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# Long-term effects of earthworms (*Lumbricus rubellus* Hoffmeister, 1843) on activity and composition of soil microbial community under laboratory conditions

Petr Heděnec[a,e,*], Tomáš Cajthaml[a,b], Václav Pižl[c], Károly Márialigeti[d], Erika Tóth[d], Andrea K. Borsodi[d], Alica Chroňáková[c], Václav Krištůfek[c], Jan Frouz[a,c]

[a] Institute for Environmental Studies & SoWa RI, Faculty of Science, Charles University in Prague, Benátská 2, 128 44 Prague 2, Czech Republic
[b] Laboratory of Environmental Biotechnology, Institute of Microbiology of the CAS, Vídeňská 1083, 142 20 Prague 4, Czech Republic
[c] Institute of Soil Biology & SoWa RI, Biology Centre of the CAS, Na Sádkách 7, 370 05 České Budějovice, Czech Republic
[d] Department of Microbiology, Institute of Biology, Faculty of Science, ELTE Eötvös Loránd University, Budapest, Pázmány Péter sétány 1/C, 1117 Budapest, Hungary
[e] Department of Geosciences and Natural Resource Management, Faculty of Science, University of Copenhagen, Rolighedsvej 23, 1958 Frederiksberg C, Denmark

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ABSTRACT

Observations of earthworm colonization on sites where they were absent previously, such as on post-mining heaps, indicate that they may substantially alter soil conditions as well as soil microbial communities. However, long-term effects of earthworms on microbial activity of soil ecosystems remains poorly understood. To improve this situation, we set up microcosm experiments with clay only, with clay and litter, and with clay, litter and earthworms. Microcosms were sampled after 3, 60, 180 and 395 days of cultivation and divided into three subsamples: clay, litter, and drilosphere (earthworm tunnels and associates casts), when present. Microbial respiration, microbial biomass carbon, species composition, metabolic activity using BIOLOG, PLFA concentration, isoprenoid quinones content and DGGE fingerprints were analysed in all microcosms and at all subsamples. Microbial biomass carbon and basal soil respiration were significantly affected by type of microcosms and time of sampling. Microbial biomass in drilosphere was significantly higher than in clay and litter. The CFU (colony forming units) were significantly affected by presence of earthworms having significantly higher CFU in drilosphere than in all other subsamples. Analysis of PLFA indicated highest relative biomass of fungi and bacteria in clay from microcosms with earthworms. The DGGE analysis showed various compositions of microbial communities among subsamples from various treatments taken at different time. Finally, our results revealed significant effect of earthworms on activity and biomass of soil microbial community under long-term laboratory incubation.

## 1. Introduction

Soil fauna, in particular earthworms play an important role in litter removal by the transformation of organic material throughout the soil profile (Frouz, 2018; Frouz et al., 2013). Bioturbation is a process in which earthworms transport organic material from top layers to deeper layers of the soil profile and vice-versa (Frouz et al., 2009). In addition, the passage of soil through the digestive tract of earthworms causes changes in the chemical structure of soil particles and thus plays an important role in the formation of humus and soil micro-aggregates (Frouz, 2018). Earthworms ingest a mixture of organic matter, soil mineral components, and microorganisms (Lavelle et al., 1997). Lavelle and Spain (2001) suggests 'earthworm casting' as the most important process in turning over soil horizons, which ranges between 36 and 108 Mg $ha^{-1}$ $year^{-1}$.

The part of soil initially affected by earthworms, such as the walls of earthworm channels and worm coprolites, is called the 'drilosphere' (Lipiec et al., 2015; Stromberger et al., 2012). Gómez-Brandón et al. (2012) showed that the type of earthworm-induced modifications of the microbial communities depend on the type of substrate ingested. Observations of earthworm colonization on sites where they were absent previously, such as boreal forest in USA or post-mining heaps, assume that earthworms may substantially alter soil conditions (Bohlen et al., 2004; Frouz et al., 2008). By mixing litter into mineral soil they remove

* Corresponding author at: Institute for Environmental Studies & SoWa RI, Faculty of Science, Charles University in Prague, Benátská 2, 128 44 Prague 2, Czech Republic.
*E-mail address:* petr.hedenec@centrum.cz (P. Heděnec).

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litter and the fermented soil layer, and alter the water holding capacity of soil, organic matter content, pH, and many other soil properties (Bohlen et al., 2004; Frouz et al., 2009, 2008, 2006).

Alterations of soil properties by earthworms may substantially alter plant communities (Bohlen et al., 2004; Frouz et al., 2008). Roubickova et al. (2009) also showed that earthworms significantly changed the soil substrate by producing excrement and performing vertical transport of organic matter and thus increased the capacity of soil to support plant species typical for latter successional stages in the Sokolov mining area (Czech Republic). Despite clear effects of earthworms on activity and structure of soil microbiota, little is known about the long-term variation of activity and the structure of soil microbiota in soil ecosystem.

Soil communities are extremely complex and diverse, including millions of species and billions of individual organisms found within a single soil ecosystem (Fierer, 2017; Lavelle et al., 1997). Last decades showed growing number of studies showing how soil microbial communities play significant role in biogeochemical cycling and nutrient transformation (Fierer, 2017). Recent progress in high-throughput DNA sequencing methods allow detailed insight into vast taxonomic diversity of soil microbes (Fierer, 2017; Li et al., 2014) while functional response, changes in biomass or metabolic activity of soil microbes remains omitted. Progress in analytical methods such as PLFA analysis (Frostegård et al., 1993), ergosterol detection (Frouz et al., 2014), BIOLOG method (Stefanowicz, 2006), provides more detailed insight into the biomass and activity of microbial communities.

Although effect of earthworms on plant dynamics, organic matter transformation and altering of soil microbial communities is well documented, temporal dynamics of metabolic activity, biomass of soil microbial community remains unknown. To address this question, we used a mineral layer from Sokolov lignite spoil heap to reveal the long-term effects of earthworms on activity and structure of soil microbial communities under laboratory conditions. Our study aims to answer the following question: What are the temporal effects of earthworms on bacterial biomass, activity, diversity, and composition under laboratory incubation? We expect significant effect of earthworms on microbial activity and structure at longer time scale. We also expect temporal variation of soil microbial activity and biomass.

