., 2001; Griffiths et al., 1998). The persistence of these introduced microorganisms and their effects on functioning of SMC are still poorly understood, as is the impact of the introduced OM.

The present field study focused on SMC and OM changes in upland grassland soil under impact of "cattle outdoor over-wintering practice", in which the cattle are confined to a relatively small area of a pasture typically from October to May periodically each year (Šimek et al., 2006; Wassmuth et al., 1999). This practice results in a high input of cattle excreta to the soil during winter when pasture plants are not growing. Previous investigations showed that due to the huge amount of organic material accumulated during winter as well as the changed soil structure (the soil structure damaged by the cattle), high amounts of greenhouse gases (GHG) like CH_4 and N_2O were emitted from an over-wintering study site (Šimek et al., 2006; Hynšt et al., 2007). Various differences between the soils impacted by the cattle and the control soils

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indicated significant shift in function and structure of the original SMC. The SMC impacted by the cattle was characterized (i) by high potential to metabolize large nitrogen inputs over short periods (Brůček et al., 2009; Hynšt et al., 2007), (ii) by increased abundance of the key GHG producers, i.e., methanogenic archaea (Radl et al., 2007) and denitrifying community (Chroňáková et al., 2009); (iii) by an abundance shift of main soil bacterial as well as fungal taxons (Philippot et al., 2009; Jirout et al., 2011) and (iv) suppression of arbuscular mycorrhizal fungi (Jirout et al., 2009).

The objective of this study was to complete information on quality and quantity shifts within the total soil microbial community and soil organic matter (SOM) changes as a result of the cattle outdoor over-wintering practice. To investigate changes in the SMC, the extended polar lipids analysis (PLA) was used (Zelles, 1999a). The extended PLAs are based on analysis of lipid components of membranes that differ between phylogenetically and metabolically defined groups (Zelles, 1999a). Within minutes of cell death, enzymes start to degrade the phospholipids (White, 1994), so profiles obtained from the PLA are primarily the product of cells present in the living, functional biomass. It involves all three domains of life (Archaea, Bacteria, and Eukaryotes) and hence it guarantees the investigation of complete microbial community profiles in soils. The extended PLA is also one of a few methods, that allow to evaluate the proportion of aerobic and anaerobic biomass and quality of each of them (Zelles, 1999a). The PLA, thanks to the above listed advantages, is one of the most suitable methods for monitoring complex changes of complete soil microbial community in response to environmental influences. To characterize quantity and quality of SOM, a measurement of organic carbon (C_{org}) content and GC–MS pyrolysis of SOM were used. The GC–MS pyrolysis is a rapid analytical technique that allows the study of changes in the inner composition of biopolymers (Steffen et al., 2007). Based on a profile of pyrolytic fragments of the organic matter, it is suitable method to investigate a complex composition change of SOM. In addition to soil analyses, the fresh cattle excrements were subjected to microbial and chemical analyses to characterize the material entering the soil.

2. Materials and methods

2.1. Study site

The investigation was carried out at a cattle over-wintering area on an organic farm in Borová near Český Krumlov in South Bohemia [Czech Republic, 170 km south of Prague; 48°52′N, 14°13′E; see Šimek et al. (2006) for details]. Between the end of October and beginning of May in each year since 1994/1995, the 4.04-ha pasture had been used to over-winter about 90 cows. Before that, this site was used as permanent grassland for hay production. The soil type at the site is a sandy loam, originally classified as Cambisol and recently re-classified as Haplic Phaeozem (arenic; WRB system), containing 60–80% sand, 14–32% silt, and 6–14% clay (USDA classification system). The mean annual precipitation at the site is 650 mm, and the average annual temperature is 7 °C (data from the meteorological station located 7 km from the experimental site). At the end of the winter season, the gradient of impact due to the cattle husbandry is visible. Based on our previous research conducted on the same over-wintering pasture (Šimek et al., 2006; Hynšt et al., 2007; Radl et al., 2007; Chroňáková et al., 2009), we investigated two impacted sites and one non-impacted site that served as a control. The most affected section (0.5 ha) was situated near the barn (SI = severe impact). The soil surface and plant cover at the SI site were totally destroyed by the end of winter season. A less impacted section (1.5 ha) in the middle of the gradient (MI = moderate impact) was characterized by partly destroyed vegetation. The third section (2 ha) at the opposite side of the over-wintering area was used as a control (NI = no impact) because cattle impact was minimal and the soil and original vegetation were unaffected. A phytocoenology study of the dominant plant species at sites SI, MI, and NI during the plant growing season (July 2006) was done by Jirout et al. (2009). The original plant cover was a perennial mixture of grasses, clovers, and other dicotyledonous plants, which do not recover fully at sites SI and MI over the cattle-free period; at these sites, the original vegetation was replaced with a mixture of fast growing plants including ruderal herbs (*Polygonum* sp., *Plantago major*, *Galinsoga parviflora*), grasses (*Echinochloa cruss-galli*), and red clover (*Trifolium pratense*). No additional fertilization was used at the sites.

2.2. Soil and excrement samples

Nine random sampling points were selected per site (SI, MI, and NI). At each sampling point, seven random single samples of soil from the top 0–20 cm were collected. These single samples were taken using a cylindrical soil corer (cross sectional area 36.3 cm^2), immediately homogenized by passing through a 5-mm mesh sieve, and mixed for obtaining a composite sample. Thus, nine composite samples per site were collected and stored at 4 °C until they were analyzed. IUPAC Recommendations 2005 were used for the terminology of soil sampling description (de Zorzi et al., 2005). The soils from all three sites were sampled in spring (11 May 2005), which was just after the animals were removed from the over-wintering area, and in fall (18 October 2005), which was just before the animals were brought back. Fresh cattle excrements from nine cows were sampled in spring (5 May 2005). Gravimetric moisture content of samples was determined after drying at 105 °C for 48 h. pH was measured using a pH meter in 1:2.5 (w/w) soil/excrement: 0.01 M $CaCl_2$ suspension. Total organic carbon (C_{org}) content was determined by wet oxidation with acid dichromate (Jackson, 1958), and total nitrogen (N_{tot}) content was measured by Kjeldahl digestion (Zbíral, 1995). Basic soil and excrement characteristics are given in Table 1.

2.3. Microbial community analyses—phospho(polar)lipid analyses

Lipids were extracted from fresh soil samples equivalent to 12 g dry weight (dw) and from fresh excrement samples equivalent to 0.8 g dw with a one-phase mixture of 25 ml chloroform, 50 ml methanol, and 20 ml phosphate buffer (pH 7.4, 0.05 M; the volume of water in fresh samples was subtracted from the phosphate buffer volume). After 2 h of horizontal shaking, 63 ml of chloroform and 63 ml of distilled water were added. After a 24-h separation time, the organic phase was recovered and dried over anhydrous sodium sulfate and concentrated in a rotary evaporator. The lipid material was fractionated into neutral lipids, glycolipids, and phospho(polar)lipids (PLs) on a silica-bounded phase column (Mega Be–Si, 1 g, 6 ml, 60 Å, Varian, USA) by consecutive elution with chloroform, acetone, and finally methanol (Gattinger et al., 2003). Aliquots of the polar-lipid fraction equivalent to 5 g and 7 g dw of soil were taken for analysis of phospholipid fatty acids (PLFA) and for phospholipid ether lipid (PLEL), respectively. Both PLFA and PLEL assays are based on the determination of polar-lipid side-chains, detected in form of fatty acid methyl esters and isoprenoid hydrocarbons, respectively.

The phospholipid fraction was subjected to mild alkaline methanolysis to liberate ester-linked fatty acids methylesters (EL-PLFA), which were separated from the unsaponifiable lipids by the solid-phase extraction (Chromabond SPE-NH_2, Macherey-Nagel, Germany). The non-ester-linked phospholipid fatty acids (NEL-PLFA) were obtained through the acidic hydrolysis and methylation

Table 1
Basic properties of soils (from site SI, MI, and NI) and excrements (mean ± SD; $n = 9$).

Characteristic	Sample						
	Excrements	Soil SI		Soil MI		Soil NI	
	Spring	Spring	Fall	Spring	Fall	Spring	Fall
pH [$CaCl_2$]	7.8 ± 0.1a	9.0 ± 0.2b	8.8 ± 0.2b	7.3 ± 0.2ac	6.9 ± 0.3c	5.9 ± 0.2d	6.6 ± 0.3d
N_{tot} [g 100 g^{-1}]^a	2.4 ± 0.1a	1.9 ± 0.3a	1.7 ± 0.3ab	1.2 ± 0.5b	0.5 ± 0.3c	0.7 ± 0.2c	0.5 ± 0.2c
C_{org} [g 100 g^{-1}]^a	40.7 ± 1.6a	5.8 ± 0.3b	5.8 ± 0.6b	4.1 ± 0.8c	1.3 ± 0.1d	2.3 ± 0.3e	1.8 ± 0.3de
Water content [%]	85.0 ± 1.8a	63.9 ± 6.9b	47.4 ± 7.3bc	39.1 ± 7.9ce	17.3 ± 1.3d	30.4 ± 5.3fe	23.1 ± 2.8fd

Soil samples were collected from three sites: SI = severe impact; MI = moderate impact; NI = no impact.
Note: Means in a row followed by different letters are significantly different at $p \leq 0.05$. Differences between means were evaluated by one-way ANOVA followed with all pairwise multiple comparison procedure (Student–Newman–Keuls test). Kruskal–Wallis one-way ANOVA followed by cross-testing of individual treatments by non-parametric post hoc Mann–Whitney *U*-test was applied for cases where normality test of data distribution failed (for C_{org} values of spring and fall samples).
[a] Based on soil or excrement dry weight (dw).

of the unsaponifiable fraction according to Zelles (1999a,b). The EL-PLFA profile was identified by gas chromatography (Agilent 6850, Agilent Technologies, USA) with a flame ionization detector on a capillary column (Ultra 2, 25 m, 0.20 mm, 0.33 μm, Agilent Technologies, USA). The samples (1 μl) were injected in split mode (1:100) at an injection temperature of 250 °C using hydrogen as carrier gas. The GC-temperature regime was 170 °C – 5 °C min^{-1} – 260 °C – 40 °C min^{-1} – 310 °C – 1.5 min. Individual peaks were identified using an automatic identification system (MIDI Inc., USA). The TSBA6 (MIS Sherlock System, ver. 6.0) was used for identification of the EL-PLFAs. The NEL-PLFAs were separated and identified by gas chromatography/mass spectrometry (GC/MS; see Zelles and Bai, 1993 for details).

Another aliquot of the phospholipid fraction equivalent to 7 g soil dw was used for PLEL analysis according to Gattinger et al. (2003). After the formation of ether core lipids, ether-linked isoprenoids were released following cleavage of ether bonds with HI and reductive dehalogenation with Zn in glacial acetic acid. The resulting isoprenoid hydrocarbons were dissolved in a 100-μl internal standard solution (nonadecanoic methyl ester) and subjected to gas chromatography–mass spectrometry analysis at operating conditions described by Gattinger et al. (2003).

Fatty acids were designated as the total number of carbon atoms: the number of double bonds followed by the position of the double bond from the methyl end (ω) of the molecule. The prefixes *i* – and *a* – indicate terminal *iso*-branched and *anteiso*-branched PLFA, respectively. 10Me – indicates a methyl group at the 10th carbon atom from the carboxyl end of the molecule (i.e., mid branching), and *cyc* – refers to cyclopropane fatty acids. *Br* – indicates an unknown methyl branch position; the prefixes 2OH – and 3OH – indicate 2- and 3-hydroxy fatty acids, respectively. The prefix *nel* – is used to distinguish NEL-PLFA from the EL-PLFA. The prefix *ip* indicates the PLEL-derived isoprenoids: ip20:0 indicates a saturated and ip20:1 a monounsaturated isoprenoid chain with 20 C atoms; ip40:0 indicates an isoprenoid chain with 40 C atoms.

The individual PLs were classified according to their source and structure into following groups: EL-STFA (saturated EL-PLFA), EL-MUFA (monounsaturated EL-PLFA), EL-TerBr (terminally branched saturated and monounsaturated EL-PLFA), EL-OH (hydroxylated saturated and monounsaturated EL-PLFA), EL-MBr (mid-branched saturated and monounsaturated EL-PLFA), EL-cyc-STFA (cyclopropyl branched saturated EL-PLFA), EL-PUFA (polyunsaturated EL-PLFA), NEL-STFA (saturated NEL-PLFA), NEL-Br-STFA (branched saturated NEL-PLFA), and NEL-PUFA (polyunsaturated NEL-PLFA).

Data for the individual PLFA/PLEL compounds (expressed in nmol g^{-1} sample dw) were summarized to obtain the contents of total phospholipids (PLs), a measure of total microbial biomass in soil and excrements (Zelles, 1999a,b). The changes of the most important structural or functional microbial groups were evaluated based on the concentration of the PLs-specific biomarkers (nmol g^{-1} dw) or the relative abundance within the PL profile (%), indicating the actual size and the relative abundance of the microbial groups, respectively. The PLs biomarkers used in this study are listed in Table 2.

2.4. Pyrolysis of organic matter

Pyrolysis with in situ methylation using tetramethyl ammonium hydroxide combined with gas chromatographic separation of the methylated pyrolytic products is a rapid analytical technique that allows the study of changes in the inner composition of biopolymers, e.g., ligninocellulose, lignin, or other non-soluble organic materials. The mass spectrometry detection enables the identification of the pyrolytic fragments. GC–MS pyrolysis was performed as previously reported by Steffen et al. (2007). In particular, after samples (1 mg) of either soil or excrements were treated by adding an excess of tetramethylammonium hydroxide (TMAH, 25% aqueous solution), they were placed on Wolfram wire spirals and then dried in a desiccator overnight at room temperature. Pyrolysis together with in situ TMAH methylation (thermochemolysis) was performed with a PYR-01 pyrolyzer (Labio, Czech Republic). Each sample on the Wolfram support was inserted within a coil of platinum filament, and the probe was then placed into the injector port of a GC–MS system (Varian 3400, Finnigan IS40). The samples were analyzed in triplicate at 550 °C for 10 s using a platinum-heated filament pyrolyzer with a quartz sample holder. The GC instrument was equipped with a split injector (split ratio 1/40), and a DB-5MS column was used for separation (30 m, inner diameter 0.25 mm, film thickness 0.25 μm) with helium as the carrier gas (1 ml min^{-1}). The temperature program started at 45 °C, and the oven was heated to 240 °C at a rate of 5 °C min^{-1}. The detector delay time was 2 min. The injector and transfer line temperature were set at 240 °C. Mass spectra were recorded at 1 scan s^{-1} under an electron impact at 70 eV, mass range 50–450 amu. The pyrolysis products were identified by comparing the mass spectra with the data in the NIST 02 library, and independently by interpreting the fragmentation pattern. The relative percentages of pyrolysis products were calculated from the relative areas of the peaks. Individual fragments detected after pyrolysis were grouped according to their source compounds: humus containing aromatic compounds (H), light humus/polysacharides (L), nitrogen-containing compounds (N), phosphorus-containing compounds (P), and fatty acids (FA).

