

Degradation of 4-Chloro-2-Methylphenoxyacetic Acid in Top- and Subsoil Is Quantitatively Linked to the Class III *tfdA* Gene

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The *tfdA* gene is known to be involved in the first step of the degradation of the phenoxy acid herbicide 4-chloro-2-methylphenoxyacetic acid (MCPA) in several soil bacteria, but bacteria containing other *tfdA*-like genes have been isolated as well. A quantitative real-time PCR method was used to monitor the increase in the concentration of *tfdA* genes during degradation of MCPA in sandy topsoil and subsoil over a period of 115 days. Quantitative PCR revealed growth in the *tfdA*-containing bacterial community, from 500 genes g⁻¹ soil to approximately 3 × 10⁴ genes g⁻¹ soil and to 7 × 10⁵ genes g⁻¹ soil for topsoil initially added to 2.3 mg MCPA kg⁻¹ (dry weight) soil and 20 mg MCPA kg⁻¹ (dry weight) soil, respectively. We analyzed the diversity of the *tfdA* gene during the degradation experiment. Analyses of melting curves of real-time PCR amplification products showed that a shift in the dominant *tfdA* population structure occurred during the degradation period. Further denaturing gradient gel electrophoresis and sequence analysis revealed that the *tfdA* genes responsible for the degradation of MCPA belonged to the class III *tfdA* genes, while the *tfdA* genes present in the soil before the occurrence of degradation belonged to the class I *tfdA* genes. The implications of these results is that the initial assessment of functional genes in soils does not necessarily reflect the organisms or genes that would carry out the degradation of the compounds in question.

Phenoxy acid herbicides have been widely used for agricultural purposes throughout the world since their development in the 1940s. In Denmark, 4-chloro-2-methylphenoxyacetic acid (MCPA) has been among the five top-selling herbicides during the last five decades. The relatively large amounts applied (1,500 to 2,000 g/ha), the weak retention of the compound in soils, and the unrestricted use during cold autumns, when degradation is slow, have resulted in frequent occurrences of MCPA as a groundwater contaminant (50). Groundwater is an important drinking water resource, especially in Denmark, where 99% of the drinking water is provided by groundwater. Hence, studies that explore the fate of potential groundwater-polluting compounds are important.

The degradation of phenoxy herbicides such as 2,4-dichlorophenoxyacetic acid (2,4-D), MCPA, and related compounds has been studied intensively during the last 40 years, with the majority of the work being done on 2,4-D. This, among other studies, has revealed detailed knowledge about the degradation and mineralization kinetics (27, 38), the catabolic pathways (6, 21, 46), the genes and enzymatic systems involved in the degradation (6, 20, 24, 35), and the phylogenetic composition of isolated microbial degraders (25, 53).

Numerous kinetic models have been developed to describe the mineralization of xenobiotic compounds in the environment (for a review, see reference 17). Typically, the models are divided into different forms of degradation kinetics that either take the growth of microorganisms into account or not. Furthermore, different models that take different forms of growth

into account, depending on initial cell and substrate concentrations, have been developed (7, 52).

The soil bacterium *Ralstonia eutropha* JMP134, originally isolated from an Australian agricultural soil sample (44), contains genes for the complete pathway of 2,4-D and MCPA degradation. These genes are the ones most extensively studied and have become a model for the study of phenoxy acid degradation (11, 55). This strain harbors plasmid pJP4, which contains all of the structural and regulatory genes needed to convert these compounds to 2-chloromaleylacetic acid, which is further metabolized by chromosomally encoded gene products to finally yield CO₂ and chloride (14, 15, 22, 31, 32, 34). The first step in the degradation pathway is initiated by an α -ketoglutarate-dependent dioxygenase, encoded by the *tfdA* gene, which cleaves the acetate side chain to produce the corresponding phenol 4-chloro-2-methylphenol (MCP) (Fig. 1) (18, 19, 54). Further degradation steps are initiated and regulated by gene products of the genes *tfdB* (45), *tfdCDEF* (35, 45), *tfdR*, and *tfdS* (37, 39).

Numerous strains capable of degrading compounds such as 2,4-D and MCPA have been isolated from various environments and have been found to be distributed over many different phylogenetic groups (4, 13, 24, 25, 33, 35, 55, 57). Itoh et al. (25) suggested classifying the strains into three groups. The first group, which can be further divided into three classes (classes I to III) based on their *tfdA* gene sequence (40), consists of β - and γ -proteobacteria containing *tfdA* genes related to that of *R. eutropha* JMP134. The second group consists of α -proteobacteria that are closely related to *Bradyrhizobium* spp. and contains the *tfdA*-like gene *tfdA α* (60% identical to the canonical *tfdA* gene). The strains in this group were first isolated from pristine environments in Hawaii, Canada, and

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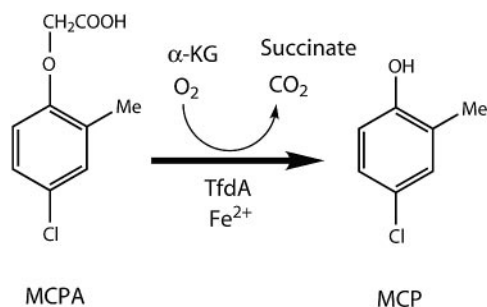


FIG. 1. Catabolic activity of the TfdA enzyme in the degradation of MCPA. α -KG, α -ketoglutarate.

Chile (30) and subsequently from arable soil in Japan (24). Members of the third group are α -proteobacteria belonging to a species of the genus *Sphingomonas*. They are also characterized by using the *tfdA* gene product during the initial step of the pathway instead of the TfdA enzyme (20). As well as this, several strains in the second and the third groups are known to contain the *cadA* gene, which is related to the *tfdA* gene involved in the degradation of 2,4,5-trichlorophenoxyacetic acid (25).

The main objective of this paper was to describe the functional diversity of MCPA degraders in a profile of MCPA-treated Danish soil. Furthermore, PCR using primers targeting the three groups of phenoxy acid degradation genes was performed to investigate whether the genes were present and which primer set revealed the most specific amplification product. In light of this, one degenerated primer set (this work) targeting the canonical *tfdA* gene for real-time quantitative PCR was chosen. The relative increase in the *tfdA* gene concentration was monitored during a degradation experiment in microcosms. Denaturing gradient gel electrophoresis (DGGE) analyses of the *tfdA* amplification product were used to elucidate whether only one or several homologues of the *tfdA* gene were involved in the MCPA degradation. This revealed a number of clearly separated bands, which were sequenced and compared to known *tfdA* sequences. We further examined the mineralization kinetics of MCPA depending on the effects of the initial substrate and cell concentrations in top- and subsoil. The topsoil was suggested to contain a higher initial cell concentration than the subsoil. The initial cell concentration and the growth of MCPA degraders were determined and monitored during a degradation period by quantitative real-time PCR of the *tfdA* gene. Microbial growth during degradation of MCPA in natural soil has previously been suggested but has only been quantified using less accurate techniques (12, 41).

MATERIALS AND METHODS

Bacteria. *Ralstonia eutropha* JMP134 (13), which carries the *tfdA* gene on the pJP4 plasmid, was used as a positive control and for standard curve preparation in the quantitative real-time PCR assay. Furthermore, it was used as a model organism in the DGGE analysis. DNA from *Burkholderia cepacia* DB01(pRO101), which carries the same *tfdA* gene as pJP4 (22), was used to create a 32 P-labeled probe for Southern blot hybridization. Both strains were propagated using standard techniques (27).

