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RESEARCH ARTICLE

Evidence for the importance of litter as a co-substrate for MCPA dissipation in an agricultural soil

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Abstract Environmental controls of 2-methyl-4-chlorophenoxyacetic acid (MCPA) degradation are poorly understood. We investigated whether microbial MCPA degraders are stimulated by (maize) litter and whether this process depends on concentrations of MCPA and litter. In a microcosm experiment, different amounts of litter (0, 10 and 20 g kg^{-1}) were added to soils exposed to three levels of the herbicide (0, 5 and 30 mg kg^{-1}). The treated soils were incubated at 20 °C for 6 weeks, and samples were taken after 1, 3 and 6 weeks of incubation. In soils with 5 mg kg^{-1} MCPA, about 50 % of the MCPA was dissipated within 1 week of the incubation. Almost complete dissipation of the herbicide had occurred by the end of the incubation with no differences between the three litter amendments. At the higher concentration (30 mg kg^{-1}), MCPA endured longer in the soil, with only 31 % of the initial amount being removed at the end of the experiment in the absence of litter. Litter addition greatly increased the dissipation rate with 70 and 80 % of the herbicide being dissipated in the 10 and 20 g kg^{-1} litter treatments, respectively. Signs of toxic effects of MCPA on soil bacteria were observed from related phospholipid fatty acid (PLFA) analyses, while fungi showed higher tolerance to the increased MCPA levels. The abundance of bacterial *tfdA* genes in soil increased with the co-occurrence of litter and high MCPA concentration, indicating the importance of substrate availability in fostering MCPA-degrading bacteria and thereby improving the potential for removal of MCPA in the environment.

Keywords MCPA · *tfdA* gene · PLFAs · Degradation · Litter · Toxicity

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Introduction

The use of herbicides in agriculture has significantly increased in the past five decades. 2-Methyl-4-chlorophenoxyacetic acid (MCPA) and its related compound 2,4-dichlorophenoxyacetic acid (2,4-D) belong to the phenoxy acid herbicide family and are world widely used for postemergence control of annual and perennial broad-leaf weeds in agricultural fields (Caux et al. 1995; Roberts et al. 1998). Following application to soil, phenoxy acids which are analogue to auxin may trigger specific degradation pathways playing a key role in their attenuation.

MCPA is mainly degraded by soil microorganisms (McGhee and Burns 1995; Thorstensen and Lode 2001), and its half-life in soil ranges between 1 and 16 weeks depending on soil conditions (Audus 1964; Helweg 1987; Sandmann et al. 1988). Several bacterial strains have been identified as MCPA and 2,4-D degraders (Bollag et al. 1967; Horvath et al. 1990; Kilpi et al. 1980; Oh et al. 1995; Pieper et al. 1988). Most of these bacterial strains harbour the *tfd* pathway known

for phenoxy acid herbicide degradation (Chaudhry and Huang 1988; Streber et al. 1987). The functional gene *tfdA* encodes an α-ketoglutarate-dependent dioxygenase responsible for the cleavage of the acetate side chain during the initial degradation step (Fukumori and Hausinger 1993; Streber et al. 1987). Unlike bacterial degradation of phenoxy acids, which is well known, the degradation of the herbicide by soil fungi has received less attention. Vroumsia et al. (2005) screened 90 fungal species belonging to ten taxonomic groups for their potential to degrade 2,4-D. Two of the species attained a dissipation potential of about 50 % within 5 days.

Many of the studies which have been conducted to investigate microbial degradation of MCPA or similar phenoxy acid herbicides were done either in pure cultures or in bioreactors that are vastly different from field conditions. The regulation of herbicide degradation in soils, however, may differ from artificial systems, and specific soil conditions like oligotrophy could play an important role. While the degradation of these herbicides, including MCPA, has been found to follow first-order kinetics (Crespin et al. 2001; Fu et al. 2009), the degradation rates seem to be affected by various environmental factors. These factors include, among others, initial pesticide concentration (Fomsgaard 1997) and organic matter content. Organic matter especially has been shown to enhance MCPA and 2,4-D dissipation in soil (e.g., López-Piñeiro et al. 2013). For example, an enhanced degradation of MCPA and other herbicides was observed in organic-rich Norwegian soils by Thorstensen and Lode (2001). Nutrient-rich rhizodeposits enhanced the dissipation of phenoxy acetic acids (Merini et al. 2007; Shaw and Burns 2004). In addition, Duah-Yentumi and Kuwatsuka (1982) showed that plant residue addition increased MCPA degradation in paddy fields. Therefore, substrate availability is important for the growth of indigenous phenoxy acid-degrading microbial populations, which are primarily the agents for pesticide degradation. In a soil microcosm study, Poll et al. (2010a) showed that MCPA degradation was improved in soil amended with maize litter. The upregulation of the MCPA-degrading community in soil was suggested to be the result of the presence of metabolic precursors of the co-substrate α-ketoglutarate in the litter. Vieublé Gonod et al. (2003) showed that uneven distribution of 2,4-D degradation at the microscale could be due to heterogeneous distribution of either the degrading community and/or organic carbon source necessary for co-metabolic degradation of 2,4-D.

The above studies of Poll et al. (2010a) and Vieublé Gonod et al. (2003) were carried out at a millimetre scale, and direct contact of soil with plant litter was restricted to the surface of soil cores. Incorporation of litter into soil increases the contact to organic sources and, consequently, the volume and number of affected soil and MCPA degraders, respectively. Furthermore, the amount of added litter in these studies was constant, and the MCPA concentration was relatively high. The objective of this study was therefore to prove the relevance of the previously observed effects for soil remediation by estimating the influence of substrate availability (litter) on MCPA degradation and on the microbial community involved. Specifically, we focused on the effect of different MCPA concentrations and litter amounts. We hypothesize that litter addition (1) will increase MCPA degradation in the soil and (2) favour the growth of specific MCPA degraders and that (3) the litter effect on MCPA degradation may be controlled by litter quantity and MCPA concentration. To address these objectives, we incubated soil spiked with different amounts of MCPA followed by litter in three quantities in a microcosm experiment. The effect of litter addition on MCPA degradation was estimated after 1, 3 and 6 weeks of incubation. The effect of MCPA and litter addition on the soil microbial community was analyzed by extraction of phospholipid fatty acids (PLFAs), whereas the evolution of the abundance of MCPA degraders was estimated by quantifying the number of *tfdA* gene sequences.

