Title: Biodegradation of the herbicide mecoprop-p with soil depth and its relationship with class III *tfdA* genes

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Abstract

Mecoprop-p [(R)-2-(4-chloro-2-methylphenoxy) propanoic acid) is widely used in agriculture and poses an environmental concern because of its susceptibility to leach from soil to water. We investigated the effect of soil depth on mecoprop-p biodegradation and its relationship with the number and diversity of *tfdA* related genes, which are the most widely known genes involved in degradation of the phenoxyalkanoic acid group of herbicides by bacteria. Mecoprop-p half-life (DT_{50}) was approximately 12 days in soil sampled from <30 cm depth, and increased progressively with soil depth, reaching over 84 days at 70-80 cm. In sub-soil there was a lag period of between 23 and 34 days prior to a phase of rapid degradation. No lag phase occurred in top-soil samples prior to the onset of degradation. The maximum degradation rate was the same in top-soil and sub-soil samples. Although diverse *tfdAα* and *tfdA* genes were present prior to mecoprop-p degradation, real time PCR revealed that degradation was associated with proliferation of *tfdA* genes. The number of *tfdA* genes and the most probable number of mecoprop-p degrading organisms in soil prior to mecoprop-p addition were below the limit of quantification and detection respectively. Melting curves from the real time PCR analysis showed that prior to mecoprop-p degradation both class I and class III *tfdA* genes were present in top- and sub-soil samples. However at all soil depths only *tfdA* class III genes proliferated during degradation. Denaturing gradient gel electrophoresis confirmed that class III *tfdA* genes were associated with mecoprop-p degradation. Degradation was not associated with the induction of novel *tfdA* genes in top- or sub-soil samples, and there were no apparent differences in *tfdA* gene diversity with soil depth prior to or following degradation.

Keywords: mecoprop-p, soil depth, biodegradation, *tfdA*, quantitative PCR, diversity

1. Introduction

Mecoprop-p ((R)-2-(4-chloro-2-methylphenoxy)propanoic acid) is a phenoxyalkanoic acid herbicide used widely for post-emergence control of broad-leaved weeds in cereal crops in autumn and spring. Mecoprop-p has a high water solubility and poses environmental concern because its low sorption, high mobility and slow degradation in soil make it susceptible to leaching from soil to water. This pesticide has been widely reported at concentrations above the EU guideline value of 0.1µg l^{-1} in groundwater for public water supply (European Environment Agency, 1999;

Environment Agency, 2003; Buss et al., 2006). This raises concerns especially where groundwater is used as the main source for drinking water.

Because of its limited susceptibility to abiotic degradation, microbial biodegradation is the major process controlling mecoprop-p dissipation in soils, and thereby the extent to which the compound is able to leach through soil to contaminate groundwater (Buss et al., 2006). Biodegradation of mecoprop-p in agricultural top-soil typically occurs through growth-linked metabolism and is rapid, with time to 50% degradation typically less than 25 days (Rodriguez-Cruz et al. 2006) and degradation rates increasing with time as degraders profilerate. However, biodegradation rates decline with soil depth; the slower degradation in sub-soil reflecting either an extended lag phase prior to growth-linked metabolism, or first order kinetics, suggesting cometabolic degradation without extensive proliferation of degradative organisms (Buss et al., 2006; Rodriguez-Cruz et al., 2006). The compound may be highly persistent in sub-soil (Reffstrup et al., 1998; Buss et al., 2006) and in aquifers (Johnson et al., 2003). Furthermore, there may be considerable horizontal as well as vertical variation in degradation rates within single agricultural fields (Rodriguez-Cruz et al., 2006).

In order to predict the fate of pesticides in the environment, it is important to understand the factors which control differences in the biodegradation rate of pesticides with soil depth. Since mecoprop-p shows very low sorption in soil, bioavailability is unlikely to change with soil depth (Kristensen et al., 2001; Johannesen and Aamand, 2003). Reduced biodegradation rates with soil depth are therefore likely to reflect differences in the abundance of degraders or the functional genes they carry. Furthermore, variability in degradation rates could be the result of direct impacts of soil properties on the proliferation of degraders or the expression of their catabolic genes.

Bacterial strains capable of degrading the phenoxyalkanoic herbicide 2,4-dichlorophenoxyacetic acid (2,4-D) have been isolated from many different phylogenetic groups (Tonso et al., 1995, Suwa et al., 1996, Itoh et al., 2002, Kitagawa et al., 2002), and a number of strains able to grow on mecoprop-p as a sole carbon source have also been isolated (Zakaria et al., 2007). Mecoprop-p is biodegraded in soil to 4-chloro-2-methylphenol, followed by ring hydroxylation at the 6-position and ring opening (Fomsgaard and Kristensen, 1999). Thus, the common first step in the biodegradation of phenoxyalkanoic acids is the cleavage of the ether bond of the alkanoic acid side chain, and a diversity of genes have been discovered that encode the responsible enzymes (Streber et al. 1987; Itoh et al. 2002; Kitagawa et al., 2002;

Schleinitz et al. 2004). The best studied genes that mediate this first catalytic step belong to the *tfdA* group and encode an α-ketoglutarate-dependent dioxygenase. The *tfdA* group includes three classes of genes, termed I, II and III, which show more than 80% sequence homology to each other (McGowan et al., 1998), and which are found within the β- and γ- proteobacteria.

tfdA-like genes whose product accepts 2,4-D as a substrate have also been detected in oligotrophic α-proteobacteria, in particular the genus *Bradyrhizobium* (Itoh et al. 2002; 2004). These have 46 to 60 % similarity to the canonical *tfdA* types (Itoh et al., 2002; 2004) and are distinguished by the alpha suffix in *tfdAα*. Also implicated in the first step of 2,4-D catabolism in the α-proteobacteria are *cadABC* genes, which are predicted to encode functional subunits of a multicomponent 2,4-D oxygenase (Kitagawa et al. 2002). In the case of the chiral phenoxypropionic acids (e.g. mecoprop), genes *rdp*A and *sdp*A, which encode enantiospecific α-ketoglutarate-dependent dioxygenases for cleavage of R (mecoprop-p) and S enatiomers, respectively, have also been discovered (Schleinitz et al., 2004).