Soils. An organic topsoil sample (10 to 25 cm) and a subsoil sample (35 to 50 cm) from a sandy profile were used for the experiments. The profile was taken from the Danish agricultural experimental station in Jyndevad, located on the outwash plain in southern Jutland. The soil has been used for organic farming

TABLE 1. Soil characteristics

Soil	Depth (cm)	pH	C (%)	Clay (%)	Silt (%)	Sand (%)	WHC ^a (% [vol/vol])
Topsoil	10–25	6.9	2.4	5	3	92	19.25
Subsoil	35–50	6.1	1.0	5	1	94	17.76

^a WHC, water-holding capacity, measured at a -10 log water potential of 2.0 cm.

since 1995, and additionally, it has not been treated with any form of phenoxy acid herbicide since at least 1988. The soils were collected and mixed manually in October 2003 and stored in the dark for 2 months at 5°C before they were passed through a 2-mm sieve. The soils were thereafter stored in the dark at -18°C until 10 days before use. Defrosting and acclimatization were performed in the dark for 10 days at 10°C (41) (for further soil characteristics, see Table 1).

Microcosm setup for degradation experiment. Before experiment setup, stock solutions of MCPA were prepared. To ensure even moistening of the soils to a water content of approximately the water-holding capacity, stock solutions of 20, 174, and 23 mg/liter MCPA were required to obtain concentrations of 2.3 mg MCPA kg⁻¹ (dry weight) soil in the topsoil (Top_{2,3}), 20 mg MCPA kg⁻¹ (dry weight) soil in the topsoil (Top₂₀), and 2.3 mg MCPA kg⁻¹ (dry weight) soil in the subsoil (Sub_{2,3}), respectively. The stock solutions were prepared by dissolution of MCPA (97.5% purity; Dr. Ehrenstorfer GmbH, Augsburg, Germany) in MilliQ water (Millipore, Birkerød, Denmark). To ensure the complete dissolution of MCPA and to avoid disturbance of the soil pH, the pH in the solutions was adjusted to 6.5 with NaOH.

Subsequently, 20 g (dry weight) of the topsoil and 15 g (dry weight) of the subsoil were transferred to 250-ml Pyrex red cap bottles (airtight). The soils were spiked with 2.3 ml (for the topsoils) and 1.5 ml (for the subsoils) of the respective stock solutions. Stock solutions with MCPA were carefully mixed into the soils with a sterile glass pipette, and the microcosms were incubated at 10°C in the dark. Triplicate bottles were set up for each soil scenario for eight sampling points, and in order to prevent the development of anaerobic conditions in the microcosms, they were aerated by leaving the flasks open for 20 min in a sterile hood every 14 days.

Microcosm setup for mineralization experiment. A mineralization experiment was set up with ^{14}C -labeled MCPA at the same concentrations described above. Stock solutions were prepared as described above for the degradation experiment. [^{14}C]MCPA (specific activity of 159.7 $\mu\text{Ci}/\text{mg}$, radiochemical purity of >95%; Izotop, Budapest, Hungary) was then added to the stock solutions in trace amounts to achieve an initial radioactivity in each microcosm of 60,000 dpm. The mineralization experiment was performed essentially as described previously by Mortensen and Jacobsen (41), except that 10 g of soil was used and no soil was removed during the experiment. We have previously determined the mass balance for the experimental setup, and recoveries of 93% \pm 1.5% were found (5, 9).

MCPA extraction and quantification. On days 0, 6, 12, 22, 33, 50, 68, and 115 after incubation, triplicates of each soil-MCPA degradation series (Top_{2,3}, Top₂₀, and Sub_{2,3}) were extracted by pressurized liquid extraction for the determination of MCPA amounts. The extractions were performed with an ASE 200 system (Dionex, Sunnyvale, CA). The soils from the degradation assay were packed in 33-ml extraction cells sufficient to contain 40 to 50 g soil. To assist the extractions, Ottawa sand (Fisher scientific, Loughborough, United Kingdom), sufficient to fill out the remaining volume (approximately 1:1 [wt/wt]), was mixed into the soil samples. The entire extraction process was carried out at 50°C. The solvent used for the extractions was a 50:50 (vol/vol) mixture of methanol-water (high-performance liquid chromatography-grade methanol; Fisher Scientific, Loughborough, United Kingdom). Other extraction conditions were as follows: a 40% solvent volume was used for a 10-min static time in one extraction cycle and a 60-s purge time with nitrogen at 1,500 lb/in² pressure (1 lb/in² = 6,894.76 Pa). The extraction method was optimized for soil samples spiked with MCPA, resulting in extraction efficiencies of 80% \pm 2% for the topsoil and 92% \pm 4% for the subsoil.

A soil extract of 15 to 20 ml was obtained in 50-ml glass vials. After being thoroughly mixed, an aliquot was filtered through a 0.45- μm polytetrafluoroethylene filter (Titan2; Sun-Sri) to remove particles. To avoid overloading of the liquid chromatography (LC) mass spectrometry system, the filtrates were diluted with a methanol-water (50:50) solution to obtain MCPA concentrations in the range of 50 to 150 $\mu\text{g}/\text{liter}$.

The quantification of MCPA in the soil extracts was performed using an LC tandem mass spectrometry (MS/MS) system. Twenty microliters of the samples

TABLE 2. PCR primers

Primer set	Model gene(s)	Primer (5'-3') ^c	Optimal annealing temp used (°C) ^c	Characteristic ^f	Reference or source
<i>tfdA</i> *	<i>R. eutropha</i> JMP134/ <i>Burkholderia</i> sp. strain RASC	F, AACGCAGCGRTTTRTCCCA ^a R, ACGGAGTTCTGYGAYATG ^a	58	Unspecific binding; qPCR not possible	59
<i>tfdA</i> -intern	<i>R. eutropha</i> JMP134	F, GCATACGACGCGCTGCCTCG R, CTTCGGCCACCGGAAGGCCT	65	Used for probe preparation for Southern blot hybridization	This work
<i>tfdA</i> **	Sequence alignment of 23 known <i>tfdA</i> genes	F, GAGCACTACGCRCTGAAYTCCCG ^{a,b} R, CTTCGGCCACCGGAAGGCCT	64	One band on agarose gel (approx. 220 bp); qPCR possible	This work
<i>tfdA</i> α*	Alignment of RD5-C2, HWK12, and HW13 (<i>tfdA</i> α genes)	F, CCGGCGTTCGATCTGCGCAAG R, GTTGACGACGCGCGCCGA	ND ^d	No clear band on the agarose gel; fluffy PCR products	51
<i>tfdA</i> α**	Alignment of HW13, HWK12, and BTH (<i>tfdA</i> α genes)	F, ACGAGTTCKSCGACATGCG ^a R, GCGGTTGTCCACATCAC	65	One band (approx. 350 bp); qPCR may be possible	25
<i>cadA</i>	HW13 (<i>cadA</i> gene)	F, AAGCTGCARTTTGARAAY ^a R, MGGATTGAAGAAATCCTGRTA ^a	58	No PCR product	24

^a R, G/A; Y, T/C; S, G/C; K, G/T; M, A/C.

^b AGC clamp was added to the 5' end of the primer for the PCR/DGGE (5'-CGCCCCCGCGCGCGGGCGGGCGGGGGCACGGGGGG-3').

^c The optimal annealing temperature is based on gradient ramping (58 to 69°C).

^d ND, no optimal annealing temperature could be determined.

^e F, forward; R, reverse.

^f qPCR, quantitative PCR.

was injected, and the compounds were separated on a 100- by 2.1-mm Xterra RP₁₈ column (3.5- μ m particle size) from Waters (Milford, MA) using a Waters Alliance model 2695 high-performance liquid chromatography system. A mobile phase composed of methanol-water-0.2% acetic acid (78:20:2) at 25°C with a flow rate of 0.18 ml/min was used. For detection and quantification of the MCPA compound, a Quattro Ultima triple quadrupole mass spectrometer from Micro-mass (Manchester, United Kingdom) equipped with an electrospray ionization unit was used. Electrospray ionization in the negative mode was used, while further conditions were as follows: capillary voltage was 3.20 kV, cone voltage was 80 V, and collision energy for MS/MS was 20 eV. Furthermore, nitrogen was used as the desolvation gas at a flow rate of 600 liters/h. A limit of detection of 1 μ g MCPA liter⁻¹ was determined for the LC-MS/MS system.