Material and methods

Study site and soil sampling

The soil for the incubation experiment was obtained from an experimental station for agricultural research in Scheyern, north of Munich, Germany (48° 30′ N, 11° 21′ E). It was not treated with MCPA or 2,4-D since 1998. The soil is a silty loam classified as Luvisol (World Reference Base for Soil Resources) with the following characteristics: pH ($CaCl_2$) 5.3, total C content 13.6 g kg^{-1}, total N 1.32 g kg^{-1} and water holding capacity (WHC) 55.6 %. In July 2007, soil was sampled from the upper horizon (0–30 cm), sieved (2 mm), homogenized and stored at −20 °C. Maize litter with a C/N ratio of 48 (47.4 % C, 1.0 % N) was chosen for the incubation. Litter of maize leaves and stems was cut into pieces of 2–10-mm length and stored air-dried until the start of the experiment.

Experimental setup

In order to study the combined effects of substrate availability and herbicide concentrations on the soil microbial community, nine treatments were setup. Three MCPA concentrations (0, 5 and 30 mg kg^{-1}) and three different amounts of maize litter (0, 10 and 20 g kg^{-1}) were arranged in a factorial design in triplicate. The MCPA concentration of 5 mg kg^{-1} is in the range of measured field concentrations after herbicide application (Johnsen et al. 2013; Smith et al. 1989), whereas 30 mg kg^{-1} was considered as an upper limit for environmental concentrations of MCPA. Thirty grams of soil per replicate was spiked with MCPA (98.9 % purity, Sigma-Aldrich) to the

above-mentioned concentrations. The water content of soil was adjusted to 50 % WHC. The spiked soil was then thoroughly mixed with the maize litter, which had been rewetted with 1 ml 0.01 M $CaCl_2$ the previous day. Soil and litter were moistened using a 0.01-M $CaCl_2$ solution to avoid dispersion of soil particles. The microcosms (750 ml) were closed tightly and incubated at +20 °C in the dark for 1, 3 and 6 weeks to avoid photodegradation. A total of 27 microcosms were setup per incubation time. Soil respiration and, consequently, oxygen depletion were followed by trapping evolved CO_2 in 1-M NaOH solution (data not shown). All microcosms were opened every 2–3 days for 30–60 min for aeration; loss of soil water was minimal. At the end of each incubation time, the litter was removed and soils were stored at −20 °C for later analysis.

MCPA dissipation

Residual MCPA was extracted from 3 g of soil with 14 ml of methanol/water (1:1) at 50 °C in a water bath for 30 min. The soil suspension was shaken before and after incubation to improve homogenization. Following centrifugation for 15 min at 25 °C, 1.5 ml of supernatant was filtered (0.45-mm pore size) into HPLC vials. MCPA concentration was estimated by HPLC (System Gold, Beckman Instruments) according to the method of Moret et al. (2006). The separation was carried out on a 200 mm×4.6 mm column packed with 5 mm Kromasil 100 C18 (MZ-Analysentechnik GmbH) at 20 °C with tetrabutyl ammonium hydroxide as ion-pairing reagent dissolved at a concentration of 10 mM in acetonitrile/water (30:70, *v*/*v*) as mobile phase. The flow rate was 1 ml min^{-1}. Detection of MCPA was done at a wavelength of 228 nm with a UV detector. Identification of MCPA was determined by peak retention time and quantification by peak area of pure MCPA standards; the extraction recovery of MCPA from freshly spiked soil extracted after storage at 4 °C for 24 h was 96 %. Metabolites were not analyzed. Decrease in residual MCPA concentrations is considered as dissipation below since the measured data did not allow separation between MCPA degradation and sorption to soil particles.

We used two different kinetic models based on simplifications of Monod kinetics by Simkins and Alexander (1984) to fit the residual MCPA concentrations extracted from soils initially spiked with 5 mg kg^{-1} MCPA (first order, Eqs. 1a, 2a and 3a) and 30 mg kg^{-1} MCPA (zero order, Eqs. 1b, 2b and 3b):

$$\frac{dC}{dt} = -k_1 C \tag{1a}$$

$$\frac{dC}{dt} = -k_2 \tag{1b}$$

Here, C (mg kg^{-1}) stands for the residual concentration of MCPA in soil at time t (days), k_1 (day^{-1}) is the first-order dissipation coefficient, and k_2 (mg kg^{-1} day^{-1}) is the zero-order dissipation coefficient.

With the initial condition that $C(t=0)=C_0$, the solutions of Eqs. 1a, b read as follows:

$$C = C_0 \cdot e^{-k_1 \cdot t} \tag{2a}$$

$$C = C_0 - k_2 \cdot t \tag{2b}$$

Equations 2a, b can be transformed to directly include the half-life ($T_{50} = t\left(C = \frac{C_0}{2}\right)$) of MCPA:

$$C = C_0 \cdot e^{-\frac{\ln(2) \cdot t}{T_{50}}} \tag{3a}$$

$$C = C_0 \left(1 - \frac{1}{2 \cdot T_{50}} \cdot t\right) \tag{3b}$$

The two parameters for the MCPA dissipation kinetics (C_0 and T_{50}; Eq. 3a, b) were estimated by fitting an extended nonlinear regression model to the MCPA data as implemented in the *nlme* library (Pinheiro and Bates 2002) of the statistical software package R (R Development Core Team 2011):

$$C_{i,j} = b_{1-i} \cdot C_{0-i} \cdot e^{-\frac{ln(2) \cdot t}{T_{50-i,j}}} + b_{2-i} \cdot C_{0-i} \cdot \left(1 - \frac{t}{2 \cdot T_{50-i,j}}\right) + \varepsilon_{i,j}, \tag{4}$$

where $C_{i,j}$ is the predicted concentration of MCPA in soil (mg kg^{-1}) at time t (days) of the ith MCPA (5 or 30 mg kg^{-1}) and jth litter (0, 0.3 or 0.6 g) treatments and $\varepsilon_{i,j}$ is the residual error. A power variance model (chapter 5.2.1, Pinheiro and Bates 2002) was used to account for heteroscedastic residual error variances, which varied with MCPA concentration and sampling date. The dissipation coefficients are related to the initial MCPA concentration, and therefore, k_1 and k_2 of the three corresponding litter treatments were closely linked to each other. To account for this, we used the "dummy" variables b_{1-i} and b_{2-i} as blocking factors (chapter 14, Draper and Smith 1998) to separate the MCPA data into two blocks and to apply two different model equations (Eqs. 2b and 3b) depending on the initial MCPA addition in one common regression analysis. Accordingly, for soils with 5 mg kg^{-1} MCPA, we set $b_1=1$ and $b_2=0$, whereas for soils with 30 mg kg^{-1} MCPA, the blocking factors were set to $b_1=0$ and $b_2=1$. As evident from Eq. 4, we fitted a common value of C_0 for 5 and 30 mg MCPA kg^{-1} treatments, respectively. The half-lives T_{50} were estimated individually for each combination of MCPA treatment and litter amendment. In total, we estimated eight parameters using 60 data points of residual MCPA concentrations in soil (including three repetitions per sampling date). Significant differences between

half-lives were estimated by a Tukey's range test employing the *glht* function of the *multcomp* R package (Bretz et al. 2011).