Lee et al. (2005) quantified functional genes known to be involved in different phenoxy acid pathways during an enrichment study with the compound 2,4-D and found that the number of the *tfdA* genes was several orders of magnitude higher than other types of metabolic genes known to be involved in 2,4-D degradation. Isolated strains capable of degrading 2,4-D have been found to be distributed among all three *tfdA* classes, plus the *tfdAα* group (Itoh et al., 2002). Knowledge of the relative role of genes associated with degradation of other phenoxy acid herbicides is more limited. Class III *tfdA* genes have been found exclusively in β- and γ- proteobacterial strains isolated from a mecoprop-p degrading soil enrichment culture (Zakaria et al., 2007). However, despite the importance of mecoprop-p as an environmental pollutant, the distribution and diversity of mecoprop-p degradative genes in the environment remains to be elucidated.

The current study focussed on the *tfdA* group of genes. The overall aim was to investigate the relationships between soil depth, the biodegradation of mecoprop-p and the copy number and diversity of the *tfdA* gene group.

2. Materials and methods

2.1. Soil collection

Sampling occurred in Long Close field on the farm at Warwick HRI, Wellesbourne, Warwickshire, UK. The soil is a sandy loam of the Wick series (Whitfield, 1974). Mecoprop-P had been applied to the field 3 years prior to sampling, and the related herbicide fenoxaprop-P-ethyl ((RS-2-[4-(6-chloro-1,3-benzoxazol-2-yloxy)phenoxy]propionic acid) had been applied 5 years previously. No other applications of phenoxyalkanoic acid herbicides had been applied in the 10 years prior to sampling. Soil was collected from five depths at three sampling locations. Three pits (1-3) separated by 60 m were excavated to 1 m depth using a mechanical digger, in February 2003. One side of each pit was further excavated using a surface sterilised trowel, so that the face was free of loose soil. Soil was collected from 0-10, 20-30, 40-50, 60-70 and 70-80 cm depth. From each depth approximately 2 kg soil was collected using a trowel and placed into a polythene bag. The trowel was surface sterilised with ethanol between the collection of each soil sample. Soil was spread onto clean polythene bags and left on the bench overnight to reduce moisture content, before being passed through surface sterilised 3 mm sieves. In the sieved soil, total organic matter and microbial biomass-C were measured, as presented and described in Bending et al. (2007).

2.2. Pesticide application and analysis

Commercial mecoprop-p formulation (Duplosan, Mirfield Sales Services Ltd., Doncaster, UK; 48% w/w) was dissolved in distilled water and added to single 300 g fresh weight portions of soil from each location to provide 5 mg pesticide kg^{-1} soil, and further water was added to bring the water holding capacity to 40%. Each soil was mixed thoroughly by hand, and then further mixed by passing through a <3 mm sieve five times. Soil was transferred to a sterile polypropylene container which was loosely capped and incubated at 15^{o}C. Moisture content was maintained by the addition of sterile distilled water as necessary (usually once each week).

The soils were sampled at regular intervals over a 3-month period, with extraction and HPLC analysis as described by Rodriguez-Cruz et al. (2006). Sorption of mecoprop-p was determined using a batch mixing method, and adsorption distribution coefficients (K_d) measured as described by Rodriguez-Cruz et al. (2006).

2.3. Most probable number of mecoprop-p degrading organisms

The number of mecoprop-p degrading organisms was determined in soil immediately following mecoprop-p addition and at the point of 100 % degradation. The size of the mecoprop-p degrading community was determined using the most probable number method, as described in Bending et al. (2003).

2.4. DNA extraction

DNA was extracted from 1 g fresh weight portions of soil taken immediately following mecoprop-p addition, and at the point of 100 % degradation, by bead beating using a MoBio (Carlsbad, California, USA) Ultraclean soil DNA extraction kit as described by the manufacturer.

2.5. Diversity of tfdA and tfdAα genes

Initial studies used primers described by Itoh et al. (2002) to amplify both *tfdA* and *tfdAα* from DNA extracts. 10-fold diluted DNA extracts from pooled 0-10 cm depth samples, taken immediately following mecoprop-p addition or at the point of 100 % degradation, were amplified using the primers 5'-AC(C/G)GAGTTC(G/T)(C/G)CGACATGCG-3' and 5'-GCGGTTGTCCCACATCAC-3'. The PCR reaction mixture and reaction conditions were as described by Bending et al. (2003) and Itoh et al. (2002) respectively. The PCR reactions were purified using a QIAquick PCR Purification Kit (Qiagen Ltd, Dorking, UK) and then cloned using a TOPO Cloning Kit (Invitrogen, Paisley, UK). For each sample, plasmid DNA was extracted from 25 clones containing an insert using a QIAprep Spin Miniprep Kit. Sequencing was performed using M13 forward and reverse primers and a PRISM BigDye Terminator Cycle Sequence Reaction Kit (Applied Biosystems, Warrington, UK), with products sequenced on an Applied Biosystems 3700 automated sequencer.

tfdA-like sequences cloned in this study were compared with selected reference *tfdA* and *tfdAα* sequences available in the Genbank database. A neighbour-joining dendrogram (Jukes Cantor distances; Phylip 3.6a3) was constructed from common partial sequences (c. 356 bp) following alignment in ClustalX1.81. Bootstrap analysis (Seqboot, Phylip 3.6a3) was conducted with 1000 replicates. The resulting trees and consensus were viewed using TreeExplorer 2.12. Sequences for the *tfdAα* and *tfdA* related clones sequenced in this research have been deposited in Genbank under accession numbers EU878493 to EU878531.