DNA extraction. Soils for DNA extractions were set up parallel to the degradation experiment. Red cap bottles with 20 g (dry weight) of soil amended with 0, 2.3, and 20 mg MCPA kg⁻¹ (dry weight) soil for the topsoil (Top₀, Top_{2.3}, and Top₂₀) and 0 and 2.3 mg MCPA kg⁻¹ (dry weight) soil for the subsoil (Sub₀ and Sub_{2.3}) were set up in triplicate. The amount of soil sampled for DNA extractions was 0.5 g (wet weight) to ~0.4 g (dry weight), which was accomplished using a sterile stainless steel sampler designed to collect a composite sample of 0.5 g soil from 5 to 10 distinct spots in the bottle. The same bottle was used throughout the experiment and was aerated parallel to the bottles in the degradation experiment as well as for sample collection on the same days as in the degradation experiment. Whole-community DNA was extracted from the 0.5-g wet soil samples with the FastDNA SPIN kit for soil (Bio101, Inc., Carlsbad, CA). The protocol recommended by the manufacturer was followed, except that the bead-beating step was modified and a freeze-thaw step was included. The bead-beating step was modified to four 30-s pulses at speed 4 instead of one 30-s pulse at speed 5.5 in the FastPrep FP 120 instrument (Bio101), and the freeze-thaw steps were performed for 1 h at -80°C and subsequently for 30 min at 37°C. DNA was finally eluted in 100 μ l DNase/RNase-free water (Bio101, Carlsbad, CA) and stored at -80°C.

Primer testing. All PCRs were carried out as real-time PCR assays in an iCycler iQ (Bio-Rad, Hercules, CA). In order to select the most effective primer set, five primer sets were tested (Table 2) using two different commercial PCR

master mix kits. *tfdA** and *tfdA*** were the most intensively tested primers. *tfdA** yielded a 307-bp fragment, while the *tfdA*** yielded a 210-bp fragment. *tfdA*** was designed in connection with this work by BLAST alignment analysis (3) of 22 known *tfdA* gene sequences found with *R. eutropha* JMP134 as the search sequence. The GenBank accession numbers for the 22 *tfdA* genes were as follows: M16730, AY238497, AY238496, U43197, U43276, AF439768, AY078159, AF029344, U32188, U25717, U87394, U22499, AF181982, AF182758, AF176240, U65531, U43196, AY238495, AY238494, AY238493, AY238492, and AB074491. Primers were designed to attach to most conserved regions, and since the primer set was designed on the basis of *tfdA* gene sequences belonging to class I and class III, it was expected that it was highly specific for these genes. Primers were obtained from MWG Biotech (Ebersberg, Germany). The first of the two commercial SYBR green PCR master mix kits we tested was iQ SYBR green supermix (Bio-Rad, Hercules, CA) containing PCR buffer, 0.4 mM of each deoxynucleoside triphosphate, 50 U/ml of *iTaq* DNA polymerase, and 6 mM MgCl₂. This kit was used mainly with the *tfdA** primer set and through the testing of *tfdA*α*, *tfdA*α**, and *cadA* primer sets. The other kit we tested was the QuantiTect SYBR green PCR kit (QIAGEN, Crawley, United Kingdom) containing deoxynucleoside triphosphate mix, HotStar *Taq* DNA polymerase, PCR buffer, Rox, and 2.5 mM MgCl₂. This kit was only used for PCR with the *tfdA** and *tfdA*** primer sets. The 25- μ l reaction mixtures contained 0.4 μ M of each primers, 12.5 μ l of the respective SYBR green mix, 25.5 μ g bovine serum albumin (Amersham Bioscience, Buckinghamshire, United Kingdom), 2.5 μ l of 1:10-diluted DNA extract, and RNase/DNase-free water to complete the 25- μ l volume. The DNA extracts were diluted 1:10 in RNase/DNase-free water to reduce the influence of PCR-disturbing factors from the soil, such as humic acids.

The conditions for the real-time PCR were as follows: 6 min at 95°C for enzyme activation; 50 cycles of 45 s at 94°C for denaturation, 30 s at the annealing temperature specified in Table 2, and 2 min at 72°C for elongation; and a final 6-min step at 72°C for extension. To obtain a specific melting profile for the real-time PCR product, an 80-cycle protocol of 45 s at 58°C; 30 s at 58°C to 98°C, increasing 0.5°C every cycle; and 45 s at 58°C was used after the real-time PCR. The melting profile was used to determine the presence of the

specific product and to determine if the PCR product was composed of DNA sequences of more than one length.

Further investigations of the PCR products were done by gel electrophoresis of 8- μ l PCR products on a 1.5% agarose gel in 1 \times Tris-acetate-EDTA buffer. The gels were stained in ethidium bromide and visualized under UV light.

In case the gels revealed more than one band, a Southern blot hybridization was performed as described previously by Jacobsen (26) and Sambrook and Russell (49), except that the digestion reaction was excluded. The 32 P *tfdA* probe for the hybridization was generated using [32 P]dCTP (Amersham, United Kingdom) and the Random Primed DNA Labeling kit (Roche Applied Science, Penzberg, Germany) based on the method described previously by Feinberg and Vogelstein (16). A PCR product derived from DNA of the *tfdA*-containing strain *B. cepacia* DB01(pRO101) with the *tfdA*-intern primer set (Table 2) was used as a template.

Quantitative real-time PCR. After optimization of primers, quantitative real-time PCR was performed only with the *tfdA*** primer set as described above on the triplicate samples from days 0, 6, 12, 22, 33, 50, 68, and 115. All quantitative real-time PCRs were set up in triplicate and included three negative control samples in each PCR setup.

Standards for the quantitative PCR were prepared using the well-characterized phenoxy acid degrader *R. eutropha* JMP134. After inoculation into 0.5 g of the topsoil in amounts of 8×10^6 , 8×10^5 , 8×10^4 , 8×10^3 , 8×10^2 , and 8×10^1 cells g $^{-1}$ soil, the DNA was extracted from the soils as described above, and the extracts were diluted 10-fold to reduce the effect of humic acid disturbances. *R. eutropha* JMP134 was used as the positive control strain as well.

DGGE analysis. PCR for the DGGE analysis was performed as described above with the QuantiTect mastermix kit. The *tfdA*** primer set was used with a GC clamp attached to the 5' end of the forward primer (Table 2). DGGE analyses were performed on real triplicates of DNA extracts from days 0, 6, 12, 33, and 68 in order to secure enough wells for one data series on one gel. The technique described previously by Muyzer et al. (42) was used for separating and visualizing the *tfdA* PCR products of the DNA extracts. The specific details of the method used are as follows. Gels contained 8% acrylamide and a urea-formamide gradient of 55 to 70% (where 100% denaturant contained 7 M urea and 40% formamide). Electrophoresis was carried out at 70 V for 17 h in 1 \times Tris-acetate-EDTA buffer using a D-code apparatus (Bio-Rad, Hercules, CA). After electrophoresis, the gels were stained with SYBR gold (Molecular Probes, Eugene, OR) for 45 min, followed by a brief rinse with MilliQ water, and then visualized under UV light and finally photographed. Bands that appeared to have a unique pattern for the soil scenarios were stabbed with sterile pipette tips, which were placed in 20 μ l RNase/DNase-free water and rinsed repeatedly. These served as templates in further PCRs using the *tfdA*** primer set (Table 2) without the GC clamp to obtain enough DNA material for sequencing. The nucleotide sequencings were performed by MWG Biotech (Ebersberg, Germany).

Data analysis. The amounts of extracted MCPA were corrected for the extraction efficiency, after which linear regression analyses were performed on the steep segment of the degradation curves to fit it to zero-order kinetics with the following equation:

$$C_t = C_0 - k_0 t \quad (1)$$

where C_t and C_0 are the amounts of herbicide remaining (percentage of initial concentration) at time t and at time zero, respectively; t is the incubation period (days); and k_0 is the zero-order rate constant. The linear regressions were performed only for the steep segment of the degradation curve excluding sampling points from the lag phase and points where MCPA concentrations were below the detection limit.