16S rRNA gene and *tfdA* gene quantification

Total community DNA was extracted from 0.3-g soil using the FastDNA Spin Kit for soil (BIO101, MP Biomedicals) according to the manufacturer's instructions. Soil DNA extracts were quantified with a NanoDrop ND1000 Spectrophotometer (NanoDrop Technologies Inc.), diluted to 10 ng μl^{-1} in molecular biology-grade water and stored at −20 °C until use.

Prior to qPCR assays, the presence of qPCR inhibitors in soil DNA was checked by comparison of standard plasmid DNA quantification in the presence or absence of soil DNA (Philippot et al. 2009). DNA dilutions showing no inhibition were used to quantify copy numbers of *tfdA* and 16S ribosomal RNA (rRNA) genes by qPCR using an ABI Prism 7900 (Applied Biosystems) with a SYBR Green PCR Master Mix (QuantiTect SYBR Green PCR Kit, QIAGEN). The primer set for quantifying *tfdA* gene was taken from Bælum et al. (2006). 16S rRNA gene was quantified by qPCR according to López-Gutiérrez et al. (2004). Each qPCR reaction contained 2 μl of each primer (10 mM), 10 μl of SYBR Green PCR Master Mix, 0.5 μl of T4gp32 (Qbiogene), 3.5 μl water and 2 μl of diluted soil DNA corresponding to 20 ng of soil DNA. 16S rRNA amplifications were carried out using the following program: denaturation at 95 °C for 900 s, followed by 35 cycles at 95 °C for 15 s, 60 °C for 30 s, 72 °C for 30 s and 80 °C for 30 s (detection). *tfdA* amplifications were as follows: denaturation at 95 °C for 900 s, followed by 40 cycles at 95 °C for 15 s, 64 °C for 30 s and 72 °C for 30 s (detection). For both qPCR assays, a final dissociation stage was performed by increasing the temperature from 80 to 95 °C. Selected *tfdA* PCR products were sequenced to confirm their specificity. 16S rRNA gene standard curves were obtained using tenfold serial dilutions of a linearized plasmid containing cloned 16S rRNA genes from *Pseudomonas aeruginosa* PAO1 (Bru et al. 2008). *tfdA* standard curves were prepared by serial dilution of JMP134 clones of PCR products resulting from soil DNA amplification. Efficiencies of the qPCR reaction were 90 and 98 % for 16S rRNA and *tfdA* genes, respectively. The relative abundance of MCPA degraders among the bacterial community was calculated using the ratio *tfdA*/16S rRNA.

Fungal biomass

Soil fungal biomass was determined by extraction and quantification of ergosterol content using the method of Djajakirana et al. (1996). One gram of soil was suspended in 50 ml ethanol (HPLC-grade, Merck) in 100 ml wide-mouth brown bottles and extracted in a shaker for 30 min at 250 rev. min^{-1} followed by centrifugation in 50-ml tubes at 4422×*g* for 30 min. An aliquot of 20 ml was transferred into a test tube and evaporated in a rotary evaporator at 50 °C under vacuum. Evaporation was repeated with 3 ml ethanol to wash tube walls. The dry extract was then dissolved in 1 ml methanol (gradient grade LiChrosolv, Merck) and percolated through a syringe filter (cellulose acetate, 0.45-mm pore size) into brown glass HPLC vials. Extracts were measured by injection of 20 μl into an HPLC autosampler (Beckmann Coulter, System Gold 125 Solvent Module). Extracts were passed through a column (250 mm×4.6 mm, Spherisorb ODS II 5 μm). Pure methanol was used as mobile phase at a flow rate of 1 ml min^{-1}. The detection was carried out with an UV detector (Beckmann Coulter, System Gold 166) at a wavelength of 282 nm. Identification of ergosterol was determined by peak retention time and quantification by peak area of pure ergosterol standards.

PLFA content

Two grams of soil was used to extract the PLFAs. Lipids were extracted, fractionated and methylated following the procedure used by Frostegård et al. (1993). Fatty acid methyl esters (FAMEs) were identified by chromatographic retention time comparison with a standard mixture composed of 37 different FAMEs that ranged from C11 to C24 (Sigma-Aldrich, St. Louis). Analysis was performed by GC using an Auto System XL (Perkin Elmer Corporation, Norwalk) equipped with an HP-5 capillary column (50 m×0.2 mm i.d., film thickness 0.33 mm). The temperature program started with 70 °C (hold time 2 min) and increased by 30 °C min^{-1} to 160 °C, then by 3 °C min^{-1} to 280 °C and held for 15 min. The injection temperature was 260 °C, and helium was used as carrier gas. For each sample, the abundance of individual FAMEs was expressed in nanomole per gram soil. The fatty acids i15:0, a15:0, i16:0, 16:1ω7, i17:0, cy17:0, 18:1ω7 and cy19:0 were regarded as bacterial PLFAs ($PLFA_{bac}$) of which i15:0, a15:0, i16:0 and i17:0 represent Gram-positive bacteria and cy17:0 and cy19:0 represent Gram-negative bacteria (Kandeler et al. 2008). 18:2ω6 was considered as fungal signature PLFA ($PLFA_{fung}$) (Frostegård et al. 1993) and, in addition to ergosterol, taken as a second independent bioindicator for the fungal biomass. The ratio of 18:2ω6 to bacterial PLFAs represents the ratio of fungal to bacterial biomass in the soil (Frostegård and Bååth 1996).

Statistical analysis

The software R (R Development Core Team 2011) was used for statistical analysis. Significance was tested at $P \leq 0.05$ in all cases. Ergosterol content, the ratio of *tfdA*/16S rRNA genes and PLFA content of bacteria and fungi were tested for significant differences by three-way ANOVA with the fixed factors

MCPA, litter and incubation time. MCPA content was tested by two-way ANOVA for each MCPA concentration separately. When significant differences were observed, a two-way ANOVA was performed at each sampling date to distinguish between effects of MCPA and litter addition. The data of fungal PLFA and *tfdA*/16S rRNA genes were log transformed to achieve homogeneity of variances. The latter was tested with Levene's test.