204 *2.6. Quantitative PCR of tfdA genes*

205 Quantitative PCR focussed on the *tfdA* gene group only. Primers used were
206 selective for *tfdA* genes and did not amplify *tfdAα* (Baelum et al., 2006). *Cupriavidus*
207 *necator* JMP134(pJP4) (Pemberton et al., 1979) was used for standard curve preparation
208 in the quantitative real time PCR assays. *C. necator* JMP134(pJP4), *Burkholderia* sp.
209 RASC (Fulthorpe et al., 1995), and an unclassified bacterial strain (Tonso et al., 1995)
210 were used for positive controls in melting curve analyses. All of the bacterial strains
211 were propagated in MMO medium (Stanier et al., 1966) supplemented with 500 mg l^{-1}
212 of 2,4-D. DNA sequence analysis confirmed that these strains contained *tfdA* class I, II
213 and III genes, respectively.

214 Standards for quantitative real time PCR (qPCR) with known quantities of the
215 bacterium *C. necator* AEO106 harboring the class I *tfdA* gene and qPCR with DNA
216 from the standards and from the soils treated with mecoprop-p, were made as described
217 previously (Fredslund et al. 2008). Briefly, the Quantitect SYBR green PCR kit
218 (Qiagen, Crawley, UK) was used for the mastermix. The reaction contained 0.4 μM of
219 the *tfdA* primers 5'-GAG CAC TAC GC(AG) CTG AA(CT) TCC CG-3' and 5'-GTC
220 GCG TGC TCG AGA AG-3' and 1 μl of 10-fold diluted DNA extract. In order to
221 ensure a highly specific reaction 25.5 μg bovine serum albumin (Amersham Bioscience,
222 Buckinghamshire, UK) was added to each reaction mixture to avoid unspecific bindings
223 and to ensure as efficient reaction conditions as possible. The PCR conditions were as
224 follows: 6 min at 95^{o}C; 50 cycles of 45 s at 94^{o}C, 30 s at 64^{o}C, and 2 min at 72^{o}C; and a
225 final step of 6 min at 72^{o}C. Subsequently, temperature ramping was performed to
226 analyse melting curve profiles of the PCR products. The conditions were as follows: 80
227 cycles of 30 s starting at 58^{o}C with an increase in temperature of 0.5^{o}C for every cycle
228 to a temperature of 98^{o}C at the final cycle. The melting curves were used to verify
229 presence of the specific real time PCR product.

230

231 *2.7. Denaturing gradient gel electrophoresis of tfdA genes*

232 To provide phylogenetic information about the *tfdA* genes associated with
233 mecoprop-p degradation, *tfdA* genes were amplified from soil taken immediately
234 following mecoprop-p addition, and at the point of 100 % degradation, using GC
235 clamped *tfdA* primers. PCR products were separated by Denaturing Gradient Gel
236 Electrophoresis (DGGE), as described previously (Bælum et al., 2006) except that
237 PuReTaq™ Ready-To-Go PCR beads (GE Healthcare, Buckinghamshire, UK) were

used to produce the PCR product. Bands excised from the gel were re-amplified and sequenced by MWG (Ebersberg, Germany).

2.8. Statistical analysis

Analysis of variance was used to determine the significance of differences in soil parameters and degradation characteristics between soil depths. Time to 50 % degradation (DT_{50}) and MPN data were not normally distributed, and were log transformed prior to analysis in order to confer normality. The model of best fit to the degradation kinetics was determined for each sample, as described by Rodriguez-Cruz et al. (2006), and this was used to obtain time to 50% degradation (DT_{50}) values, the length of lag phase prior to exponential degradation and the maximum mineralization rate (i.e. the rate of decline of mecoprop-p concentration during the exponential degradation phase). All statistical analyses were performed using GenStat (7^{th} edition, VSN International Ltd.).

3. Results

3.1. Variation in mecoprop-p degradation rates and adsorption down the soil profile

There were significant progressive declines in percentage of organic matter (OM) and biomass down the soil profile, demonstrating a clear gradient in soil chemical and biological properties with depth (Table 1). In top-soil (depths above 30 cm), mecoprop-p degradation rates were similar in soil from all three sampling locations and proceeded rapidly without a lag phase (Fig. 1a-c). Top-soil biodegradation kinetics were most closely fitted to a linear model, and DT_{50} occurred within 13 d (Table 1). In sub-soil (depths below 30 cm), kinetics most closely followed the Gompertz model (Fig. 1). There was a lag phase of between 23.3 and 33.4 d prior to a phase of rapid degradation (Fig. 1, Table 1). However, there was substantial variability in degradation rate between the sampling locations and at site 3 in samples taken from below 60 cm depth, there had been no rapid phase of degradation after 80 d (Fig. 1c). DT_{50} in sub-soils increased from 30.8 d at 40-50 cm depth to 83.6 d at 70-80 cm depth. Soil depth had no significant effect on the maximum degradation rate, which averaged at 0.59 µg mecoprop-p g^{-1} soil d^{-1}. K_d averaged 0.15 g^{-1} ml^{-1} and was not significantly affected by depth (data not shown).