For the results from the mineralization experiment, the calculated cumulative mineralization values were corrected for background radioactivity. After this, nonlinear regression analyses were performed as described previously by Mortensen and Jacobsen (41).

Nucleotide sequence accession numbers. The GenBank accession numbers for the DNA sequences reported in this study are DQ272405, DQ272406, DQ272407, DQ272408, DQ272409, DQ272410, DQ272411, DQ272412, DQ272413, DQ272414, DQ272415, and DQ272416 for A1, A2, A3, A4, A5, A6, A7, A8, A9, B1, B2, and B3, respectively.

RESULTS

MCPA degradation. The form of the curves for the disappearance of MCPA can be divided into three segments: first, a

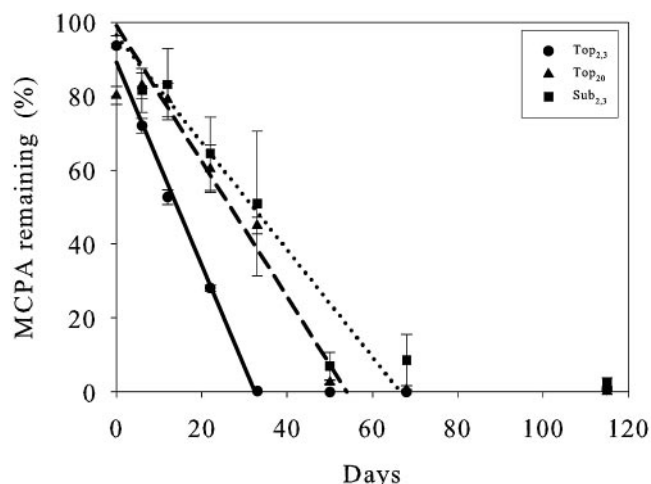


FIG. 2. Degradation of MCPA in the three soil scenarios measured by LC-MS/MS. Linear regression lines of the steep segments are shown. Solid line, Top_{2,3}; dashed line, Top₂₀; dotted line, Sub_{2,3}. Error bars indicate standard errors.

lag phase, where no or little degradation took place; second, a steep part with relatively fast degradation of MCPA (equation 1); and finally, the last segment, where almost all MCPA (>98%) had disappeared. The lag phases for Top₂₀ and Sub_{2,3} both had durations of 12 days, while in Top_{2,3}, no lag phase was observed. The steep part of the curve, where MCPA disappeared, shows a large degree of linearity. Therefore, this part was fitted to zero-order kinetics by means of linear regression (Fig. 2), and zero-order rate constants along with DT₅₀ values were determined (Table 3). Degradation was fast and resulted in time to 50% degradation (DT₅₀) values of 16, 33, and 37 days for Top_{2,3}, Top₂₀, and Sub_{2,3}, respectively. However, MCPA could be detected in the soil for rather longer times, until days 33, 50, and 68 for Top_{2,3}, Top₂₀, and Sub_{2,3}, respectively. In addition to detection and quantification of MCPA, we also detected contents of the first degradation product, MCP. In Top_{2,3} at days 0 to 22 and in Top₂₀ at days 22 to 33, we found MCP in concentrations up to 5% of the initial MCPA concentration (data not shown). MCP was not detected in Sub_{2,3} and was not detected after days 22 and 33 for Top_{2,3} and Top₂₀, respectively.

Mineralization studies. The mineralization curves showing accumulated amounts of 14 CO₂ released from mineralization of 14 C-MCPA versus time were all sigmoid, and, like the degradation curves, they could be divided into three segments: a lag phase, a steep segment, and a plateau (Fig. 3A). The lag

TABLE 3. Parameter estimates from the zero-order kinetics model^a of the steep part of the degradation curves and calculated DT₅₀ values

Soil	MCPA (mg/kg)	R ²	k ₀ (% × day ⁻¹)	DT ₅₀ (days)
Topsoil	2.3	0.990	-2.75	16.2
Topsoil	20	0.941	-1.83	33
Subsoil	2.3	0.711	-1.45	37

^a See equation 1.

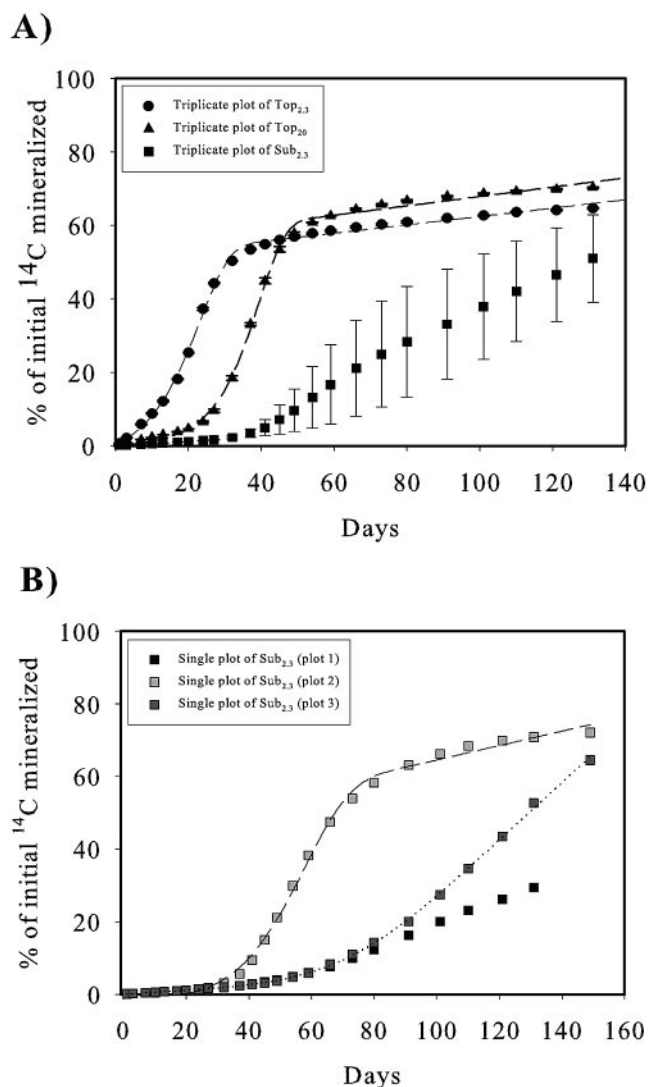


FIG. 3. Mineralization of ¹⁴C-MCPA in the three soil scenarios. (A) Curves show triplicate cumulative data from Top_{2.3}, Top₂₀, and Sub_{2.3}. The dashed line shows a nonlinear fit to the exponential form of the 3/2-order model. Due to the large standard deviation, the plot of the triplicates for Sub_{2.3} was not fitted. (B) Single plots of the Sub_{2.3} replicates. The dotted line shows nonlinear fits to the linear form of 3/2-order model, and the dashed line shows a nonlinear fit to the exponential form of the 3/2-order model (plots 2 and 3). Plot 1 was not satisfactorily fitted to any model. Error bars in A indicate standard errors.

phase was significantly longer for Top₂₀ and Sub_{2.3} than for Top_{2.3}. Furthermore, the lag phase was longer for Sub_{2.3} than for Top₂₀, a pattern similar to that observed in the degradation curves (Fig. 2). An *F* test ($P < 0.05$) showed that the mineralization curves for Top_{2.3} and Top₂₀ were best fitted to the exponential growth form of the 3/2-order model (7) indicating growth during the degradation. The plateau of the curves for Top_{2.3} and Top₂₀ occurred after approximately days 37 and 54, respectively, where 55% and 61% of the initially added ¹⁴C had been mineralized. Furthermore, the *F* test and other model parameters (data not shown) showed that Top₂₀ fitted better to the model than Top_{2.3}.