2.5. Data analysis

All statistical analyses were performed with SPSS software package release 12.0 (SPSS Inc., Chicago, IL, USA). The analysis of variance (ANOVA) was followed by an all pairwise multiple comparison procedure, the Student–Newman–Keuls test was used to determine differences among the three sites. A non-parametric

Table 2
Phospholipid (PL) bio-markers used in this study and their interpretation.

Indicator	Interpretation	Source
PLA sum	Total biomass	Zelles (1999a,b)
EL-PLFA sum	Aerobic microbiota	Zelles (1999a,b)
NEL-PLFA sum	Anaerobic microbiota	Zelles (1999a,b)
PLEL sum	Archaea	Zelles (1999a,b) and Gattinger et al. (2002b, 2003)
Sum of EL-PLFA$_{bact}$ (i-15:0, a-15:0, 15:0, i-16:0, 17:0, i-17:0, cyc-17:0, cyc-19:0, 16:1ω7 and 18:1ω7)	Aerobic bacteria	Frostegård and Bååth (1996)
Sum of mid branched EL-PLFA (10Me 18:0, 10Me 17:0 and 10Me 16:0)	Actinomycetes	Kroppenstedt (1985)
EL-PUFA sum	Aerobic microeukaryotes	Zelles (1999a,b)
EL-PLFAfu 18:2(6	Fungi	Federle (1986)
Sum of EL-MUFA 16:1(11; 16: 1(9; 16:1(7; 16:1(5, 17:1(8; 18:1(9; 18:1(7c; 18:1(5	Methhylotrophic bacteria	Makula (1978), Hanson and Hanson (1996), Virtue et al. (1996), and Gebert et al. (2004)
Sum of EL-MUFA 18:1/sum of EL-MUFA 16:1	Ratio of Type I/Type II methanotrophs	Hanson and Hanson (1996), He et al. (2008), and Gebert et al. (2004)
PLEL (ip20:1)	Methanogenic archaea	Gattinger et al. (2002a,b), and Radl et al. (2007)
Increase of EL-cyclopropyl-STFA	Growth stress indications	Guckert et al. (1986)
Number of PLA	Qualitative indicator of microbial community richness	Laczko et al. (1997)

Kruskal–Wallis ANOVA followed by cross-testing of individual treatments by non-parametric post hoc Mann–Whitney *U*-test was applied when data were not normally distributed. Means were considered to be significantly different at $p \leq 0.05$. Principal component analyses (PCA) were performed on the complex PL profiles to identify differences in microbial communities in cattle affected and non-affected soils and excrements, (log [mol% + 1] values were used; CANOCO for Windows version 4.5, Plant Research International, Wageningen; ter Braak and Šmilauer, 2002). PCA was also used on data from the whole pyrolytic fragments profiles to identify the OM qualitative differences of cattle affected and non-affected soils and the excrements. Results are presented as the means ± SD of nine replicates. The Pearson correlation coefficient (r) was used to define the correlations between PL markers and selected abiotic conditions. The significant p-level was at $p \leq 0.05$.

3. Results

3.1. Microbial biomass

At the end of the six months overwintering period (spring), soil microbial biomass (SMB) was two-times higher at the MI site and four-times higher at the SI site than at the control NI site (Table 3). Surprisingly, SMB values at the SI site remained very high in fall despite reduced plant growth and the absence of animals in summer. In contrast to this, the SMB at the MI site decreased significantly during vegetation season on the level of the control NI site. No significant SMB differences were found at the control NI site in spring and fall. Microbial biomass was three times higher in cattle excreta than in SI soil.

3.2. Microbial community composition

According to PCA comparing PL profiles, the composition of the soil microbial community after 10 years of cattle outdoor overwintering practice was significantly changed at the SI site (Fig. 1). The observed changes were stable in many aspects, i.e., they did not disappear after the cattle-free summer period in contrast to the MI site, where the resilience effect after the cattle-free summer period was apparent (Table 3, Fig. 1). Only in the MI site the soil microbial community showed a PL profile similar to that of NI site. PC1 and PC2 explained 85% of the qualitative differences between compared microbial communities in the soil and excrements.

The soil PL profile was dominated by EL-PLFA (91.8–97%), followed by NEL-PLFA (2.3–4.3%), and PLEL (0.6–4.2%) in contrast to excrement PL profile enriched by NEL-PLFA (51.5%) at the expense of EL-PLFA (43.3%). The PLEL in excrements represented 5.2% of the total PL profile (Table 3). In addition to that, significant differences among studied soils under three different levels of cattle impact were found; details are listed below in the text. The microbial community richness was highest in the highly impacted soil at site SI than in the soil from the other two sites and the excrements.

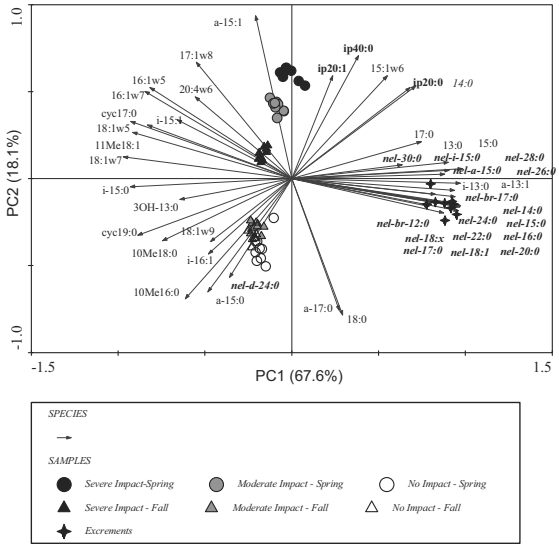

Fig. 1. PCA diagram of complex phospholipid (PL) profiles segregating the microbial community of non-impacted soils from the severely impacted soils and excrements. Based on PL fingerprints, in total 91 PLFA/PLEL. Only PLFA-PLEL fitted with PC1 and PC2 more than 30% is projected in the figure. The abbreviation ip- was used for PLEL-derived isoprenoid hydrocarbons. The abbreviation nel- was used for NEL-PLFA.

Table 3
Total active phospholipid (PL) community biomass and content of PL microbial group markers, PL ecophysiology bioindicators (nmol g^{-1} dw), and PL community richness (number of PLs) in excrements and in soil samples from three sites (SI, MI, NI) (mean ± SD; $n = 9$).

PLA functional groups	Samples analyzed						
		Soil SI		Soil MI		Soil NI	
	Excrements	Spring	Fall	Spring	Fall	Spring	Fall
Total biomass	1717.5 ± 190.7a	600.8 ± 130.3b	598.4 ± 108.5b	310.8 ± 103.5c	109.9 ± 31.9d	150.6 ± 32.0d	135.0 ± 29.6d
Aerobic microbiota	744.0 ± 25.0a	551.8 ± 121.5b	557.5 ± 98.7b	296.5 ± 94.6c	105.9 ± 29.7d	146.8 ± 30.4d	131.3 ± 27.8d
Anaerobic microbiota	884.0 ± 183.4a	24.3 ± 7.9b	16.4 ± 3.2b	6.8 ± 2.7c	3.0 ± 0.7d	3.2 ± 0.6d	3.0 ± 0.6d
Archaea	89.0 ± 37.1a	24.0 ± 5.0b	24.5 ± 3.3b	7.4 ± 2.2c	1.0 ± 0.2d	0.6 ± 0.2d	0.8 ± 0.2d
Aerobic bacteria	258.3 ± 9.5a	189.1 ± 42.1b	178.6 ± 33.2bc	93.0 ± 30.6c	31.7 ± 9.4d	42.8 ± 8.2d	36.9 ± 7.9d
Actinomycetes	0.0 ± 0.0a	5.7 ± 1.5b	46.8 ± 9.5c	9.0 ± 1.9bd	11.5 ± 3.5d	15.9 ± 2.8d	15.5 ± 3.7d
Aerobic microeukaryotes	34.1 ± 2.3a	21.6 ± 6.6b	17.9 ± 3.4b	15.4 ± 6.3b	4.1 ± 2.7c	3.9 ± 2.3c	5.2 ± 2.3c
Fungi	34.1 ± 2.3a	15.1 ± 5.4b	13.0 ± 2.2b	11.3 ± 4.5b	2.6 ± 2.7c	3.9 ± 2.4c	3.8 ± 2.1c
Methanogens	4.2 ± 1.5a	2.1 ± 1.3b	1.8 ± 0.2b	0.7 ± 0.4c	0.1 ± 0.0d	0.1 ± 0.0d	0.1 ± 0.0d
Methanotrophs	134.7 ± 5.6a	244.6 ± 59.1b	237.2 ± 45.0b	110.7 ± 40.9a	32.5 ± 10.5c	46.3 ± 12.6c	42.9 ± 11.3c
Type I/Type II methanotrophs	12.7 ± 2.9a	0.8 ± 0.1b	0.9 ± 0.1b	1.2 ± 0.1b	1.6 ± 0.1c	2.3 ± 0.3d	1.9 ± 0.1dc
Community richness	42.2 ± 3.2a	68.9 ± 9.5b	70.3 ± 4.4b	62.9 ± 4.9bc	54.0 ± 2.4c	47.4 ± 6.7ac	54.8 ± 3.7c

Soil samples were collected from three sites: SI = severe impact; MI = moderate impact; NI = no impact.
Note: Means in a row followed by different letters are significantly different at $p \leq 0.05$. Differences between means were evaluated by one-way ANOVA followed with all pairwise multiple comparison procedure (Student–Newman–Keuls test).

The specific occurrence of PLA markers indicated that the new microorganisms established in the SI soil originated from cattle excrements. The EL-PLFAs anteiso-13:1, iso-13:1, anteiso-15:1, iso-19:1, iso-19:0, 3OH-iso-11:0, 15:1ω6, 20:1ω9, 14:1ω5, 2OH-16:0, 3OH-16:0, and 13:0, and the NEL-PLFAs iso-16:0, iso-15:0, iso-17:0, and 16:2 were detected only in the group of impacted SI and MI soils (in MI soil only in spring). The EL-PLFAs 13:0, anteiso-13:1, iso-13:1, and 15:1ω6, and the NEL-PLFAs iso-15:0, iso-16:0, iso-17:0, and 16:2 clearly originated from the cattle excrements. The impacted soils (SI-spring, SI-fall and MI-spring) were enriched also by methanogenes markers, the content of which was higher approximately about one order in comparison to MI-fall and both NI soils. The size of methanogenic population in the impacted soils represented less than half-size of methanogenic population in the excrements (Table 3). Also PL markers of methanotrophic bacteria Type I and Type II indicated an interesting difference among the samples. The impacted soils (SI-spring, SI-fall and MI-spring) were characterized by equable markers content of both groups in contrast to the excrements and non-impacted soils. The content of Type I markers was approximately doubled in the non-impacted soils NI than Type II and even about one order higher in the excrement.

With respect to the differences between spring and fall data, the SI soil had a higher content of anaerobic indicators (NEL-PLFA) in spring than in fall (Table 3). This indicator represented 4.3% in the spring samples and 2.8% in the fall sample from the SI site, the latter percentage being statistically similar to that in the other soils (2.3–2.9%), including the non-impacted control. In fall, the development of actinomycetes (Table 3) in both SI and MI soils represented a statistically important shift in microbial community structure. The EL-MBr, actinomycetes indicators, increased from 1% to 8% and from 3% to 10.5% in SI and MI soils, respectively. The levels of the actinomycetes indicators in the NI soil were 10.5% in spring and 11.5% in fall. The MI soil had a higher content of biomass as well as a higher abundance of all observed microbial groups except the actinomycetes in spring than in fall (Table 3). Both the SI and MI soils in fall were also characterized by reduced growth status of the microorganisms, indicated by a significant increase of the stress marker EL-cyc-STFA which rose from 6% in spring to 9% in fall in the SI soil and from 7% in spring to 12% in fall in the MI soil. The EL-cyc-STFA value in the NI soil was 11% in spring and fall.

In contrast to soil PL profiles, the excrement PL profile was characterized by higher relative content of NEL-STFA and EL-STFA, PLEL, EL-PUFA and NEL-PUFA, NEL-br-STFA and lower content of EL-MUFA and absence of PL markers of actinomycetes (i.e., EL-MBr-STFA). The total number of PLs detected indicate that microbial community richness was lower in excrements than in soils (Table 3).

3.3. Impact of cattle husbandry on abiotic soil properties

Soil pH was one to two units greater in MI and SI soils than in the control NI soil (Table 1). The pH of the SI soil was even significantly higher than that of the fresh cattle excrements. N_{tot} content was significantly higher in the SI soil in spring and fall and in the MI soil in spring than in the NI soil. The N_{tot} content of the SI soil in spring and fall was not statistically different than that in the excrement (Table 1). There was also no statistical difference between N_{tot} content in SI soil in spring and fall. The opposite situation was observed at the MI site, where N_{tot} content decreased more than 50% during the plant growth period and reached the level of the control soil (NI) in fall. The changes of organic carbon content (C_{org}) in both impacted soils and in both seasons were similar to the changes in N_{tot} content. C_{org} content was significantly higher in SI soil (spring and fall) and in MI soil (spring) than in NI (control) soil. As was the case for N_{tot} content, C_{org} content in MI soil was higher in spring than in fall, when values were similar to those in the NI soil. The C/N ratio of the fall MI samples (2.5) was the lowest among all analyzed soil samples.

The increase of the soil C_{org} content in soil was accompanied by an increase in soil water content; this was most evident in the SI soil, which contained twice as much water as the NI soil. The water content in the MI soil was significantly lower than that in the SI soil

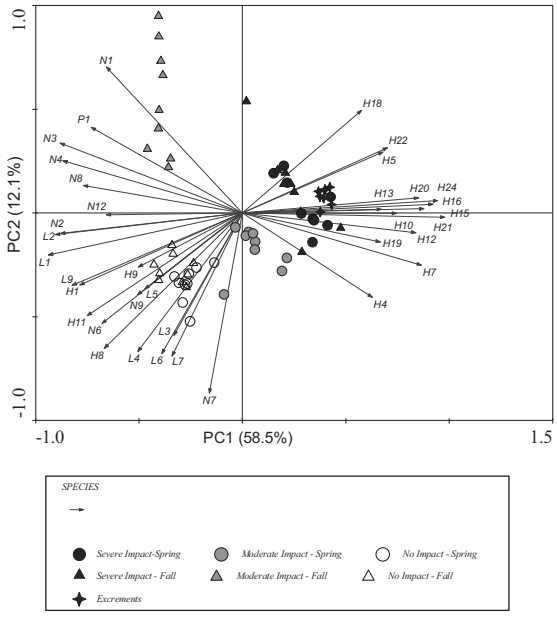

Fig. 2. Qualitative comparison of the profiles of organic matter pyrolytic products of soils and excrements based on principal component analysis. *H* = humus/aromatic compounds; *L* = light humus polysacharides; *N* = N-organic derivates; *P* = P-organic derivates; *FA* = fatty acids.

and did not differ significantly from that in the NI soil in spring or in fall. Although water content tended to be lower in fall than in spring at all three sites, the difference between spring and fall was significant only at the MI site, which was the driest of the sites.