Results

MCPA dissipation and dissipation kinetics

MCPA dissipation was dependent on MCPA concentration and litter addition (Table 1; Fig. 1). In soils with 5 mg kg^{-1} MCPA, about 50 and 95 % of MCPA dissipated within 1 and 3 weeks of incubation, respectively. Addition of 20 g kg^{-1} litter slightly but significantly reduced MCPA dissipation after 1 week ($F_{2,6}$=11.2, $P \leq 0.01$). These differences disappeared after 3 and 6 weeks. However, soils with 30 mg kg^{-1} MCPA showed different dissipation patterns. In these treatments, dissipation of the herbicide was affected by litter after 3 ($F_{2,6}$=18.2, $P \leq 0.01$) and 6 weeks ($F_{2,6}$=32.0, $P \leq 0.001$) of incubation. Litter amendment significantly enhanced the dissipation of the herbicide compared to the control treatment (Table 2). Despite higher dissipation in the case of 20 g kg^{-1} litter addition, litter treatments were not significantly different from each other.

The dissipation of MCPA could be represented reasonably well by first-order kinetics (Eq. 3a) for soils spiked with 5 mg kg^{-1} MCPA and by zero-order kinetics (Eq. 3b) for soils spiked with 30 mg kg^{-1} MCPA (Fig. 1). As indicated by 95 % confidence intervals given in Table 2, the estimated parameter values of both kinetic models differed significantly from zero and showed acceptable uncertainties. Absolute dissipation rate of MCPA (mg $time^{-1}$ MCPA loss) was lower in soils with 5 mg kg^{-1} MCPA, whereas the relative dissipation rate (mg initial $concentration^{-1}$ MCPA loss) was lower in soils with 30 mg kg^{-1} MCPA. Consequently, estimated half-lives were shorter in soils with less MCPA addition. Litter had no effect on the estimated half-lives (T_{50} ~7 days) at a low MCPA application rate, whereas the half-lives at a high MCPA application rate decreased significantly with litter addition from 51.5 days without litter addition to 29.6 and 26.3 days for the 10 and the 20 g kg^{-1} litter treatments, respectively (Table 2).

tfdA/16S rRNA genes

Copy numbers of the *tfdA* gene were within the range of 5.9×10^4 to 1.4×10^7 g^{-1}, and those of 16S rRNA gene were in the range of 2.1×10^9 to 1.5×10^{10} g^{-1} soil (Tables S1 and S2; Figs. S1 and S2). The relative abundance of the MCPA-degrading bacterial community significantly increased after MCPA addition (Table 1; Fig. 2). This effect was dependent on incubation time and the presence of litter. In soils without litter, the addition of 5 mg kg^{-1} MCPA slightly increased relative abundance of *tfdA* gene copy numbers after 3 and 6 weeks, whereas the addition of 30 mg kg^{-1} MCPA showed no effect. In the presence of litter, MCPA addition strongly increased *tfdA* gene abundance after 3- and 6-week incubation. This effect was pronounced in soils with 30 mg kg^{-1} MCPA (+ 409 to 1405 %). Highest relative abundance of the *tfdA* gene in the litter treatments with 5 mg kg^{-1} MCPA was detected after 3 weeks, whereas in soils with high MCPA concentration, gene abundance peaked at the end of the incubation.

Ergosterol

There were significant effects of MCPA, litter and incubation time on ergosterol levels (Table 1). MCPA increased

Table 1 Three-way ANOVA analyses showing the *P* values for the effect of MCPA, litter and incubation time (time) on ergosterol, bacterial and fungal PLFAs and *tfdA* gene sequences

	Two-way ANOVA		Three-way ANOVA			
	MCPA concentration 5 mg kg^{-1}	30 mg kg^{-1}	Ergosterol content	Bacterial PLFA	Fungal PLFA	*tfdA*/16S rRNA gene
MCPA	–	–	*0.002*	*0.000*	0.905	*0.000*
Litter	*0.010*	*0.000*	*0.000*	*0.000*	*0.000*	*0.000*
Time	*0.000*	*0.000*	*0.001*	*0.000*	*0.012*	*0.000*
MCPA×litter	–	–	*0.022*	0.566	0.319	*0.000*
MCPA×time	–	–	0.097	0.797	0.093	*0.000*
Litter×time	*0.003*	*0.000*	0.167	0.992	0.054	*0.001*
MCPA×litter×time	–	–	0.282	0.513	0.998	*0.000*

MCPA concentrations as a function of litter and incubation time were analyzed by two-way ANOVA. Significant ($P \leq 0.05$) values are indicated in italics

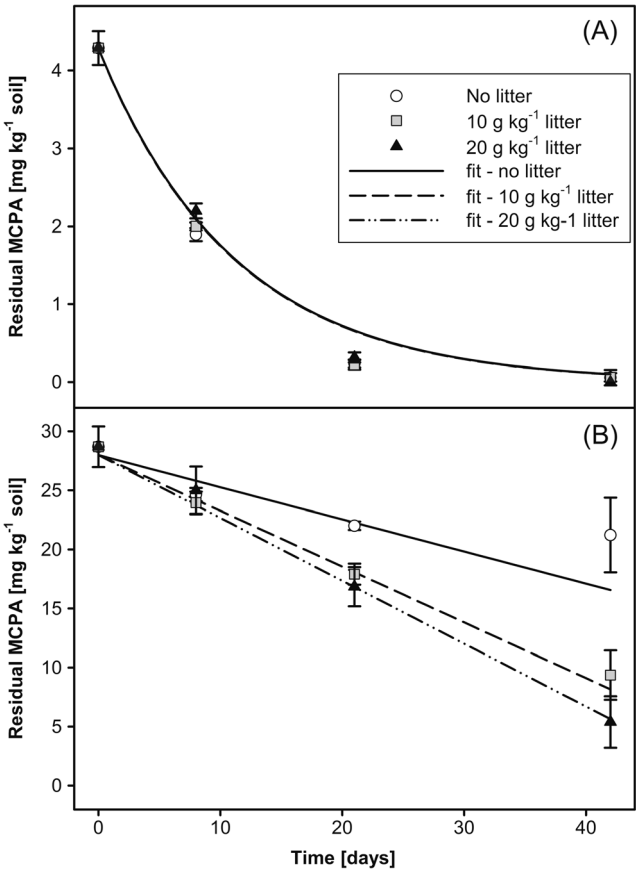

Fig. 1 Residual MCPA concentration in soil **a** spiked with 5 mg kg^{-1} MCPA and fitted with first-order dissipation kinetics and **b** spiked with 30 mg kg^{-1} MCPA and fitted with zero-order dissipation kinetics (see Table 2 for estimated parameter values)

ergosterol contents in the presence of litter (Table 1), which was pronounced at the end of both the first and the third weeks (Fig. 3). At the end of 6-week incubation, signs of a deleterious effect of MCPA on ergosterol were observed (Table 1; Fig. 3), with reduction by the addition of 30 mg kg^{-1} MCPA in the no-litter treatment. Litter addition generally increased ergosterol concentrations.

