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ORIGINAL ARTICLE

The earthworm *Aporrectodea caliginosa* stimulates abundance and activity of phenoxyalkanoic acid herbicide degraders

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2-Methyl-4-chlorophenoxyacetic acid (MCPA) is a widely used phenoxyalkanoic acid (PAA) herbicide. Earthworms represent the dominant macrofauna and enhance microbial activities in many soils. Thus, the effect of the model earthworm *Aporrectodea caliginosa* (Oligochaeta, Lumbricidae) on microbial MCPA degradation was assessed in soil columns with agricultural soil. MCPA degradation was quicker in soil with earthworms than without earthworms. Quantitative PCR was inhibition-corrected per nucleic acid extract and indicated that copy numbers of *tfdA*-like and *cadA* genes (both encoding oxygenases initiating aerobic PAA degradation) in soil with earthworms were up to three and four times higher than without earthworms, respectively. *tfdA*-like and 16S rRNA gene transcript copy numbers in soil with earthworms were two and six times higher than without earthworms, respectively. Most probable numbers (MPNs) of MCPA degraders approximated 4×10^5 g_{dw}^{-1} in soil before incubation and in soil treated without earthworms, whereas MPNs of earthworm-treated soils were approximately 150× higher. The aerobic capacity of soil to degrade MCPA was higher in earthworm-treated soils than in earthworm-untreated soils. Burrow walls and 0–5 cm depth bulk soil displayed higher capacities to degrade MCPA than did soil from 5–10 cm depth bulk soil, expression of *tfdA*-like genes in burrow walls was five times higher than in bulk soil and MCPA degraders were abundant in burrow walls (MPNs of 5×10^7 g_{dw}^{-1}). The collective data indicate that earthworms stimulate abundance and activity of MCPA degraders endogenous to soil by their burrowing activities and might thus be advantageous for enhancing PAA degradation in soil.
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Introduction

2-Methyl-4-chlorophenoxyacetic acid (MCPA) is a phenoxyalkanoic acid (PAA) herbicide used worldwide to control broad leaf weeds and has been introduced into the environment in large amounts (Smith, 1985; Worthing and Hance, 1991; Gintautas *et al.*, 1992; Donaldson *et al.*, 2002; Sorensen *et al.*, 2006). Both MCPA and the structurally related PAA 2,4-dichlorophenoxyacetic-acid (2,4-D) are contaminants in ground water and soil (Thompson *et al.*, 1984; Bryant, 1992; Scheidleder, 2000). Although MCPA is relatively rapidly degraded in soil, it is usually more persistent than 2,4-D (Audus, 1964; Gaunt and Evans, 1970; Ka *et al.*, 1994; McGhee and Burns, 1995; Bælum and Jacobsen, 2009). MCPA degradation in soil is mainly biotic, and MCPA-degrading bacteria are ubiquitous in soil (Oh *et al.*, 1995). Microorganisms that utilize and degrade MCPA have been isolated from different environmental samples and are aerobes mainly of the Alpha-, Beta- and Gammaproteobacteria (Bollag *et al.*, 1967; Müller and Babel, 1999; Marriott *et al.*, 2000; Kleinsteuber *et al.*, 2001; Kitagawa *et al.*, 2002; Hoffmann *et al.*, 2003; Macur *et al.*, 2007).

The first step in the degradation of MCPA is initiated by oxygenases encoded by *cadAB* or *tfdA*-like genes (that is, *tfdA* and *tfdAα*; Streber *et al.*, 1987; Itoh *et al.*, 2002, 2004; Kitagawa *et al.*, 2002). Such oxygenases cleave the ether-bonded acetate side chain of MCPA to produce 2-methyl-4-chlorophenol (MCP) (Fukumori and Hausinger, 1993a, b; McGowan *et al.*, 1998). Major PAA-degrading microorganisms are categorized into three groups on an evolutionary and physiological basis (McGowan *et al.*, 1998; Itoh *et al.*, 2002, 2004). Group 1 consists of Beta- and Gammaproteobacteria, which harbor *tfdA*-like genes. Groups 2 and 3

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consist of alphaproteobacterial *Bradyrhizobium*- and *Sphingomonas*-related organisms, respectively, including potential novel organisms (Fulthorpe *et al.*, 1995; Kamagata *et al.*, 1997; Zaprasis *et al.*, 2010). Group 2 organisms harbor both *tfdA*-like and *cadA* genes, whereas only *cadA* was detected in group 3 organisms (Itoh *et al.*, 2002, 2004; Kitagawa *et al.*, 2002; Huong *et al.*, 2007, 2008). As transcription of *cadA* is induced by 2,4-D in pure cultures, groups 2 and 3 might employ *cadA* gene homologs for PAA herbicide degradation (Itoh *et al.*, 2002, 2004; Kitagawa *et al.*, 2002). Diverse and new genes encoding putative PAA oxygenases were recently identified in an agricultural soil (Zaprasis *et al.*, 2010). However, to date only group 1 *tfdA*-like genes are associated with PAA degradation in soil (Bælum *et al.*, 2006, 2008; Bælum and Jacobsen, 2009; Rodriguez-Cruz *et al.*, 2010).

MCPA degradation requires molecular oxygen (Kuhlmann *et al.*, 1995; Vink and vanderZee, 1997). Thus, increasing soil aeration by macropore formation might stimulate MCPA degradation and associated organisms. Burrows generated by earthworms enhance aeration, water capacity, water infiltration and gas exchange of soils (Edwards, 2004). Burrows likewise affect the transfer of nutrients and herbicides in soil (Farenhorst and Bowman, 2000; Farenhorst *et al.*, 2000). Thus, earthworms are recognized as soil engineers (Scheu 1987a, b, 1996; Jones *et al.*, 1994). Soil that is influenced by earthworms (that is, the drilosphere, including burrow wall, gut content and cast) is characterized by high nutrient availability and microbial activity (Lavelle, 1988; Scheu, 1991; Brown, 1995; Schmidt *et al.*, 1999; Tiunov and Scheu 1999; Brown *et al.*, 2000; Horn *et al.*, 2003; Edwards, 2004; Drake and Horn, 2007), suggesting that earthworms might be used to promote biodegradation of herbicides in soils. However, whether earthworms are stimulatory for herbicide degradation is still under debate, information on the interaction of earthworms and herbicide degraders in soils is scarce and the impact of earthworms on microbial PAA degraders in soil remains unknown (Hickman and Reid, 2008).

Thus, the objectives of this study were to (i) determine the effect of the soil-feeding model earthworm *Aporrectodea caliginosa* (Savigny) on the abundance and activity of MCPA degraders in soil; (ii) evaluate the relevance of gut contents and burrow walls for hosting MCPA degraders in soil; and (iii) determine if *cadA* and non-group 1 *tfdA*-like gene hosting microorganisms are associated with MCPA degradation in soil.