The qualitative comparison of the profiles of organic matter pyrolytic products (OMPP) of soils illustrated the impact of the cattle outdoor over-wintering practice on soil organic matter quality. An important influence of the excrement organic matter on the composition of the SOM was confirmed by the multivariate PCA analysis (Fig. 2), which showed that the OM profile of the most impacted soil (SI, spring and fall) was not discriminated from the profile of the cattle excrements. These profiles were dominated by high contents of aromatic humus structures (H), representing more than 50% of the total OM profile (Table 4). No seasonal changes were observed in the relative contents of the OMPP groups at the SI site. This finding agrees with the other findings that the conditions and soil microbial community at site SI were relatively stable. The OMPP profile was also stable in the NI soil, which formed a separate cluster in the PCA plot (Fig. 2). In contrast to the OMPP profiles in SI and NI soils, the OMPP profiles in the MI soil were substantially different in spring than in fall (Fig. 2). While the MI soil in spring was enriched with relatively large amounts of both H and L compounds, the MI soil in fall was dominated by compounds containing N and P (Table 4). The correlation between relative portion of OMPP groups and relative content of PL microbial markers revealed the best correspondence of the actinomycetes to the qualitative changes of the OM. Significant positive correlation ($r = +0.84$, $p < 0.001$) was found between relative abundance of actinomycetes and both L ($r = +0.90$, $p < 0.001$), N ($r = +0.54$, $p < 0.001$) and P compounds ($r = +0.40$, $p < 0.001$) and strong negative correlation between actinomycetes and H compounds ($r = -0.78$, $p < 0.0001$). On the other side significant positive correlation was found between the relative content of H compounds and relative abundance of anaerobes in the community ($r = +0.53$, $p < 0.0001$) (Table 5).

4. Discussion

Similar significant impact of cattle excrement on grassland soil microbial biomass was found in a study by Gattinger et al. (2007), who reported a four-fold greater microbial biomass in grassland soil treated with cattle manure (200 t ha^{-1} a^{-1}, periodically 21 years) than in unfertilized soil. The soil microbial biomass reported by Gattinger et al. (2007), however, was about one order of magnitude lower than in the present study although both studies reported similar levels of microbial biomass and total amount of C in the manure or cattle excrements. While content and activity of the soil microbial biomass can be stimulated by an increase in available C when animal excreta are incorporated into soil, plants and microorganisms compete for nutrients in grassland soils, which can reduce the nutrients available to microorganisms and consequently reduce microbial utilization of available C (Lovell and Jarvis, 1996). Bol et al. (2000) reported that only ~20% of cattle dung C could be recovered in soil microbial biomass. Hatch et al. (2000) also documented that the short-term (i.e., ~3 months) effect of surface dung deposition on microbial activity varied with soil fertility. It follows that animal excreta deposited on the soil surface may not directly affect the dynamics of microbial growth and activity but may contribute to stabilized soil C pools, and thus only indirectly affect soil microbial biomass and activity. In contrast, the excreta deposits on our study sites were more efficiently incorporated into the soil and affected microbial biomass. Two factors seemed to be important for and specific to our study: (1) the input of microorganisms and nutrients to the pasture soil via cattle excreta occurred during winter, when the plants do not compete with the soil microorganisms for nutrients; and (2) the repeated overwintering of cattle at the same sites for the previous 10 years probably caused the soil microorganisms to adapt to the corresponding high organic loads, modified soil properties and plant cover. Our study also documented significant increase of the anaerobes in the severely cattle impacted soil after the winter period. It is in accordance with Dowd et al. (2008) who indicated, that the microbial population of lower intestinal bacteria of cattle are dominated by the anaerobes. There is a scarcity of information on the biomass and diversity of anaerobic microorganisms in the excrements of livestock species and especially their surviving in the environment after defecation. Our study showed that important parts of fecal anaerobes, which enriched the soil community, did not survive the summer period. The anaerobes' relative abundance positively correlated with the moisture, organic carbon content, and relative content of H-humus and C/N ratio (Table 5).

A qualitatively new microbial community profile at the site SI was enriched by terminally branched EL- and NEL-STFA/MUFA and their hydroxylated derivates, typical for g. *Bacteriodes*, *Clostridium*, *Porphyromonas*, *Prevotella*, *Enterobacter*, and *Flavobacterium*, *Cytophaga*, *Cellulomonas*, respectively (BHIBLA Library, MIDI, Inc., USA; Suzuki et al., 1993; Oyaizu and Komagata, 1981; Zelles, 1999a,b), which are the ubiquitous bacteria of cattle feces (Dowd et al., 2008). High content of monounsaturated terminally branched acids is also specific for *Bacillus firmus* or *Bacillus alcalophilus* (Kaneda, 1991), the bacteria tolerant to high pH and anaerobic conditions. Next unique FA of the cattle-impacted soils was the NEL 16:2. The NEL-PUFAs are indicative for fungi (Zelles, 1997, 1999a,b) and were found in significant portion in the cattle excrements. The study by Jirout et al. (2011) based on comparison 18S rDNA confirmed that rumen-born anaerobic fungi *Neocallimastix* and *Cyllamyces* (originated in the excrement) enriched the SI soil. The anaerobic fungi specifically occur in the intestine of ruminants (Orpin, 1975), freshly defecated excrements (Bauchop, 1979) and

Table 4
The relative content of functional groups of substances in the pyrolysis–GC–MS analyses (mean ± SD; $n = 9$).

Functional group of substance	Samples analyzed						
		Soil SI		Soil MI		Soil NI	
	Excrements	Spring	Fall	Spring	Fall	Spring	Fall
%Humus/aromatic compounds (H)	63.8 ± 1.9a	61.6 ± 1.8a	55.5 ± 3.6ab	50.0 ± 3.8b	29.8 ± 2.6c	37.1 ± 1.4d	36.3 ± 2.8d
%Light humus/polysaccharides (L)	12.3 ± 0.7a	13.3 ± 1.6a	14.1 ± 1.0a	21.5 ± 2.1b	21.3 ± 2.0b	28.5 ± 1.2c	29.2 ± 2.7c
%N-containing compounds (N)	10.9 ± 0.6a	12.6 ± 2.1a	14.6 ± 3.8a	16.1 ± 1.9a	32.1 ± 4.2b	21.0 ± 1.1c	21.9 ± 1.6c
%P-containing compound (P)	0.8 ± 0.3a	1.3 ± 0.8a	1.9 ± 1.2a	3.4 ± 1.2a	7.0 ± 1.3b	2.4 ± 0.5a	2.0 ± 0.4a
%Fatty acids (FA)	12.2 ± 1.3a	11.3 ± 2.4a	13.9 ± 3.2a	9.0 ± 2.9a	9.8 ± 2.6a	11.0 ± 0.9a	10.6 ± 1.8a

Soil samples were collected from three sites: SI = severe impact; MI = moderate impact; NI = no impact.
Note: Means in a row followed by different letters are significantly different at $p \leq 0.05$. Differences between means were evaluated by one-way ANOVA followed with all pairwise multiple comparison procedure (Student–Newman–Keuls test).

they are able to survive several months in soil environments with high moisture content (McGranaghan et al., 1999). The PLA as well as DNA comparison indicated that these anaerobic fungi did not survive the dry summer period, since they were not detected in the fall samples (Jirout et al., 2011).

The most obvious change at the SI site was the high abundance of lipids indicative of archaea. Increased abundances of archaeal communities resulting from cyclic long-term application of manure was also shown by Gattinger et al. (2007), who postulated that high organic fertilization increases archaeal biomass, which could be mainly related to methanogen *Methanoculleus* and *Methanosarcina* species (Gattinger et al., 2007). These results were in accordance with phylogenetic analysis of the *mcrA* genes isolated from SI soil (Radl et al., 2007), which indicated that 33% of the analyzed clones belonged to the genus *Methanosarcina*, 15% to g. *Methanococcus*, 11% to g. *Methanosaeta* whereas many of the sequenced clones (41%) showed close affiliations with uncultured rumen archaeons, suggesting a transfer of archaea from the rumen to soil. High methanogenic diversity in a cattle-impacted soil at the SI site of the Borová farm was revealed with phylogenetic microarray AnaeroChip by Koubová et al. (2012). They found predominance of *Methanosarcina*, and presence of *Methanoculleus*, *Methanobacterium*, *Methanocalculus*, *Methanobrevibacter*, *Methanosaeta*, *Methanothermobacter*, *Methanogenium*, *Methanohalobium*, and *Methanolobus* in SI soil.

Using PLEL indicator lipids for methanogen archaea (ip20:1), we documented a high abundance and high survival of methanogenic populations in the SI soil (Table 3). The relative abundance of methanogen marker at SI soil both spring and fall (0.40% and 0.32%, respectively) was even higher in comparison to the abundance in the cattle excrement (0.24%). Similar results were referred by Gattinger et al. (2007), who found significantly higher relative abundance of Methanosarcina-related archaea based on PLEL indicator as well as 16SrcDNA quantification in soil with high and long cattle manure application compared to the cattle manure. Soils which are nutrient-saturated and replenished by organic carbon and nitrogen from cattle dung and urine represented suitable habitat for Methanosarcinales, that need anaerobic habitat, are halotolerant and alkaliphilic (Kendall and Boone, 2006; Jones et al., 1998). In accordance with this, we found significant correlation of the relative abundance of methanogens with the pH of soil/excrement samples (Table 5). Sheppard et al. (2005), who studied impact of sewage sludge application on methanogen community of upland soils, indicated a pH decrease in the sludge amended soil as factor responsible for reduction of methanogen diversity. The positive correlation was found also for the total archaeal lipidic biomarkers and the pH and N_{tot} (Table 5). Nicol et al. (2004) studied the response of the archaeal community to nitrogen and pH changes in upland grassland soil. They did not find a significant effect of these factors on archaeal community, which dominated by non-thermophilic Crenarchaeota in contrast to our cattle impacted soils. Lehtovirta et al. (2009) showed that nonthermophilic Crenarchaeota group 1.1c, declined as the pH increased (in scale 4.5–7.5). The pH higher than 6 was found as a limit value for the Crenarchaeota detection. Based on these findings we can conclude that the massive and long-term application of the cattle manure or excrements can affect the native upland soil archaea community in two ways: (i) by an inoculation of the rumen-born Euryarchaeota and (ii) by reduction of the authochthonous Crenarchaoeta via increasing soil pH. Soil pH was also shown of

Table 5
The strong correlation between relative abundance of microbial groups in the community and measured chemical parameters of soils and excrements. The significant p-level ($p \leq 0.001$) and the Pearson correlation coefficient ($r \geq 0.5$) were used.

Correlated variables		Correlation parameters	
		r	p
%Actinomycetes	%Light humus/polysaccharides (L)	+0.90	0.001
	%N-containing compounds (N)	+0.54	0.001
	N_{tot}	−0.82	0.0001
	%Humus/aromatic compounds (H)	−0.78	0.0001
%Anaerobic microorganisms	Moisture	+0.99	0.0001
	C_{org}	+0.98	0.001
	C/N	+0.97	0.001
	%Humus/aromatic compounds (H)	+0.53	0.0001
%Archaea	N_{tot}	+0.81	0.001
	pH	+0.75	0.001
%Methanogenes	pH	+0.66	0.001
%Methanotrophs	C/N	−0.90	0.001
	Moisture	−0.89	0.001
	C_{org}	−0.85	0.0001

importance in determining the nature of denitrification end products in cattle impacted soil (Čuhel et al., 2010) and in an affecting the intrinsic capability of soil denitrificators (Čuhel and Šimek, 2011). The decrease of $N_2O/(N_2O + N_2)$ ratio was found in the higher pH of these pasture soils.

The impacted soils contained increased concentrations of EL-MUFA, indicative for the Gram-negative bacteria including the aerobic methanotrophs. Based on the ratio of EL-MUFA typical for methanotrophs Type I to Type II, we found differences between impacted and non-impacted soil. The significantly lower ratio in the SI soil was related to an equable markers content of both Type I and Type II or slight predominance of the Type II methanotrophic bacteria, which require much higher concentration of CH_4 to thrive (Knief et al., 2003). In contrast, Type I methanotrophic bacteria, which are active and proliferate quickly regardless of the CH_4 or O_2 mixing ratio (Knief et al., 2003), predominated in the non-impacted soil. These findings are in good agreement with the production and emission of the CH_4 as previously reported for the soils from the same experimental microsites (Radl et al., 2007; Šimek et al., 2006; Hynšt et al., 2007; Koubová et al., 2012). A significant negative correlation was found between relative abundance of methanotrophic bacteria and moisture, organic carbon content, and C/N ratio of the studied soils. All three factors are closely related to the enrichment of soil by cattle excrements organic matter. The majority of researchers attribute the main negative relationship of methanotrophy and soil moisture (Whalen et al., 1991) connected with slower diffusion in wet soil limiting CH_4 supply. The soil C/N ratio may affect methanotrophs since it is an important control factor of the N-transformation in soil. The stimulation of N-mineralization, resulted in high NH_4^+ content can inhibit methanotrophs and CH_4 oxidation (Hanson and Hanson, 1996). This situation probably occurred in MI site during the growing season.

Fungal dynamics were evident at site MI. The most suitable conditions for fungal growth were given in spring. Jirout et al. (2011), who studied the diversity of fungal community at the same experimental over-wintering pasture in year 2006–2008, found also significant seasonal effect of the cattle disturbances both on cultivable and genotype level of soil micromycetes composition. The seasonal changes in abundance of fungi as well as other soil microbial taxons were undoubtedly affected by the plant community. Jirout et al. (2009) showed different plant regeneration at site SI (60%) and MI (85%) in relation to control non-disturbed NI site (100%). In addition, the highest mycorrhizal fungi colonization of plant roots was measured at MI site in comparison to SI and even NI site. The antagonistic interaction between mycorrhizal and saprotrophic fungi (Krashevska et al., 2010) could explain the seasonal shift in micromycetes abundance at MI site. The most seasonal changes were found at MI site due to the intensive interaction between plant roots and soil microorganisms. The rhizodepositions and their "priming effect" on soil microbial activity (Fontaine et al., 2003) can intensify or modify the SOM decomposition. Also an improved soil aeration due to roots growth can help the degradation of some SOM complex compounds, like aromatic H compounds detected in cattle impacted soil and excrements. They contained mainly lignin-related structures including syringyl, guaiacyl, and p-hydroxyphenyl units. Syringyl and guaiacyl compounds belong to recalcitrant lignin structures and can be found mainly in gymnosperms and angiosperms, respectively (Martínez et al., 2005). All three substances are suggested as building blocks of graminoids lignin (Bland and Logan, 1965). Kirk and Farrell (1987) described negligible losses of lignin compounds under anaerobic conditions, which would help to explain high and relatively stabile content of H compounds in SI soil typical by increased anaerobes abundance. The reduction of zooedaphon diversity in the most loaded SI soil, as was described on the example of earthworms by Koubová et al. (2012), related to the distress of permanent grassland is partly responsible for slowing down the SOM transformation. On the other side, significant reduction of H compound was observed accompanied by the significant decrease of anaerobic biomass as well as by important increase of SOM enriched by N and P derivates during vegetation season at moderately impacted MI site, wich confirmed the high degree of SOM decomposition and the high microbial activity (Schulten and Schnitzer, 1992; Nierop et al., 2001).