PLFA contents

Increasing MCPA addition reduced the content of bacterial-derived PLFAs at each of the three sampling dates (Table 1; Fig. 4). Litter addition increased the content of bacterial PLFAs; nevertheless, litter addition could not compensate for the negative effect of higher MCPA loads (Fig. 4). In all treatments, concentrations of $PLFA_{bac}$ declined significantly with incubation time. Both gram-positive and gram-negative bacteria responded similarly to MCPA and litter addition (data not shown).

MCPA concentrations had no effect on the content of fungus-derived PLFA, although there was a trend towards increased $PLFA_{fung}$ contents observed after 1 and 3 weeks of incubation (Table 1; Fig. 5). Signs of deleterious effects of MCPA on $PLFA_{fung}$ appeared at the end of the incubation. Litter addition significantly increased the contents of fungal PLFA. The content of $PLFA_{fung}$ appeared to be increased by MCPA in the presence of litter.

Discussion

Dissipation of MCPA

MCPA dissipation in soil differed with the applied amounts of MCPA and litter. In this study, we did not separate MCPA dissipation into MCPA degradation and other contributing processes like sorption. However, Nowak et al. (2011) showed that most of bound residues of 2,4-D in soils were biogenic residues, i.e., MCPA-derived C which was assimilated by 2,4-D degraders. Since 2,4-D and MCPA belong to the phenoxy acid herbicide family, they share important chemical properties and, consequently, their degradation pathway. We, therefore, hypothesize that degradation is the main process for MCPA dissipation in our study. The degradation rates of many phenoxy acid herbicides have been shown to depend on their concentrations (deLipthay et al. 2007; Smith 1989). Fast

Table 2 Estimated parameter values of first-order (Eq. 3a) and zero-order (Eq. 3b) kinetics for the 5 mg kg^{-1} MCPA and 30 mg kg^{-1} MCPA treatment, respectively

	Treatment		
	No litter	10 g kg^{-1} litter	20 g kg^{-1} litter
Estimated initial MCPA concentration C_0 (mg kg^{-1})			
5 mg MCPA kg^{-1}	4.28 (4.12–4.44)		
30 mg MCPA kg^{-1}	27.98 (26.9–29.1)		
Estimated half-life (days)			
5 mg MCPA kg^{-1}	7.78 a (7.57–7.98)	7.73 a (7.55–7.91)	7.77 a (7.59–7.95)
30 mg MCPA kg^{-1}	51.5 b (39.7–63.2)	29.6 c (26.3–32.9)	26.3 c (24.0–28.6)

Values of half-life followed by a common letter are not significantly different ($p<0.001$). Values in brackets give 95 % confidence intervals of estimated means

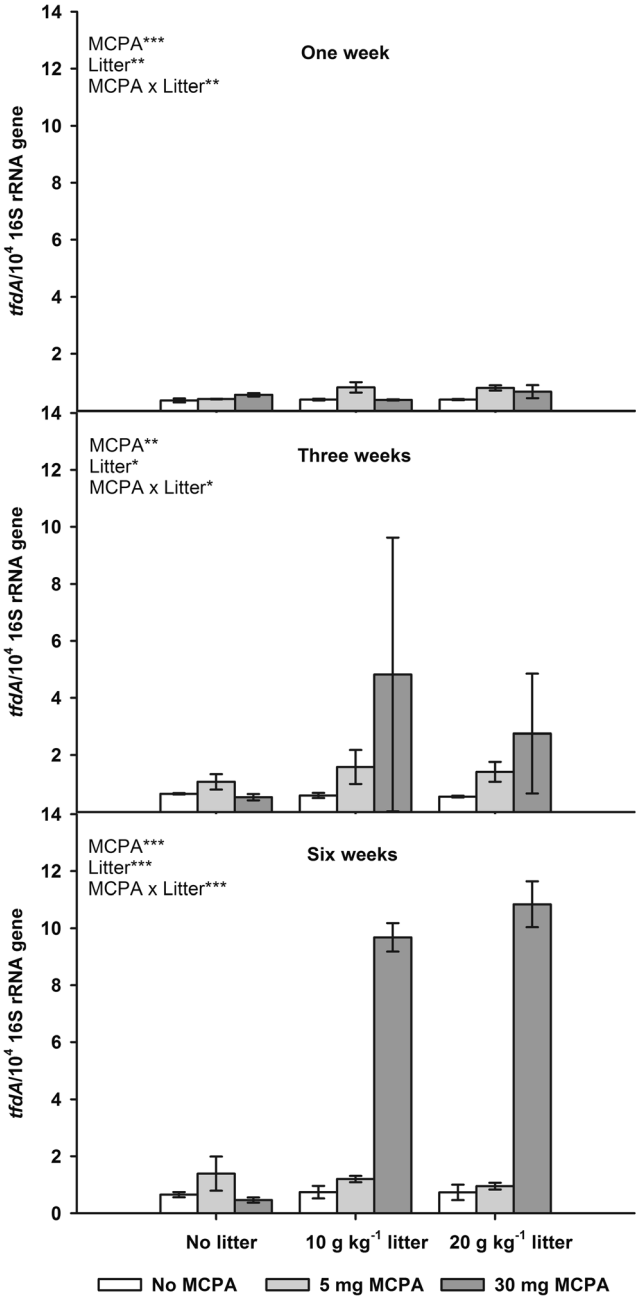

Fig. 2 MCPA effect on *tfdA* per 10^4 16S rRNA sequences as a function of litter amendments at 1-, 3- and 6-week incubation. Data were evaluated with a two-way ANOVA for each sampling date. Significant effects are given in the *insets* (*$P<0.05$, **$P<0.01$, ***$P<0.001$). Values are means (±SD) of three replicates