## 2. Material and methods

### 2.1. General experimental design

Clay, litter and earthworms (*Lumbricus rubellus* Hoffmeister, 1843) were sampled in post-mining sites after brown coal mining near the town of Sokolov (NW Bohemia, Czech Republic; 50°12′14.8″N and12°38′13.8″E) at an approximately 20-year-old alder (*Alnus glutinosa* Linne) plantation on alkaline (pH 7–8) tertiary clay material (Frouz et al., 2002, 2001). Clay material for the mineral layer of microcosms was sampled from the deeper layer (20–40 cm, CN ratio and C content were 9.2 and 2.2% respectively, the top having been removed before sampling to avoid enrichment by recent organic matter) (Frouz et al., 2002, 2001). Litter was collected using a nylon mesh bag on a wooden frame (0.5 × 0.5 m) exposed 0.5 m above the soil surface (Frouz et al., 2001). CN ratio and C content of litter were 20 and 9.18% respectively. Earthworms were collected on the same site using mustard-water solution. All materials were collected in the last third of April 2005. The litter was hand-sorted to remove wooden debris and cut into pieces of about 2 × 3 cm and homogenized. Mineral soil was homogenized by passing it through a 2 mm sieve. Litter and clay were sterilized separately by carrying out two 24 h freezing/defrosting cycles with freezing temperature −40 °C.

### 2.2. Construction and incubation of microcosms

Three types of microcosms were constructed: (i) with clay only, (ii) with clay and litter, and (iii) with clay, litter and earthworms. In all types of microcosms, 100 g of clay was added into plastic boxes of 10 × 12 × 6 cm to form an 8–12 mm layer. In litter and worm microcosms, the clay layer was then covered by 25 g of litter (7.5 g of dry weight equivalent). Two mature individuals of epigeic earthworms (*Lumbricus rubellus*; 50 mm long and 10 g fresh weight in average) were added to each box of worm treatment. Sixteen replicates were prepared for each microcosm whereas four replicates from each treatment were taken after 3, 60, 180 and 395 days. Microcosms were gently sprayed with water using a spray bottle to keep stable moisture conditions. Microcosms were closed with non-hermetic plastic lids and kept in the dark at 15 °C for 395 days. All microcosms were checked weekly and gently sprayed with water using a spray bottle (about 1 ml of water per week and per microcosm). In earthworm treatment, the litter disappeared from the soil surface in about 60 days, after which another 7.5 g of dry weight equivalent of litter was added. Microcosms with clay and clay with litter were used as control.

### 2.3. Material sampling

Microcosms were continually sampled after 3, 60, 180 and 395 days of incubation. Clay only microcosms produced only one subsample: clay (C); clay and litter microcosms were separated into two subsamples: litter (L) and clay (CL); and worm treatments were separated into three subsamples: litter (LW), clay (CW), and drilosphere (D). The drilosphere is the part of soil immediately affected by worms, such as walls of earthworm tunnels and worm coprolites. These were carefully separated with sterile scalpels from the rest of the clay layer. During the processing of worm treatments, worms and worm cocoons were recovered to assess the status of the worm population. In samples taken after 3 and 60 days, two mature worms were recovered in all worm microcosms. After 60 days cocoons were found in all sampled microcosms. During sampling after 180 days, no worms but only cocoons were found, and during sampling after 395 days, juvenile worms were found in all sampled microcosms. Individual microsites separated from various microcosms were divided in aseptic plastic boxes at the same sites as microcosms and 5 g from each subsample was used for measurement of microbial respiration, biomass, direct counts of bacteria colony-forming units (CFU), analysis of phenotypic fingerprinting using BIOLOG plates, concentration of phospholipid fatty acid (PLFA), isoprenoid quinones, and denaturing gradient gel electrophoresis (DGGE). Because there was not enough litter (LW) to conduct all analyses, the analyses described below deal only with mineral treatments (C, CL, CW and D).

### 2.4. Total direct counts, cultivation, and isolation of cultivable fraction of soil bacteria

Bacterial CFU were estimated from freshly-collected clay and drilosphere re-suspended in water and serially diluted ($10^{-4}$) in low-nutrient agar (R2A, Oxoid CM0906), as described by Krištůfek et al. (2001). Total direct counts were estimated with DAPI (4′, 6-diamidino-2-phenylindole) staining and microscopic counting according to Bloem (1995). The ratio of culturable cells to total cells (C:T) was calculated to determine the index of succession state of microbial communities (Bloem, 1995).

### 2.5. Basal soil respiration, microbial biomass C and ergosterol measurement

The 4 g of each subsample were used for measurement of each microbial biomass and respiration. Microbial respiration was measured as $CO_2$ production, by the trapping of $CO_2$ with NaOH in an air-tight vial and consequent titration of NaOH by HCl after $BaCl_2$ addition. As the soils were alkaline, the amount of respired $CO_2$ was corrected according to Shan-Min et al. (1987), assuming an equilibrium between the gaseous and solution phases within the system (aqueous carbonate equilibrium). The microbial biomass was quantified using the fumigation

method (Jenkinson and Powlson, 1976). For microbial measurement, the material was used in original (field) moisture.

### 2.6. BIOLOG fingerprint measurement

For the phenotypic fingerprinting of microbial communities, BIOLOG GN2 micro plates (BIOLOG Inc., Hayward, CA., USA) containing a low-concentration mineral buffer system, a tetrazolium redox dye and 95 different sole-carbon-sources in dehydrated form, were used. Each well of the microplates was inoculated with 150 µl of diluted samples (suspended in physiological saline, pH 7.0), corresponding to the GN-NENT turbidity standard. All plates were incubated for 5 days at 25 °C and the colour development, as well as the reduction of the tetrazolium dye due to the substrate oxidation, was both measured with an ELISA Reader (Labsystems Multiscan PLUS) at $OD_{590}$ after 24, 48, 72, 96 and 120 h of incubation.

### 2.7. PLFA extraction and analysis

To analyse microbial membrane lipids (phospholipid fatty acids – PLFA) samples of mineral layer equivalent to a dry weight of 1 g were extracted according to the Bligh-Dyer method as described in Oravecz et al. (2004). The resulting lipid material was fractionated into neutral lipids, glycolipids and phospholipids on a silica-bonded phase column (SPE-SI; LiChrolute, Merck, Germany) by elution with chloroform, acetone, and methanol, respectively.