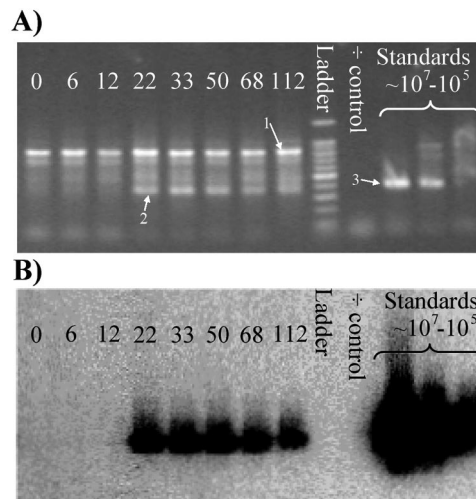


FIG. 4. Primer optimization. (A) Standard agarose gel electrophoresis of real-time PCR amplification products using the *tfdA** primer; the iQ SYBR green supermix and soil DNA from days 0, 6, 12, 33, 50, 68, and 115 of Top₂₀ were used as a template. The standard is *R. eutropha* JMP134 inoculated into the soil in concentrations of 8×10^6 , 8×10^5 , and 8×10^4 cells/g soil, with 8×10^6 cells/g soil shown to the left. Bands 1 and 2 represent the most characteristic bands, as described in the text. Band 3 represents the strain known to contain the *tfdA* gene *R. eutropha* JMP134. The ladder is a 100-bp DNA ladder. (B) Southern hybridization analysis of the agarose gel shown in A using a *tfdA* probe. Similarity can be detected for bands 2 and 3.

Due to large standard errors, the average mineralization curve of the triplicates for Sub_{2.3} was not fitted to any kinetic model. The plot of the individual replicates (Fig. 3B) shows three different mineralization curves, only two of which (plots 2 and 3) had sigmoid forms and could be fitted as the topsoil experiments. According to the *F* test ($P < 0.05$) (48), plot 2 was best fitted to the exponential form of the 3/2-order model as for the topsoil scenario, while plot 3 was best fitted to the linear 3/2-order model (7), indicating no or slight growth. Plot 1 showed no sigmoid curvature and was therefore not fitted to any model.

Primer testing. To make the real-time PCR quantification technique as accurate as possible, the choice of primer set was critical. Therefore, a well-characterized primer set covering a broad range of known *tfdA* sequences was chosen (59). Unfortunately, we found that this primer set was likely to generate diverse bands, indicating that the PCR product contained genes of more than one length (Fig. 4A). One band (band 2) had the same length, approximately 350 bp, as the positive control strain, *R. eutropha* JMP134 (band 3).

In comparison to the topsoil, the subsoil showed no clear pattern in the bands from the PCR product, even though there were several unspecific bands on the gel (data not shown). To determine which bands were related to the *tfdA* gene, a Southern blot assay was performed with the gels (Fig. 4B). This revealed that only bands 2 and 3 contained DNA that matched the *tfdA* probe designed from the *R. eutropha* JMP134 strain. Several optimization experiments, including increasing annealing temperature, were made to get rid of the unspecific amplification products. Among these, we tried to use another commercial real-time PCR master mix kit, which decreased the

unspecific bands (data not shown), but still not satisfactorily, which was expressed by a high detection limit and low R^2 values (<0.90) for the standard curve. Other optimization steps included concentration of $MgCl_2$ and concentration of the primers in the reaction mixture, but we still had no success (data not shown). Therefore, we designed a new set of primers, *tfda*** (Table 2), which revealed PCR products with no unspecific bindings and a decrease in the cycle threshold values during real-time PCR. Lower cycle threshold values indicate a more specific and effective PCR. The detection limit in the real-time PCR assay for this primer was rather low (800 genes g^{-1} soil). Furthermore, the R^2 values for the standard curves were high (0.999 to 0.990).

Consequently, we used the *tfda*** primer set for further quantitative real-time PCR assays. Primer testing of primer sets *tfda* α * (51), *tfda* α ** (24), and *cadA* (25) were performed as well (Table 2). Only *tfda* α ** amplified a PCR product that may be possible to use for quantitative real-time PCR (data not shown).

Quantitative real-time PCR analysis. The quantitative real-time PCR analysis indicated that growth occurred in all three soil scenarios during the degradation period (Fig. 5). In $Top_{2,3}$, the *tfda* concentration increased to a maximum of 3.0×10^4 genes g^{-1} soil at day 12 (Fig. 5A), while the maximum gene concentrations in Top_{20} and $Sub_{2,3}$ were 7.0×10^5 and 2.6×10^4 genes g^{-1} soil, obtained at days 22 to 33 and 50 to 68, respectively (Fig. 5B and C). The number of genes is based on the assumption that *R. eutropha* JMP134(pJP4) grown on rich media and used for real-time PCR standard curve preparation only contains one copy of the *tfda* gene. The increase in the number of *tfda* genes is inversely related to the concentration of MCPA in the three soils (Fig. 5A to C). Lag phases were clearly expressed and were shortest for $Top_{2,3}$ and Top_{20} and longest for $Sub_{2,3}$. It is interesting, however, that the increase in the number of *tfda* genes occurred before the exponential mineralization phases did. It is also interesting that the maximum concentrations of the *tfda* genes were reached while half of the MCPA remained and that a decrease in concentrations of *tfda* genes was subsequently observed in the topsoils.

Functional diversity of *tfda* genes. Two analyses of the functional diversity of the *tfda* genes were performed, and both indicated a shift during the degradation period. First, an analysis of the melting profiles of the real-time PCR amplification products was performed. A shift in the temperature optimum of the melting profile was observed when the concentration of *tfda* genes increased. This was observed as a melting optimum of $90^\circ C$ at day 0 and a melting optimum of $87^\circ C$ at day 22 (Fig. 6). Therefore, a DGGE analysis of PCR products using the GC-clamped *tfda*** primer set (Table 2) was performed to obtain more detailed information about the shift in the degrader population with time. This analysis revealed several different bands, which grew up and grew stronger during the degradation experiment. In the $Top_{2,3}$ and Top_{20} analyses, the overall pattern was about the same, with at least five dominating bands (Fig. 7A and B). However, the variation of band intensity differed slightly between treatments. In $Top_{2,3}$, the bands grew up between days 6 and 12, while in Top_{20} , the bands were weak at day 12 and first became strong at day 33, corresponding to the longer lag phase in Top_{20} . For comparison, the melting optimum of the DNA amplified from the