Materials and methods

Field site and sampling

Agricultural soil with MCPA application history (in 2002) was collected from the top 0–10 cm depth at the experimental farm 'Klostergut Scheyern' in Germany (48°30′00″N, 11°20′07″E;(Fuka *et al.*, 2008; Zaprasis *et al.*, 2010). Soil pH is 5.8, clay, silt and sand contents are 22%, 36%, and 42%, respectively, and the C/N content is 10 (Fuka *et al.*, 2008; Zaprasis *et al.*, 2010). Soil samples were stored in the dark at 4 °C for less than 1 month, and were sieved (2 mm) before use. Adult earthworms of the soil-feeding, endogenic species *A. caliginosa* were collected from the upper 0–15 cm of soil from the meadow 'Trafo Wiese' near Bayreuth, Germany (Horn *et al.*, 2003, 2006), and were identified by standard protocols (Brohmer, 1984). Worms were washed with tap water and placed on wet filter paper in the dark at 15 °C for 3 days to defecate before use.

Soil column experiments

Polyvinyl chloride columns (14 (inner diameter) × 30 (height) cm^2; Franken *et al.*, 1995) were packed to 15 cm height, with approximately 2.4 kg (fresh weight) soil that was adjusted to a final moisture content of 21% ($w\,w^{-1}$) with sterile deionized water and mixed with 2% ($w\,w^{-1}$) grass pieces. Soil was supplemented with: (i) earthworms and MCPA; (ii) worms only; (iii) MCPA only; and (iv) water only. Worm containing soil columns were set up in triplicates. Earthworms (11–16) with the total average biomass of 10.0 ± 0.3 g were added per soil column, which is in the upper range of earthworm densities occurring *in situ* (Edwards, 2004). Soil that received MCPA was dispersed in plastic containers, sprayed with a 3 mM stock solution of MCPA to reach a final concentration of 20 mg MCPA per kg soil (approximating agriculturally relevant concentrations; Smith, 1982) and subsequently homogenized by hand before soil column setup. Control columns received a similar amount of deionized water. All columns were covered by black cloth and incubated at 15 °C in dark for 37 days. Approximately 10 g (fresh weight) of bulk soil (that is, mixed soil that did not contain gut content or burrow walls) were collected in triplicates from the upper 0–1 cm of soil every 2–3 days and analyzed for MCPA. When no MCPA was detected, soil columns were destructively sampled. Bulk soil from the upper 0–5 and 5–10 cm were collected from every soil column. Burrow walls were separated carefully (< 2 mm thickness; Bouche, 1975) from bulk soil. Gut content was obtained by squeezing earthworm gut material out of the anus. Bulk soil and drilosphere samples from all time points were stored at 4 °C in the dark for up to 24 h for most probable number (MPN) analyses and analyses of MCPA degradation capacity, up to 3 days for MCPA determinations, and at −80 °C for DNA/RNA extraction.

Microcosm experiments with MCPA

The capacity of soil to degrade MCPA was determined in microcosms (that is, soil slurries). Ten grams (fresh weight) of soil before soil column

experiments were diluted with 40 ml MMS (Müller *et al.*, 2001) that contained 30 μM MCPA (approximating 30 μg g^{-1} soil) in sterile 125-ml infusion flasks (Merck ABS, Dietikon, Switzerland) that were sealed with rubber stoppers and aluminum caps. Incubations were performed under oxic or anoxic conditions with air or dinitrogen as gas phases, respectively. In total, 0.5 g (fresh weight) of bulk soil or drilosphere material after soil column experiments were diluted with 2.0 ml MMS (Müller *et al.*, 2001) that contained 100 or 300 μM MCPA in sterile 10 ml glass tubes sealed with rubber stoppers and plastic caps, with air as gas phase. All incubations were performed at 15 °C in the dark and agitated at 150 r.p.m. in a KS 501 digital orbital shaker (IKA Werke GmbH & Co KG, Staufen, Germany). Samples were collected with a 1-ml sterile syringe (BD Plastipak, Franklin Lakes, NJ, USA) every 2 or 3 days and stored at 4 °C and −80 °C for MCPA analysis and nucleic acid extraction, respectively. Oxic microcosms with [U-^{14}C]MCPA were likewise set up with bulk soil or drilosphere material from soil pre-exposed to MCPA and earthworms in soil columns to determine the fate of MCPA (see online Supplementary Data for details).

Most probable numbers

MCPA degraders were enumerated by the MPN method before and after soil column experiments (Alef, 1991). Ten-fold serial dilutions (from 10^{-1} to 10^{-8}) were prepared in MMS (Müller *et al.*, 2001) with 300 μM MCPA. Dilutions were homogenized by vortexing for 10 s. Fifty microliters of each dilution were then inoculated in five parallels into 150 μl MMS containing 300 μM MCPA in 96-well plates and were incubated at 15 °C in the dark for 50–60 days. Wells were scored positive for MCPA degradation when MCPA concentrations were lower than 20 μM. Sixteen non-inoculated wells containing MMS with MCPA were included per 96-well plate. MCPA was essentially not consumed in such wells. MPNs were calculated according to de Man (1975).

Analytical techniques

MCPA was extracted from solid material with two volumes of a 10 mM NaOH and solid-phase extraction (BAKERBOND SPE, Mallinackrodt Backer B.V., Deventer, The Netherlands; Thorstensen and Christiansen, 2001; Moret *et al.*, 2005). The recovery of MCPA was >95%. Liquid samples from MPNs and microcosms were centrifuged (12 000 r.p.m., 5 min) and the supernatants were microfiltrated (0.22 μM) to remove particles.

MCPA was determined by high-performance liquid chromatography (Agilent 1200 series, Agilent Technologies, Santa Clara, CA, USA) equipped with a 1200 series diode array detector, quaternary pump, vacuum degasser, standard autosampler, column oven and a MultoHigh 100 RP 18–5 μ column ($250 \times 4\,mm^2$) with pre-column ($20 \times 4\,mm^2$) (both CS-Chromatographie Service GmbH, Langerwehe, Germany) at 30 °C. The injection volume was 50 μl, and the mobile phase was 20 mM sodium acetate (pH 3)−50% acetonitrile at 1 ml min^{-1}. Online spectra from 220 to 340 nm were used to confirm peak purity, and the signal at 230 nm was used for quantification with external standards. Moisture content of soil was determined by weighing triplicate samples before and after drying at 65 °C for 24 h.

Nucleic acid extraction

DNA and RNA were co-extracted from 0.5 g (wet weight) of samples as described (Zaprasis *et al.*, 2010). DNA and RNA were obtained by RNase A and DNase I (Fermentas GmbH, St Leon-Rot, Germany) treatments of aliquoted nucleic acids, respectively (Griffiths *et al.*, 2000). DNA was further purified with a MiniElute PCR product purification kit (Qiagen, Hilden, Germany).

Reverse transcription and PCR

Reverse transcription of RNA was performed with random hexamer primers and SuperScript III First-Strand Synthesis Supermix (Invitrogen, Karlsruhe, Germany) according to the manufacturer's protocol. Two microliters of each cDNA product was used as template in 25 μl PCR mixture with appropriate primers (Table 1). PCR was performed in a peqSTAR 96 Model Thermal Cycler as described (Zaprasis *et al.*, 2010), with annealing at temperatures appropriate for each primer set (Table 1). RNA samples did not yield a PCR product, indicating that the RNA was DNA free.