Membrane phospholipids were subjected to a mild alkaline methanolysis (Oravecz et al., 2004). The resulting fatty acid methyl esters were separated by gas chromatography (Agilent 6850, FID), using a fused silica capillary column (Ultra-2, crosslinked 5% PH ME Siloxane, 25 m, 0.2 mm, 0.33 µm; Agilent Technologies, USA). The fatty acid profiles of phospholipids (PLFA) were identified and their peak areas determined using the MIS Aerobe method of the MIDI System (Microbial ID, Inc., Newark, DE, USA). The fatty acids nomenclature follows the pattern of A:BwC, described by Frostegård et al. (2011). The complete PLFA profiles were used for microbial community composition evaluation as well as for microbial biomass estimation (Frostegård et al., 2011). The ratio of monounsaturated and saturated PLFA (MUFA/STFA ratio) was used as an indicator of substrate availability in microbial communities (Bossio and Scow, 1998).

### 2.8. Respiratory quinone extraction and analysis

Samples used for the analysis of quinones were stored in glass bottles free of contamination or confounding lipids, in Bligh and Dyer solution (Bligh and Dyer, 1959), at −20 °C for the time of processing (maximum 1–5 days). The extraction of soil lipid constituents was started in dichloromethane: methanol: 50 mM phosphate buffer (pH:7.4) (1.5:3:1) solution, shaken in the dark overnight at 4 °C. It was followed by an ultrasonication for 10 min and phases were split by adding 15 ml dichloromethane: deionized water (1:1) and letting to stand overnight at 4 °C. The upper water-methanol phase was removed with an aspirator then the dichloromethane phase was filtered on Whatman No. 2 chromatography paper. Dichloromethane was evaporated with a rotary evaporator (Rotadest 2118) at 37 °C. For the separation of lipid components, the extracted lipids were dissolved in 300 ml chloroform, and separated on BB C18 octadecyl-sulphate column (Merck): Sample was washed with chloroform to collect respiratory quinones. This fraction was concentrated with a rotary evaporator at 37 °C, then quinones were purified with thin layer chromatography on Kieselgel 60 F254 sheets. (This fraction contains the majority of non-lipid contaminants such as humic acids, fulvic acids, etc.). The quinones were identified with HPLC (pump: HP 9001, column: ODS Spherisorb) at 270 nm based on the quinone profile of the following authentic bacterial strains: *E. coli* DSM 1116; *Pseudomonas fragi* DSM 3456; *Bacillus sphaericus* DSM28; *Arthrobacter variabilis* DSM 20132; *Brevibacterium linens* DSM 20426; *Nocardioides simplex* DSM 10368; *Micromonospora olivoasterospora* ATCC 21819, *Actinomadura verucosospora* DSM 43358. Only menaquinones were used for analysis.

### 2.9. DNA extraction and DGGE analysis

Genomic DNA was extracted from soil samples using Fast DNA Spin kit for soil DNA extraction (MP Biolabs, Solon, OH, USA) according to the manufacturer's instruction and then amplified by PCR method (Henckel et al., 2000). PCR amplification of bacterial 16S rRNA genes from soil genomic DNA for DGGE analysis was done using the bacteria-specific for-ward primer 357F containing a GC-clamp and the reverse primer 907R (Henckel et al., 2000). The PCR mixture (50 µl) contained 1 × PCR buffer, 1.5 mM $MgCl_2$, each deoxynucleoside triphosphate at 0.4 mM, each primer at 1.0 mM and 1 U of Ex Taq polymerase (TaKaRa Bio Inc., Japan) and 20 ng soil genomic DNA. The PCR amplification program included initial denaturation at 94 °C for 3 min, followed by 30 cycles of 94 °C for 40 s, 56 °C for 60 s, and 72 °C for 60 s, and a final extension at 72 °C for 10 min. PCR products were subjected to electrophoresis using 1.5% agarose gel. The band with a correct size was excised and purified using Wizard® SV Gel and PCR Clean-Up System (Promega, USA). DGGE was performed using a DCode system (Bio-Rad, California). An 8% polyacrylamide gel with a linear denaturant concentration from 30 to 55% (where 100% denaturant contains 7 M urea and 40% formamide (v:v)) was used to separate the PCR products. Approximately 300 ng of PCR product per lane was loaded onto the DGGE gel. The gel was electrophoresed for 4 h at 60 °C at a constant voltage of 200 V, stained for 1 h with SYBR Green I (Molecular Probes, Eugene, Org. USA), and illuminated on a transilluminator under UV-light. A matrix was constructed for all lanes, taking into account the presence or absence of the individual bands and the relative contribution of each band to the total intensity of the lane. A Dice's coefficient similarity matrix was generated based on the above matrix. Finally, DGGE profiles for all samples were clustered based on Dice's similarity coefficient using the complete-linkage method.

### 2.10. Statistical analysis

Two-way ANOVA was used to determine the content of PLFA biomarkers, microbial biomass, and microbial respiration, using four sampling treatments and four sampling times. Sheffe post hoc test was used to test the differences between individual sampling treatments. Shannon-Wiener index of diversity (H), number of species (S) evenness (E), diversity (D) and equitability (Eq) based on PLFA, BIOLOG and quinones data were calculated in SPSS 11.0 (SPSS Inc., Chicago, IL, USA). All these indices were calculated separately for each sample and two-way ANOVA was used to evaluate the effect of treatments and sampling times on these indices. Sheffe post hoc test was used to test differences between individual sampling treatments. The SPSS 11.0 (SPSS Inc., Chicago, IL, USA) was used for ANOVA. Differences were considered statistically significant at $P < 0.05$. Principal component analysis (PCA) centered with Euclidean biplot was applied to compare PLFA, BIOLOG and quinones data using a CANOCO software package (Lepš and Šmilauer, 2003). Percentages of molar concentration were used for PLFA and quinones. Absorbance of individual wells in plates with ACWD 0.8 was used for PCA. Differences in positions of individual samples on first ordination axis were evaluated by two-way ANOVA using treatment and sampling term as a factor. The DGGE patterns were analysed by using the TL120 version 2006 software and an UPGMA (Unweighted Pair Group Method with Arithmetic mean) dendrogram was created.