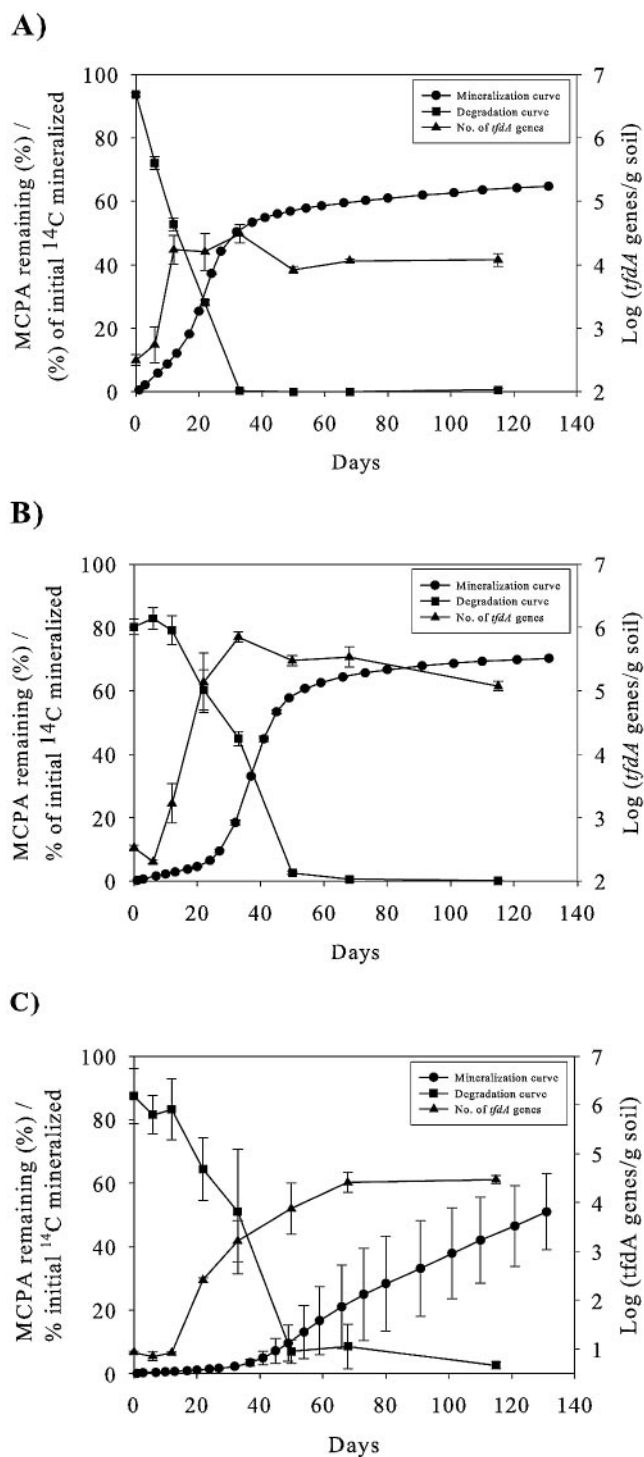


FIG. 5. Nonfitted mineralization and degradation curves compared with the log number of *tfda* genes using the *tfda*** primer set in the soil. (A) $Top_{2,3}$; (B) Top_{20} ; (C) $Sub_{2,3}$. Error bars indicate standard errors.

standards containing *R. eutropha* strain JMP134 was $90^\circ C$. The same changes in melting profiles were observed for all degradation experiments. The melting profile for day 12 in Top_{20} , however, seemed to evolve a double top, which indicated a

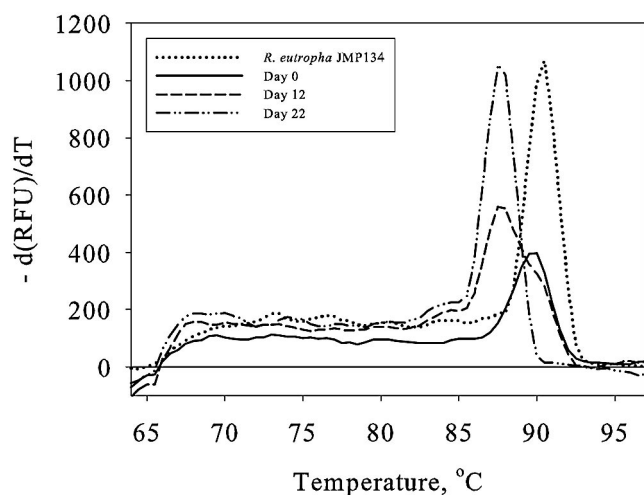


FIG. 6. Melting profiles of real-time PCR amplification products of soil DNA from days 0, 12, and 22 in Top₂₀ using the *tfdA*-II primer set and the QuantiTect SYBR green PCR kit. Furthermore, the melting profile of the PCR amplification product from the *tfdA*-containing strain *R. eutropha* JMP134 is shown. The profile displays the negative first derivative of temperature versus relative fluorescence units (RFU) [$-d(\text{RFU})/dT$] plotted against temperature.

beginning shift in the composition of the degrader population (Fig. 6 and see Fig. 8). Moreover, the intensities of the bands in Top_{2,3} were strongest at day 12, after which the intensity declined throughout the experiment; however, this was not seen in Top₂₀.

The DGGE profiles of Top_{2,3} and Top₂₀ from days 0 to 6 and 0 to 12, respectively, were very similar to those of the DNA extracts from the topsoils without MCPA (Fig. 7D). A number of bands were visible on the gel containing the Top₀ DNA extracts, where some of them were common bands that were represented in all lanes throughout the experiment and where some of them were distinct bands represented in only single lanes. The overall pattern in Top₀ was that no change in the population containing the *tfdA* gene occurred over time and that the soil had a natural content of *tfdA* genes that denaturalized in the same way as the *tfdA* gene from *R. eutropha* strain JMP134. Interestingly, the organisms represented by this band did not grow up during the experiments when MCPA was applied; however, the intensity of the band remained the same or had a slight decrease (Fig. 7A and B). The DGGE patterns from the subsoil were different from those from the topsoil. First, there were no bands in Sub₀ (Fig. 7E) or on days 0 to 33 for Sub_{2,3} (Fig. 7C). Second, the intensity of the bands from Sub_{2,3} at days 33 and 68 varied between the triplicates, especially for day 33. Furthermore, the intensity of the upper two bands, if any, was weak. Despite this, the other bands were present and had about the same intensity as those for Top_{2,3} (Fig. 7A).

The sequences obtained from the bands stabbed from the DGGE gels were compared to existing sequences encoding α -ketoglutarate-dioxygenase obtained from GenBank, and a phylogenetic tree was constructed (data not shown). The phylogenetic analysis showed that bands A1 to A7, A10, and B1 to B3 (Fig. 7) were located in the same cluster as the class III *tfdA* genes showing 96 to 99% homology to the indigenous genes in

this class (Fig. 8). Bands A8 and A9, on the other hand, were located in the same cluster as the class I *tfdA* genes showing 98 and 99% homology, respectively, to the *R. eutropha* JMP134 *tfdA* gene (Fig. 8). Furthermore, the GC content of bands A1 to A7, A10, and B1 to B3 was approximately 63%, while it was 69% for bands A8 and A9.

DISCUSSION

The carbon added in the form of the MCPA substrate should be sufficient to sustain the growth of approximately 10^7 new cells g^{-1} soil in Top₂₀ and 1.2×10^6 cells g^{-1} in Top_{2,3} and Sub_{2,3}. This is based on the assumption that one new cell requires approximately 1 pg of carbon to evolve (2). In further calculations, we assumed that one cell (JMP134 as well as naturally occurring bacterial populations) only contains a single *tfdA* gene. As the *tfdA* gene is plasmid borne, a single cell can possibly hold more than one *tfdA* gene; this latter assumption may be quite untenable (58). Furthermore, different cells may require different amounts of carbon to evolve, and different substrates may cause populations to grow differently as well (1, 2). When the uncertainties caused by the assumptions described above are taken into account, the maximum concentrations of 3.0×10^4 , 7.0×10^5 , and 2.6×10^4 genes g^{-1} soil observed in Top_{2,3}, Top₂₀, and Sub_{2,3}, respectively, correspond to less than 10% of the theoretical numbers. This may be explained in two ways. First, predation by protozoa and amoebae may be responsible for a large decrease of the cell number. This was indicated in a study reported previously by Thirup et al. (56), which showed that the number of culturable microorganisms first increased as a result of amendment with carbon substrate and subsequently decreased as a result of predation. A corresponding pattern was seen in the present study, where numbers of *tfdA* genes, especially in the topsoil experiments, decreased by 0.5 to 1 order of magnitude throughout the experiment after the maximum gene concentration was observed. Second, the primer set used may not have targeted the entire *tfdA* degrader population. Recently, Shaw and Burns (51) found that the canonical *tfdA* gene could not be detected using the *tfdA** primer set on DNA extracted from a 2,4-D degradation experiment for which the soil had not been preexposed to phenoxy acid herbicides but that the *tfdA* α -I primer set yielded a PCR product for the same soil. This indicates that the latter group of phenoxy acid degraders may be involved in the degradation of 2,4-D and probably also in the degradation of MCPA, especially in soils that are not frequently exposed to the herbicide. However, during the test of primers targeting the canonical *tfdA* gene, the *tfdA* α gene, and the *cadA* gene, we observed that PCR products were yielded with the canonical *tfdA* primer and with the *tfdA* α primer but not with the *cadA* primer set. This indicates that organisms that use the *tfdA* α gene product in MCPA metabolism may be present as well.

