University of South Bohemia in České Budějovice
Faculty of Science

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

RNDr. Thesis

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**Annotation**

The potential of earthworms *Eisenia andrei* to regulate functional microbiota in cattle-impacted soil was investigated in the laboratory microcosm experiment. Methanogenic archaea and anaerobes were characterised using phylogenetic microarray and phospholipid fatty acid analysis, respectively.

**Declaration [in Czech]**

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České Budějovice, 20. 7. 2016

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Anna Koubová

**Co-author`s agreement**

We hereby declare that Anna Koubová had major contribution of paper Effects of the earthworm *Eisenia andrei* on methanogens in a cattle-impacted soil: A microcosm study. European Journal of Soil Biology 48, 32–40.

*Anna Koubová was responsible for establishement and maintaining of the experiment, sample preparation, PLFA procedure, DNA isolation, microarray analyses, real-time PCR preparation, data evaluation, and writing of the manuscript.*

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Original article

# 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 ($F = 0.03$, $P = 0.97$).

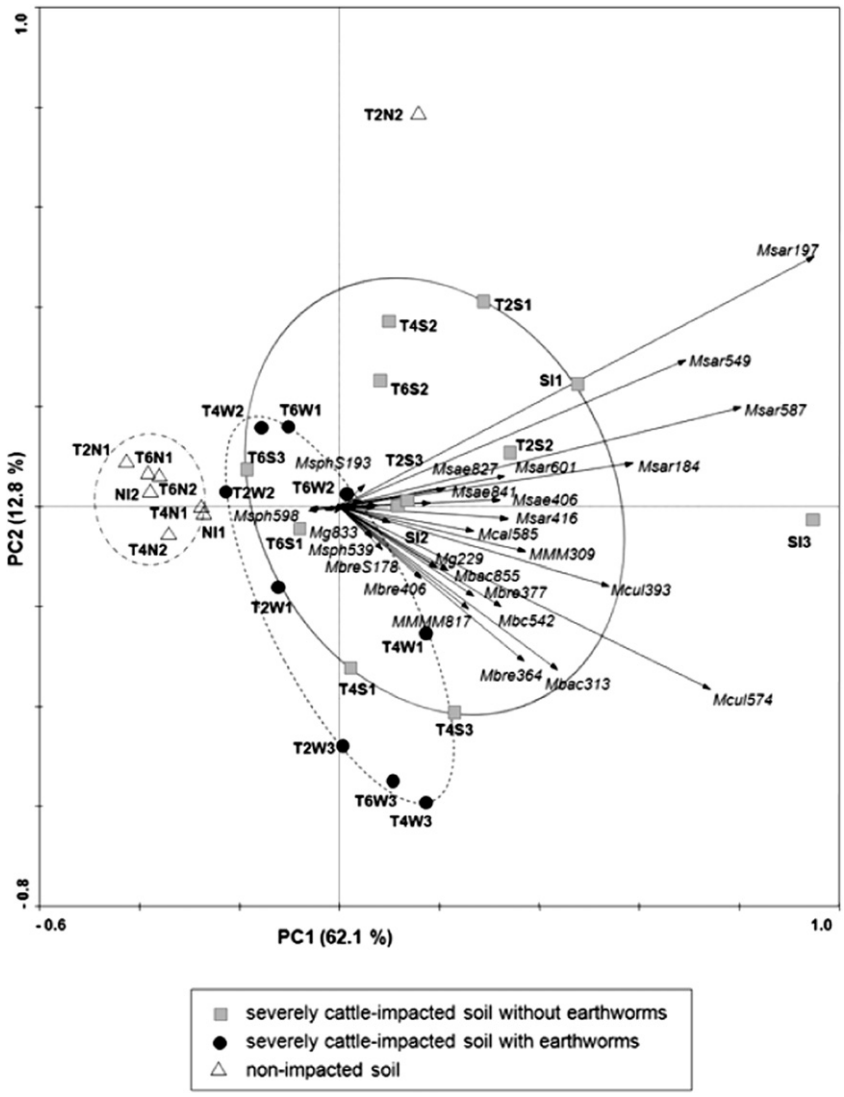


**Fig. 1.** Ordination plot of sample distribution after principal component analysis (PCA) of signal-to-noise ratio (SNR) values of methanogenic probes. The symbols represent soil samples: non-incubated severely cattle-impacted soil (SI), non-incubated non-impacted soil (NI), severely cattle-impacted 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

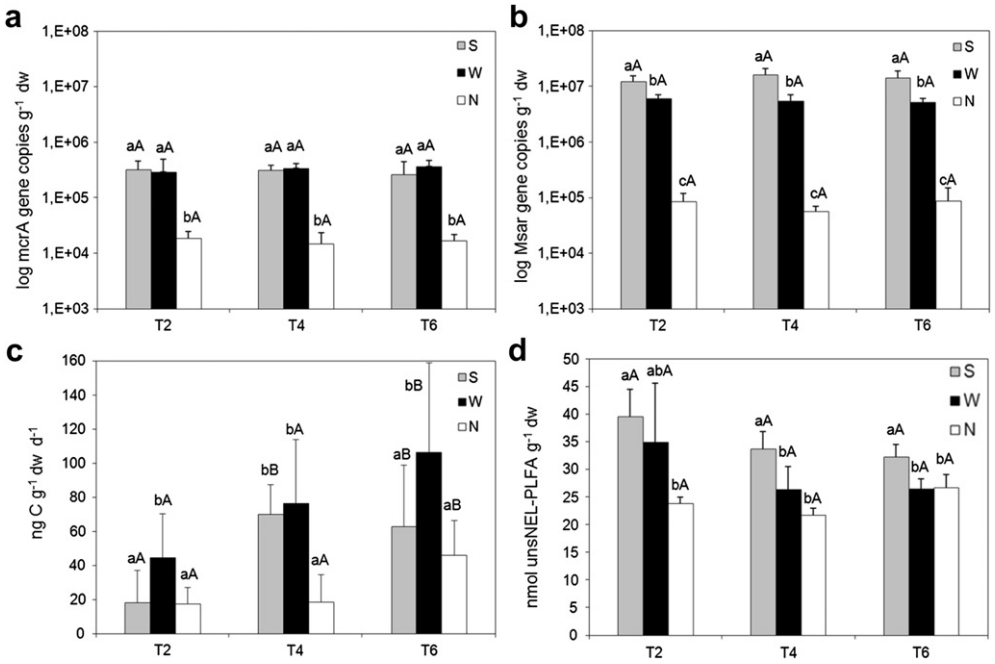


**Fig. 2.** Key indicators of the methanogenic and anaerobic microbial communities as affected by the following treatments: severely cattle-impacted soil incubated without earthworms (S), severely cattle-impacted soil incubated with earthworms (W), and incubated non-impacted soil (N) sampled after 2, 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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