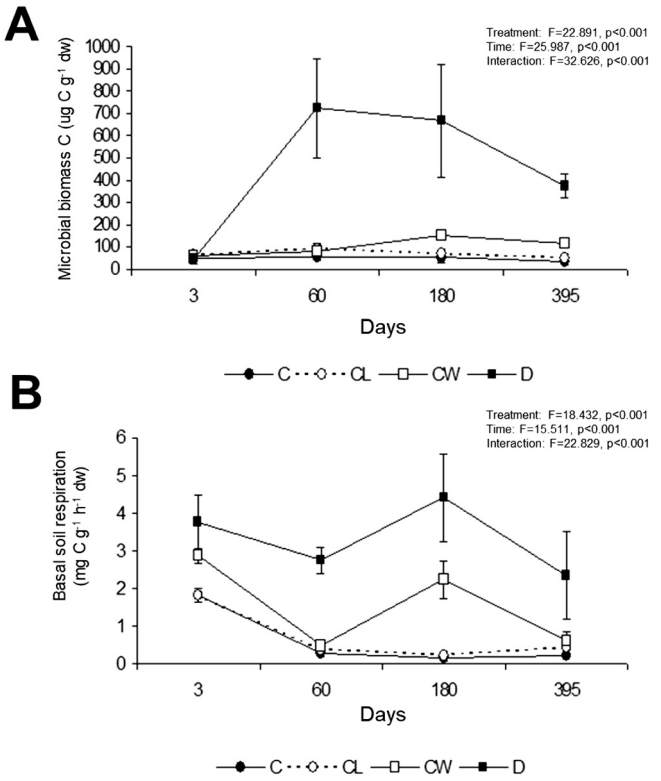


**Fig. 1.** Biomass carbon and basal soil respiration among treatments during 395 day's incubation. Subsamples are abbreviated as follows, C-clay, CL-clay in microcosms with litter, CW-clay remaining after drilosphere separation in worm microcosm, D drilosphere, dw indicates dry weight. Bars indicate SD.

## 3. Results

### *3.1. Long term effect of earthworms on basal soil respiration and microbial biomass C*

Microbial biomass C (Fig. 1A) was significantly affected by both type of microcosms and sampling time ($p < 0.001$). Microbial biomass in drilosphere was significantly higher than in all other subsamples from different microcosms. The lowest microbial biomass was found at the start of the experiment, which was significantly lower than values after 60 and 180 days of cultivation with values at the end of cultivation being intermediate do not differ significantly from others. Microbial respiration was also significantly affected by microcosms and time of incubation ($p < 0.001$). Respiration of drilosphere was significantly higher than in all other subsamples. Respiration in of clay in worm microcosms was lower than drilosphere but significantly higher than subsamples from remaining microsites (Fig. 1B). In contrary to biomass, the highest respiration was found at the start of experiment (3rd day) which was significantly higher than all other treatment, respiration in 180 days was lover but significantly higher than respiration in other two sampling terms. Microbial biomass negatively correlated with specific microbial respiration (Fig. 2).

### *3.2. Long term effect of earthworms on cultivable fraction of soil bacterial community*

Both direct counts of bacterial cells and CFU were significantly affected by microcosms where earthworms were present ($p < 0.001$) being significantly highest in drilosphere (Fig. 3AB). There was significant ($p < 0.001$ for direct counts for cells, and $p = 0.037$ for direct counts of CFU respectively) effect of time on direct counts of bacteria which decreased over time in all microsites but not on CFU. Proportion

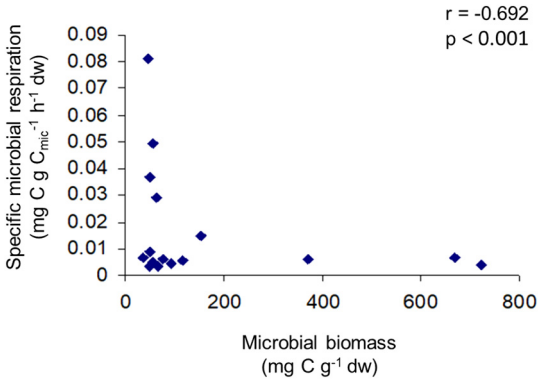


**Fig. 2.** Correlation of microbial biomass C and specific microbial respiration (dw indicates dry weight).

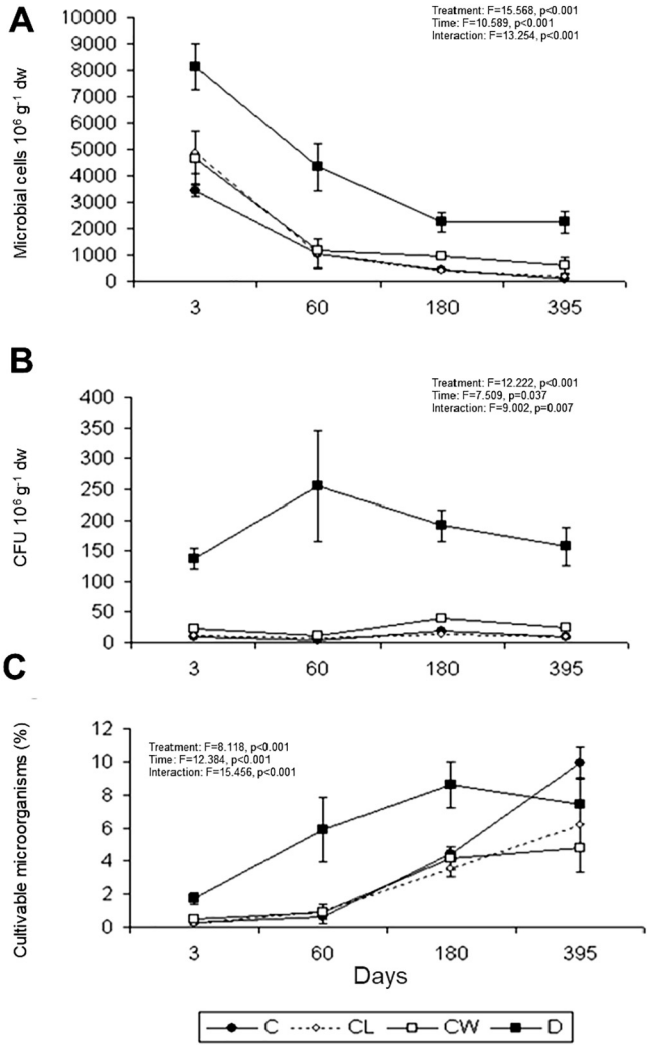


**Fig. 3.** Number of bacterial cells (a), colony forming units (b) and proportion of cultivable fraction of soil bacteria (c) among treatments during 395 day's incubation. Subsamples are abbreviated as follows, C-clay, CL-clay in microcosms with litter, CW-clay remaining after drilosphere separation in worm microcosm, D drilosphere, dw indicates dry weight. Bars indicate SD.