3.2. Number of Mecoprop-p degraders

Prior to mecoprop-p application the most probable number (MPN) of mecoprop-p degrading organisms was lower than the detection limit of 100 degraders g^{-1} soil in all samples. At the point of 100% degradation numbers of mecoprop-p degrading organisms had increased in all samples to between 4.0 to 5.9 log cells g^{-1} soil, although there were no significant differences in the number proliferating at the different soil depths (Table 1), and no relationship between the number of degraders and DT_{50}.

3.3. Diversity and relative abundance of tfdAα and tfdA genes

Using the Itoh et al. (2002) primers, which amplify both *tfdAα* and *tfdA* genes, products of the correct size (356 bp) could be amplified from the pooled 0-10 cm sample prior to mecoprop-p addition and at the point of 100% mecoprop-p degradation and these were cloned. Phylogenetic analysis of cloned sequences is shown in Fig. 2. The data indicates that the soil supported diverse *tfdAα* and *tfdA* sequences, although some of the branches of the phylogenetic tree were not well supported using the neighbour joining method with bootstrap percentages less than 50%. Clones with high homology to the *tfdAα* gene were found with the same abundance prior to and after the degradation of mecoprop-p. The tree shows fairly strong support (92%) for 27 of the soil clones from both mecoprop-p treated and untreated soil clustering with between 69% (clone U20) and 91% (clone M22) identity to known *tfdAα* sequences from bradyrhizobial isolates (e.g. *Bradyrhizobium* strain RD5-C2) and also with sequences amplified from enrichment cultures from other UK soils. There was also strong support (100%) for one clone sequence from mecoprop-p treated soil (clone M1) clustering with 99% identity to the *tfdA* of *Achromobacter xylosoxidans* EST4002, a known class III *tfdA*, and, with 78% identity to *tfdA* from *C. necator* JMP134 pJP4 (class I). The analysis also identified that the remainder of the clones (both mecoprop-p-treated and untreated; M20, U15, U26, U14, M23, U18, U1, M15, U17, M9, M24) did not cluster with *tfdA* or *tfdAα* from cultured strains.

3.4. Quantitative PCR of tfdA genes

Prior to mecoprop-p treatment, the number of *tfdA* genes in the soils was below 400 g^{-1} soil. Even though it was possible to detect *tfdA* genes in the soils prior to mecoprop-p application (Fig. 3), the reliability of the PCR decreases below 400 *tfdA* genes g^{-1} soil so that quantification was not possible. Subsequent to mecoprop-p

degradation we observed a significant increase in the *tfdA* genes with numbers ranging from 4.74×10^4-7.66×10^4 genes g^{-1} soil (Table 1). ANOVA revealed that there was no significant difference in the number of *tfdA* genes in soil from different depths. Furthermore there was no significant relationship between the number of *tfdA* genes and DT_{50} or the MPN of mecoprop-p degrading organisms.

In addition to the quantitative data obtained from the real time PCR, we were able to investigate diversity in the *tfdA* genes present prior and subsequent to mecoprop-p degradation (Fig. 3). Prior to the mecoprop-p treatment class I as well as class III *tfdA* genes were detectable in the soils. However only the class III *tfdA* gene was detectable at the point of 100% mecoprop-p degradation, although the possible presence of class I sequences cannot be excluded. Identical melting curve profiles were obtained for all samples prior to and after degradation, but in order to simplify the results one representative profile is presented for DNA extracts prior to mecoprop-p application and one profile for DNA extracts after 100 % degradation.

3.5. Denaturing gradient gel electrophoresis of tfdA genes

In order to investigate the dynamics of *tfdA* genes during mecoprop-p degradation DGGE analysis was performed. Samples had between 4 and 5 separate DGGE bands (data not shown), but there was no difference in banding number or pattern either between sampling times, depth or location. However, bands were observed to be stronger in samples taken at 100% degradation than at 0% degradation. BLAST searching showed that all bands present on DGGE gels in samples at 100% mecoprop-p degradation showed >99% homology to *Burkholderia cepacia* plasmid pIJB class III *tfdA* (EMBL accession U87394), with bands also showing >99% homology to *tfdA* Class III DGGE bands A2-6 and B1 (EMBL accessions DG272406-DQ272414) described by Baelum et al. (2006).

4. Discussion

4.1. Biodegradation kinetics

Mecoprop-p degradation rates were slower in sub-soils relative to top-soils. Similarly, Helweg (1993) found a decline in mecoprop-p dedgradation with soil depth, with DT_{50} increasing from 7 d at 0-33 cm depth to between 34 d and 70 d in sub-soil samples at 33-100 cm depth. The fact that only the lag phase length and not the maximum degradation rate was different in top- and sub- soil samples suggests that

biodegradation in subsoil was not differentially limited by soil physical or chemical properties. In particular, the low sorption of mecoprop-p and the use of standardised soil particle size and bulk density in experiments suggest that differences in bioavailability or dispersion of mecoprop-p cannot account for the difference in biodegradation kinetics.

The length of the lag phase is thought to reflect the time taken for adaptation to produce a catabolic population or for the growth of an initially small adapted population to a size which produces measurable biodegradation (Alexander, 1994). Given the short length of the lag phase, in the current study it most likely reflected growth of adapted strains. Using quantitative PCR and DGGE we were able to detect *tfdA* genes in both top- and sub- soil prior to mecoprop addition. Thus, we know that the catabolic potential, at least with respect to the first degradative step, was initially present at all soil depths. Although detectable, the number of *tfdA* genes was below the limit of quantification for the qPCR method and therefore we were not able to define a relationship between biodegradation kinetics and the initial number of catabolic microbes. Similarly, Bending et al. (2007) found that a decline in degradation rates of the pesticide isoproturon with soil depth could not easily be attributed to differences in the number of isoproturon catabolising organisms present prior to addition of the compound. However, it is possible that differences, beyond our detection limit, in the initial number of catabolic organisms could have resulted in the contrasting degradation rates between soil depths.