The OMPP of the MI soil compared to SI soil was enriched also by fragments of nonaromatic compounds (L), e.g., polysaccharides or polymaleic acid related to soil fulvic acids (Saiz-Jimenez, 1994). Polysaccharide compounds are usually linked with the fresh SOM (Vancampenhout et al., 2009) and accumulate under conditions favorable for decomposition processes (Grandy et al., 2009). Ascomycetous, mycorrhizal fungi (Hatakka, 2001) and actinomycetes (McCarthy, 1987), that are able to degrade the lignin material and were significantly promoted during vegetation period (Jirout et al., 2009, 2011; Table 3), seem to be together with plants important drivers of SOM transformation in the studied soil. The ongoing research on the quantity and diversity of zooedaphon will contribute to complex information about the interaction between the edaphon and the SOM transformation within this ecosystem.

5. Conclusions

The in situ changes of soil microbial communities under the impact of intensive cattle husbandry were investigated. The study described specific phenomena of over-wintering pastures, where the cyclical high input of cattle excreta into the soil during the dormant season led to the development of a qualitatively new microbial community profile derived from ruminant microflora. The typical change of the cattle-impacted soils consisted of archaeal enrichment, which was responsible for the previously documented increases of CH_4 greenhouse gas emission from the soil at the end of the over-wintering period. The stability of the microbial changes due to the cattle outdoor over-wintering practice was dependent on the stability of the quantitative and qualitative changes of the SOM-impacted soil. This study increases our understanding of the persistence and activity of ruminant enteric microorganisms that are introduced into soil.

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Paper IV

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Anaerobic oxidation of methane in grassland soils used for cattle husbandry

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Abstract. While the importance of anaerobic methane oxidation has been reported for marine ecosystems, the role of this process in soils is still questionable. Grasslands used as pastures for cattle overwintering show an increase in anaerobic soil micro-sites caused by animal treading and excrement deposition. Therefore, anaerobic potential methane oxidation activity of severely impacted soil from a cattle winter pasture was investigated in an incubation experiment under anaerobic conditions using ^{13}C-labelled methane. We were able to detect a high microbial activity utilizing CH_4 as nutrient source shown by the respiration of $^{13}CO_2$. Measurements of possible terminal electron acceptors for anaerobic oxidation of methane were carried out. Soil sulfate concentrations were too low to explain the oxidation of the amount of methane added, but enough nitrate and iron(III) were detected. However, only nitrate was consumed during the experiment. ^{13}C-PLFA analyses clearly showed the utilization of CH_4 as nutrient source mainly by organisms harbouring 16 : 1ω7 PLFAs. These lipids were also found as most ^{13}C-enriched fatty acids by Raghoebarsing et al. (2006) after addition of $^{13}CH_4$ to an enrichment culture coupling denitrification of nitrate to anaerobic oxidation of methane. This might be an indication for anaerobic oxidation of methane by relatives of "*Candidatus* Methylomirabilis oxyfera" in the investigated grassland soil under the conditions of the incubation experiment.

1 Introduction

Methane is well known as a carbon and energy source for a specific group of methanotrophic microorganisms under aerobic conditions (Mancinelli, 1995). Today, however, a new role of methane has attracted the focus of research: the importance for the growth of microorganisms under anaerobic conditions (Thauer and Shima, 2008). Though the anaerobic oxidation of methane (AOM) was thought to be biochemically impossible for a long time and thus absent in nature (reviewed by Strous and Jetten, 2004), there has been recent evidence that this microbial mediated process could be a major factor in global carbon cycling (Shima and Thauer, 2005; Strous and Jetten, 2004; Valentine and Reeburgh, 2000; Smemo and Yavitt, 2011).

Different terminal electron acceptors have been described so far for AOM (Thauer and Shima, 2008; Smemo and Yavitt, 2011). Studies on marine sediments indicated the use of sulphate by a consortium of anaerobic methanotrophic archaea (so-called ANME) and sulphate-reducing bacteria (Strous and Jetten, 2004; Valentine and Reeburgh, 2000; Boetius et al., 2000; Hinrichs et al., 1999). This process is considered to consume most of the methane produced in marine sediments (Valentine, 2002). The reaction mechanism, its biochemistry and physiology are still under discussion and apparently involve reverse methanogenesis (Thauer and Shima, 2008).

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The second electron acceptor described so far is nitrate/nitrite (Ettwig et al., 2009, 2010; Raghoebarsing et al., 2006). In contrast to the microbial consortium using sulphate as electron acceptor under anaerobic conditions, which requires the close interaction of bacteria and archaea, it has been postulated that AOM in the presence of nitrate/nitrite can be catalyzed by one single bacterium which belongs to the so far uncultured phylum NC10 (Ettwig et al., 2009; Thauer and Shima, 2008). One of the very few studies focusing on anaerobic oxidation of methane investigates isotope fractionation factors for carbon and hydrogen during methane oxidation by an enrichment culture of "*Candidatus* M. oxyfera" (Rasigraf et al., 2012). Values were similar to those found for aerobic and other anaerobic methanotrophs. The results show that biological methane oxidation has a narrow range of fractionation factors for carbon and hydrogen irrespective of the underlying biochemical mechanism.

AOM coupled to nitrate reduction has been reported for various nutrient-rich aquatic habitats, including contaminated groundwater (Smith et al., 1991), sewage sludge (Islas-Lima et al., 2004), eutrophic canals and ditches (Ettwig et al., 2009), but also sediments of an oligotrophic freshwater lake (Deutzmann and Schink, 2011). Next to these aquatic habitats, the AOM was also recently described in drained peat and automorphic sod-podzol soils. The addition of oxidized compounds (e.g. nitrate) had a stimulatory effect on methane consumption in these soils (Pozdnyakov et al., 2011) under anaerobic conditions. The latter finding indicates that AOM might not be limited to aquatic systems only, but might also occur in other (soil) ecosystems, especially in soils with prevailing or frequently occurring larger anaerobic compartments, e.g. submerged soils, subsoils or mechanically compacted soils.

Pasture sites used for cattle overwintering in Central Europe are highly influenced by the animals. Compared to ordinary pastures, which are used for grazing during summer time, a much higher number of animals are present on a small area for a relatively long period. This causes a reduction in soil aeration through soil compaction (Menneer et al., 2005). The presence of animals is accompanied by a high input of organic material through excrements which consequently stimulates microbial metabolism, increases oxygen demand and supports the occurrence of anoxic (micro)sites in the soil (Šimek et al., 2006). Not surprisingly, Radl et al. (2007) could therefore demonstrate high abundance of methanogens in these soils during winter time as well as high methane production rates. Due to the prevailing anaerobic conditions and the high concentrations of ammonia from excrements in the soil, abundances of aerobic methane oxidizers carrying the *pmoA* gene were low (Radl et al., 2007). Nevertheless, in-situ methane oxidation rates in those soils were high; therefore, it has been postulated that AOM might play an important role in the investigated site for methane consumption.

To test this hypothesis, we incubated severely impacted soils from a cattle winter pasture under anaerobic conditions using ^{13}C-labelled methane. Soil from the same area, which was not used for cattle overwintering, served as control. Measurements of gases were performed using isotope ratio mass spectrometry (IRMS). Subsequent phospholipid fatty acid (PLFA) analysis provided insights into the microorganisms possibly involved in anaerobic methane oxidation.

2 Materials and methods

2.1 Site description and soil sampling

Soil samples were taken from a cattle-grazed farmland in Borová, South Bohemia, Czech Republic (48°52′ N, 14°13′ E), characterized by a mean annual temperature of 7 °C and a sum of precipitation of 650 mm. The soil is characterized as sandy loam and classified as Cambisol containing 60–80 % sand, 14–32 % silt and 6–14 % clay (for other details on the site and soil, see Hynšt et al., 2007 and Radl et al., 2007).

For the present study we sampled soils in April 2009, at the end of the overwintering period 2008/2009. Soils from two sites were sampled: a severely impacted site (SI) located in close proximity to the stables and being the overwintering area for the cattle, and a control plot (CO) which had not been used for cattle grazing and overwintering since 1999. The control had an intact plant cover consisting of a perennial mixture of grasses and dicotyledonous plants such as clovers (Jirout et al., 2009), while the plant cover of the severely impacted site was completely destroyed at the time of sampling, exposing the surface soil. Five independent field replicates (200 g each) from each site were randomly taken with a soil auger from 0–20 cm depth pooled for further analysis to reduce field heterogeneity, and stored at 4 °C for 2 weeks before the incubation experiment started.

2.2 ^{13}C labelling experiment

To generate anaerobic conditions, 10 g of soil were filled into a serum bottle (100 ml), followed by the adjustment of the water content to 100 % of the maximum water holding capacity using distilled water. Overall 13 bottles of SI and CO soil were prepared. Finally, the bottles were sealed with a butyl rubber septum. To ensure the absence of oxygen also in soil pores, bottles were purged with nitrogen (N_2, 5.0 grade) twice within 25 h. At each time the added nitrogen replaced the headspace atmosphere two times. Finally, 500 µl of labelled methane (20.2 % ^{13}C-enriched) were injected into 8 serum bottles with SI soil (SI_{CH_4}) and 8 bottles with CO soil (CO_{CH_4}). As a control, 5 replicates of SI and 5 replicates of CO soil were left without methane amendments (SI, CO).

The samples were subsequently maintained at a constant temperature of 14 °C on a CombiPAL Autosampler (CTC, Zwingen, Switzerland) for 7 days.

During the incubation, CO_2 and CH_4 concentrations, as well as their corresponding $^{13}C:^{12}C$ ratios in

headspaces, were determined on-line using a gas chromatography / isotope ratio mass spectrometer system (GC / IRMS; Finnigan MAT, delta plus, Germany). Part of the assembly was a modified PreCon Interface (Finnigan MAT) which enables also measurements of high CO_2 and CH_4 concentrations by varying the injection volumes. The fraction of CO_2 originating from oxidized CH_4 (f_{CH_4}) was calculated from

$$f_{CH_4} = \frac{^{13}C_{\text{emission treatment}} - ^{13}C_{\text{emission control}}}{^{13}C_{CH_4} - ^{13}C_{\text{emission control}}} \quad (1)$$

where $^{13}C_{CH_4}$ is the ^{13}C amount of the added CH_4 and $^{13}C_{\text{emission}}$ is the ^{13}C amount in the CO_2 emitted during a certain time period. $^{13}C_{\text{emission}}$ was calculated as follows:

$$^{13}C_{\text{emission}} = \frac{C_{n/n+1} \times ^{13}C_{n/n+1} - C_n \times ^{13}C_n}{C_{n/n+1} - C_n} \quad (2)$$

in which $C_{n/n+1}$ are the CO_2 concentration at points in time $n/n+1$ and $^{13}C_{n/n+1}$ are the ^{13}C amounts at points in time $n/n+1$.

At the end of the incubation experiment, soil subsamples for PLFA analysis were stored at −80 °C, while subsamples subjected to measurements of microbial biomass as well as abiotic soil properties were analyzed immediately.

2.3 Soil physical and chemical properties

Soils from both the incubation experiment (SI, CO, SI_{CH_4}, CO_{CH_4}) as well as from the time before the incubation experiment started (SI_{T0}, CO_{T0}) were extracted with 0.01 M $CaCl_2$ at a soil-to-liquid ratio of 1 : 2. For the determination of water extractable organic carbon (WEOC) and nitrogen (WEON), an analyzer DIMATOC 100 (DIMATEC Analysentechnik GmbH, Essen, Germany) was used. Determination of ammonium (NH_4^+-N) and nitrate (NO_3^--N) soil concentrations was done by continuous flow analysis with a photometric autoanalyzer (CFA-SAN Plus/Skalar Analytik, Germany). The content of reducible iron [Fe(III)] was determined using the hydroxylamine extraction method and spectrophotometric analysis (Lovley and Phillips, 1987). Sulphate was measured with the NANOCOLOR Sulphate 200 kit (Macherey-Nagel, Germany) at a soil-to-liquid ratio of 1 : 9.

Microbial biomass was determined by the chloroform-fumigation extraction method (Vance et al., 1987) with 1 g of soil in 16 ml 0.5 M K_2SO_4 solution. For the determination of microbial biomass carbon (C_{mic}), aliquots of the soils were fumigated with chloroform for 24 h prior to $CaCl_2$ extraction (Jörgensen and Brookes, 1991). C concentration and ^{13}C abundance were measured by a liquid chromatography/isotope ratio mass spectrometer system (LC/IRMS) (LC IsoLink coupled with MAT 253, both Thermo Finnigan, Bremen) as described by Krummen et al. (2004) and Marx et al. (2007). ^{13}C abundance was calculated by allegation alternate (Marx et al., 2007).

2.4 Phospholipid fatty acid analysis

Phospholipid fatty acid extraction was performed according to Zelles et al. (1995). Replicates from incubated SI soils with as well as respectively without methane (SI_{CH_4}, SI) were pooled and an equivalent of 10 g of dry soil were extracted with a mixture of 125 ml methanol, 63 ml chloroform and 50 ml phosphate buffer (0.05 M, pH 7).

After 2 h of horizontal shaking, 63 ml water and 63 ml chloroform were added to promote phase separation. After 24 h the water phase was removed and discarded. The total lipid extract was separated into neutral lipids, glycolipids and phospholipids on a silica-bonded phase column (SPE-SI 2 g / 12 ml; Bond Elut, Analytical Chem International, CA, USA). The phospholipid fraction was further separated into saturated (SATFA), monounsaturated (MUFA), polyunsaturated (PUFA), non-ester-linked unsubstituted (NEL-UNSFA) and hydroxy-substituted (NEL-UNOH) fatty acids (see Zelles et al., 1995 and Zelles, 1999 for details). PLFAs were analyzed as fatty acid methyl esters (FAME) on a gas chromatograph / mass spectrometry system (5973 MSD GC / MS Agilent Technologies, Palo Alto, USA) linked via a combustion unit to an isotope ratio mass spectrometer (GC / MS-C-IRMS, DeltaPlus^{Advantage}, Thermo Finnigan, Bremen, Germany). Separation and detection of FAME was performed via GC / MS, while the isotopic composition of fatty acids was detected after combustion (GC Combustion III, Thermo Finnigan, Bremen, Germany) in the IRMS. Columns and temperature programs were used according to Esperschuetz et al. (2009). The lipid fraction of monounsaturated fatty acid was measured underivatized to obtain a correct isotopic signal. After the first measurement, the sample was subjected to a DMDS-derivatization to identify the position of the double bond. The mass spectra of the individual FAMEs were identified by comparison with established fatty acid libraries (Solvit, Luzern, Switzerland) using MSD ChemStation (Version D.02.00.237).

Standard nomenclature for PLFA was used according to Frostegard et al. (1993): the number before the colon represents the number of C-atoms; the number after the colon gives the number of double bonds and their location from the aliphatic end (ω). The prefixes "cy-", "iso-" and "ant-" indicate cyclopropyl-groups, and iso- and anteiso-branching, respectively. Saturated straight-chained fatty acids were indicated by "n". The "br" and the number before a fatty acid indicate methyl-branching at the individual C-atom. The prefixes α and β indicate that the OH groups of an unsaponifiable OH fatty acid are located at positions 2 and 3, respectively, while numbers preceded by ω indicate the position of OH groups from the aliphatic end. Non-ester-linked (unsaponifiable) fatty acids were indicated by NEL (Zelles, 1999).