Growth among bacterial cells and increases in catabolic genes during degradation of phenoxy acids have been reported elsewhere previously (see, e.g., references 12, 28, and 36), but only Lee et al. (36) used a highly accurate quantitative real-time PCR method. They reported concentrations of 2.0×10^7 *tfdA* genes g^{-1} in activated sludge sequentially amended with 300 mg 2,4-D kg^{-1} . Concerning the higher amount of carbon substrate, this corresponds to what was found in the present

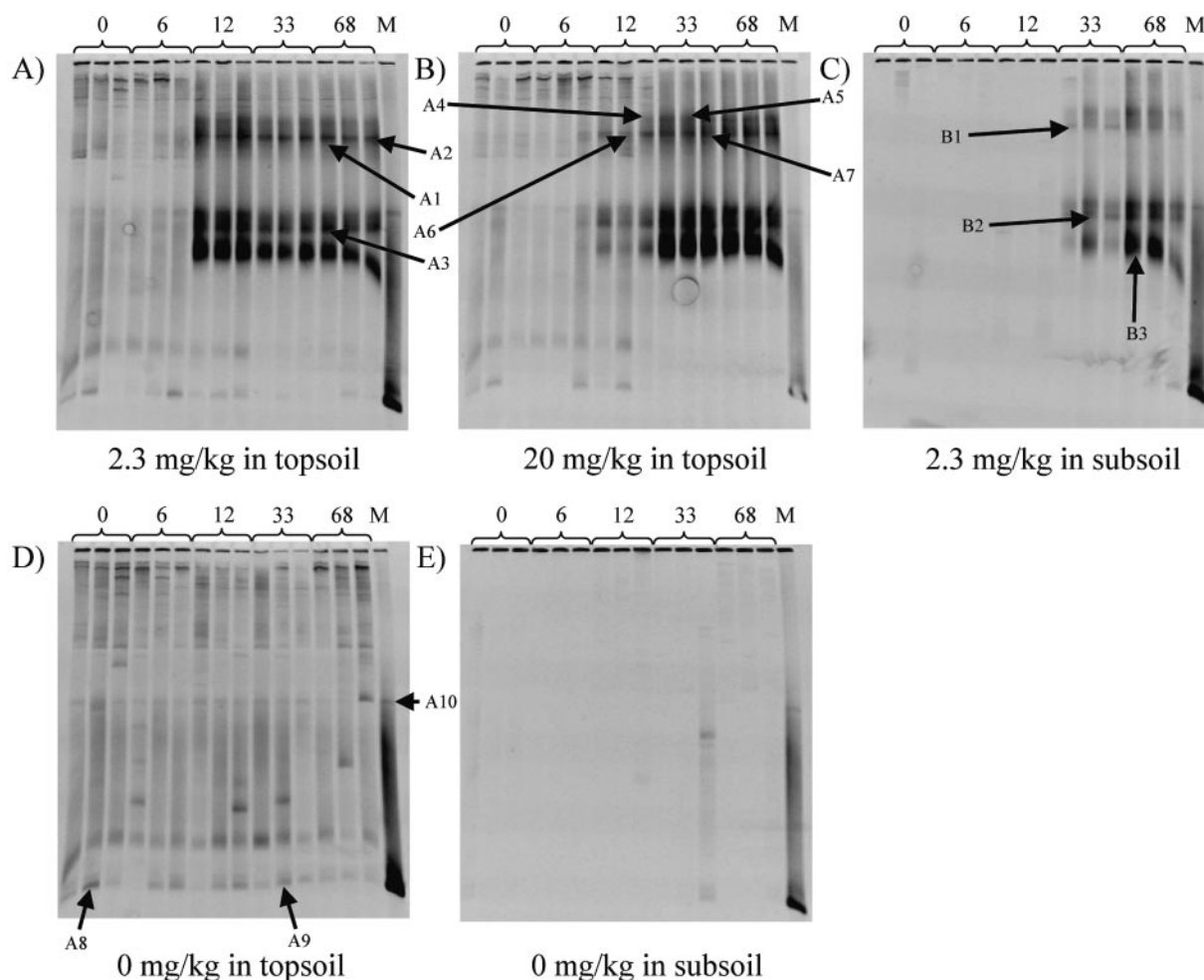


FIG. 7. Functional diversity of MCPA degrader genes using PCR/DGGE analysis with the *tfdA*** primer set. The gene product was separated using 55 to 70% urea gels. DNA extracts from days 0, 6, 12, 33, and 68 were analyzed in real triplicates. For the marker profile (M), DNA extracts from soil samples inoculated with the *tfdA*-containing strain *R. eutropha* JMP134 were used. A1 to A10 indicate bands selected for sequence analysis from topsoil DNA, and B1 to B3 indicate bands selected for sequence analysis from subsoil DNA. A shows the DGGE profile of DNA extracted from Top_{2,3}, B shows the DGGE profile of DNA extracted from Top₂₀, and C shows the DGGE profile of DNA extracted from Sub_{2,3}. D shows the DGGE profile of DNA extracted from Top₀, and E shows the DGGE profile of DNA extracted from Sub₀.

study. de Liphthay et al. (12), however, reported slightly lower increasing concentrations of the *tfdA* gene in water samples amended with approximately 20 mg 2,4-D liter⁻¹, as they observed maximum concentrations of $\sim 10^3$ genes ml⁻¹.

Analysis of the functional diversity of the indigenous MCPA degrader community by DGGE on the *tfdA* genes extracted has to our knowledge not previously been published. In this study, results from two different methods both indicated a change in the community of the bacteria containing the *tfdA* gene during a degradation period of 68 days. First, we saw a change in the maximum of the melting profiles of the PCR products from the quantitative PCR assay of the topsoil DNA from 90°C to 87.5°C. This indicates that the *tfdA* PCR products from the organisms grown on MCPA had a lower GC content and therefore were different from the genes present in the soil not exposed to MCPA. This corresponds with the results from the DGGE analyses in which the PCR products from the DNA sampled during the first days of the experiment had a tendency to denaturize at a higher denaturation grade. Interestingly, the

maximum of the melting profiles from the first sampling days of the experiment were similar to that of the standard strain *R. eutrophus* JMP134. This was seen on the DGGE gels as well, where a major part of the DNA from the first sampling days seemed to denaturize in the lower part of the gels where DNA from the *R. eutropha* JMP134 also denaturated. The presence of *tfdA* genes in the control topsoil was interesting as well. The soil used for the experiments had not been exposed to phenoxy acid herbicides for at least 15 years; however, a background population of *tfdA* genes is maintained even in the absence of appropriate pesticide substrates. These *tfdA* genes did not increase in numbers during the degradation period, which indicates that they were placed in populations that did not grow actively.

Phylogenetic analyses of nucleotide sequences stabled from bands on the gels revealed a large degree of homology to what was seen on the melting profiles and on the DGGE gels. All the sequences stabled from the DGGE gels belonged to the first group of phenoxy acid-degrading organisms. The se-

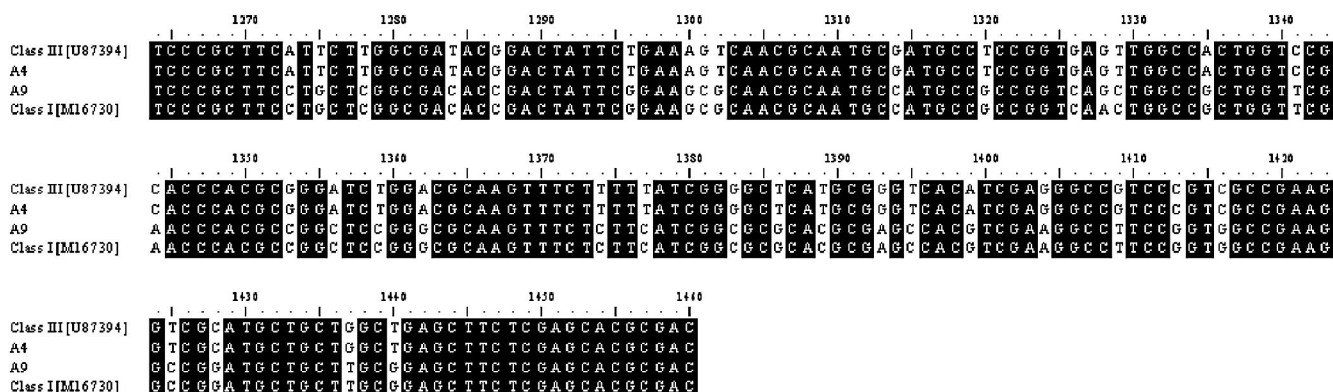


FIG. 8. Sequence alignment of the DNA obtained with the *tfdA*** primer set. The position number relates to GenBank accession number M16730 (*R. eutropha* JMP134). The class I *tfdA* gene sequence represented by *R. eutropha* JMP134 and the class III *tfdA* sequence represented by *Burkholderia cepacia* pJJB were both obtained from GenBank. A2 and A9 correspond to the sequences obtained at day 68 and day 0, respectively. These products were run on a DGGE gel and stabbed prior to sequencing as indicated in the legend of Fig. 7. A2 is highly homologous to bands A1, A3 to A7, and B1 to B3, while A9 and A8 are homologous. Black shading shows highly conserved regions. Designations in brackets are GenBank database accession numbers.