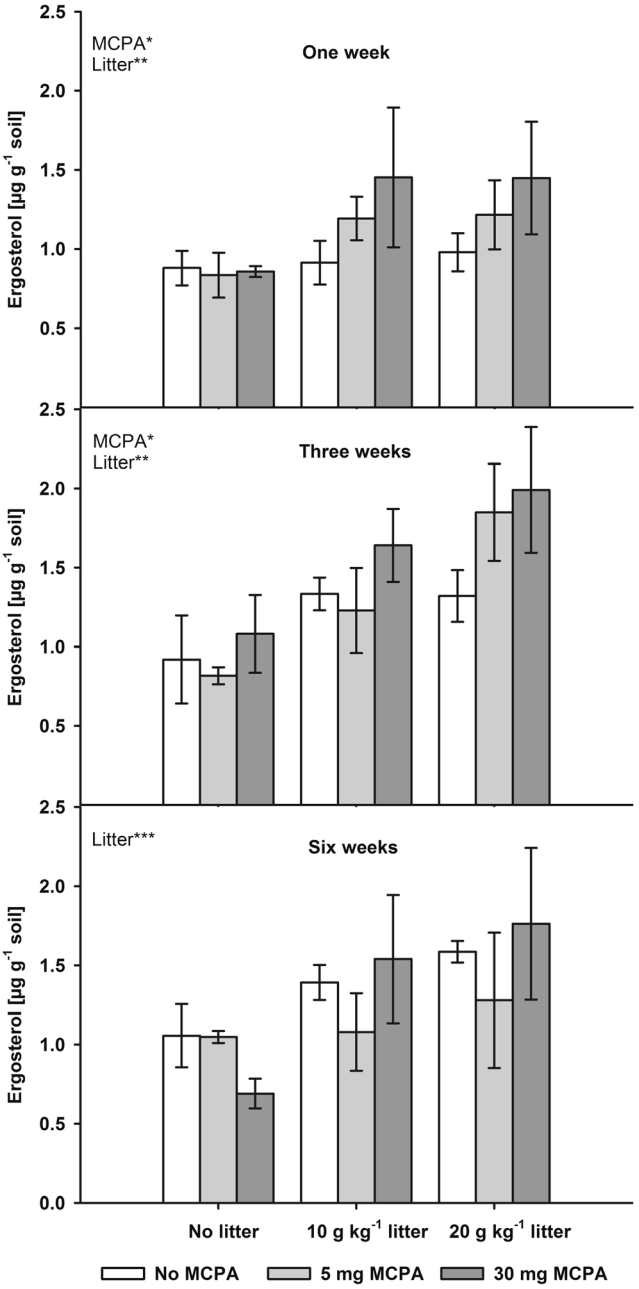

Fig. 3 MCPA effect on ergosterol as a function of litter amendments at 1-, 3- and 6-week incubation. Data were evaluated with a two-way ANOVA for each sampling date. Significant effects are given in the *insets* (*$P<0.05$, **$P<0.01$, ***$P<0.001$). Values are means (±SD) of three replicates

removal of phenoxy acid herbicides at soil concentrations below 10 mg kg^{-1} has been frequently reported (McCall et al. 1981; Ou 1984) whereas at higher concentrations, biphasic degradation has been observed (Smith 1989). In our experiment, 5 mg kg^{-1} MCPA rapidly dissipated in soils without litter addition and dissipation was best described by first-order kinetics (Fig. 1a; Table 2), although the relatively low temporal resolution of measured residual MCPA concentrations limited the unambiguous identification of a kinetic model of MCPA dissipation. However, previous studies have shown first-order kinetics of herbicide degradation at concentrations below 5 mg kg^{-1} (Alton and Stritzke 1973; Smith and Hayden 1980). We, therefore, assume that the applied first-order kinetics is valid and suggest that MCPA dissipation in soils with 5 mg kg^{-1} MCPA was predominantly limited by MCPA bioavailability and not by the abundance of MCPA degraders. Dissipation of 30 mg kg^{-1} MCPA was best described by zero-order kinetics

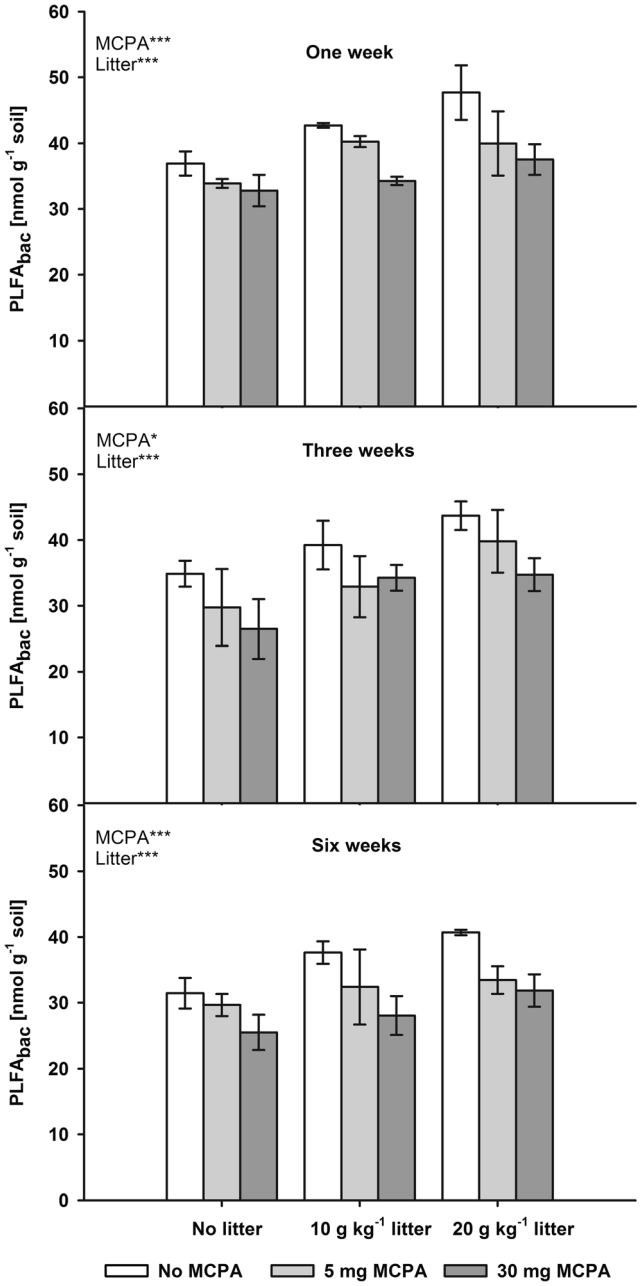

Fig. 4 MCPA effect on bacterial PLFA ($PLFA_{bac}$) as a function of litter amendments at 1-, 3- and 6-week incubation. Data were evaluated with a two-way ANOVA for each sampling date. Significant effects are given in the *insets* (*P<0.05, **P<0.01, ***P<0.001). Values are means (±SD) of three replicates