2.5 Statistical analysis

Prior to analysis, data were tested for normal distribution by Q-Q plots and the Kolmogorov-Smirnov test. Homogeneity of variances was checked by Levene's test. Soil chemical parameters were subjected to analysis of variance (two-factor ANOVA). Means were considered significantly different at $P \leq 0.05$. A linear mixed-effects model was fit to time series of CO_2 concentration originating from methane (Fig. 1). The model considered the fixed effects soil, time, and their interaction; repeated measures were considered as a random effect. Temporal autocorrelation of residuals was not significant and, consequently, was not modelled. All statistical calculations were carried out using SPSS software version 13.0 (SPSS Inc, Chicago, IL), except the linear mixed-effects model for which R 2.14.1 (www.R-project.org) with library *nlme* was used.

3 Results

3.1 Consumption of methane

In contrast to CO, SI showed a significant consumption of the added methane and production of carbon dioxide under anaerobic conditions (Fig. 1). Whereas in CO a maximum of 0.83 % of C incorporation after 6.4 days of methane addition was measured, the incorporation of carbon derived from CH_4 into CO_2 in SI samples was observed already after 2.8 days (2.4 %). The incorporation reached the maximum of 8.5 % after 4.4 days and then declined to 4.0 % after 6.2 days. The high standard deviations of mean C incorporated from CH_4 into CO_2 might be related to the fact that methane consumption in some replicates started later than in others.

Concentration increase of CO_2 originating from CH_4, measured by ^{13}C incorporation, with time was significantly ($p < 0.0001$) higher for SI soil than for CO soil. In total, the percentage of C in CO_2 originating from CH_4 oxidation was 22 % for the SI soil and 3.7 % for the CO soil. The methane added to SI soil samples incubated under anaerobic conditions was almost completely consumed (91.3 %). In the control treatments of SI soil without methane addition, the share of CH_4-C incorporated into CO_2 was below 0.03 % over the whole experimental period

3.2 Soil chemical properties

Content of water-extractable organic carbon (WEOC) was about ten times higher in SI soils compared to CO soils and was neither affected by the addition of methane nor by the incubation (Table 1). Content of water-extractable organic nitrogen (WEON) significantly decreased in SI soil during anaerobic incubation, from 13 to 8.2 and 9.7 $\mu g\,N\,g^{-1}$ dw in the treatment without methane and with methane, respectively. Nitrate concentrations in SI and CO soils were under or near the detection limit in the anaerobic treatments; however, values in the original soils were significantly higher and accounted for 23 $\mu g\ NO_3^-$-N g^{-1} of soil in SI and 9.0 $\mu g\ NO_3^-$-N g^{-1} in CO. Ammonium concentration increased significantly in SI soil samples from 1.0 $\mu g\,N\,g^{-1}$ dw before anaerobic incubation to 13 and 12 $\mu g\,N\,g^{-1}$ dw in treatments without and with methane amendment. Ammonium concentration in CO soils, however, did not change during the incubation (Table 1). Reducible iron was significantly higher in CO than in SI soils, but no significant difference between the treatments was found. The content of sulphate was below the detection limit (0.13 $mg\,S\,g^{-1}$ dw) in all soil samples.

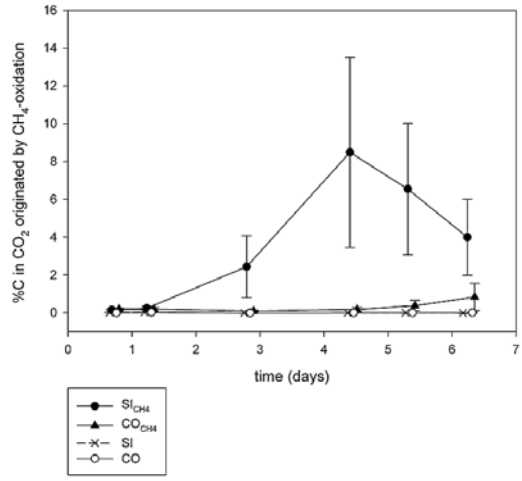

Fig. 1. Percentage of C incorporated into CO_2 originated from CH_4 at different time points ($f_{CH_4} \times 100\,\%$) for the severely impacted soil with methane (SI_{CH_4}), control soil with methane (CO_{CH_4}) and treatments without methane (SI, CO). Results are presented as means $\pm$ standard deviation ($n = 8$ for SI_{CH_4} and CO_{CH_4}; $n = 5$ for SI and CO).

3.3 Microbial biomass and microbial community structure

Microbial biomass (C_{mic}) was 3.7 times higher in SI soil compared to CO soil (Table 1). After methane addition and anaerobic incubation, C_{mic} was significantly enriched in ^{13}C (+19 ‰ VPDB) compared to the treatment without methane (−28 ‰ VPDB) in SI soil, while no enrichment was found in soil CO (Fig. 2).

In total, 69 different PLFAs were detected in SI_{CH_4} and SI treatments. Since CH_4 was enriched with ^{13}C, PLFAs of methane-oxidizing microbial communities showed increased $\delta^{13}C$ values compared to the controls. In Fig. 3, a ranking is given of PLFAs with high ^{13}C incorporation. Highest ^{13}C enrichment was detected in monounsaturated

Table 1. Soil characteristics of SI and CO before the incubation experiment started (CO_{T0}, SI_{T0}) and after major anaerobic experiment without (CO, SI) and with methane (CO_{CH_4}, SI_{CH_4}). Different parameters were measured: pH value ($CaCl_2$), water extractable organic carbon (WEOC) and nitrogen (WEON), nitrate and ammonium concentrations, reducible iron [Fe(III)], microbial biomass (C_{mic}) and $\delta^{13}C_{mic}$. Means and standard deviations, significant differences among treatments at $p < 0.05$ are indicated by different letters (a, b, c).

Soil parameters	CO_{T0}	CO	CO_{CH_4}	SI_{T0}	SI	SI_{CH_4}
pH	5.3	5.8	5.8	7.3	7.1	7.1
WEOC ($\mu g\,g^{-1}$ dw)	27*a* (2.6)	23*a* (8.2)	23*a* (4.6)	250*b* (39)	220*b* (13)	230*b* (23)
WEON ($\mu g\,g^{-1}$ dw)	2.6*a* (0.89)	b.d.l.[a]	0.35*b* (0.63)	13*c* (1.2)	8.2*d* (0.67)	9.7*e* (0.55)
Nitrate ($\mu g\,N\,g^{-1}$ dw)	9.0*a* (0.0)	0.25*b* (0.43)	b.d.l.[a]	23*c* (2.1)	b.d.l.	b.d.l.
Ammonium ($\mu g\,N\,g^{-1}$ dw)	1.2*a* (0.24)	2.4*a* (0.56)	1.7*a* (0.38)	1.0*a* (0.82)	13*b* (1.5)	12*b* (1.5)
Iron ($\mu g\,g^{-1}$ dw)	140*a* (15)	130*a* (8.6)	120*a* (21)	83*b* (20)	69*b* (7.7)	63*b* (6.6)
C_{mic} ($\mu g\,g^{-1}$ dw)	150*a* (16)	140*a* (16)	140*a* (16)	560*b* (58)	490*b* (35)	460*b* (63)
δ^{13}Cmic (‰ VPDB[b])	−26*a* (0.21)	−26*a* (0.25)	−24*a* (1.8)	−28*b* (0.19)	−28*b* (0.27)	19*c* (31)

[a] Below detection limit, meaning below 0.2 $\mu g\,N\,g^{-1}$ soil dry weight.
[b] Vienna Pee Dee Belemnite.

16 : 1ω7 (+330 ‰ δ^{13}C) and two unsaponifiable, non-ester-linked PLFAs of unknown branching (ubr16 : 0, +284 ‰ and +288 ‰ δ^{13}C). The absolute amount of labelled carbon incorporation into PLFAs is shown in Fig. 4. By far highest values were found for 16 : 1ω7 (0.36 nmol C g^{-1} dw out of CH_4), followed by n14 : 0, n16 : 0 16 : 1ω5 and iso 15 : 0 where uptake rates were all more than a factor of 10 lower compared to 16 : 1ω7.

4 Discussion

Anaerobic methane oxidation (AOM) has been reported as important methane-consuming process in aquatic habitats, mainly marine ecosystems (Valentine, 2002). For soils the importance of this process is still questionable. Anaerobic conditions in soils might not only be caused by flooding, but also by soil compaction and aggregation. Grasslands used as pastures for cattle overwintering show an increase in anaerobic soil micro-sites caused by animal treading and excrement deposition. Not only the high content of carbon itself, but also the resulting increases in microbial biomass (72 % higher in cattle highly impacted SI soil compared to unimpacted CO soil) and activity may stimulate soil aggregation (Martens and Frankenberger, 1992). An increase of the microbial biomass in SI was confirmed in the study of Elhottová et al. (2012). The latter study also showed different microbial community diversity in SI compared to CO based on extended polar lipid analysis.

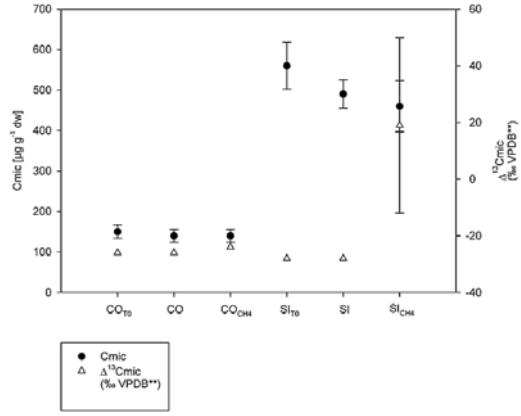

Fig. 2. Microbial biomass (Cmic) and δ^{13}C content of microbial biomass (δ^{13}Cmic) before (CO_{T0}, SI_{T0}) and after the incubation experiment without (CO, SI) and with methane (CO_{CH_4}, SI_{CH_4}). Results are presented as means ± standard deviation ($n = 8$ for SI_{CH_4} and CO_{CH_4}; $n = 5$ for SI_{T0}, CO_{T0} and SI, CO).
** Vienna Pee Dee Belemnite.

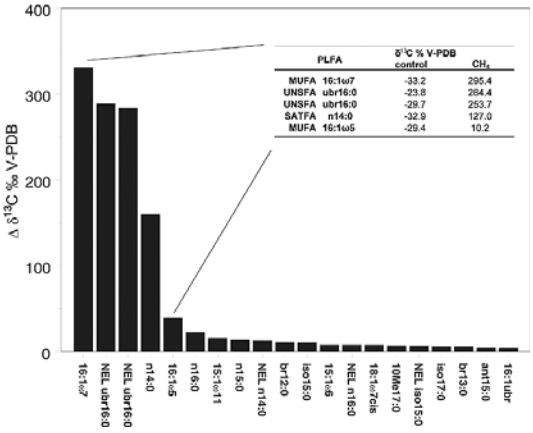

Fig. 3. Ranking of the δ^{13}C enrichment in PLFA of SI_{CH_4} against SI. Values of the five most ^{13}C-enriched lipids are given in the inset table.

Our stable isotope experiment, using ^{13}C-labelled methane, allowed us to separate methane production and consumption processes occurring simultaneously. The percentage of CH_4-C incorporated into CO_2 was significantly increased in anaerobically incubated SI soil compared to

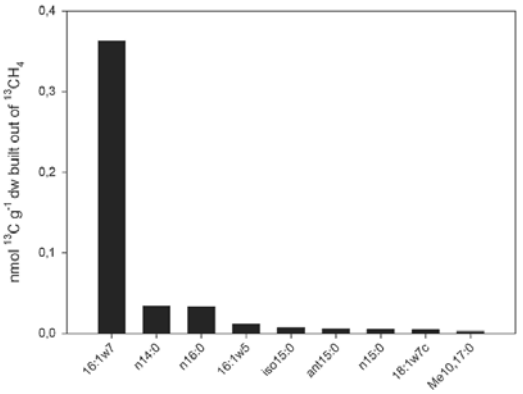

Fig. 4. Ranking of the absolute carbon incorporation from labelled methane into PLFAs of SI_{CH_4}.

CO soil (Fig. 1). This gives clear evidence that AOM occurs in the severely impacted soil under the conditions of the incubation experiment.

No oxygen is supposed to be left in soil pores, as a slurry was used and flushed in addition repeatedly using O_2-free nitrogen. However, the possible amount of oxygen in the soil pores was calculated theoretically. At a conservatively estimated dry bulk density of 1.0 g cm^{-3}, if all water-free pores were filled with air containing 21 % oxygen, 6.2 mmol O_2 would be available as oxidizing reactant. However, 22.4 mmol O_2 are necessary to fully oxidize 500 µl of CH_4. Thus, oxidation of 73 % of the 500 µl CH_4 added cannot be fully explained by aerobic oxidation, even if one assumes that free oxygen was present in soil and that it would not be consumed by oxidation of other substrates. Both of these assumptions are extremely unlikely. Furthermore, decreasing amounts of nitrate and increasing amounts of ammonium (Table 1) indicate absence of nitrification and prevalence of denitrification or other anaerobic processes during incubation, which also confirms that the soil was actually anaerobic.

The presence of other possible electron acceptors for AOM was checked. Sulphate concentrations in SI and CO soils were below the detection limit of 0.13 mg S g^{-1} dw. Theoretical calculations showed that at least 126 µg SO_4^{2-} g^{-1} soil would have been necessary to oxidize the added 500 µl CH_4 (for reaction equation see Scheller et al., 2010). Therefore, it is very likely that sulphate was not the terminal electron acceptor for AOM in the investigated grassland soil. Furthermore, so far AOM coupled to sulphate reduction was only described for marine systems (Hinrichs et al., 1999; Boetius et al., 2000; Strous and Jetten, 2004; Valentine and Reeburgh, 2000).

It was also proposed that Fe(III) could serve as electron acceptor for AOM in freshwater wetlands (Murase and Kimura, 1994; Daniel et al., 1999). The reaction is energetically favourable (Smemo and Yavitt, 2011). In the present experiment, iron concentrations ranged between 63 and 83 µg g^{-1} in the different treatments of the SI soil and between 120 and 140 µg g^{-1} in the CO soil. The theoretical amount of Fe(III) necessary for the oxidation of 500 µl methane is ca 73 µg g^{-1} soil (for reaction equation, see Daniel et al., 1999). Thus, enough Fe(III) has been in SI soil to serve as terminal electron acceptor for anaerobic oxidation of methane. But only 20 µg g^{-1} dw were consumed in SI_{CH_4} during the experiment.

Taking the recent results into account, it is most likely that nitrate/nitrite functioned as electron acceptor for AOM in the SI soil. So far, investigated enrichment cultures preferred nitrite over nitrate. But it remains to be shown whether nitrate can actually be used by "*Candidatus* Methylomirabilis oxyfera" (Wu et al., 2011). The concentrations of nitrate in the severely impacted soil declined from 23 µg NO_3^--N g^{-1} dw soil to below detection limit after anaerobic conditions were established, but a significant decline was also observed for the control soil and no difference was found between the treatments with and without methane. This might be due to a high activity of denitrifying organisms previously found in the soil SI (Chronáková et al., 2009). The amount of NO_3^--N necessary to oxidize 500 µl of methane would theoretically be 29 µg g^{-1} soil (for reaction equation, see Raghoebarsing et al., 2006). Taking methodological biases into account, there might have been enough nitrate for the oxidation of the added methane in our incubation experiment.