of cultivable bacteria was significantly ($p < 0.001$) affected by both type of microcosms and time. The highest proportion of cultivable bacteria was in drilosphere, which was significantly higher than all other subsamples earthworms free microcosms, which do not differ significantly among each other (Fig. 3C). Proportion of cultivable

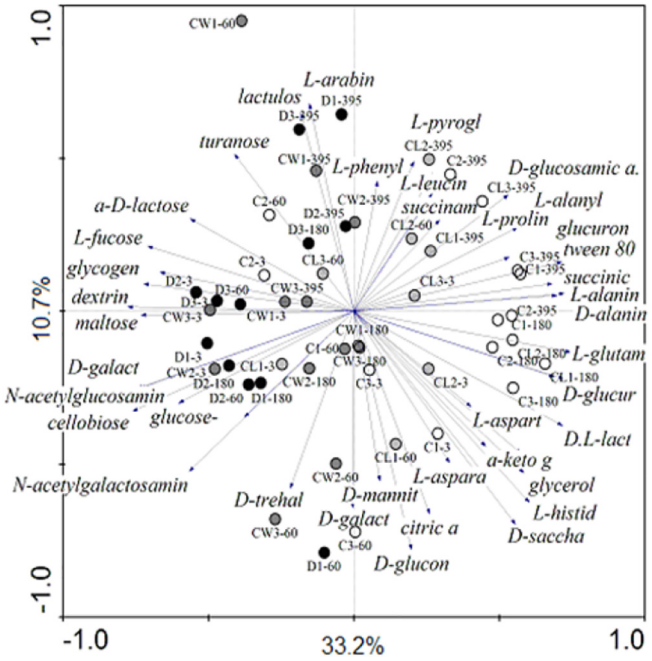


**Fig. 4.** Ordination diagram (PCA) of phenotypic fingerprint based on BIOLOG data among treatments during 395 day's incubation. Microsites are abbreviated as follows, C-clay, CL-clay in microcosms with litter, CW-clay remaining after drilosphere separation in worm microcosm, D drilosphere.

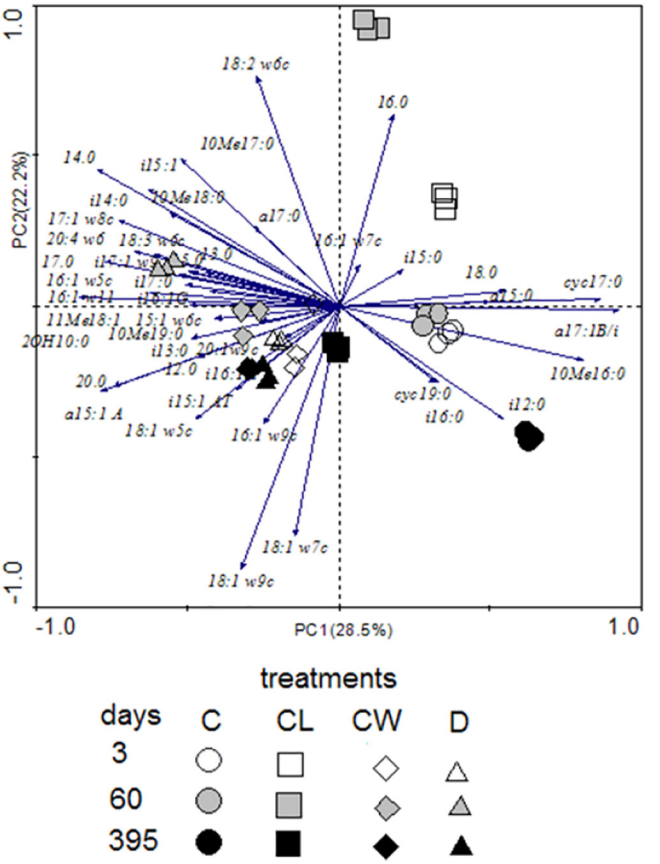


**Fig. 5.** Ordination diagram (PCA) of PLFA fingerprint among treatments during 395 day's incubation. Treatments are abbreviated as follows, C-clay, CL-clay in treatment with litter, CW-clay remaining after drilosphere separation in worm treatment, D-drilosphere.

bacteria increased sequentially during the experiment being, highest in last sampling time.

### 3.3. Long-term effects of earthworms on metabolic activity

Ordination plot based on PCA of BIOLOG data showed differences between subsamples from various microcosms and sampling times (Fig. 4) There were significant differences between worm-affected microcosms (D and CW) and worm-unaffected microsites (C and CL). Two-way ANOVA followed by post hoc test showed significant differences between sampling times when mature earthworms were present (3 and 60 days) and sampling terms where only cocoons or juvenile worms were found (180 and 395 days). Amino acids were characteristic for treatments without earthworms while easily available carbohydrates and monomers of complex polymers such as cellobiose or *N*-acetylglucosamide were characteristic for worm treatments showing significantly higher activity in the drilosphere than in other microcosms.

### 3.4. Relative abundance and structure of microbial communities

The ordination plot based on PCA of PLFA fingerprint data showed differences between type of microcosms and sampling time (Fig. 5). Subsamples from microcosms with earthworms showed higher concentration of PLFA than subsamples from microcosms without earthworms. Similarly, the differences between individual sampling showed higher PLFA concentration in samples taken after 3 days of incubation than samples taken after 60 days incubation.