4.2. Dynamics of tfdA genes and most probable number of mecoprop degraders

Degradation of mecoprop-p was shown to be associated with a significant increase in numbers of *tfdA* genes in the soils. Increasing numbers of *tfdA* genes as a response to phenoxyalkanoic acid degradation has been shown for the related herbicides MCPA (Bælum et al., 2006; Bælum et al., 2008) and 2,4-D (Lee et al., 2005). The number of *tfdA* genes at 100% degradation reported in the present work is very similar to numbers reported by Bælum et al. (2006), where ~3×10^4 *tfdA* genes g^{-1} soil were reported after mineralization of 2.3 mg MCPA kg^{-1} soil. In the present work we report numbers of 4.74×10^4-7.66×10^4 for 5 mg mecoprop-p kg^{-1} soil. The MPN of degraders at 100% degradation was not related to DT_{50} values, and showed far greater variability than the number of *tfdA* genes. Furthermore, it can be seen that ratio of MPN mecoprop-p degraders to the number of *tfdA* genes at the different soil depths varied considerably,

374 from below 1 to over 12. The lack of a relationship between MPN mecoprop-p
375 degraders and the number of *tfdA* genes could be due to multiple sources. A *tfdA* copy
376 number-to-MPN mecoprop degrader ratio greater than 1 could reflect variation in the
377 *tfdA* gene copy number per bacterial cell or the inability of a subset of *tfdA* positive
378 organisms to catabolise mecoprop in the MPN test medium. A *tfdA* copy number-to-
379 MPN mecoprop degrader ratio less than 1 could reflect the contribution of microbial
380 groups not possessing *tfdA* genes to the MPN score.
381 The amount of carbon added as mecoprop-p was the same for all soil-depths,
382 and therefore it will, independent of DT_{50} values, potentially support more or less the
383 same amount of growth. However, a range of factors could affect the bacterial
384 population size or catabolic gene number reached following degradation of a defined
385 quantity of mecoprop-p, including predation, the use of additional substrates by the
386 degrader population and differences between catabolic strains in the efficiency with
387 which carbon in mecoprop-p is converted into biomass. Our data, in which all locations
388 supported similar proliferation of catabolic genes following complete degradation of
389 mecoprop-p, irrespective of DT50, suggests that that these factors were not important in
390 determining population sizes of mecoprop-p degraders. This suggests that the lag phase
391 reflected the rate of development of populations with appropriate catabolic genes rather
392 than differences between locations with respect to other processes such as predation.
393 Further work should test this by relating numbers of degraders and catabolic genes at
394 defined time intervals during degradation to the rate of degradation, and particularly the
395 length of the lag phase.
396
397 *4.3. Diversity of tfdA and tfdA-alpha genes*
398 In addition to the quantitative data, the real time PCR assay revealed data on
399 functional diversity among the mecoprop-p degraders based on *tfdA* gene sequences.
400 The *tfdA* primers used for PCR in the present study were originally designed to target
401 the three different classes of the *tfdA* gene as proposed by McGowan et al. (1998), and
402 by studying melting curve profiles of PCR products we were able to establish
403 specifically which classes proliferated during the experiment. We found that both class I
404 and III *tfdA* genes were present in soil prior to biodegradation. The fact that the class III
405 *tfdA* genes proliferated during the experiment indicates that the organisms harbouring
406 the class I *tfdA* gene were not able to grow on mecoprop-p as a carbon source. It is not
407 possible to prove inactivity of class I harbouring organisms definitively based on the

data available in the present study, as these genes in theory can be expressed without resulting in growth. Bælum et al. (2006) revealed a similar pattern in the case of MCPA degradation as they found increased abundance of class III *tfdA* genes during degradation. Furthermore, Zakaria et al. (2007) investigated the diversity of *tfdA* genes in a mecoprop-p enrichment culture and similarly revealed growth of bacteria harbouring class III genes only. In the current study the reason for the lack of detectability of class I *tfdA* genes at the end of the experiment is presumably that the increased density of class III genes shadowed their presence in the PCR.

The melting curve analysis which linked mecoprop-p degradation to class III *tfdA* genes was supported by the DGGE analysis. All bands were excised and all sequences obtained had 99-100% homology to class III *tfdA* genes, supporting the findings obtained by melting curve analysis. In particular, the *tfdA* genes associated with mecoprop-p degradation in the current study showed >99 % homology to *tfdA* genes associated with MCPA degradation in a Danish agricultural field (Bælum et al., 2006), suggesting conservation of genes involved in degradation of related compounds in different geographical locations.

Interestingly, when we used primers that targeted both *tfdA* and *tfdAα*, we only recovered one clone (M1), from soil treated with mecoprop-p, which was closely related (99% sequence identity) to class III *tfdA*. That the only *tfdA* sequence to be detected was class III is in agreement with the melting profile analysis which indicated that the class III *tfdA* became enriched in response to mecoprop-p addition. However, the low recovery ratios of *tfdA*-to-*tfdAα* in the clone libraries suggests that *tfdAα* appears to be more abundant than *tfdA* both before and after mecoprop-p addition. The finding here of considerable *tfdAα* abundance in soil, even before mecoprop-p addition, is in agreement with the conclusions of other research which suggests that *tfdAα* is present in *Bradyrhizobia* and possibly other genera in the α-proteobacteria within the soil community independently of phenoxyacetic acid herbicide exposure (Itoh et al. 2002, 2004; Parker and Kennedy, 2006).