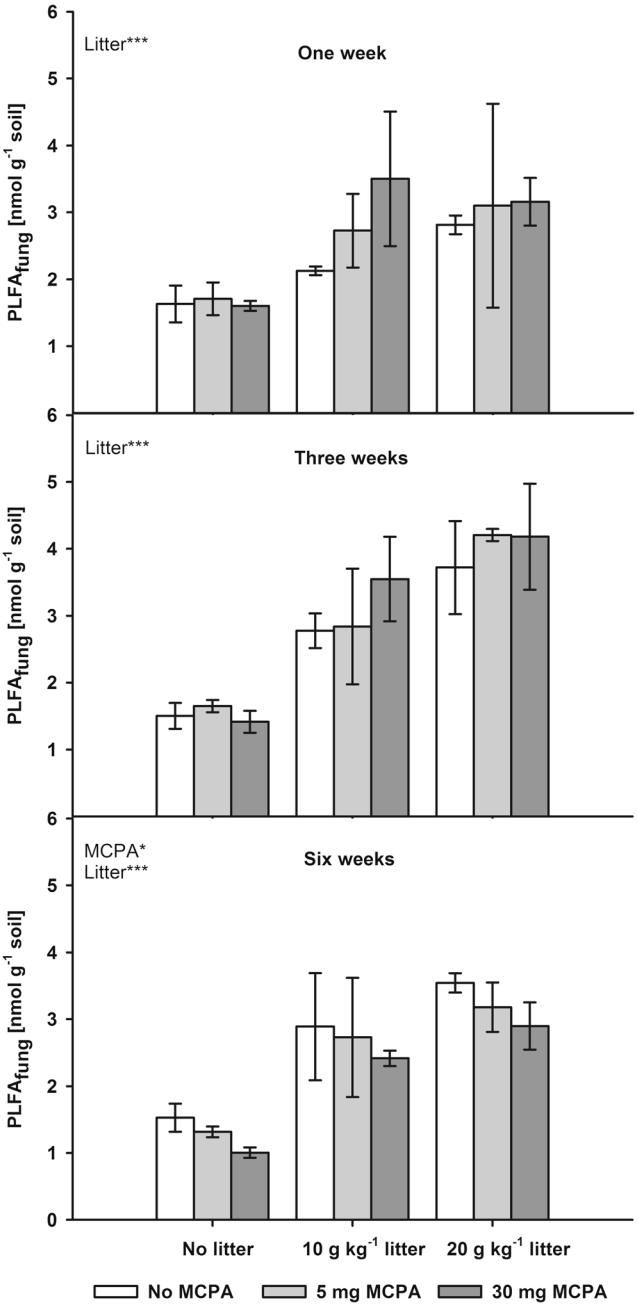

Fig. 5 MCPA effect on fungal PLFA ($PLFA_{fung}$) as a function of litter amendments at 1-, 3- and 6-week incubation. Data were evaluated with a two-way ANOVA for each sampling date. Significant effects are given in the *insets* (*P<0.05, **P<0.01, ***P<0.001). Values are means (±SD) of three replicates

(Fig. 1b; Table 2), which implies that dissipation was probably not limited by MCPA availability but rather by the abundance of MCPA degraders. This suggests that the initial abundance of MCPA degraders in soils with 30 mg kg^{-1} MCPA was not sufficient to degrade MCPA at a relative rate comparable to that in soils with 5 mg kg^{-1} MCPA. Our results are in accordance with those of Johnsen et al. (2013) and Rosenbom et al. (2014), who found that the initial degrader population was an important factor controlling pesticide degradation in soil.

The long half-life of 30 mg kg^{-1} MCPA compared to the half-life of 5 mg kg^{-1} MCPA might be attributed to the lack of pre-exposure of the soil to MCPA. Pre-exposure to 2,4-D and MCPA has been reported to boost degrading populations, thereby increasing the dissipation of these herbicides in agricultural soils (Bælum et al. 2008; Smith and Aubin 1991). Differences in dissipation between both MCPA levels underline the importance of initial herbicide concentrations in

determining the potential proliferation of the MCPA-degrading microbial community.

Several studies have shown that degradation of organic compounds in soils can be improved by adding raw organic matter or compost (e.g., Barker and Bryson 2002; López-Piñeiro et al. 2013). In our study, litter addition significantly accelerated MCPA dissipation only in soils exposed to the high MCPA concentration (Fig. 1; Table 2). A similar effect of rice straw addition on MCPA degradation in a perfused soil system was reported by Duah-Yentumi and Kuwatsuka (1982). Berger (1999) and Cederlund et al. (2007) also found that the addition of straw enhanced the degradation of diuron by stimulating microbial activity. Poll et al. (2010a) found significantly more MCPA-derived C bound to microbial biomass in litter-treated than in litter-untreated soils and suggested that litter addition favoured activity and C assimilation of MCPA-degrading microbes.

MCPA-degrading community

We analyzed the abundance of specific MCPA degraders to test whether the effect of litter addition on MCPA dissipation in soils spiked with 30 mg kg^{-1} MCPA was related to growth of MCPA degraders. The *tfdA* gene encodes for the initial degradation step of the *tfd* pathway, which is the best-known pathway for MCPA degradation (Chaudhry and Huang 1988; Streber et al. 1987). As was the case for the dissipation rate, the effect of MCPA on the MCPA-degrading communities depended on herbicide concentration and litter addition.