Significantly increased amounts of ammonium concentrations in the anaerobically incubated SI soils (13 and 12 µg g^{-1} soil) compared to SI soil after aerobic preincubation (1 µg g^{-1} soil) might indicate that dissimilatory nitrate reduction to ammonia (DNRA) occurred. This process takes place under low oxygen conditions like denitrification and might be favoured under high C / NO_3^- ratios when the electron acceptor (NO_3^-) becomes limiting (Tiedje et al., 1983), as it has been reported for SI. If nitrate/nitrite is the electron acceptor for AOM in the system, there might be a competition for electron acceptors between the two processes. Another possible explanation for the increasing amount of ammonia points towards the mineralization of organic nitrogen and its accumulation resulting from the lack of oxygen (Ghosh and Bhat, 1998).

The finding that the ^{13}C-label of methane finally appeared in the microbial biomass (Fig. 2) in soils from SI under anaerobic conditions confirms that CH_4-C was not only oxidized, but also incorporated by the respective organisms. This provided the opportunity to analyze ^{13}C-marked phospholipid fatty acids. Highest relative and absolute label incorporation was detected in PLFA 16 : 1ω7. This indicates that organisms harbouring 16 : 1ω7 are highly active in degrading methane under anaerobic conditions. This assumption is supported by the ratio of ω7 monounsaturated fatty acids (ω7) to

cyclopropylic fatty acids (cy). The latter named cyclic derivatives of $\omega 7$ MUFAs operate as membrane-stabilizing fatty acids. They are reported to be formed under environmental stress conditions such as starvation (Findlay and Dobbs, 1993; Frostegard et al., 1993). While in the SI soil without methane a $\omega 7/\mathrm{cy}$ ratio of 1.7 was observed, it increased to values between 3.1 and 5.3 in the methane-treated SI soil. This indicates that methane was used as nutrient source by these organisms, and hence the nutritional stress decreased.

PLFA $16:1\omega 7$ was detected as the major PLFA in different *Methylococcus* and *Methylomonas* strains belonging to the type I methane-utilizing bacteria by Bowman et al. (1991). But it was also found in strictly and syntrophic anaerobes (Leckie, 2005; Evershed et al., 2006; Zelles, 1997; Henson et al., 1988) and different Gram-negative organisms (Boschker et al., 1998; Holmes et al., 1999; White, 1994). The lack of specific biomarkers for type I methanotrophs, that is to say $16:1\omega 8$, as well as type II methanotrophs ($18:1\omega 8$) (Bowman et al., 1991) in the soil of the incubation experiment is an indication that common aerobic methanotrophs of the type I and II cluster are unlikely to explain the observed methane uptake pattern. In contrast, Raghoebarsing et al. (2006) found $16:1\omega 7$ also as the highest enriched PLFA in a ^{13}C labelling experiment with an enrichment culture coupling denitrification of nitrate to anaerobic oxidation of methane. In addition, n14 : 0, n16 : 0 and iso 15 : 0 which incorporated next to $16:1\omega 7$ the highest amount of C out of CH_4 in this incubation experiment (Fig. 4), were also found to be ^{13}C-enriched in the study of Raghoebarsing et al. (2006). This is an indication of nitrate/nitrite-based AOM in the incubation experiment of this study, maybe by relatives of "*Candidatus* Methylomirabilis oxyfera".

A high absolute degree of label incorporation was also observed for $16:1\omega 5$ which is supposed to be a specific component of type I methanotrophs (Bowman et al., 1991). The unknown branched, unsaponifiable 16 : 0 PLFAs are highly ^{13}C-enriched, but low abundant. This does not exclude that they play a role in oxidizing methane under anaerobic conditions. These lipids were found in different anaerobe organisms (Ratledge and Wilkinson, 1988; Henson et al., 1988). It cannot be excluded that the incorporation could also be due to the consumption of ^{13}C-CH_4-derived ^{13}C-CO_2 or feeding on intermediate products. The intensity of the labelling, however, suggests that the lipids in question must be at least partially of methanotrophic origin.

5 Conclusions

Results from the present study give clear evidence for an anaerobic activity of methane-oxidizing microorganisms in severely cattle-impacted grassland soil. In a stable isotope experiment using ^{13}C-enriched CH_4, we were able to detect a high microbial activity utilizing CH_4 as carbon source shown by respiration of $^{13}CO_2$. Thus, there is evidence that soil microorganisms have the potential to perform anaerobic oxidation of methane. Moreover, ^{13}C-PLFA analyses clearly showed the utilization of CH_4 as carbon source mainly by organisms harbouring $16:1\omega 7$ PLFAs. These lipids were also found as most ^{13}C-enriched fatty acids by Raghoebarsing et al. (2006) after addition of $^{13}CH_4$ to an enrichment culture coupling denitrification of nitrate to anaerobic oxidation of methane. This might be an indication for anaerobic oxidation of methane by relatives of "*Candidatus* Methylomirabilis oxyfera" in the investigated grassland soil under the conditions of the incubation experiment. However, the used approach in this experiment does not allow differentiating if the metabolized methane is used by the corresponding microbes for an increase in biomass or changed ecophysiological properties (and consequently changed PLFA profiles). Furthermore, it is unclear how much of the applied methane is transformed into CO_2 or other metabolites, which are not integrated into the cell-derived carbon. Therefore, a quantitative assessment of the process is not possible based on our data. To overcome this problem, further molecular analyses, including sequencing approaches, are necessary to characterize anaerobic methane oxidizers in different soils.

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Paper V

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Effects of the earthworm *Eisenia andrei* on methanogens in a cattle-impacted soil: A microcosm study

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ABSTRACT

Cattle treading accompanied by a high input of organic matter was previously found to favour methanogenesis in soils at a site used as winter pasture in outdoor cattle husbandry. In this current study, the phylogenetic microarray AnaeroChip revealed high methanogenic diversity in a cattle-impacted soil with predominance of *Methanosarcina*, and presence of *Methanoculleus*, *Methanobacterium*, *Methanocalculus*, *Methanobrevibacter*, *Methanosaeta*, *Methanothermobacter*, *Methanogenium*, *Methanohalobium*, and *Methanolobus*. The bioturbation effects of an epigeic earthworm, *Eisenia andrei*, on the methanogenic microbial community in cattle-impacted soil were studied in a 6-month laboratory microcosm experiment. The microarray showed that the methanogenic community was changed by addition of earthworms to the soil. The abundance of 16S rRNA *Methanosarcina* gene copies decreased two fold in soil with worms compared to soil without worms after 2 months of incubation and decreased three fold after 4 and 6 months of incubation. The biomass of anaerobic microorganisms, as determined by unsubstituted non-ester-linked phospholipid fatty acid analysis, decreased in soil incubated for 4 and 6 months with worms. The abundance of the methyl-coenzyme M reductase (*mcrA*) gene, which is involved in CH_4 production and is present in all methanogens, was not, however, changed by worms, and addition of worms even increased the rate of methane production. This study provides the first data concerning interactions between earthworms and methanogens in cattle-impacted pasture soil. The results of this laboratory microcosm experiment indicate that *E. andrei* changes the composition of the soil methanogenic community but does not reduce the total abundance of the methanogenic community and methane production rate.

1. Introduction

Agricultural practices in Central Europe, especially in the submontane areas, are characterized by outdoor cattle husbandry [45,53]. Soils at the winter pastures, immediately near cattle barns, are nutrient-saturated and replenished by organic carbon and nitrogen from cattle dung and urine which accumulate during the winter [21,26,58]. Increased organic matter, higher water content, and soil compaction resulting from animal trampling likely reduce soil aeration. Consequently, anaerobic microorganisms including denitrifiers [7] and methanogens [46] proliferate in such soils. Promotion of anaerobic processes increases emission of the greenhouse gases methane and nitrous oxide, especially at the end of the overwintering period [25,46,50].

Methanogenic archaea including representatives derived from the ruminant microflora are often a relatively abundant and stable component of soils fertilised with cattle manure [16]. Soils in pasture ecosystems with a high supply of nutrients revert from a sink to a source of methane [8]. Radl et al. [46] revealed that cattle-impacted soils at a winter pasture showed huge CH_4 production, reaching 30–60 mg C–CH_4 m^{-2} h^{-1} at the end of the overwintering season when the conditions were suitable for microbial activity. Moreover, the analyses of the microbial community in a severely cattle-impacted soil indicated a high abundance of archaeal polar lipids and the methyl-coenzyme M reductase (*mcrA*) gene [46].

Restoration of soils at winter pastures is required for sustaining a healthy and productive agricultural system [26]. Recovery of the

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original aeration status seems to be crucial in the greenhouse gas balance at a global level [56]. Aeration of soil and therefore the activity of microorganisms involved in methane emission from soil are affected by earthworms [51]. Several studies have focused on the effects of earthworm on methanotrophs but the direct effects of earthworms on the soil methanogenic community remain unclear. Park et al. [42] revealed that amendments of landfill cover soils with earthworm casts increased the abundance of methanotrophs and stimulated the CH_4-oxidizing capacity. Moon et al. [38] obtained similar results and highlighted the role of casts as a filter-bed material to reduce methane emissions from landfills. Héry et al. [23] confirmed the stimulation of net methane consumption in landfill cover soil inoculated with the earthworm *Eisenia fetida*; the active methanotrophic community, however, did not differ significantly in the presence or absence of earthworms. Singer et al. [51] showed that the earthworm *Pheretima hawayana* enriched PCB-contaminated soils with microorganisms capable of methane oxidation.

The direct interactions between earthworms and methanogens have been studied at the level of methanogen detection in the earthworm intestine tract. Although considerable amounts of methane are produced in the intestines of many soil invertebrates, e.g., diplopods and larvae of tropical scarabaeid beetles [11,34,54], no methane release has so far been detected from the earthworm gut [31,54]. In addition, methanogens could not be isolated from the intestines of *Lumbricus rubellus* or *Octolasion lacteum* [31] or detected with a phylogenetic microarray in *L. rubellus* guts (B. Knapp and M. Goberna, personal observation).

The epigeic earthworm *Eisenia andrei* inhabits organic composts and manure heaps [44]. This species is valuable in vermicomposting [57] and has often been used in bioremediation of contaminated soils [9,24,48]. In this study, we hypothesised that *E. andrei* might reduce the diversity and biomass of the methanogenic community in a severely cattle-impacted soil with high methane emission. To test this hypothesis, we conducted a 6-month laboratory microcosm experiment that measured the bioturbation effects of *E. andrei* on the methanogenic community in the cattle-impacted soil collected from a cattle winter pasture. The 6-month incubation of soil simulated the approximately 6-month period when the cattle-impacted soils typically regenerate after the winter period in Central Europe. The objective of this study was to describe the methanogenic community in cattle-impacted soil and to determine whether addition of the compost earthworm *E. andrei* to cattle-impacted soils changes (i) methanogenic diversity, (ii) the abundance of the most common methanogenic species, (iii) the abundance of the methanogens, (iv) the microbial biomass of the anaerobic microbial community, and (v) the potential methane production rate.

2. Materials and methods

2.1. Soils and earthworms

Pasture soil for the experiment was obtained from an organically managed farm (Borová) near Český Krumlov, South Bohemia, Czech Republic. Further details about the study area are given by Radl et al. [46] and Šimek et al. [50]. Soil was collected from two sites at the end of the overwintering period in May 2008: (i) severely cattle-impacted (**SI**) soil was collected near the cattle barn, and (ii) control non-impacted (**NI**) soil was collected from a fenced, cattle-free area about 300 m from the cattle barn. The soil was a sandy loam originally classified as Cambisol and recently reclassified as Haplic Phaeozem (arenic; WRB system) containing 60–80% sand, 14–32% silt, and 6–14% clay (USDA classification system). The control site (NI) had 100% vegetation cover that included a mixture of perennial grasses, clovers, and other dicotyledonous plants. Site SI, in contrast, had only 60% vegetation cover during the vegetation period [28], and this cover was enriched by a mixture of fast growing plants including ruderal herbs (*Polygonum* sp., *Plantago major*, *Galinsoga parviflora*), grass (*Echinochloa cruss-galli*), and red clover (*Trifolium pratense*). By the end of the overwintering period, the vegetation at site SI was trampled into the soil, and the soil surface was destroyed and saturated with cattle excrements. The earthworm community at site NI consisted of 8 species (*Aporrectodea caliginosa*, *A. trapezoides*, *A. rosea*, *Dendrobaena octaedra*, *Lumbricus castaneus*, *L. rubellus*, *L. terrestris*, and *O. lacteum*) with 198–259 individuals m^{-2}. Site SI, in contrast, contained only one species (*E. fetida*) and only 3 individuals m^{-2}.

Soil was taken from the upper 0–20 cm layer, and sieved samples (5-mm mesh) were stored at 4 °C until the start of the experiment. The characteristics of SI and NI soil were determined immediately before the experiment (Table 1). Soil moisture (g of water per g of dry soil) was assessed gravimetrically by drying the soil at 105 °C for 5 h. Total organic carbon (C_{org}) was determined by wet oxidation with acid dichromate, and total nitrogen (N_{tot}) was determined by Kjeldahl digestion [59]. The methods used to determine the other soil characteristics are given in detail below. Specimens of the earthworm *E. andrei* were obtained from a managed grass vermicompost (Biology Centre AS CR, České Budějovice, Czech Republic).

2.2. Microcosms and experimental design

The microcosms consisted of 1000-ml serum bottles filled with 500 g of fresh soil. Three treatments were established: (i) SI soil with earthworms (**W**), (ii) SI soil without earthworms (**S**), and (iii) NI soil without earthworms (**N**). Each treatment was represented by nine microcosms. For treatment W, 10 immature worms of similar body weight were cleaned in sterile water and added to the microcosms. Total initial fresh weight of worms per bottle was 3.3 ± 0.3 g (mean ± SD). The bottles were covered with a plastic mesh held tightly with a rubber band to prevent earthworms from escaping and to allow aeration. All microcosms were placed in an incubator at 15 °C in the dark. Original soil moisture (Table 1) was kept constant during the experiment by weighing and regularly adding sterile tap water as needed. Soils were incubated for 2 (T2), 4 (T4), and 6 months (T6). At the end of each incubation period, three microcosms per treatment were examined; the earthworms

Table 1
Main properties of severely cattle-impacted soil (SI) and non-impacted soil (NI) evaluated before the setup of the microcosm experiment. Values are means (±SD) of triplicates. Different lowercase letters in a row indicate significant differences between soils SI and NI at $P \leq 0.05$. Differences between means were evaluated using Student's *t*-test for independent samples.

Characteristics	Treatments	
	SI	NI
Moisture [g H_2O g^{-1} dw]	0.58 ± 0.10 [a]	0.28 ± 0.02 [b]
Organic carbon C_{org} [mg g^{-1} dw]	64.9 ± 3.7 [a]	17.1 ± 1.4 [b]
Total nitrogen N_{tot} [mg g^{-1} dw]	10.1 ± 0.5 [a]	5.0 ± 1.0 [b]
CH_4 production rate [ng C g^{-1} dw d^{-1}]	853.2 ± 278.3 [a]	16.8 ± 11.4 [b]
CO_2 production rate [μg C g^{-1} dw d^{-1}]	605.2 ± 18.3 [a]	19.1 ± 0.8 [b]
Total microbial biomass [nmol $PLFA_{tot}$ g^{-1} dw]	132.6 ± 6.1 [a]	23.7 ± 9.9 [b]
Biomass of anaerobes [nmol unsNEL-PLFA g^{-1} dw]	7.0 ± 1.3 [a]	1.6 ± 0.6 [b]
mcrA gene copy number [*mcrA* copies g^{-1} dw]	1.7 ± 0.3 × 10^{6} [a]	9.7 ± 9.8 × 10^{3} [b]
Methanosarcina 16S rRNA gene copy number [Msar copies g^{-1} dw]	3.4 ± 0.6 × 10^{7} [a]	6.8 ± 4.0 × 10^{4} [b]

Note: dw = dry weight.

were cleaned on a wet filter paper and weighed. Soils were gently homogenised and immediately used for biochemical analyses and measurement of methane production or were frozen at −20 °C for molecular analyses.