The clay subsamples were characterized by 10Me16:0, which is a marker of Actinobacteria. The CL subsamples indicated clear succession from the position close to C subsamples through to the stage characterized by fungal marker (18:2w6) to the stage characterized by enrichment by monounsaturated fatty acids (MUFA), that indicated improved substrate availability in positions close to worm microsites. Both subsamples of worm microsites (CW and D) showed complex fingerprints and MUFA enrichment, corresponding to a complex community with Gram negative bacteria domination and good substrate availability (18,1w7c, 18:1w9c, 16:1w9c).

Evaluation of PLFA markers for individual subsamples from earthworm affected/unaffected microcosms indicated a clear trend of increase in MUFA/STFA ratio, from clay to drilosphere, which indicates increasing C substrate availability in drilosphere (Table 1). Fungal markers indicate significantly lower content of fungi in C than in all other microsites. Total PLFA as well as content of bacterial, actinobacterial and microeukaryote markers peaked in the CW microsite (Table 1).

The number of PLFAs and both diversity indices increased in worm microsites in comparison to no worm microsites (Table 2). Both the Shannon Wiener diversity index and evenness were significantly higher in the clay only (C) microsite than in drilosphere (Table 2).

Neither diversity indices nor species numbers were significantly affected by microsites, however, H and Eq indices were significantly affected by sampling time (Table 2). Content of ergosterol in earthworm treatment was significantly higher than in no earthworm treatment (C and CL).

In total, eighteen DGGE profiles were identified based on highest Dice's coefficient similarity matrix (Fig. 6). DGGE clearly separated microbial communities according to individual subsamples from earthworm affected/unaffected microcosms and sampling time (Fig. 6). After 60 days of the experiment, drilosphere (D) was clearly separated from other microsites and microbial communities in the rest of the soil in earthworm microcosms (CW) were more similar to no worm microsites (C and CL). On the other hand, after 180 days of cultivation, worm-affected microsites (CW and D) were grouped together in one cluster and clearly separated from no worm microcosms. In all cases individual sampling times fell in separate microcosms, which indicates

**Table 1**
Distribution of PLFA markers (mean ± SD) for individual groups of microbiota fungal/bacterial ratio (F/B) MUFA/STFA ratio and total content of PLFA in individual subsamples.

| | Fungi | Actinomycetes | Bacteria | Microeukaryotes | F/B | MUFA/STFA | PLFA (total) |
|---|---|---|---|---|---|---|---|
| C | 0.170 ± 0.13a | 0.740 ± 0.16a | 3.050 ± 0.61a | 0.170 ± 0.13a | 0.060 ± 0.04a | 1.780 ± 0.11b | 8.500 ± 1.370a |
| CL | 1.690 ± 0.68b | 1.240 ± 0.75ab | 6.040 ± 4.12a | 2.030 ± 0.94b | 0.350 ± 0.18c | 1.110 ± 0.42a | 21.250 ± 13.43a |
| CW | 2.390 ± 1.94b | 3.560 ± 3.71c | 21.190 ± 19.88c | 3.690 ± 3.54c | 0.140 ± 0.05b | 1.980 ± 0.33bc | 64.670 ± 62.40c |
| D | 1.580 ± 0.64b | 1.860 ± 0.80b | 13.740 ± 4.51b | 2.350 ± 1.03b | 0.110 ± 0.03b | 2.160 ± 0.21c | 44.960 ± 16.47b |
| | F(p) | F(p) | F(p) | F(p) | F(p) | F(p) | F(p) |
| Treatment | 17.049(< 0.001) | 62.654(< 0.001) | 26.531(< 0.001) | 25.513(< 0.001) | 87.845(< 0.001) | 49.083(< 0.001) | 32.23(< 0.001) |
| Time | 8.2267(0.002) | 123.49(< 0.001) | 25.45(< 0.001) | 24.434(< 0.001) | 33.896(< 0.001) | 2.4203(< 0.001) | 34.288(< 0.001) |
| Interaction | 8.5059(< 0.001) | 58.625(< 0.001) | 16.327(< 0.001) | 17.071(< 0.001) | 16.009(< 0.001) | 8.3866(< 0.001) | 22.021(< 0.001) |

In bottom F and *p* values of two-way ANOVA evaluating effect of individual microcosms, time and its interaction id show.
Statistically homogenous are marked by the same letter (Sheffe, $P < 0.05$).
Subsamples are abbreviated as follows, C-clay, CL clay in microcosm with litter, CW – clay remaining after drilosphere separation in worm microsites, D drilosphere. F/B refers fungal-bacterial ratio.

a clear temporal dynamic in microbial community composition.

## 4. Discussion

### 4.1. Long-term effects of earthworms on microbial activity

Our study revealed long term effect of earthworms incubated under laboratory condition on activity of soil microbial community. The lowest microbial biomass was found at the start of the experiment, peaking after 60 and 180 days of incubation. This pattern corroborates the study of Frouz et al. (2014) where microbial activity and content of organic C changed with duration of fauna presence. In agreement with the other studies (Frouz et al., 2014; Lavelle et al., 1997), soil organic matter may be stabilized by earthworm activity via physical binding inside aggregates resulting from coating by clay minerals, which may slow down microbial decomposition.

In clay soils, earthworms ingest clay particles with litter and coat the organic matter with clay as it passes through their guts; as noted earlier, the clay-coating may slow the decomposition of organic matter and enhance C storage in the long term (Frouz et al., 2006, 2007). We hypothesize about role of chemical composition of leaf litter (Ponge, 2013). For example, earthworm processing of litter with a low C:N ratio resulted in high C storage in microcosms containing clay (Ponge, 2013).

In agreement with the study reported by Lipiec et al. (2015), microbial organic carbon significantly increased in drilosphere compared with litter and mineral layer. The same pattern has been found for basal soil respiration. According to Stromberger et al. (2012) we hypothesize a different composition of microbial community exists in drilosphere than in other soil compartments. The different composition and structure of microbial communities in drilosphere strongly influence basal soil respiration and microbial biomass C (Stromberger et al., 2012).