Quantification of tfdA*-like,* cadA *and 16S rRNA genes and transcripts*

Quantitative kinetic PCRs (qPCRs) and evaluation of assay specificities were routinely performed with DNA and cDNA (that is, reverse transcriptase qPCR) as described (Zaprasis *et al.*, 2010). All qPCR reactions were set up in duplicates. Negative controls with sterilized water instead of DNA template were included in every PCR setup. Standard curves were set up by serially diluting M13uni/rev PCR products of a pGEM-T vector, with the appropriate insert from 10^8 to 10^1 target gene copies per μl for every primer set. qPCR reactions were performed in 20 μl reaction mixtures that were composed of iQ SYBR Green Supermix (BioRad, München, Germany), 60 ng μl^{-1} bovine serum albumin, 0.2–1.6 pM of each primers (Biomers, Ulm, Germany), 5 μl of template (c)DNA and sterilized deionized water. Initial denaturation was at 95 °C for 8 min, followed by 45 cycles of denaturation at 95 °C for 40 s, annealing at temperatures appropriate for each primer set (Table 1) for 30 s and elongation at 72 °C for 15 s when fluorescence signal was recorded. The final PCR elongation step was at

Table 1 PCR primers and annealing temperatures

Primer set[a]	*Sequence (5′–3′)*[b]	*Annealing at (°C)*	*Target genes*	*Product (bp)*	*Reference*
1. TfdAα45F TfdAα408R	GGCGTCGATCTGCGCAAGCC CAGCGGTTGTCCCACATCAC	65.0	*tfdA*-like (subset of *Bradyrhizobium*-related group 2 Alphaproteobacteria)	~360	(Zaprasis *et al.*, 2010)
2. TfdAα423F TfdAα777R	ACSGAGTTCGSIGAYATSC CAGCGGTTGTCCCACATCAC	68.0	*tfdA*-like (groups 2–5, including Alphaproteobacteria)	~360	(Zaprasis *et al.*, 2010)
3. TfdA421dF[c] TfdA778vkR	ACSGARTTCKSIGACATGC AGCGGTTGTCCCACATCAC	53.0	*tfdA*-like (groups 1–5, including Alpha-, Beta- and Gammaproteobacteria)	~360	(Zaprasis *et al.*, 2010)
4. Eub341F Eub534R	CCTACGGGAGGCAGCAG ATTACCGCGGCTGCTGG	55.7	16S rRNA (bacteria)	~190	(Muyzer *et al.*, 1993)
5. CadA610F CadA1054R	AAGCTGCARTTTGARAAYGG MGGATTGAAATCCTGRTA	50.0	*cadA (Sphingomonas-, Bradyrhizobium*-related Alphaproteobacteria*)*	~460	(Kitagawa *et al.*, 2002)

Abbreviations: F, forward; R, reverse; T-RFLP, terminal restriction fragment length polymorphism.
[a]Numbers in primer names indicate position of binding sites relative to the reference sequence of *Cupriavidus necator* JMP134 (AY365053) (TfdAα423F/TfdAα777R), *Alphaproteobacterium* RD5-C2 [AB074490] (TfdAα45F/TfdAα408R) and *Bradyrhizobium* sp. HW13 (AB062679) (CadA610F/CadA1054R).
[b]S, C/G; Y, C/T; R, A/G; I, inosine.
[c]Primer was labeled with fluorescent dye DY681 for T-RFLP analysis.

72 °C for 5 min. Melt-curve analyses were performed from 70 °C to 95 °C with increments of 0.2 °C per cycle. qPCR with *tfdA*-like gene-specific primer set 2 (Table 1) and *cadA*-specific primer set 5 (Table 1) yielded specific products with a T_m of approximately 91 °C and 90 °C, respectively. Agarose gel electrophoresis of selected qPCR products supported this finding (data not shown). Normalized transcript and gene copy numbers are less sensitive to varying DNA extraction efficiencies (that is, DNA extraction bias) than copy numbers per gram dry weight soil. Thus, unless otherwise indicated, gene and transcript copy numbers were normalized to ng DNA and RNA, respectively. Transcript copy numbers per gram dry weight were normalized to corresponding gene copy numbers. Transcript and gene copy numbers were used to calculate relative gene frequency ratios (RGFRs) that are indicative of earthworm impact on gene and transcript copy numbers according to the following equation:

$$RGFR = \frac{CN_{+\text{earthworms}}}{CN_{-\text{earthworms}}}$$

where $CN_{+\text{earthworms}}$ and $CN_{-\text{earthworms}}$ are copy numbers from soil incubated in the presence and absence of earthworms, respectively. RGFRs of >1, <1 and 1 indicate earthworm mediated stimulation, reduction or no detectable earthworm effect, respectively, on gene or transcript copy numbers.

Inhibition of qPCR and reverse transcriptase qPCR by environmental nucleic acid extracts

All environmental DNA samples were diluted 50-fold to reduce potential inhibition of qPCR by co-extracted PCR-inhibiting substances. Gene copy numbers were corrected for inhibition of qPCR by determining inhibition factors for every DNA sample (Zaprasis *et al.*, 2010). *IF* was 0.03–0.93 and 0.63–1.00 for structural genes (that is, *tfdA*-like and *cadA* genes) and 16S rRNA genes, respectively, indicating variable degrees of qPCR inhibition associated with different DNA extracts.

The inhibitory effect of environmental RNA extracts on reverse transcriptase qPCR was likewise evaluated. pGEM-T vectors (Promega, Mannheim, Germany) with correct inserts were linearized by digestion with *Sac*I (New England Biolabs) for overnight at 37 °C and were purified with a PCR product purification kit (Qiagen). Linearized vectors were then used as template for *in vitro* transcription with T7 RNA polymerase (Fermentas GmbH) according to the manufacturer's protocol. RNA transcripts were quantified by recording the absorbance at 260 nm (ND1000, Peqlab, Erlangen, Germany) and used as control RNA. Soil RNA was spiked with control RNA to increase target gene copy numbers per reaction by approximately 100-fold. Reverse transcriptions were performed simultaneously with: (i) soil RNA spiked with control RNA (*S*); (ii) non-spiked soil RNA (*NS*); and (iii) control RNA only (*C*). All cDNA products were used for qPCR and transcript copy numbers were used for the calculation of inhibition factors ($IF = (S-NS)/C$; ranges from 0 for complete to 1 for no inhibition; Zaprasis *et al.*, 2010). *IFs* were 0.001–0.370 and 0.730–1.000 for transcripts of structural genes (that is, *tfdA*-like and *cadA* genes) and 16S rRNA genes, respectively, indicating severe and variable degrees of inhibition of reverse transcription associated with different RNA extracts. *NS* was divided by *IF* to obtain corrected transcript numbers in RNA extracts. Such findings underscore the necessity to analyze qPCR inhibition per nucleic acid extract and to correct copy numbers accordingly.