quences stabbed from the bands located at the same denaturation grade as *R. eutropha* strain JMP134 were closely related to this strain and other class I strains as well. In contrast, the sequences stabbed from the bands that increased during the degradation period were related to the class III *tfdA* genes. In general, the role of those organisms containing class I *tfdA* genes in this particular soil remains unclear. A likely and quite interesting explanation may be that they are organisms that can only perform one or a few steps of the degradation pathway, while the class III *tfdA* gene-containing strains are capable of degrading the compound completely. If the class I gene-containing organisms are only capable of degrading the first step, for instance, it may not be likely to grow as a result of the degradation process. If the class III gene-containing bacteria, on the other hand, are capable of degrading MCPA completely, they may be able to grow as a result of the degradation process. This explanation is further supported by the fact that we actually were able to detect the degradation product MCP at days 0 to 22 of the degradation experiment in Top_{2,3} and at days 22 and 33 in Top₂₀. Neither this compound nor the class I *tfdA* gene was found in Sub_{2,3}. Until now, the majority of the research on catabolic genes involved in phenoxyacetic acid degradation has been done with respect to 2,4-D, and in general, it has been accepted that organisms containing the class I *tfdA* gene were capable of degrading this particular compound completely. However, it may be suggested that minor differences in the metabolism between MCPA and 2,4-D have made the class I *tfdA* gene-containing bacteria in this particular soil incapable of degrading MCPA completely. Classes I to III were previously defined by Fulthorpe et al. (20) and Itoh et al. (24) based on homology and whether the *tfdA* gene is carried on a plasmid or not. The class III *tfdA* genes are 77% identical to the class I genes, whereas class II only consists of *Burkholderia* sp. strain RASC and a few strains that are 76% identical to the class I strains and 80% identical to the class III strains (24). As is the case for the strains containing the class I gene, the organisms containing the class III gene belong to a broad range of β - and γ -proteobacteria. Even though the diversity of the *tfdA* gene is not yet fully understood, the DGGE analysis

indicated that up to five different *tfdA* genes were involved in the degradation of MCPA. However, no clear phylogenetic differences could be determined between those strains represented by the strong bands on the DGGE gels. This is probably due to comigration and other types of bias likely to be observed during DGGE analysis (43). Vallaeys et al. (60) reported that strains isolated from the same soil microcosm containing highly homologous *tfdA* genes were different based on their 16S rRNA genes. This may improve the hypothesis that several different species may contain the catabolic gene and be involved in the degradation and that the five bands sequenced from the DGGE gels represent *tfdA* genes from different strains of MCPA-degrading organisms.

The kinetics of mineralization and degradation observed in this study seem to be slightly slower, especially in the subsoil experiment, than what was reported previously by Caux et al. (8) and Mortensen and Jacobsen (41) but equal to what was reported previously by Helweg (23). This rather slow degradation/mineralization may be partly due to the high MCPA concentrations used, which, as seen in this study, results in a longer lag phase. As adaptation has been shown to increase the rate of mineralization (12), a more likely explanation may be the lack of exposure to MCPA during the last 15 years. Similar results were seen for the soil not previously exposed to phenoxy acid herbicides used by Helweg (23).

For the topsoils, the mineralization curve is shifted towards longer reaction times compared with the degradation curves, indicating metabolite formation during degradation. Even though the delay of mineralization compared to degradation was quite small, we detected the first degradation product, MCP, in Top_{2,3} and Top₂₀ but not in Sub_{2,3}. However, no proper accumulation of the metabolite was observed, as the compound was not detected after days 22 and 33 in Top_{2,3} and Top₂₀, respectively. It was rather surprising that we did not detect the metabolite in the subsoil, as the delay of the mineralization was more pronounced for this scenario. It is possible that other metabolites evolve in the subsoil, as we only analyzed this particular compound. This indicates that the degradation of MCP may be rate limiting in topsoils. This is

further supported by the increase in the number of *tfdA* genes, which occurred in advance of the exponential phase of the mineralization. Detection of MCP and other unidentified metabolites during the degradation of MCPA have been reported in recent studies as well (10, 29).

In general, the amounts of MCPA mineralized in topsoils did not exceed a maximum of 60 to 70% of the initial concentrations. The remaining 30 to 40% can be explained by the incorporation of ^{14}C from MCPA into biomass and into soil organic matter. Mineralization potentials of this magnitude have been reported elsewhere previously (41, 61). Additionally, there is a tendency towards a slightly higher total mineralization in the subsoil experiment than in the topsoil, which can be explained by a larger degree of adsorption in the topsoil than in the subsoil. This was previously shown by Jensen et al. (29) to result in a larger bioavailability in the subsoil and thereby higher mineralization potentials, corresponding to the findings previously reported by Mortensen and Jacobsen (41) and Willems et al. (61).

Using kinetic modeling, we found that the mineralization in $\text{Top}_{2,3}$ and Top_{20} was best described by the exponential form of the 3/2-order model, in agreement with results reported previously Mortensen and Jacobsen (41), who used the exponential form for soils from three depths amended with 1 mg MCPA kg^{-1} soil. Similarly, Reffstrup et al. (47) fitted the mineralization of the phenoxypropionic herbicide mecoprop amended at 5 to 500 mg kg^{-1} to the exponential form of the 3/2-order model as well, while experiments amended with 0.0005 to 0.5 mg mecoprop kg^{-1} were fitted to the linear form of the 3/2-order model. This indicates that the initial concentration of the substrate may be important for whether growth occurs or not. However, this was not observed in the present study, which may be due to substrate concentrations that were too high. The fact that Top_{20} fitted better to the model than $\text{Top}_{2,3}$ may be a consequence of the relatively higher degree of growth in Top_{20} than in $\text{Top}_{2,3}$ compared to the substrate concentration.

In the subsoil, the result of the present study differs from what was found previously by Fomsgaard (17) and Mortensen and Jacobsen (41). They found that growth was more likely to occur in subsoil than in topsoil because the initial population of degraders in topsoils may be able to degrade the pesticide without significant growth.

Conclusion. In summary, we found that growth of microbial degraders occurs during the degradation of MCPA. The growth was more pronounced during the degradation of high concentrations (20 mg kg^{-1}) than during the degradation of low concentrations (2.3 mg kg^{-1}). This led to better fitting of mineralization data of the high-concentration scenario to a model taking exponential growth into account. In addition, we found that a shift in the degrader population occurred during the degradation period. Class I *tfdA* genes were found to be present among the naturally occurring microbial population in the topsoil, which had not been exposed to MCPA for 15 years. The concentration of these genes did not increase during the degradation period. Contrary to this, we found that degraders containing *tfdA* genes belonging to class III grew up and became dominant. We found that at least five different *tfdA*-containing genotypes grew up during the degradation period. Thus, the results of an analysis of functional genes present in

soil prior to exposure with a contaminant might not necessarily reflect the actual population carrying out the degradation. Last, in soils containing an initial load of *tfdA* class I genes, the metabolite MCP was detected during the degradation experiment, but no proper accumulation of metabolites from the MCPA degradation was observed.

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