The experimental design was based on previous studies involving addition of earthworms to soil [1,2,61] and on a pilot microcosm experiment conducted in our laboratory. This pilot experiment tested the growth of *E. andrei* in both NI and SI soils sampled in May 2007. The mentioned trial, which used the same environmental conditions as the main experiment, indicated that *E. andrei* grew and reproduced satisfactorily when 10 individuals were added per 500 g of fresh SI soil (unpublished data). On the other hand, *E. andrei* suffered high mortality (50%) in the NI soil (unpublished data), and we inferred that non-impacted grassland soil provided unsuitable conditions for this compost earthworm. Consequently, the microcosm experiment did not include the incubation of the earthworms in NI soil.

2.3. DNA extraction

Total DNA was extracted from 0.25 g of soil using the Power Soil DNA Isolation Kit (MoBio Laboratories, Carlsbad, USA) according to the manufacturer's protocol. Extracted DNA was quantified with a spectrophotometer (Genesys 6, Thermo Spectronic, USA) and subjected to electrophoresis on 1% agarose gel using 1× TAE buffer and ethidium bromide staining [49].

2.4. Screening of methanogenic diversity with the microarray

To screen the methanogenic diversity, we used the Anaero-Chip, a phylogenetic microarray targeting the 16S rRNA gene of most lineages of methanogens [15]. Soil DNA was PCR-amplified using 5′ Cy5-labeled 109F (5′- ACKGCTCAGTAACACGT) and 5′ PO_4^{2-}-labeled 934R primers (5′- GTGCTCCCCCGCCAATTCCT) [19]., digested, hybridised with the arrays, and fluorescently scanned as described in detail by Franke-Whittle et al. [15]. Microarray slides were scanned with a ScanArray Express microarray scanner (Perkin Elmer, Shelton, USA) at wavelengths of 543 nm and 633 nm. Fluorescent images were evaluated with ScanArray Express software (Perkin Elmer, Shelton, USA). The signal-to-noise ratio (SNR) was calculated, and all the positive probes ($SNR \geq 2$) were included in the evaluations [35]. Signals above the detection limit could be obtained when more than 0.4 pg of DNA from a pure culture is subjected to PCR amplification [15]. Array design, protocol, and experimental data can be accessed at ArrayExpress (code: E-MEXP-2843; http://www.ebi.ac.uk/arrayexpress/).

2.5. Quantification of mcrA and Methanosarcina 16S rRNA gene copies by real-time PCR

Total methanogens were quantified by real-time PCR amplification of the *mcrA* gene. The *mcrA* gene is a characteristic functional marker coding for the α-subunit of methyl-coenzyme M reductase, a key enzyme of methanogenesis [36]. Real-time PCR was performed in a StepOnePlus™ Real-Time PCR System (Applied Biosystems, USA). Reaction mixtures contained 10 μl of Fast SYBR® Green PCR Master mix (Applied Biosystems, USA), 5 μg of BSA (MBI Fermentas, Lithuania), 10 pmol of each primer ME1 (5′-CMATG-CARATHGGWATGTC-3′ [20];) and MCR1R (5′-ARCCADATYTGR TCRTA-3′ [52];), 1 μl of DNA template, and distilled water to a final volume of 20 μl. The amplification was carried out using a protocol by Kim et al. [33] with the modification of annealing temperature as follows: initial denaturation at 95 °C for 10 min; followed by 40 cycles at 94 °C for 1 min, 64 °C for 1 min (decreased by 0.5 °C each cycle for the first 10 cycles), and 72 °C for 1 min; and a final extension at 76 °C for 10 s. Standard curves were constructed using 10-fold serial dilutions of plasmids containing a partial sequence of the *Methanosarcina barkeri mcrA* gene.

The most abundant methanogenic archaeal genus detected by the microarray, *Methanosarcina*, was quantified using a genus-specific assay targeting 16S rRNA sequences. PCR was conducted in a Rotor-Gene 6000 (Corbett Life Sciences, Australia). Reaction mixtures included 10 μl of 1× Quantimix Easy SYG kit (Biotools, Spain), 8 μg of BSA, 2 pmol of each primer 240F (5′-CTATCAGG-TAGTAGTGGGTGTAAT-3′) and 589R (5′-CCCGGAGGACTGACCAAA-3′) [14], 2 μl of DNA template (1/10 diluted), and distilled water to a final volume of 20 μl. Thermocycling was as follows: 95 °C for 5 min followed by 40 cycles of 20 s at 95 °C, 20 s at 64 °C, and 20 s at 72 °C.

Standard curves were constructed using PCR-amplified 16S rRNA genes from a pure culture of *Methanosarcina barkeri* (DSM 800; Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany). PCR products were purified using NucleoSpin Extract II (Macherey–Nagel, Düren, Germany), and five 10-fold serial dilutions were used to construct the standard curve.

Both real-time PCR runs were completed with a melting analysis (65–95 °C, ramp 0.5 °C min^{-1}) to check for product specificity and primer dimer formation. All samples and standards were run in duplicates. Amplification efficiencies of the PCR reactions were calculated using data from the standard curves with the following formula: Efficiency $= [10^{(-1/slope)}]$-1. The quality of the amplification was evaluated by the generation of melting curves of the PCR products and confirmed by electrophoresis on 1% agarose gels stained with ethidium bromide.

2.6. Measurement of CO_2 and CH_4 production

Carbon dioxide production was evaluated in SI and NI soils at the start of the microcosm experiment. Soils were homogenised, and CO_2 production rate was determined by measuring CO_2 emitted from 15 g of wet soil incubated in 100-ml serum bottles ($n = 4$) kept at 25 °C for 24 h. After 2 and 24 h, 0.5-ml gas samples were analysed using a gas chromatograph (Hewlett–Packard 6850 equipped with TCD). CO_2 production rate was calculated from CO_2 increase during the 22 h incubation. The total headspace in each bottle was determined, and the amounts of CO_2 were corrected for gas dissolved in the soil liquid phase.

Methane production was evaluated in soils from all treatments before the start of the experiment and at the end of each incubation period. Soils were homogenised, and 15 g of wet soil was weighed into 100-ml serum bottles. Bottles were sealed with butyl rubber stoppers and were evacuated and flushed with argon four times. Each evacuation (up to −0.01 MPa) and/or flushing (up to 0.11 MPa) lasted 2 min, and at the end the internal atmosphere did not contain detectable oxygen, as confirmed by gas chromatography. An anoxic incubation atmosphere was used to optimize aeration conditions for methanogenesis; incubation temperature was adjusted to 25 °C according to our previous experience with the measurements of methane production from the soils under study. After 0, 24, 48, and 72 h, 0.5-ml gas samples were taken with a gas-tight syringe and analysed for CH_4 using a gas chromatograph (Hewlett–Packard 5890 Series II equipped with a FID). Methane production was finally calculated from CH_4 increases during the first 48 h incubation when the time course of CH_4 concentration best fitted the linear function. Headspace measurements and corrections were made as explained above for CO_2 production. The results for CO_2 and CH_4 production rates were expressed as C (g dry $soil)^{-1}$ day^{-1}.

2.7. Polar lipid analysis (PLA)

Microbial biomass of anaerobes was evaluated using an extended method of polar lipid analysis (PLA [17,60];), which allows for an evaluation of unsubstituted non-ester-linked fatty acids (unsNEL-PLFA) indicative of anaerobic microorganisms (bacteria, archaea, and microeukaryotes). Extended PLA was applied with following modifications: total lipids were extracted from a fresh soil sample equivalent to 10 g of dry soil. Phospholipid-methylesters were fractionated using an aminopropyl-bonded phase column (Chromabond SPE-NH_2, Macherey–Nagel, Germany) only in two fatty acid groups (ester- and non-ester-linked fatty acids [EL-, NEL-PLFAs]) without further separation by solid phase extraction. The EL-PLFA and NEL-PLFA profiles were identified separately by gas chromatography (Agilent 6850, Agilent Technologies, USA) with a flame ionization detector on a capillary column (Ultra 2, 25 m, 0.20 mm, 0.33 μm, Agilent Technologies, USA). The samples (1 μl) were injected in a split mode (1:100), with 250 °C injection temperature and H_2 as a carrier gas. The column temperature regime was held at 170 °C with a ramp of 5 °C min^{-1}, followed by 260 °C with a ramp of 40 °C min^{-1} and a final temperature of 310 °C for 1.5 min. The individual peaks in the EL-PLFAs were identified with the TSBA6 Library, and those in the NEL-PLFAs were identified using Anaero6 Library software of the MIS Sherlock System (ver. 6.0, MIDI, Inc., USA). The sum of EL-PLFA and NEL-PLFA content was used as an indicator of total microbial biomass and the content of the NEL-PLFAs as indicator of the anaerobic microbial biomass.

2.8. Statistical analyses

Data on SI and NI soils analysed at the beginning of the experiment were tested for differences using Student's *t*-test for independent samples. The effects of the treatments (N, S, and W) and incubation time (T2, T4, and T6) were tested by two-way analysis of variance (ANOVA). Tukey's *post hoc* test was used for mean separation. Means were considered significantly different at $P \leq 0.05$. All statistical analyses were performed in SPSS ver. 17.0. Principal component analysis (PCA) was applied to visualise the microarray data (probes with SNR $\geq$ 2 in one or more samples) based on a covariance matrix. PCA was focused on inter-sample distances and was performed with log-transformed data using Canoco for Windows 4.5 (Centre for Biometry Wageningen, the Netherlands). The ordination plot was created with CanoDraw for Windows 4.5 [3].

3. Results

3.1. Methanogenic diversity

Microarray analysis of all soils revealed 36 positive (SNR $\geq$ 2) out of a total of 98 methanogenic probes belonging to 16 genera. The number of positive probes was low in non-impacted soils: none were detected in NI soil at the start of the experiment, and 1.7 ± 2.9 (mean ± standard deviation) were detected in treatment N, regardless of incubation time (Table 2). Only two samples in treatment N included some positive probes targeting *Methanosarcina, Methanosphaera, Methanobacterium, Methanogenium,* and *Methanoculleus*, with maximal SNRs ranging from 2.0 to 31.6. In cattle-impacted soils, up to 19.7 ± 6.5 positive probes were counted in SI soil at the start of the experiment, 13.3 ± 4.4 in treatment S, and 11.1 ± 5.2 in treatment W at all incubation times. *Methanosarcina* was the most abundant genus in all samples, with maximal SNR values of 100.4 in SI soil before the setup of the experiment, and 23.2 in treatment S and 11.7 in treatment W.

Table 2
Methanogenic microorganisms detected by AnaeroChip. A signal-to-noise ratio (SNR) $\geq$ 2 was considered as a critical value for the positive signals of the probes. The symbols correspond to experimental treatments: non-incubated severely cattle-impacted soil (SI), non-incubated non-impacted soil (NI), severely cattle-impacted soil incubated without earthworms (S), severely cattle-impacted soil incubated with earthworms (W), and incubated non-impacted soil (N).

Methanogens	SNR				
	SI	NI	S	W	N
Methanobacteriaceae	+	−	+	+	+
Methanobacterium	+	−	+	+	−
Methanobrevibacter	+	−	+	+	−
Methanocalculus	+	−	+	−	−
Methanocaldococcus	−	−	+	−	−
Methanoculleus	+	−	+	+	+
Methanogenium	+	+	+	+	−
Methanohalobium	+	−	+	−	−
Methanolobus	+	−	−	−	−
Methanosaeta	+	−	+	+	−
Methanosarcina	+	−	+	+	+
Methanosphaera	−	−	−	+	+
Methanothermobacter	+	−	−	−	−
MIM[a]	+	−	−	−	−
MMM[b]	+	−	+	+	+
MMMM[c]	+	−	+	+	−

[a] MIM = Methanolobus − Methanomethylovorans group.
[b] MMM = Methanomicrobium − Methanogenium − Methanoplanus group.
[c] MMMM = Methanothermobacter − Methanobacterium − Methanosphaera − Methanobrevibacter group.

Methanoculleus, *Methanobacterium*, *Methanobrevibacter*, *Methanosaeta* and *Methanosphaera* were consistently abundant genera in all cattle-impacted samples (i.e., positive probes targeting these microorganisms appeared in most replicates), with maximal SNR values ranging from 6.4 to 37.5. Several genera with low SNR values in treatment S were below the detection level of the microarray (<0.4 pg DNA) in treatment W (e.g., *Methanocalculus*, *Methanocaldococcus*, and *Methanohalobium*; Table 2).

PCA performed with the positive SNR values of all probes that yielded positive signals in one or more samples extracted two axes explaining 74.9% of the total variance (Fig. 1). Along PC1, which explained 62.1% of the total variance, the samples were distributed as follows: (i) soil NI and treatment N were discriminated in a small cluster at the negative pole of the axis, which showed no remarkable correlation with any methanogenic probe; (ii) soil SI and treatment S were dispersed towards the positive pole of the axis, which was highly correlated with *Methanosarcina* and *Methanoculleus* probes, and moderately correlated with probes specific for *Methanosaeta*, *Methanobacterium*, and *Methanobrevibacter*; and (iii) samples corresponding to treatment W were located near the zero value of PC1. Although treatment W partly overlapped with treatment S, the difference between the location of W and S samples was mainly due to treatment W having lower average SNR values in *Methanosarcina* probes (data not shown). An enrichment in *Methanobacterium* and *Methanobrevibacter* probes was observed in samples from treatment W. No clear trend was detected in the dispersion of the samples regarding time of incubation.

3.2. Abundance of methanogens and Methanosarcina genus

The quantification of the *mcrA* gene revealed significantly higher abundance of total methanogens ($t = -7.30$, $P < 0.001$) in SI than in NI soil before the start of the experiment (Table 1). The gene *mcrA* was less abundant ($F = 17.74$, $P < 0.001$) in treatment N than in treatments S and W (Fig. 2a) but *mcrA* gene quantity did not differ between treatments W and S. Furthermore, incubation time did not change the quantity of *mcrA* gene copies in any treatment ($F = 0.03$, $P = 0.97$).