Results

MCPA degradation capacity of field fresh soil and associated structural genes

More than 95% of supplemental MCPA disappeared within 7 days of incubation in oxic soil microcosms

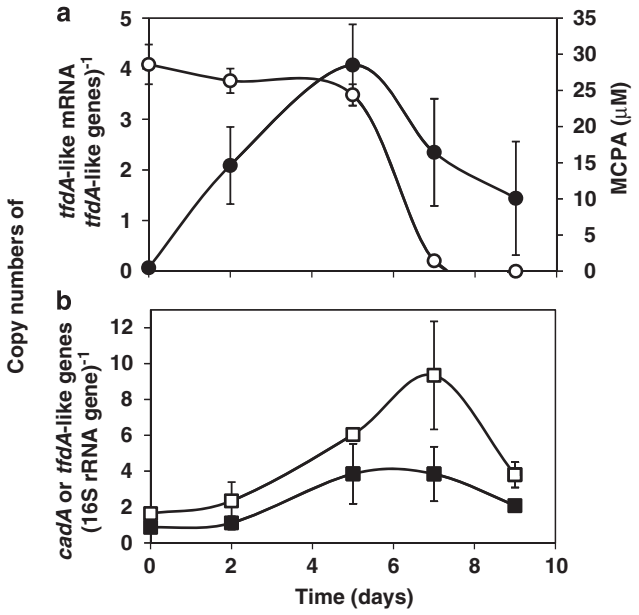

Figure 1 MCPA disappearance in oxic soil microcosms concomitant to transcription of group 2–5 *tfdA*-like genes (**a**) and the relative abundance of *cadA*- and *tfdA*-like genes per 16S rRNA genes (**b**). *tfdA*-like genes and transcripts were quantified with primer set 2 (Table 1). Symbols: ○, MCPA; ●, copy numbers of *tfdA*-like mRNA per *tfdA*-like genes $\times 10^{-1}$; □, copy numbers of *cadA* per 16S rRNA genes $\times 10^{-4}$; and ■, copy numbers of *tfdA*-like genes per 16S rRNA genes $\times 10^{-3}$. Soil was sampled in June 2008. Error bars represent standard deviations of three replicates.

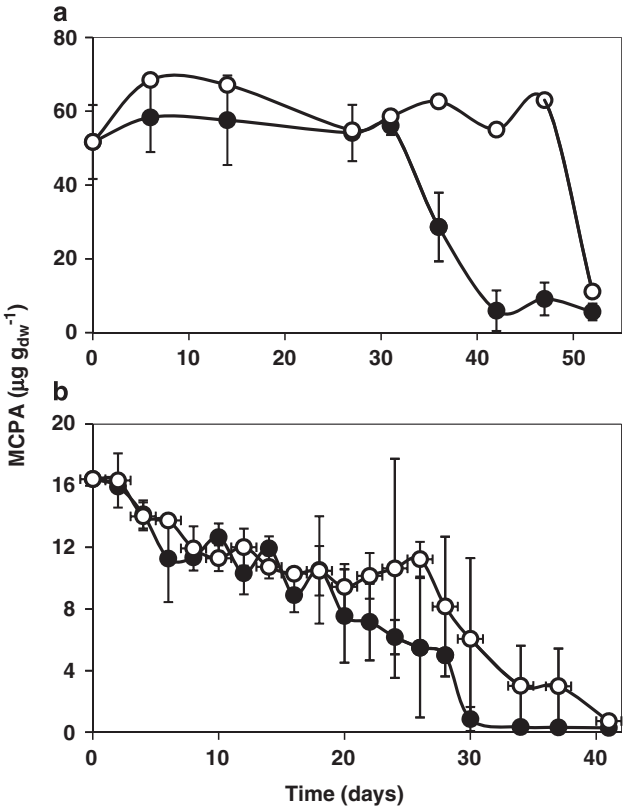

Figure 2 Effect of earthworms on MCPA disappearance in soil columns with soil sampled in March (**a**) and September 2008 (**b**). Symbols: ●, soil with earthworms; ○, soil without earthworms. Error bars represent standard deviations of three replicates. Only one replicate was analyzed for soil without earthworms in panel **a**.

(Figure 1a). Approximately 5 and 20 μM MCPA disappeared from days 0 to 5 and 5 to 7, respectively (Figure 1a). MCPA was stable within 75 days of incubation in anoxic soil microcosms (data not shown). Thus, disappearance of MCPA in oxic microcosm was interpreted as degradation. Copy numbers of *tfdA*-like mRNA per *tfdA*-like genes, and of *tfdA*-like and *cadA* per 16S rRNA genes increased concomitantly to MCPA degradation (Figure 1), indicating that *tfdA*-like and *cadA* hosting aerobes of the Alphaproteobacteria and other non-group 1 *tfdA*-like gene hosting organisms degraded MCPA in oxic Scheyern soil microcosms (for details see Supplementary Results).

MCPA degradation in soil columns

MCPA disappearance in MCPA-supplemented soil in the presence of earthworms was completed approximately 10 days before disappearance in the absence of earthworms (Figures 2 and 3a). Such results were consistently obtained in three independent sets of soil column experiments with initial MCPA concentrations ranging from 15 to 50 $\mu g\, g_{dw}^{-1}$ (Figures 2 and 3a). Given extraction efficiencies of MCPA from soil >95% (see Materials and methods), the polarity of MCPA and the stability of MCPA in sterile soil (Thorstensen and Lode, 2001), disappearance of MCPA in soil is regarded as degradation further on. MCPA was not detected after 37 days of incubation (Figure 3a) and in control soil without MCPA (data not shown). Earthworm survival rates were 72 ± 4% and 68 ± 15% for soil with and without MCPA, respectively. Bioconcentration of MCPA in earthworms was marginal (see Supplementary Figure S1, Supplementary Materials and methods and Supplementary Results), indicating that earthworms stimulated MCPA degradation in soil.

Transcript and gene dynamics in MCPA-supplemented soil columns

cadA and *tfdA*-like gene copy numbers in Scheyern soil before MCPA degradation were 3×10^4 and 4×10^6 per gram dry weight soil, respectively, indicating that *tfdA*-like genes outnumbered *cadA*. Transcript copy numbers of *tfdA*-like genes were 4×10^6 per gram dry weight soil, indicating that *tfdA*-like genes were expressed in field fresh Scheyern soil. *cadA* transcripts in soil before MCPA degradation were not detected.

cadA and *tfdA*-like genes as well as *tfdA*-like gene transcript copy numbers per gram dry weight soil increased up to 24-, five- and sixfold in MCPA-supplemented soil columns, indicating replication