Although it is known that the *tfdAα* protein can accept 2,4-D as a substrate (Itoh et al., 2002), it is not known if mecoprop-p is also a substrate for *tfdAα*. Whether *tfdAα* contributed to mecoprop-p degradation here does remain to be tested, although, in light of the evidence of non-function for 2,4-D (Itoh et al., 2004), we suggest that *tfdAα* did not contribute, despite the fact that *tfdAα* appeared to be abundant in the soil community.

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558

559 Table 1

560 Soil properties and degradation parameters of top-soil and sub-soil samples.

561 Data represent average of the three replicate sampling locations (a-c) at each depth.

Soil depth (cm)	**Organic matter (%)**	**Biomass (mg C kg^{-1} soil)**	**DT_{50} (days)[a]**	**Lag phase (days)**	**log MPN (g^{-1} dw soil)[b,c]**	***tfdA* copy no. (g^{-1} dw soil)[b,d]**	**Ratio MPN degraders: *tfdA* copy no.**
0-10	2.7	68.8	12.3 (1.09)	0.0	5.1	76591	3.1
20-30	2.4	66.9	12.7 (1.10)	0.0	5.9	47369	12.5
40-50	2.2	45.6	30.8 (1.48)	28.0	5.0	70800	11.0
60-70	1.5	16.3	61.7 (1.65)	23.3	4.0	67156	0.2
70-80	1.1	9.5	83.6 (1.77)	33.4	4.4	51244	0.8
LSD (*P*<0.05)	0.29	16.0	(0.51)	14.2	1.5	129513	21.0
Significance of effect of depth[e]	***	***	*	***	NS	NS	NS

562 [a] figures in brackets represent log transformed data to which LSD relates

563 [b] At the point of 100% mecoprop-p degradation

564 [c] Number of degraders at time 0 were below detection limits

565 [d] *tfdA* copy number at time 0 < 400 g^{-1} dw soil

566 [e] NS, not significant; *** significant *P*<0.001; * significant *P*<0.05

567

Figure Legends

Figure 1 Degradation of mecoprop-p in top- and sub-soil samples for the three sampling locations (a,b,c) studied. Soil depth: 0-10 cm(◆); 20-30 cm (■); 40-50 cm (▲); 60 - 70 cm (●); 70-80 cm (✶).

Figure 2 Phylogenetic position of cloned *tfdA*-like sequences amplified from Wellesbourne top-soil (0-10 cm) sampled prior to mecoprop-p addition (U) and at the point of 100% mecoprop-p degradation (M) in relation to reference strains and clones for which Genbank accession numbers and strain or clone name are given.
Reference clones marked with § are from the study by Shaw and Burns (2005); reference strains marked with * were recently isolated from soils in Vietnam and Japan (Sakai et al. 2007; Huong et al. 2007). *E. coli TauD* which was used as the outgroup encodes taurine/α-KG dioxygenase. Clusters representing Type I *tfdA*, Type II *tfdA* and Type III *tfdA* (McGowan et al. 1998) and *tfdAα* (Itoh et al., 2002) and the support for each major branch, where > 70%, as determined from 1000 bootstrap samples is indicated. The scale bar represents Jukes-Cantor distance.

Figure 3 Melting curve profiles of real time PCR amplification products. The profiles display the negative first derivative of temperature versus relative fluorescence units (RFU) [-d(RFU)/dT] plotted against temperature (^{o}C). All samples showed similar responses, and data for a representative top-soil sample is presented
a) Real time PCR melting curve profiles using standard sequences as template
b) Representative real time PCR melting curve profiles prior to and following degradation of mecoprop-p