Previous studies indicated that respiration is high in fresh fauna excrement but decreases with time; in old excrements, respiration is often found to be lower than in fauna unaffected control (Frouz and Simek, 2009; Lavelle et al., 1997; Špaldoňová and Frouz, 2014). In agreement with the study reported by Frouz et al. (2014), we suggest younger faeces may have been relatively more abundant in the young system while older faeces may have been more abundant in the old system and this change in combination with the difference in the respiration of old and young faeces could explain temporal decreasing in basal soil respiration. We also suggest a role-mixing effect (bioturbation) takes place, thus increasing the effect of aeration.

According Frouz and Simek (2009), we hypothesize that younger faeces contain labile compounds which are easily decomposed, while older faeces contain more recalcitrant substances. Another possible mechanism of how respiration is affected by earthworms may be connected with local aeration and compaction of soil (Frouz et al., 2007; Šimek and Pižl, 2010). When earthworms are introduced to a new system, they build a system of corridors which may increase aeration and as consequence also microbial respiration (Frouz et al., 2008). At

**Table 2**
Mean ± SD of Shannon-Wiener index of diversity (H), number of species (S) evenness (E), diversity (D) and equitability Eq for PLFA, biolog substrates and quinones.

| | | C | CL | CW | D | Treatment F(p) | Time F(p) | Interaction F(p) |
|---|---|---|---|---|---|---|---|---|
| Biolog | D | 8.390 ± 0.31b | 8.480 ± 0.45ab | 8.57 ± 0.26ab | 8.660 ± 0.33a | 4(0.02) | 15(< 0.0001) | 2(ns) |
| | Eq | 0.098 ± 0.005 | 0.099 ± 0.004 | 0.098 ± 0.004 | 0.100 ± 0.00 | 2(ns) | 10(< 0.0001) | 2(ns) |
| | H | 5.990 ± 0.12b | 6.02 ± 0.16a | 6.06 ± 0.10ab | 6.110 ± 0.12a | 4(0.01) | 14(< 0.0001) | 2(ns) |
| | E | 0.930 ± 0.02b | 0.94 ± 0.01ab | 0.94 ± 0.01ab | 0.950 ± 0.01a | 5(0.004) | 9(0.0002) | 2(ns) |
| | S | 86.7 ± 6.0 | 86.1 ± 7.0 | 87.6 ± 4.9 | 87.100 ± 5.70 | 1(ns) | 14(< 0.0001) | 2(ns) |
| PLFA | D | 16.070 ± 2.52b | 16.290 ± 2.34b | 23.680 ± 1.63a | 23.970 ± 0.58a | 34(< 0.0001) | 20 < 0.0001 | 33(< 0.0001) |
| | Eq | 0.850 ± 0.03a | 0.790 ± 0.04b | 0.750 ± 0.04c | 0.730 ± 0.03c | 47(< 0.0001) | 12(0.0002) | 1(ns) |
| | H | 3.800 ± 0.20b | 3.750 ± 0.17b | 4.190 ± 0.17a | 4.190 ± 0.09a | 61(< 0.0001) | 7(0.003) | 13(< 0.0001) |
| | E | 0.900 ± 0.020a | 0.850 ± 0.02b | 0.840 ± 0.03b | 0.830 ± 0.03b | 25(< 0.0001) | 13(0.0002) | 1(ns) |
| | S | 19.000 ± 3.60d | 21.800 ± 2.70c | 31.800 ± 1.7b | 33.100 ± 1.50a | 27(< 0.0001) | 15(< 0.0001) | 17(< 0.0001) |
| Quinones | D | 7.570 ± 2.60 | 6.90 ± 2.36 | 7.86 ± 2.40 | 7.040 ± 2.07 | 1(ns) | 6(0.003) | 1(ns) |
| | Eq | 0.640 ± 0.14 | 0.60 ± 0.13 | 0.68 ± 0.12 | 0.570 ± 0.11 | 1(ns) | 10(0.002) | 1(ns) |
| | H | 11.910 ± 1.93 | 12.18 ± 3.01 | 11.82 ± 3.43 | 12.180 ± 2.29 | 1(ns) | 2(ns) | 1(ns) |
| | E | 0.690 ± 0.18 | 0.77 ± 0.08 | 0.79 ± 0.05 | 0.680 ± 0.12 | 2(ns) | 2(ns) | 1(ns) |
| | S | 2.500 ± 0.70 | 2.7 ± 0.30 | 2.8 ± 0.30 | 2.400 ± 0.40 | 1(ns) | 1(ns) | 1(ns) |

F and *p* values of two ways ANOVA for effect of treatment, time and its interaction given at the end of each line.
Statistically homogenous groups are marked by the same letter (Sheffe, $P < 0.05$).
Subsamples are abbreviated as follows: C-clay, CL clay in microcosm with litter, CW – clay remaining after drilosphere separation in worm microcosms, D drilosphere.