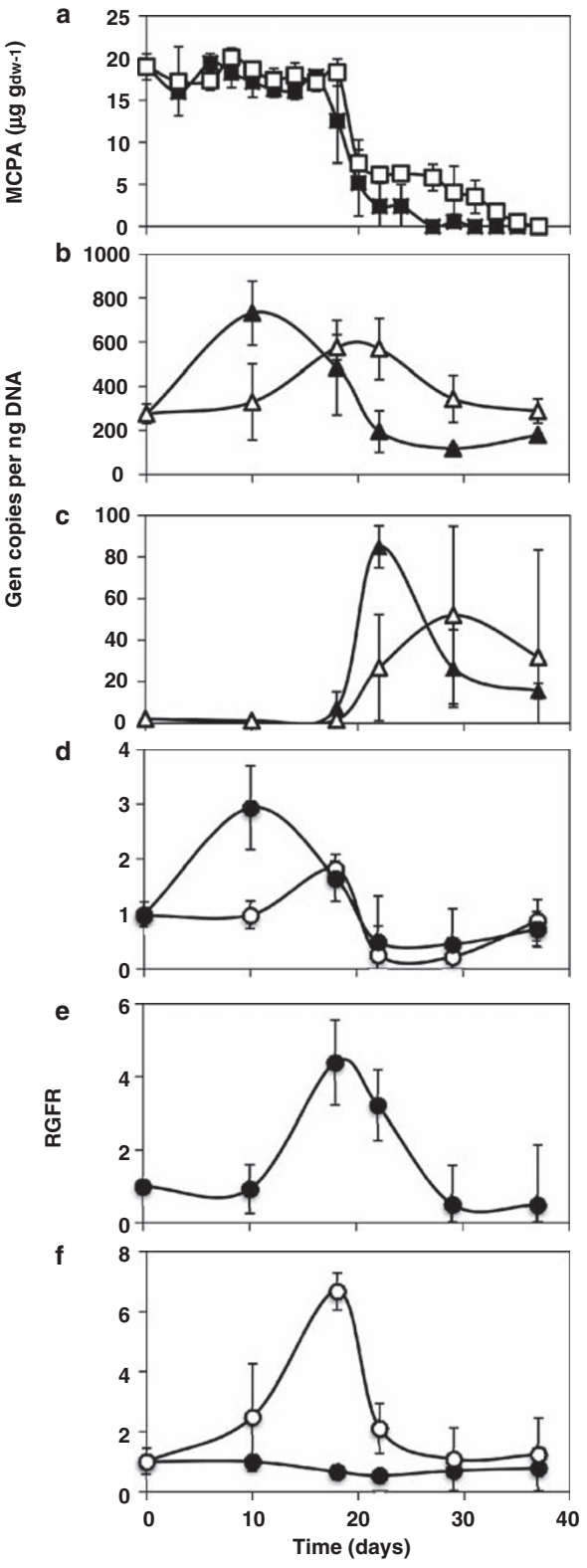

Figure 3 Effect of earthworms on MCPA disappearance (**a**), the abundance of *tfdA*-like (**b**) and *cadA* (**c**) genes per ng DNA, and gene as well as transcript abundances of *tfdA*-like (**d**), *cadA* (**e**) and 16S rRNA genes (**f**) in soil columns expressed as RGFRs. *tfdA*-like genes were quantified with primer set 2 (Table 1). Symbols: ■, ▲, soil with earthworms; □, △, soil without earthworms; ○, RGFRs of transcripts; and ●, RGFRs of genes. Soil was sampled in June 2008. Error bars represent standard deviations based on three replicates.

and activity of *cadA* and *tfdA*-like gene hosting microorganisms (data not shown). DNA-normalized *tfdA*-like gene copy numbers in soil treated with earthworms and MCPA were highest at day 10 before MCPA degradation and decreased thereafter (Figures 3a and b), indicating that earthworms rather than MCPA stimulated the abundance of potential MCPA degraders. Highest *tfdA*-like gene copy numbers in MCPA-only treated soil were detected when more than 50% of MCPA was degraded around day 20 (Figures 3a and b). Copy numbers decreased again until day 37, which co-incided with a slow down in MCPA degradation. Copy numbers of *cadA* genes were maximal when more than 80% of initial MCPA was degraded (Figures 3a and c). Thus, the data reinforce the likelihood that both *cadA*- and *tfdA*-like gene hosting microorganisms were stimulated by MCPA and involved in MCPA degradation. However, copy numbers of *cadA* genes were less than 100 per ng DNA and 10 times lower than those of *tfdA*-like genes (Figures 3b and c). Transcripts of *cadA* were not detected during MCPA degradation, suggesting that *cadA*-encoded oxygenases might have a minor role for MCPA degradation in soil compared with those encoded by *tfdA*-like genes.

RGFRs (see Materials and methods) >1 indicate a positive effect of earthworms on the abundance of a certain gene or transcript. RGFRs for *tfdA*-like genes were highest after 10 days of incubation in the presence of MCPA (Figure 3d), and RGFRs of *cadA* increased to 4 when 30% of initial MCPA was degraded in soil exposed to earthworms (Figures 3a and e), indicating that earthworms increased the abundance of *cadA*- and *tfdA*-like gene hosting microorganisms. Such results are in agreement with the findings that (i) *tfdA*-like and *cadA* genes were quantitatively linked to MCPA degradation in microcosms (Figure 1) and (ii) MCPA degradation in the presence of earthworms was completed before MCPA degradation in the absence of earthworms (Figures 2 and 3a).

RGFRs of *tfdA*-like gene transcripts were maximal when MCPA degradation started (Figures 3a and d), indicating that earthworms stimulated the expression of *tfdA*-like genes. RGFRs were smaller than 1 at days 22 and 29, when less than 13% of the initial MCPA was detected in soil treated with earthworms and MCPA (Figure 3a). Soil that was not exposed to earthworms contained 21–32% of the initial MCPA at days 22 and 29 (Figure 3a), suggesting that the high MCPA concentrations in soil not exposed to earthworms enhanced *tfdA*-like gene expression, yielding RGFRs <1. Such results are consistent with RGFRs of 1 at day 37, when MCPA concentrations approximated 0 in soil exposed and not exposed to earthworms (Figures 3a and d).

RGFRs of 16S rRNA genes increased up to 6 during incubation (Figure 3f), indicating that earthworms stimulated the activity of total soil bacteria. However, RGFRs of 16S rRNA genes approximated 1 throughout the incubation, indicating that earthworms

did not significantly affect replication of total bacteria (Figure 3f).

Transcript and gene copy numbers after column incubation

Copy numbers of *tfdA*-like genes in gut contents, burrow walls and bulk soil at day 37 approximated 10^3, 10^5 and 10^6 per gram dry weight, respectively, and were similar for soil treated with and without MCPA. *tfdA*-like gene transcripts were not detected in gut contents. *tfdA*-like gene expression in burrow walls was five times higher than in bulk soil (Figure 4a), indicating that potential MCPA degraders were highly active in burrow walls. *tfdA*-like gene expression was likewise up to 20 times higher in burrow walls and soil that were treated with MCPA than in those treated without MCPA (Figure 4a).

tfdA-like gene copy numbers per copies of 16S rRNA genes of burrow walls and bulk soil that were treated with and without MCPA were similar at day 37 (Figure 4b), indicating that the stimulatory effect of MCPA on the relative abundance of *tfdA*-hosting potential MCPA degraders (see above, and Figures 3b and c) was transient in burrow walls and bulk soil. The relative abundance of *tfdA*-like per 16S rRNA genes were generally lower in gut content than in burrow walls and bulk soil (Figure 4b). *tfdA*-like gene copy numbers per 16S rRNA genes were higher in gut content from soil treated with MCPA than those from soil treated without MCPA (Figure 4b), indicating the persistence of a stimulatory effect of MCPA on *tfdA*-hosting organisms in gut content.