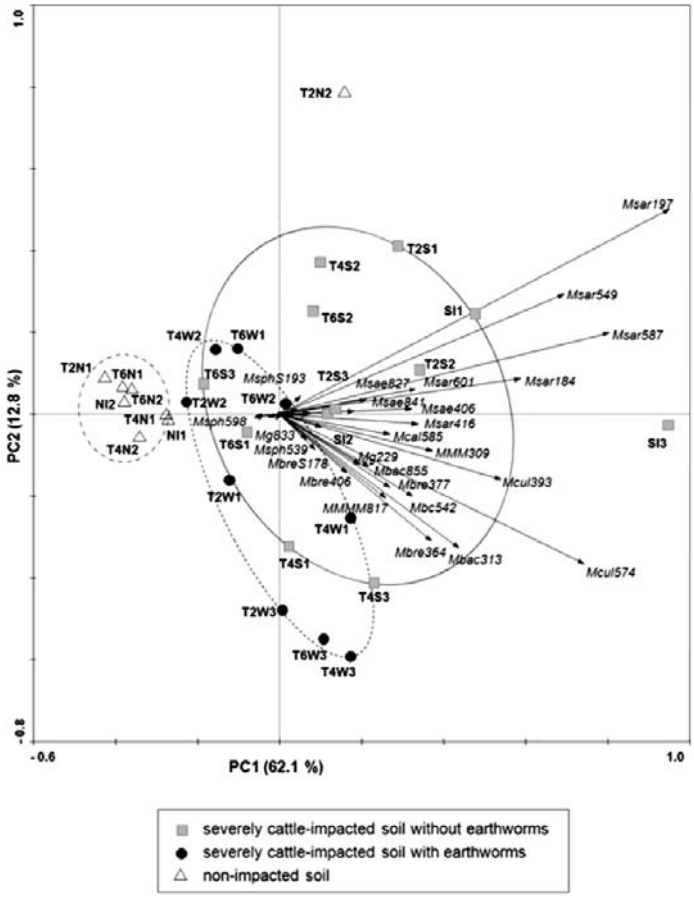

Fig. 1. Ordination plot of sample distribution after principal component analysis (PCA) of signal-to-noise ratio (SNR) values of methanogenic probes. The symbols represent soil samples: non-incubated severely cattle-impacted soil (SI), non-incubated non-impacted soil (NI), severely cattle-impacted soil incubated without earthworms (S), severely cattle-impacted soil incubated with earthworms (W), and incubated non-impacted soil (N) sampled after 2, 4, and 6 months (T2, T4, and T6, respectively). The arrows demonstrate methanogenic probes corresponding to different families, genera, and species according to Franke-Whittle et al. [15]: Methanobacteriaceae (*Mbac*), *Methanobacterium* (*Mbc*), *Methanobrevibacter* (*Mbre*), *Methanocalculus* (*Mcal*), *Methanoculleus* (*Mcul*), *Methanogenium* (*Mg*), *Methanomicrobium - Methanogenium - Methanoplanus* (*MMM*), *Methanosaeta* (*Msae*), *Methanosarcina* (*Msar*), *Methanosphaera* (*Msph*), *Methanosphaera stadtmanae* (*MsphS*), and a group of *Methanothermobacter - Methanobacterium - Methanosphaera - Methanobrevibacter* genera (*MMMM*). The length of the arrow reflects the power of the variable to differentiate the samples. Samples are encircled to facilitate visual inspection of the treatments.

The most abundant methanogen according to microarray data (i.e. SNR values), the genus *Methanosarcina*, was quantified with real-time PCR. Its 16S rRNA gene copy numbers were significantly higher ($t = -14.03$, $P < 0.001$) in non-incubated SI soil than in non-incubated NI soil (Table 1). During the incubation (Fig. 2b), *Methanosarcina* 16S rRNA gene copies differed significantly among treatments S, W, and N ($F = 551.6$, $P < 0.001$). Tukey's *post hoc* test ranked the samples as follows: S > W > N. The gene copy numbers of *Methanosarcina* were two times greater in treatment S than in treatment W at T2 and three times greater in treatment S than in treatment W at T4 and T6. The effect of incubation time within a treatment was not significant ($F = 0.22$, $P = 0.80$).

3.3. Methane production

Before the start of the experiment, potential methane production rate in the SI soil was 853 ng C g^{-1} d^{-1}, which was about 50 times higher than in the NI soil (Table 1). The potential methane production rate decreased significantly in both treatments S and W during the first 2 months of incubation, and did not reach the values of original SI soil during the duration of the experiment, with maximum values around 100 ng C g^{-1} d^{-1} (Fig. 2c). Potential methane production rates at T2 and T6 were significantly higher in treatment W than in treatments S and N. In general, methane production significantly differed among treatments W, S, and N ($F = 14.20$, $P < 0.001$), and the length of incubation significantly affected the potential methane production rate ($F = 14.89$, $P < 0.001$). Potential methane production rate increased with increasing incubation time in all treatments.

3.4. Microbial biomass of anaerobes

Microbial biomass of anaerobes was significantly higher in non-incubated SI soil (6.96 nmol unsNEL-PLFA g^{-1}) than in

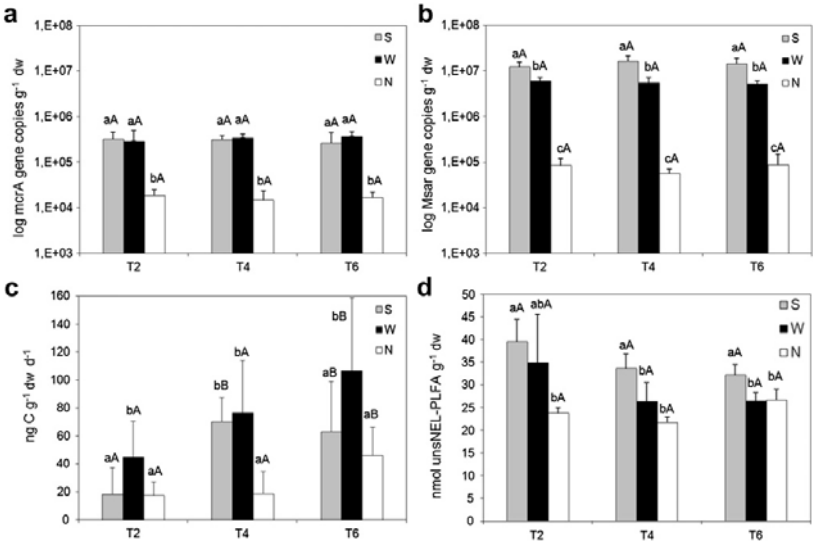

Fig. 2. Key indicators of the methanogenic and anaerobic microbial communities as affected by the following treatments: severely cattle-impacted soil incubated without earthworms (S), severely cattle-impacted soil incubated with earthworms (W), and incubated non-impacted soil (N) sampled after 2, 4, and 6 months (T2, T4, and T6, respectively). Methyl coenzyme M reductase (*mcrA*) gene copy numbers (a), *Methanosarcina* 16S rRNA gene copy numbers (b), potential methane production rate (c), and microbial biomass of anaerobes expressed as the sum of unsubstituted non-ester-linked fatty acids (unsNEL-PLFA) (d). Values are means (±SD) of triplicates. Different lowercase letters indicate significant differences ($P \leq 0.05$) between the treatments; different uppercase letters indicate significant differences between soils of the same treatment sampled at different times.

non-incubated NI soil (1.56 nmol unsNEL-PLFA g^{-1} soil; Table 1). During the incubation, this parameter decreased more in treatment W than in treatment S (Fig. 2d), and this difference was significant after 4 and 6 months of incubation ($F = 12.78$, $P < 0.001$). Biomass of anaerobic microorganisms did not significantly differ between treatment W and treatment N. The unsNEL-PLFA concentration was significantly higher after incubation in treatments S, W, and N than in non-incubated SI and NI soils (Table 1, Fig. 2d).

3.5. Growth and survival of earthworms

Cattle-impacted soil supported the growth of the earthworms to full maturity, as indicated by the presence of the clitella on all earthworms recovered at the end of the experiment. Earthworm survival ranged from 80 to 100% in all microcosms. The growth rate of earthworms during the first 2 months averaged 8.4 mg of biomass d^{-1} (live weight). The next months of incubation were characterised by the reproduction of adults followed by a decrease in earthworm biomass. Total earthworm biomass of the adults increased from the initial 3.3 ± 0.3 g (mean ± standard deviation; $n = 3$) to 8.0 ± 1.3 g per microcosm at T2, and then decreased to 4.6 ± 2.1 per microcosm at T4 and 4.8 ± 0.5 g per microcosm at T6. Based on visual inspection, soil structure was different in treatment W than in treatment S. In treatment W (with earthworms) but not in treatment S (without earthworms), soil aggregates were bonded with mucus, casts had accumulated in the top soil layer, and burrows were evident.

4. Discussion

4.1. Methanogenic diversity in cattle-impacted soil

Cattle outdoor management practices significantly change soil properties, mainly due to deposition of large quantities of cattle excreta and urine [41,58], which in turn stimulate methanogenic communities and CH_4 production [46]. Although non-methanogenic *Crenarchaeota* dominate archaeal communities in upland pasture soils [39], this study revealed a significant increase in the abundance and diversity of methanogenic *Euryarchaeota* in upland pasture soil under severe cattle influence that occurs with outdoor winter livestock management. The phylogenetic microarray AnaeroChip, which allows the identification of methanogenic microorganisms in complex environments [15], revealed high methanogenic diversity in cattle-impacted soils and a predominance of *Methanosarcina*. This is a metabolically versatile genus that uses various biochemical pathways for methanogensis. *Methanosarcina* is a specific inhabitant of the cattle rumen [27,43] and was one of the most common groups of methanogens previously found in cattle-impacted soil (33% of the total methanogens [46]), and in 24-month-old cattle dung (78.7% of the total methanogens [47]). The significant increase of *mcrA* gene copies (from 10^3 g^{-1} NI soil to 10^6 g^{-1} SI soil) indicative for total methanogens was in accordance with previous research on the same pasture area [46].

4.2. Earthworm effects on the abundance of soil methanogens and total anaerobes

The microarray provided a first view into the earthworm–methanogenic interactions in pasture soils on the basis of a laboratory incubation experiment. The inoculation of cattle-impacted soils with earthworms did not significantly reduce the total numbers of positive methanogenic probes, but semiquantitative microarray analysis and real-time PCR analysis confirmed a significant decrease in *Methanosarcina* abundance and a decrease below detectable levels of three other genera. Earthworm-mediated reduction of *Methanosarcina* abundance in treatment W correlates with the decrease of total anaerobes, which could be explained by the changes in the soil properties, including the

increased aeration due to worm burrowing; oxygen is toxic to anaerobes [4,6]. Field observations at the same winter pasture showed that aeration of the cattle-impacted soil due to plant roots reduced the abundance of total anaerobes (Elhottová et al., unpublished results).

It should be taken into account that anaerobic microorganisms in treatment W could have been affected not only due to changes in aeration conditions in the microcosm, but also due to the digestion of microorganisms during the passage of soil through the earthworm intestinal tract [32]. Although literature does not provide any specific information about effect of earthworm gut passage on the abundance of soil methanogens and other anaerobes, it can be assumed, that the methanogens, being protected within soil aggregates or organic matter, better resist to digestion in the worm intestine than the number of facultative anaerobic bacteria and fungi.

Although the reduction in *Methanosarcina* in presence of earthworms was significant, we did not confirm the earthworm effect on the abundance of total methanogens. This discrepancy between results obtained by two molecular approaches may be caused by lower sensitivity of the *mcrA*-targeted real-time PCR assay than of the 16S rRNA-based assay. We obtained a ratio of *Methanosarcina* 16S rRNA to *mcrA* gene copies of 19.5 and 7.0 for soils S and N, respectively. These ratios are much higher than what can be expected from genome-based information because all three *Methanosarcina* spp. that have been sequenced so far possess three rRNA operons and only one copy of the *mcrA* gene per genome [10,37]. For this reason, values obtained for numbers of *mcrA* copies might be greatly underestimated and thus may not show the differences between earthworm-affected and non-affected treatments. Since we only measured 16S rRNA gene copies of one genus (*Methanosarcina*), we can not discard that we are missing information related to other genera that were also abundant in the samples (e.g. *Methanoculleus*).

4.3. Earthworm effects on soil methanogenic activity and potential methane production

The decrease of the *Methanosarcina* abundance in worm-affected soils was not sufficient to reduce methane emissions. The potential methane production rate was even greater in soil with earthworms than without earthworms after 2 and 6 months of incubation. In another study, methane production was relatively constant in anaerobic digesters fed with cattle excreta despite oscillations of the gene copy numbers of the dominant *Methanosarcina* and eight other methanogenic genera [18]. The numbers of the 16S rRNA gene copies are not accurate indicators of methanogenic activity. Moreover, the number of *mcrA* gene copies was not correlated with methane emission. The quantity of methanogens (*mcrA* and 16S rRNA gene copies) cannot directly explain measured methane emission because gene copy numbers (based on DNA level) represent only the potential of the methanogenic community. Methanogenic activity seems to be easily induced by favourable environmental conditions, as was shown for thermophilic methanogens in compost. Thummes et al. [55] revealed that rewetting the compost after oxic drying induced methanogenesis, indicating the reversibility of the process. Some microorganisms ingested by earthworms find better environmental conditions in the intestine than in soil and hence proliferate during or after gut passage, while others are killed or inhibited [5,32]. The presence or absence of their metabolic products as well as earthworm burrowing might be essential for individual groups of methanogens (i.e., *Methanobacterium* and *Methanobrevibacter*) whose microarray SNRs increased in W compared to S soil. In addition, uncultured methanogens (approximately 41% of the clones reported by Radl et al. [46],) that were not detected by AnaeroChip, may have contributed to the methanogenic activity in W microcosms. The earthworm-mediated incorporation of fresh organic matter into soil in the form of polysaccharidic mucous may have promoted microbial activity and thereby intensified soil organic matter mineralization. This "priming effect" [13] would be particularly marked in a small microcosm owing to the low mobility of earthworms. Substantial earthworm activity in a small soil volume could cause large local increases of labile compounds and result in diverse aerobic/anaerobic activities and fluctuating oxic and anoxic microzones. Contrary to our results, passage of soil and related materials through the earthworm guts and the presence of their casts seem to contribute significantly to methane depletion [23,38,42]. According to Kamman et al. [29], the CH_4 flux in pasture soil depends on endogenous gross CH_4 production and gross CH_4 consumption related to the anaerobic/aerobic microsites and site-specific methanogen/methanotrophic communities.

4.4. Growth and survival of earthworms

Earthworm growth dynamics in this long-term microcosm experiment were in accordance with previous studies [22,40], and the maximum growth of earthworms (rapid growth phase) occurred during the first 2 months. The earthworm weight loss observed after 2 months of incubation might be explained by both an over-population effect [22] and the start of cocoon production [12]. In addition, self-toxicity caused by the accumulation of *E. andrei* casts in the relatively small microcosms could be partly responsible [30]. On the other hand, the earthworm mortality at 6 months was low (0–20%).

5. Conclusions

This study provides new information about the increased diversity and abundance of methanogens in pasture soil under severe cattle impact caused by outdoor winter livestock management. The phylogenetic microarray AnaeroChip, which allows the identification of methanogenic microorganisms in complex environments, provided a first view into the earthworm–methanogenic interactions in pasture soils. The laboratory microcosm experiment, which simulated the 6-month period when the cattle-impacted soils typically regenerate after the winter season, showed that the earthworms changed the methanogenic community composition, reduced the abundance of the most common methanogen *Methanosarcina* sp., and reduced the total anaerobic microbial biomass in the cattle-impacted soil. Earthworms did not reduce the total abundance of the methanogenic community, and earthworm activity increased the potential methane production rate. These results require confirmation by long-term field research.

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