Fig 1

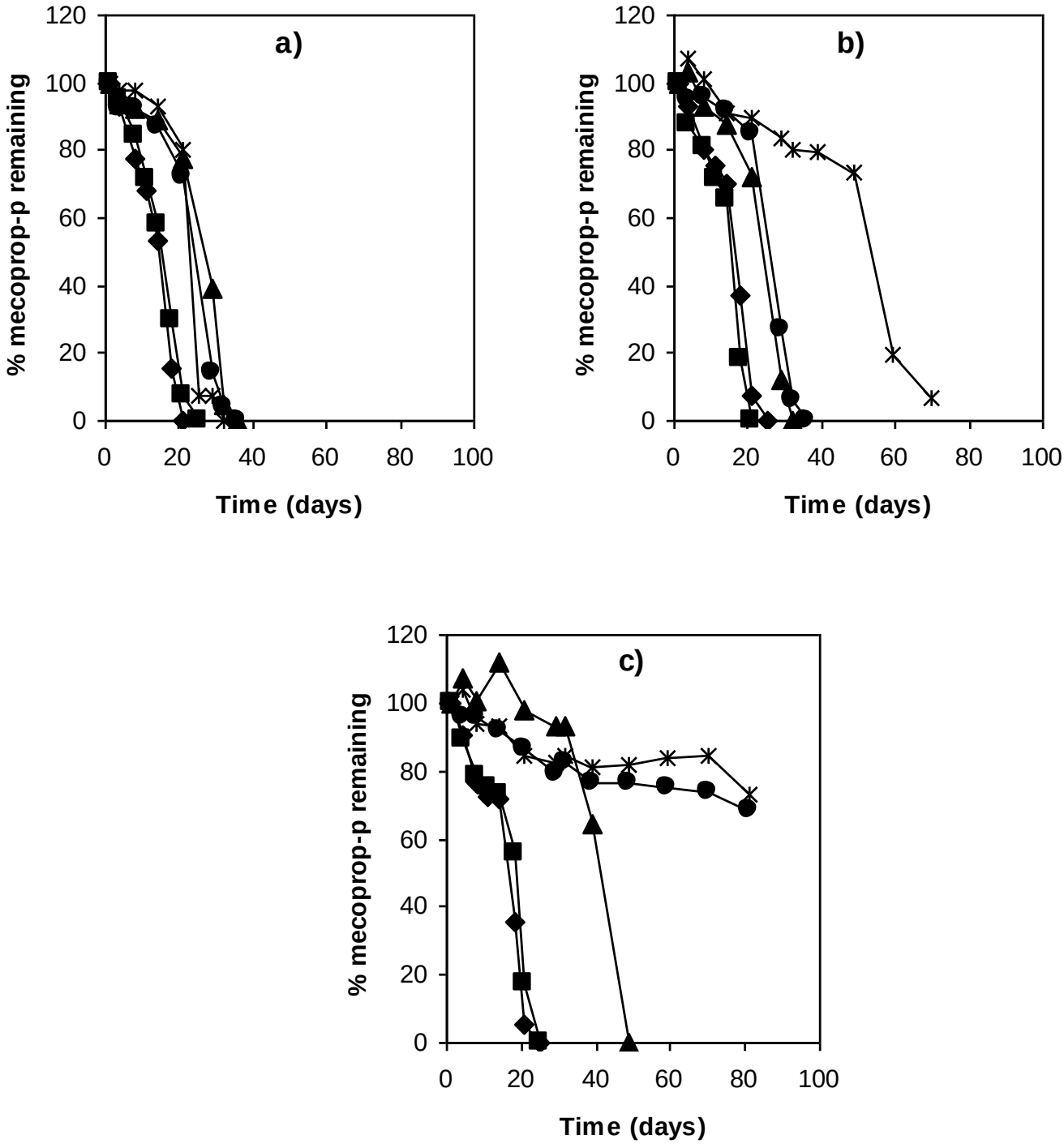


Fig 2

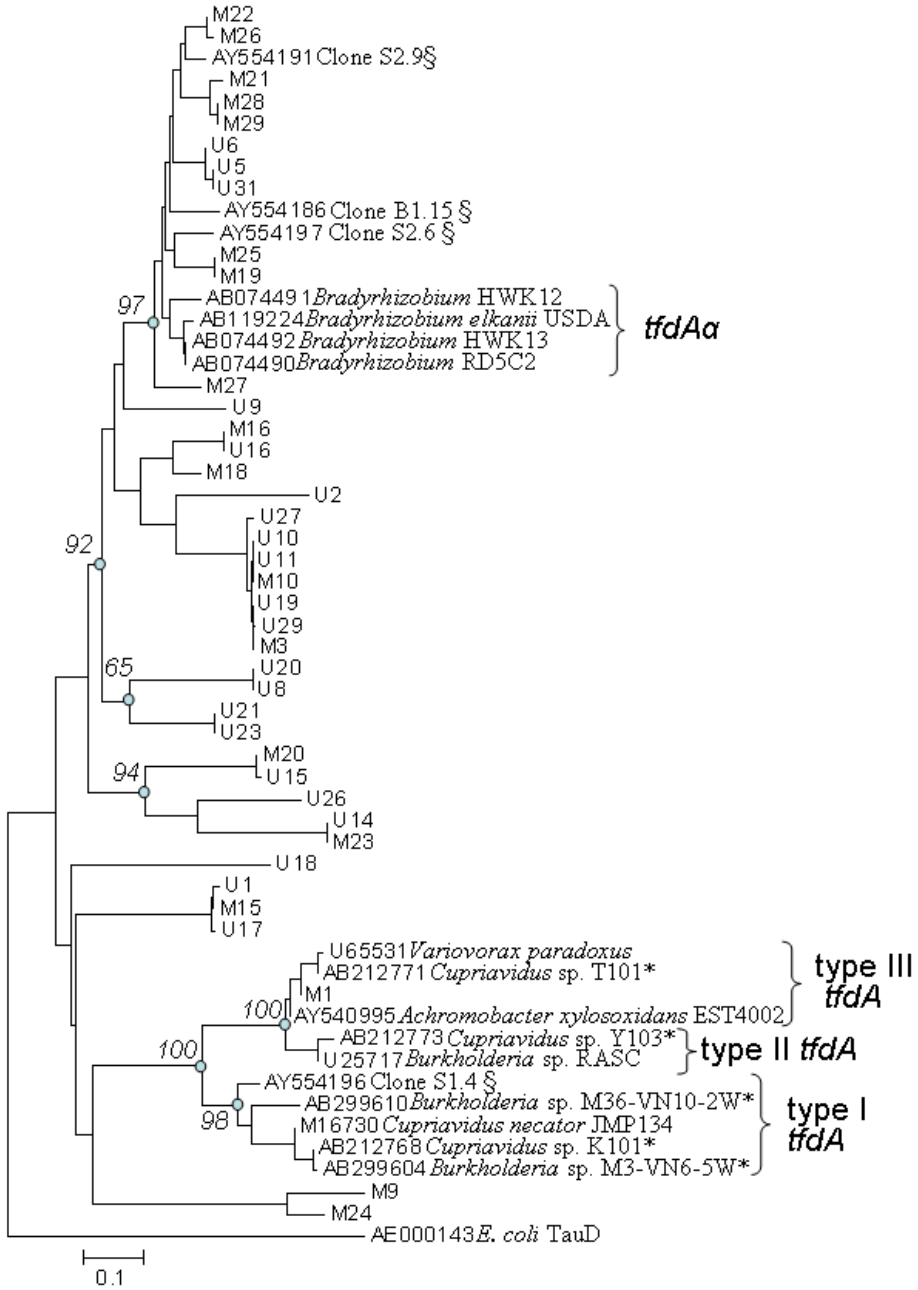
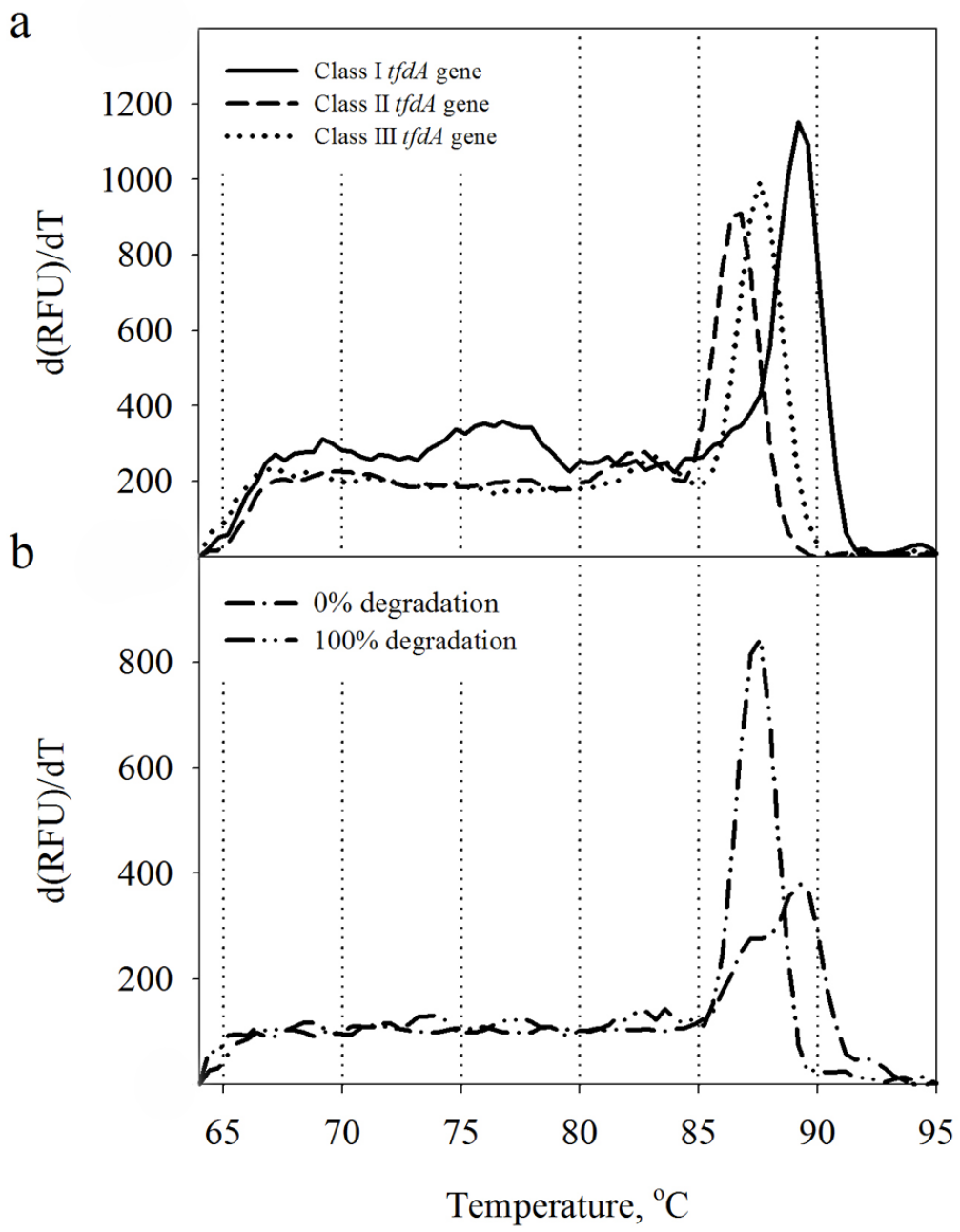

M22
M26
AY554191 Clone S2.9§
M21
M28
M29
U6
U5
U31
AY554186 Clone B1.15 §
AY554197 Clone S2.6 §
M25
M19
97
AB074491 Bradyrhizobium HWK12
AB119224 Bradyrhizobium elkanii USDA
AB074492 Bradyrhizobium HWK13
AB074490 Bradyrhizobium RD5C2
tfdAα
M27
U9
M16
U16
M18
U2
U27
92
U10
U11
M10
U19
U29
M3
65
U20
U8
U21
U23
M20
94
U15
U26
U14
M23
U18
U1
M15
U17
U65531 Variovorax paradoxus
AB212771 Cupriavidus sp. T101*
M1
100
AY540995 Achromobacter xylosoxidans EST4002
type III tfdA
AB212773 Cupriavidus sp. Y103*
100
U25717 Burkholderia sp. RASC
type II tfdA
AY554196 Clone S1.4 §
AB299610 Burkholderia sp. M36-VN10-2W*
98
M16730 Cupriavidus necator JMP134
AB212768 Cupriavidus sp. K101*
AB299604 Burkholderia sp. M3-VN6-5W*
type I tfdA
M9
M24
AE000143 E. coli TauD
0.1

Fig 3


a
Class I tfdA gene
Class II tfdA gene
Class III tfdA gene
d(RFU)/dT
1200
1000
800
600
400
200
b
0% degradation
100% degradation
d(RFU)/dT
800
600
400
200
65
70
75
80
85
90
95
Temperature, °C