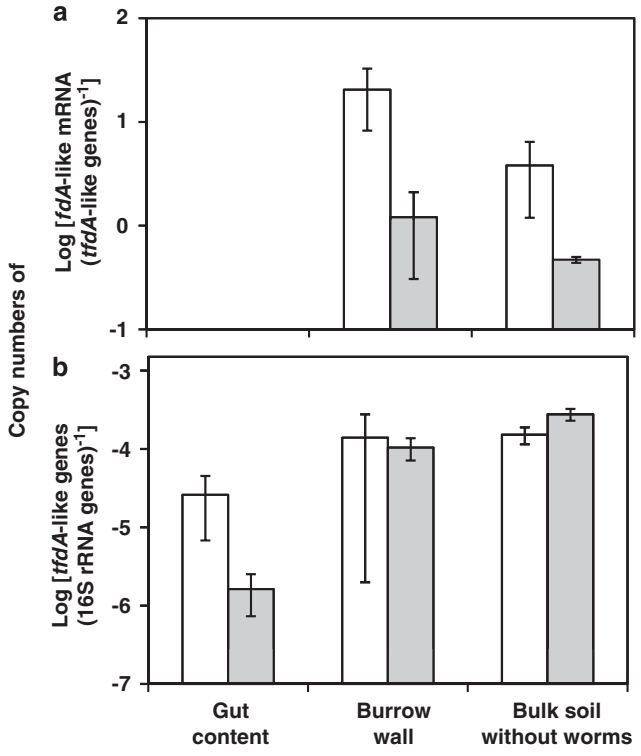

Figure 4 Effect of earthworm and MCPA treatment (Figure 3a) on the relative abundance and expression of *tfdA*-like genes in gut contents, burrow walls and bulk soil at day 37 of soil column incubation. (**a**) *tfdA*-like mRNA per *tfdA*-like genes; (**b**) *tfdA*-like genes per 16S rRNA genes. *tfdA*-like genes and transcripts were quantified with primer set 2 (Table 1). Symbols: white bars, soil treated with MCPA; gray bars, soil treated without MCPA. Error bars represent standard deviations based on three replicates.

MCPA degradation capacity of bulk soil after column incubation

MCPA degradation started without apparent delay in oxic microcosms with bulk soil (that is, mixed soil that did not contain burrow walls or gut contents) from the upper 0–10 cm of soil that was treated with MCPA and earthworms (Figure 5). Ninety percent of initial MCPA was degraded within 2 days (Figure 5). In contrast, MCPA degradation started with apparent delay in oxic microcosms with soil that was treated with MCPA in the absence of earthworms (Figure 5). MCPA degradation started earlier in 0–5 cm soil (Figure 5a) than in 5–10 cm soil (Figure 5b) and was completed after 9 days in such microcosms, indicating that initiation of MCPA degradation is soil depth dependent. MCPA degradation started at days 6–8 in oxic microcosms with control soil that was incubated without MCPA (Supplementary Figure S2). Ninety percent of initial MCPA was degraded within 9 and 12 days in such microcosms with soil that was pre-incubated in the

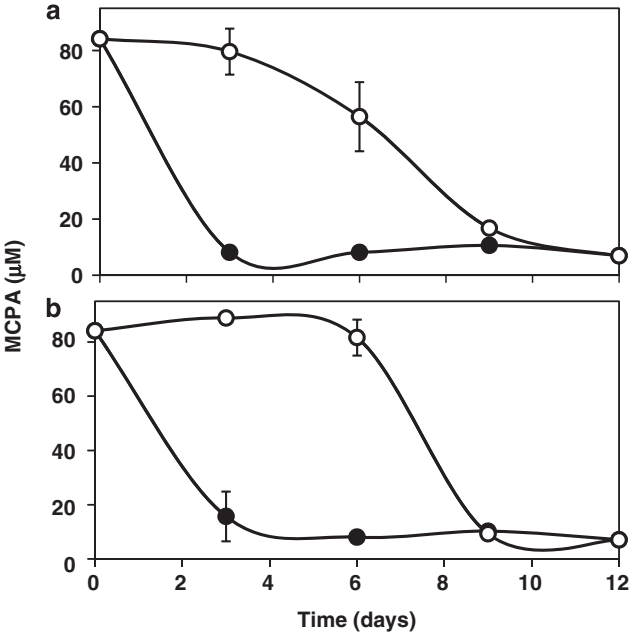

Figure 5 Effect of earthworm treatment (Figure 3a) on the capacity of soil from 0–5 cm (**a**) and 5–10 cm (**b**) depth of soil columns to degrade MCPA after 37 days of incubation. Oxic microcosms contained soil that had been treated with MCPA in the presence (●) or absence (○) of earthworms. Error bars represent standard deviations of three replicates.

presence and absence of earthworms, respectively (Supplementary Figure S2). Similar results were obtained for oxic microcosms that were supplemented with high concentrations of MCPA (approximately 300 μM; data not shown). Up to 30% of initial MCPA-^{14}C were recovered in CO_2 after 28 days of incubation in microcosms with earthworm- and MCPA-treated soil. Approximately 10% of initial MCPA-^{14}C were recovered as NaOH-extractable and -non-extractable organic residues (Supplementary Figure S3) after long-term incubation, confirming results obtained by high-performance liquid chromatography analyses.

MCPA degradation capacity of drilosphere soil after column incubation

Gut content did not facilitate MCPA degradation in oxic microcosms, indicating that MCPA is not degraded in the earthworm gut (Figure 6 and Supplementary Figure S3). MCPA increased during the incubation of gut content in microcosms (Figure 6), indicating a release of MCPA from gut content. Such data are consistent with the bioconcentration of MCPA in worms during soil column incubation (Supplementary Figure S1 and Supplementary Results). In contrast, MCPA was degraded within 3 days in oxic microcosms with burrow walls and bulk soil from 0–5 cm depth and within 6 days in oxic microcosms with bulk soil from 5–10 cm depth (Figure 6), indicating that burrow walls and soil from the upper 0–5 cm sustain MCPA degraders that are poised to degrade MCPA. MCPA degradation was completed within 9 and 12–15 days in microcosms with burrow walls and bulk soil, respectively, which were retrieved from a second set of soil columns (Supplementary Figure S4), suggesting that burrow walls might represent a 'hotspot' for MCPA degradation in soils. The low recovery of MCPA-^{14}C in NaOH-extractable and -non-extractable organic residues after long-term incubation of burrow walls supplemented with ^{14}C-MCPA in microcosms corroborates such a conclusion (Supplementary Figure S3).