In general, quantification of *tfdA* gene sequences revealed a relative increase in the abundance of bacterial MCPA degraders in samples spiked with MCPA (Fig. 2). Therefore, specific MCPA bacterial degraders were found to be supported by increasing levels of MCPA even though the total bacterial population was declining. Our results conform to the observation of Vieublé Gonod et al. (2006) stating that 2,4-D impact on the entire structure of soil microbial communities could be a result of both the growth of specific degraders and a toxic effect of the herbicide on some other guilds. In no-litter treatments, there was only a slight increase in the relative abundance of *tfdA* genes. Contrary to a previous study (Poll et al. 2010a) where soil samples were pre-incubated with MCPA to stimulate the degrading populations, the soil used in the present study had no history of MCPA application. However, rapid dissipation of 5 mg kg^{-1} MCPA (Fig. 1a) together with only a slight increase in the relative abundance of *tfdA* gene sequences (Fig. 2) suggests that the soil supports a small but significant MCPA-degrading community with a degradation potential that is high enough to degrade low herbicide concentrations. At higher concentrations (30 mg kg^{-1}), the half-life of MCPA increased (Fig. 1b). Therefore, the indigenous degrader population was probably not capable to degrade this high MCPA dose at the same relative rate as the low MCPA dose. Interestingly, bacteria harbouring the *tfdA* gene were not able to grow on MCPA in this soil, although previous studies have shown that these bacteria can grow on MCPA as a single carbon source (Bælum et al. 2008). Obviously, an external energy source was a prerequisite for a quick and strong response of the degrader community to MCPA addition in our study. In soils amended with litter, 5 mg kg^{-1} MCPA moderately increased relative abundance of *tfdA* genes by up to 170 % after 3 weeks of incubation. However, despite this increase in relative abundance of MCPA degraders, dissipation rates were not affected by litter addition. Litter amendments in combination with 30 mg kg^{-1} MCPA highly afforded the relative abundance of *tfdA* genes (Fig. 2; Table 1), which coincided with the observed increase in MCPA dissipation rates. This confirms our hypothesis that litter addition together with MCPA favours the proliferation of the MCPA-degrading community and, thus, MCPA degradation at a larger scale. Our results are in line with the study of Fredslund et al. (2008) who found that MCPA degradation in the field strongly depended on growth conditions for MCPA degraders.

The amendment of soils with litter in our study probably provided substrates for MCPA degraders other than bacteria. Fungi have been described to degrade xenobiotics by co-metabolism rather than using xenobiotics as sole carbon source (Bennett et al. 2001). Several studies have demonstrated the ability of fungi to degrade 2,4-D and MCPA, and in most cases, lignin-degrading enzymes have been implicated (Bollag et al. 2003; Sedarati et al. 2003; Singleton 2001; Vroumsia et al. 1999). Kalbitz et al. (2003) found an enrichment of lignin-derived aromatic compounds in dissolved organic carbon (DOC) originating from biodegradation of maize straw and other plant residues. This implies that in our study, litter-derived DOC might have diffused into the adjacent soil and induced the production of lignin-degrading enzymes by soil fungi. This view is supported by the significant interaction between MCPA and litter addition on ergosterol content with higher ergosterol content only in soils spiked with MCPA and litter. Combined with results of fungal PLFAs (Fig. 5; Table 1), this indicates adaptation of fungi to high MCPA concentrations. This was clearer after 1 and 3 weeks, especially in combination with litter. Castillo et al. (2001) found similar effects of wheat straw addition on fungal degradation of MCPA, with an increase in two lignin-degrading enzymes (lignin peroxidase and manganese peroxidase/laccase) coinciding with MCPA removal. Out of 90 fungal strains tested for their ability to degrade 2,4-D, one of the best-performing fungi was *Mortierellaceae* (Vroumsia et al. 2005). This fungal species was also found by Poll et al. (2010b) to be the dominant fungus during the initial phase of litter degradation, and it points to the possibility that early fungal litter degraders were also involved in MCPA degradation in our experiment.

Effects of MCPA exposure on the microbial community

Based on the extractable MCPA after 1, 3 and 6 weeks, the microbial community was only initially exposed to MCPA in the 5 mg kg^{-1} treatments, whereas exposure to MCPA lasted for the whole incubation period in the 30 mg kg^{-1} treatments (Fig. 1). According to these exposure scenarios, one could have expected that the microbial community is resilient and shows only transitional toxic effects of MCPA in soils with 5 mg kg^{-1}. In contrast, more persistent effects could have been expected in soils with higher MCPA concentrations.

The overall effect of MCPA, with or without litter, on bacterial derived PLFAs was inhibitory (Fig. 4), which was also supported by the abundance of the 16S rRNA gene copy number (Fig. S2). This negative effect seems to be dose-dependent. In the presence of 5 mg kg^{-1} MCPA, the bacterial population was only slightly affected. However, the bacterial community was not resilient and the decrease in the content of bacterial PLFAs was still obvious after 6 weeks. In the presence of 30 mg kg^{-1} MCPA, this pattern was more pronounced. Zabaloy et al. (2010) indicated that at an environmental relevant level of ≤5 mg kg^{-1}, 2,4-D induced only a slight functional shift in 2,4-D-degrading community. Similarly, Ros et al. (2006) reported that a low dose of atrazine had a negligible effect on the potential functional diversity. A probable explanation for the decrease in bacterial PLFAs could be a toxic effect on some bacterial populations at high herbicide concentrations. Smith and Lafond (1990) showed the detrimental effect of high doses of MCPA on herbicide-degrading microorganisms. De Lipthay et al. (2003) pointed out that the microbial community composition changed as a consequence of the toxic effect of 2,4-D on some populations in subsurface aquifers. In contrast to the bacterial PLFAs, contents of ergosterol and the fungal PLFA were increased in the presence of MCPA after 1 week and not affected or decreased after 6 weeks, respectively. The initial effect of MCPA on fungi could either be explained by the above-mentioned contribution of early fungal litter degraders to MCPA degradation or by higher fungal tolerance against toxic effects of MCPA compared to bacteria. As a consequence of litter and MCPA effects on bacteria and fungi in our soil, the microbial community tended to shift initially to a more fungal-dominated community, as illustrated by the bacteria to fungus ratio (Table S3; Fig. S3).

Conclusion

The extent and rate of MCPA dissipation depended on the following factors: herbicide concentration and litter quantity. At a low concentration (5 mg kg^{-1}), up to 95 % of MCPA dissipated after 3 weeks, whereas at a high concentration (30 mg kg^{-1}), persistence of the herbicide was observed with only 31 % dissipation at the end of the incubation in the no-litter treatment. Litter addition significantly enhanced MCPA dissipation by stimulating the growth of specific bacterial MCPA degraders (*tfdA* bacterial community) as well as fungal populations possibly involved in co-metabolic degradation of MCPA. Therefore, litter or substrate addition is important in soils without a history of MCPA application because it enables the degrading community to respond quickly and strongly to the addition of this herbicide.

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