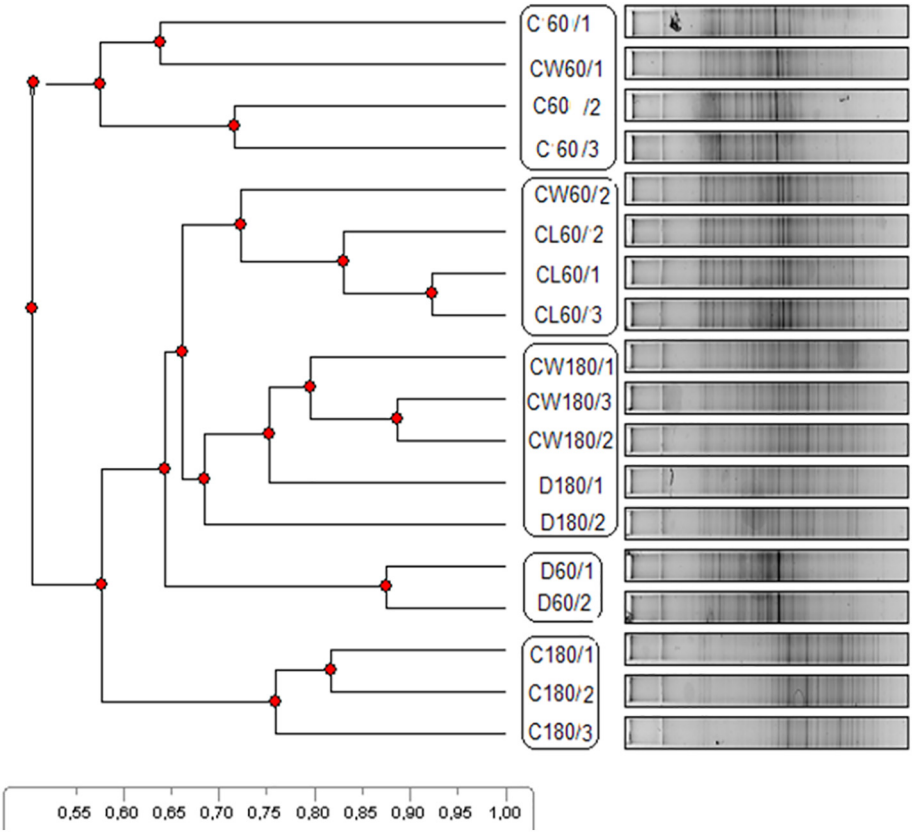


**Fig. 6.** DGGE profile of treatments during 395 day's incubation. Microsites are abbreviated as follows, C-clay, CL clay in microcosms with litter, CW-clay remaining after drilosphere separation in worm microcosms, D-drilosphere.

the same time, soil is locally compacted in earthworm casts and microbial activity inside these casts may be limited.

Biolog data revealed significant differences in metabolic activity between worm-affected microcosms and worm-unaffected microcosms. Significantly, the highest metabolic activity was found in drilosphere. According to the study reported by Lipiec et al. (2015) we hypothesize that increased moisture content in earthworms casts and drilosphere can significantly affect microbial activity (Cregger et al., 2012). In addition, Lipiec et al. (2015) showed the largest concentrations of both $C_{org}$ and microbial biomass C in the burrow wall and cast aggregates with the lowest wetting rates.

### 4.2. Temporal effects of earthworms on microbial biomass

Results of our study showed long terms effect of earthworms incubated under laboratory conditions on microbial biomass. Direct counts of bacteria and the number of CFU as well the portion of cultivable bacteria were significantly affected by presence of earthworms, being significantly higher in drilosphere than in all other subsamples which do not differ significantly between each other. We suggest that the higher number of bacterial cells could be explained by contact with earthworms' gut microbiota (Sampedro et al., 2006). According to the study of Stromberger et al. (2012), drilosphere contained greater concentrations of soil C, microbial biomass, and biomass of PLFA biomarkers compared to other treatments. We hypothesize that the higher bacterial biomass in drilosphere could also be affected by mucus produced by earthworms while burrowing to create tunnels (Scheu et al., 2002).

Analysis of PLFA indicated that clay treatment was characterized by 10Me16:0, which is marker of actinobacteria. We hypothesize that hydrophobic clay material without organic material provides better conditions for actinobacteria, which are more resilient to water stress due to their filamentous structure and capability to form spores (Fierer, 2017). In contrast, clay with litter treatment indicated a clear shift from the position close to clay treatments through to the stage characterized by fungal markers. The addition of litter to microcosms led to an increase in ergosterol content, which can be explained by the attraction of litter to fungi, as they are heterotrophs and so are able to decompose recalcitrant organic compounds (Morriën and van der Putten, 2013). We hypothesize that leaf-litter contains more recalcitrant organic compounds, which are easily decomposable by fungi (Van der Putten et al., 2013).

### 4.3. Temporal effects of earthworms on diversity of microbial communities

Our study highlighted relationship interaction of earthworms and diversity of soil microbial communities under laboratory conditions. According to the study by Gómez-Brandón et al. (2012) we consider earthworms with their associated gut microbiota to be a strong determinant shaping the structure of microbial communities. However, neither diversity indexes nor species numbers were significantly affected by treatment. Alpha diversity indices were significantly affected by sampling time. We suggest altered diversity indices are affected by experimental design due to separated and isolated microcosms. In agreement with Lindstrom and Langenheder (2012) we admit that the role of dispersal mechanisms and connectivity to be an important factor in the maintenance of local diversity.

Additionally, we agree with the other researchers (Ritz et al., 2004) who have shown dispersal mechanisms as an important factor in shaping soil microbial communities. For example, Treves et al. (2003) showed a decrease of dispersal activity of soil microbes by spatial isolation of meta-communities. On the contrary, both Shannon-Wiener evenness and equitability were significantly higher in clay-only treatment than in drilosphere. We hypothesize that nutrient rich drilosphere

supports fast growth and microorganisms with higher biomass, which can outgrow other microbial species and homogenize the population. On the other hand, nutrient-poor clays support specialized slow-growing and oligotrophic microorganisms with lower biomass which can coexist with other microbial species (Stegen et al., 2012).

The DGGE analysis showed different composition of microbial communities between subsamples from various treatments at various sampling times. After 60 days of our experiment, drilosphere (D) was clearly separated from other treatments, and microbial communities in the rest of the soil in worm microcosms (CW) were more similar to treatments without worms (C and CL), while after 180 days of incubation, worm-affected treatments (CW and D) were grouped together in one cluster and clearly separated from no-worm treatment. In agreement with Sampedro et al. (2006) we propose the existence of a specific microbial community in the earthworm gut that during passage through the gut, strongly affects the diversity of microbial communities in nearby ecosystems.

We also suggest that earthworms significantly affected microbial biomass after longer incubation and thus strongly affected the composition and structure of soil microbial communities. Similar results were shown in recent studies (Frouz et al., 2007, 2013, 2014; Špaldoňová and Frouz, 2014). For example, Frouz et al. (2013) showed that earthworm casts strongly effected changes in the composition of the bacterial community and other soil biota.

## 5. Conclusions

Microbial biomass and respiration were significantly affected earthworms and time of incubation. Both direct counts of bacteria and number of CFU were significantly affected by microsites, being significantly higher in drilosphere than in all other microsites that did not differ significantly between each other. Analysis of PLFA indicated clear differences between microsites. The DGGE analysis showed differences in composition of microbial communities between subsamples from various treatments at various sampling times. In conclusion, we suggest that presence of earthworms significantly increased metabolic activity and changed structure of soil biota during long term incubation and thus earthworms are important drivers of nutrient transformation in soil ecosystems.

## Declaration of competing interest

We declare no conflict of interest.

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