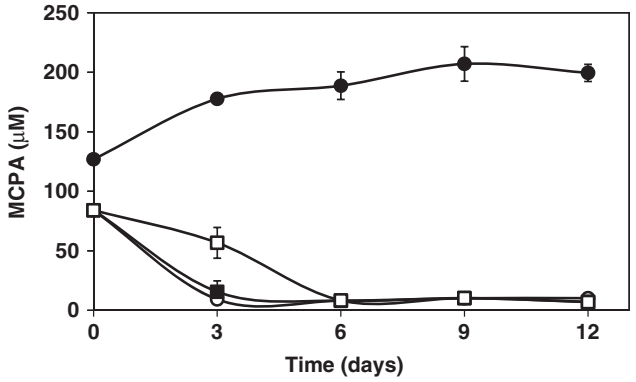

Figure 6 Capacity of gut contents (●), burrow walls (○), 0–5 cm (■) and 5–10 cm (□) depth bulk soil to degrade MCPA. Oxic microcosms contained soil that had been treated with MCPA in the presence of earthworms in soil columns (Figure 3a). Error bars represent standard deviations of three replicates.

Enumeration of MCPA-degrading aerobes

MPNs of MCPA degraders in field fresh Scheyern soil before soil column incubations were $1.5 \pm 0.5 \times 10^4\ g_{dw}^{-1}$, indicating that Scheyern soil harbors low numbers of MCPA degraders before treatment with MCPA and/or earthworms. After incubation in soil columns, MPNs occurred in the following order: (Table 2). The upper 0–5 cm of soil harbored 2–30 times more MCPA degraders than did soil from 5–10 cm depth (Table 2), indicating that the abundance of MCPA degraders is soil depth related. MPNs of MCPA degraders in bulk soil after treatment with MCPA and earthworms were 3–4 orders of magnitude higher than those obtained in the absence of earthworms and MCPA, or in field fresh soil before incubation (Table 2). These results were indicative of earthworm- and MCPA-dependent growth of MCPA degraders during soil column incubations. MPNs of MCPA degraders in bulk soil

Table 2 Average MPNs of aerobic MCPA degraders from soil and drilosphere

Sample	*+MCPA*[a] *(MPNg_{dw}^{-1} ($\times 10^5$))*		*$MPN^{+worms}_{+MCPA}/MPN^{-worms}_{+MCPA}$*[c]	*−MCPA*[a] *(MPNg_{dw}^{-1} ($\times 10^5$))*		*$MPN^{+worms}_{-MCPA}/MPN^{-worms}_{-MCPA}$*[c]
	+Worms[b]	*−Worms*[b]		*+Worms*[b]	*−Worms*[b]	
Gut content	ND	NA	NA	ND	NA	NA
Burrow wall	542 ± 154	NA	NA	4.47 ± 1.25	NA	NA
0–5 cm depth soil	1149 ± 339	7.85 ± 2.41	146	4.29 ± 1.20	0.12 ± 0.03	36
5–10 cm depth soil	150 ± 660	0.88 ± 0.28	167	0.14 ± 0.04	0.05 ± 0.02	3

Abbreviations: MCPA, 2-methyl-4-chlorophenoxyacetic acid; MPN, most probable numbers; NA, not applicable; ND, not detected, that is, MPNs were smaller than 40 g_{dw}^{-1} of gut content.
Soil and drilosphere material were obtained posterior to soil column incubations with or without MCPA in the presence or absence of earthworms (Figure 2b). Standard deviations were calculated based on MPN analyses of three replicate soil columns.
[a]MPNs of samples from soil columns supplemented with (+MCPA) or without (−MCPA) MCPA.
[b]MPNs of samples from soil columns supplemented with (+worms) or without (−worms) earthworms.
[c]Ratio of MPNs from soil incubated in the presence and absence of earthworms; values greater than 1 indicate earthworm-dependent enhanced growth of MCPA degraders.

after treatment with earthworms only were up to two orders of magnitude higher than those of soil without earthworms (Table 2), indicating that earthworms alone stimulated growth of MCPA degraders. MPNs of MCPA degraders in MCPA-treated soil were two orders of magnitude higher than those in non-MCPA-treated soil, indicating that MCPA likewise stimulated growth of MCPA degraders.

MPNs of MCPA degraders in burrow walls were in the same range as those of 0–5 cm depth bulk soil after soil column incubation, indicating that MCPA degraders were abundant in burrow walls (Table 2). Burrow walls from MCPA-treated soil harbored 100 × more MCPA degraders than burrow walls from non-MCPA-treated soil (Table 2), indicating that MCPA stimulated growth of MCPA degraders in burrow walls. MCPA degraders were not detectable in gut contents (Table 2). Thus, MPNs were consistent with the capacity of burrow walls to degrade MCPA and the virtual absence of this capacity in earthworm gut content (see Figure 6, Supplementary Figure S4 and Supplementary Figure S5).

Discussion

Earthworm-mediated stimulation of MCPA degradation

Microbial MCPA degradation in soil was stimulated by earthworms (Figures 2 and 3), which extends previous reports on earthworm-stimulated herbicide degradation in certain soils to MCPA (Pivetz and Steenhuis, 1995; Mallawatantri *et al.*, 1996; Meharg, 1996; Gevao *et al.*, 2001; Binet *et al.*, 2006). As earthworms represent the dominant macrofauna in many soils and strongly interact with the soil microbial community (Edwards and Bohlen, 1996; Drake and Horn, 2007), such findings highlight the importance of earthworms for degradation of xenobiotics.

MPNs of MCPA degraders in Scheyern soil before MCPA exposure were up to four orders of magnitude higher than those of PAA degraders in other soils (Cullimore, 1981; Holben *et al.*, 1992). Copy numbers of *tfdA*-like genes were likewise higher than or similar to those in diverse soils (Bælum *et al.*, 2006; Gonod *et al.*, 2006; Bælum and Jacobsen, 2009; Zaprasis *et al.*, 2010), indicating that Scheyern soil harbors a robust MCPA-degrading community. Exposure of such a community to earthworms stimulated the soils' MCPA degradation capacity and the growth of MCPA degraders, and also increased *cadA* abundance, *tfdA*-like gene and transcript copy numbers (Figures 3–5 and Table 2), indicating that earthworms stimulate MCPA degradation in soil by stimulating growth and activity of MCPA degraders endogenous to soil.

Structural genes associated with MCPA degradation in soil

MCPA degradation in soil is generally associated with class I and III *tfdA* genes of the betaproteobacterial group 1 organisms in soil (Bælum *et al.*, 2006, 2008) and organisms not capable of degrading the PAA 2,4-D harbor *tfdA*-like genes (Hogan *et al.*, 1997). However, gene products of *tfdA*-like genes are capable of PAA conversion (Itoh *et al.*, 2002), indicating a potential role of *tfdA*-like genes for a (co-metabolic) conversion of MCPA. Indeed, *Bradyrhizobium*-related *tfdA*-like genes are abundant in Scheyern soil (Zaprasis *et al.*, 2002, 2010) and the expression of *tfdA*-like genes was stimulated by MCPA (Figures 1a and 4a). *cadA* is induced by 2,4-D, *cadA*-encoded oxygenases are likewise capable of 2,4-D conversion and *cadA*-hosting organisms catabolize diverse PAA degradations (Kitagawa *et al.*, 2002; Huong *et al.*, 2007, 2008). *cadA*-hosting microorganisms were enriched during MCPA degradation in this study (Figures 1b and 3c), indicating that such organisms are involved in MCPA degradation. *cadA* and group 2 *tfdA*-like genes occur in PAA-degrading Alphaproteobacteria-like *Bradyrhizobium* sp. and *Sphingomonas* sp. (Itoh *et al.*, 2002, 2004; Kitagawa *et al.*, 2002). *Sphingomonas*- and *Bradyrhizobium*-related PAA degraders are often isolated from soil, are capable of MCPA degradation and *Sphingomonas* spp. numerically dominate PAA degraders in certain soils (Ka *et al.*, 1994; Kamagata *et al.*, 1997; Chung and Ka, 1998; Park and Ka, 2003; Huong *et al.*, 2007; Macur *et al.*, 2007). The qPCR assays used in this study target diverse *tfdA*-like genes (including novel and *Bradyrhizobium*-related genes) of Scheyern soil (Zaprasis *et al.*, 2010) and *cadA* of *Bradyrhizobium* and *Sphingomans* spp. (Kitagawa *et al.*, 2002; Huong *et al.*, 2007). Terminal restriction fragment length polymorphism analysis of *tfdA*-like genes indicated that groups 3 and 4, and diverse group 2 *tfdA*-like genes occurred in the drilosphere and soil, and that relative abundances of group 1 *tfdA*-like genes during terminal restriction fragment length polymorphism analysis were low (see Supplementary Materials and methods, Supplementary Results and Supplementary Figure S5). These collective findings suggest that *Sphingomonas*- and *Bradyrhizobium*-related alphaproteobacterial as well as hitherto undetected MCPA degraders in Scheyern soil were associated with MCPA degradation.

Earthworms stimulate alphaproteobacterial MCPA degraders

Alphaproteobacteria including rhizobia are active and abundant in the earthworm gut as indicated by 16S rRNA analyses and their isolation (Ihssen *et al.*, 2003; Wüst *et al.*, 2010), are abundant in isolate libraries of cast and are readily isolated from burrow walls (Furlong *et al.*, 2002; Singleton *et al.*, 2003). Alphaproteobacterial 16S rRNA genes are likewise abundant in 16S rRNA gene libraries of cast (Furlong *et al.*, 2002). Relative gene abundances of *cadA* and group 2–5 *tfdA*-like genes were enhanced by earthworms and the degradation of MCPA was

quantitatively linked to gene expression of such *tfdA*-like genes (Figures 1 and 3), indicating that earthworms stimulated *Sphingomonas*- and *Bradyrhizobium*-related, soil endogenous alphaproteobacterial MCPA degraders.

Burrow walls as 'hotspots' of MCPA degradation

Burrow walls (assuming 2 mm thickness) can have areas of up to $1.2\,m^2\,m^{-3}$ and volumes of $50\,l\,m^{-3}$, indicating that such structures are important in soil (for example, Tiunov and Scheu, 1999; Bastardie *et al*., 2005). Such surfaces in soil are exposed to oxygen, are associated with high exoenzyme activities, carbon turnover and sustain a highly respiratory active, heterotrophic microbial community (Zhang and Schrader, 1993; Tiunov and Scheu, 1999; Don *et al*., 2008). Burrow walls are rich in (dissolved) organic carbon, nutrients and moisture, and thus provide ideal conditions for soil aerobes (Zhang and Schrader, 1993; Devliegher and Verstraete, 1997; Parkin and Berry, 1999; Tiunov and Scheu, 1999; Tiunov and Dobrovolskaya, 2002; Amador *et al*., 2003). MCPA degradation is stimulated by moisture in soil (Helweg, 1987). Alphaketoglutarate is a cofactor required for the cleavage of etherbonds by many aerobic MCPA degraders and has a high probability to occur in burrow walls (Müller and Babel, 2000, 2001; Babel, 2009). Indeed, burrow walls yielded higher or similar cell numbers of MCPA degraders (Table 2), higher *tfdA*-like gene expression (Figure 4) and higher capacities to degrade MCPA (Figure 6 and Supplementary Figure S5) than did bulk soil, indicating that MCPA degraders are active in burrow walls.

Burrows of soil-feeding earthworms like *A. caliginosa* are more unstable than those of anecic worms that feed on litter (Jegou *et al*., 1998). The burrow walls are coated with casts, urine and body-surface-secreted mucus (Edwards and Bohlen, 1996; Gorres *et al*., 2001). Surface-secreted mucus selectively stimulates certain microbial taxa (Oleynik and Byzov, 2008). Thus, stabilization of burrows, continuous supply of nutrients and the stimulation of MCPA degraders would require the continuous presence of the worms. Burrows can enhance the transport, sorption and degradation of pesticides (for example, Farenhorst *et al*., 2000, 2001; Jensen *et al*., 2002; Bolduan and Zehe, 2006; Kersante *et al*., 2006; Monard *et al*., 2008). Consequently, burrows represent an important habitat sustaining active aerobic herbicide degraders in soils.

The special conditions of the burrow walls extend up to 1 cm into surrounding soil (Tiunov and Scheu, 1999; Tiunov and Dobrovolskaya, 2002), indicating that soil regarded as bulk or non-drilosphere soil might also be affected by earthworms (that is, represent, in fact, drilosphere soil). Such an assumption is corroborated by the enhanced MCPA degradation in bulk soil with earthworms (Figures 2 and 3a), the high capacity of soil pre-exposed to earthworms to degrade MCPA (Figure 5 and Supplementary Figure S3), MPNs of MCPA degraders (Table 2), relative *tfdA*-like gene abundances and expression (Figure 4), and the high *cadA* gene abundances in soil with earthworms (Figure 3c). Thus, the impact of earthworms on MCPA degraders extends from the drilosphere *sensu stricto* (that is, burrow walls <2 mm thickness; Bouche, 1975) into bulk soil, highlighting the importance of the earthworm burrowing activity for herbicide degraders in soil.

Conclusions

As earthworms stimulated the capacity of soil to degrade MCPA and likewise stimulated replication of MCPA degraders in soil incubated in the presence and absence of MCPA (Figure 5 and Table 2), it may be hypothesized that soils with high earthworm densities harbor a microbial community prone to degrade herbicides more effectively than soils with a low number of earthworms. Earthworms might be utilized for bioaugmentation (that is, the introduction of degrader strains into soil), bioturbation (that is, mixing and redistribution of contaminants in soil), and thus generally bioremediation purposes (Daane and Haggblom, 1999; Hickman and Reid, 2008; Monard *et al*., 2008). Thus, identification of important microbial taxa involved in the degradation of certain contaminants and unraveling the complex interactions of invertebrates with such degraders in soils is fodder for future research and might provide a basis for improved contaminant removal from soil.

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