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35

36 **Abstract**

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

59

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

61

62 **1. Introduction**

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

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

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

86 In order to predict the fate of pesticides in the environment, it is important to  
87 understand the factors which control differences in the biodegradation rate of pesticides  
88 with soil depth. Since mecoprop-p shows very low sorption in soil, bioavailability is  
89 unlikely to change with soil depth (Kristensen et al., 2001; Johannesen and Aamand,  
90 2003). Reduced biodegradation rates with soil depth are therefore likely to reflect  
91 differences in the abundance of degraders or the functional genes they carry.  
92 Furthermore, variability in degradation rates could be the result of direct impacts of soil  
93 properties on the proliferation of degraders or the expression of their catabolic genes.

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

104 Schleinitz et al. 2004). The best studied genes that mediate this first catalytic step  
105 belong to the *tfdA* group and encode an  $\alpha$ -ketoglutarate-dependent dioxygenase. The  
106 *tfdA* group includes three classes of genes, termed I, II and III, which show more than  
107 80% sequence homology to each other (McGowan et al., 1998), and which are found  
108 within the  $\beta$ - and  $\gamma$ - proteobacteria.

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

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

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

133

## 134 **2. Materials and methods**

135

### 136 *2.1. Soil collection*

137            Sampling occurred in Long Close field on the farm at Warwick HRI,  
138 Wellesbourne, Warwickshire, UK. The soil is a sandy loam of the Wick series  
139 (Whitfield, 1974). Mecoprop-P had been applied to the field 3 years prior to sampling,  
140 and the related herbicide fenoxaprop-P-ethyl ((RS-2-[4-(6-chloro-1,3-benzoxazol-2-  
141 yloxy)phenoxy]propionic acid) had been applied 5 years previously. No other  
142 applications of phenoxyalkanoic acid herbicides had been applied in the 10 years prior  
143 to sampling. Soil was collected from five depths at three sampling locations. Three pits  
144 (1-3) separated by 60 m were excavated to 1 m depth using a mechanical digger, in  
145 February 2003. One side of each pit was further excavated using a surface sterilised  
146 trowel, so that the face was free of loose soil. Soil was collected from 0-10, 20-30, 40-  
147 50, 60-70 and 70-80 cm depth. From each depth approximately 2 kg soil was collected  
148 using a trowel and placed into a polythene bag. The trowel was surface sterilised with  
149 ethanol between the collection of each soil sample. Soil was spread onto clean  
150 polythene bags and left on the bench overnight to reduce moisture content, before being  
151 passed through surface sterilised 3 mm sieves. In the sieved soil, total organic matter  
152 and microbial biomass-C were measured, as presented and described in Bending et al.  
153 (2007).

154

## 155 *2.2. Pesticide application and analysis*

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

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

168

## 169 *2.3. Most probable number of mecoprop-p degrading organisms*

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

174

#### 175 2.4. DNA extraction

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

180

#### 181 2.5. Diversity of *tfdA* and *tfdAα* genes

182 Initial studies used primers described by Itoh et al. (2002) to amplify both *tfdA*  
183 and *tfdAα* from DNA extracts. 10-fold diluted DNA extracts from pooled 0-10 cm depth  
184 samples, taken immediately following mecoprop-p addition or at the point of 100 %  
185 degradation, were amplified using the primers 5'-  
186 AC(C/G)GAGTTC(G/T)(C/G)CGACATGCG-3' and 5'-GCGGTTGTCCCACATCAC-  
187 3'. The PCR reaction mixture and reaction conditions were as described by Bending et  
188 al. (2003) and Itoh et al. (2002) respectively. The PCR reactions were purified using a  
189 QIAquick PCR Purification Kit (Qiagen Ltd, Dorking, UK) and then cloned using a  
190 TOPO Cloning Kit (Invitrogen, Paisley, UK). For each sample, plasmid DNA was  
191 extracted from 25 clones containing an insert using a QIAprep Spin Miniprep Kit.  
192 Sequencing was performed using M13 forward and reverse primers and a PRISM  
193 BigDye Terminator Cycle Sequence Reaction Kit (Applied Biosystems, Warrington,  
194 UK), with products sequenced on an Applied Biosystems 3700 automated sequencer.

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

203

## 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<sup>-1</sup>  
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°C; 50 cycles of 45 s at 94°C, 30 s at 64°C, and 2 min at 72°C; and a  
225 final step of 6 min at 72°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°C with an increase in temperature of 0.5°C for every cycle  
228 to a temperature of 98°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 (Baelum et al., 2006) except that  
237 PuReTaq™ Ready-To-Go PCR beads (GE Healthcare, Buckinghamshire, UK) were



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

240

## 241 2.8. *Statistical analysis*

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

252

## 253 **3. Results**

254

### 255 3.1. *Variation in mecoprop-p degradation rates and adsorption down the soil profile*

256 There were significant progressive declines in percentage of organic matter  
257 (OM) and biomass down the soil profile, demonstrating a clear gradient in soil chemical  
258 and biological properties with depth (Table 1). In top-soil (depths above 30 cm),  
259 mecoprop-p degradation rates were similar in soil from all three sampling locations and  
260 proceeded rapidly without a lag phase (Fig. 1a-c). Top-soil biodegradation kinetics were  
261 most closely fitted to a linear model, and DT<sub>50</sub> occurred within 13 d (Table 1). In sub-  
262 soil (depths below 30 cm), kinetics most closely followed the Gompertz model (Fig. 1).  
263 There was a lag phase of between 23.3 and 33.4 d prior to a phase of rapid degradation  
264 (Fig. 1, Table 1). However, there was substantial variability in degradation rate between  
265 the sampling locations and at site 3 in samples taken from below 60 cm depth, there had  
266 been no rapid phase of degradation after 80 d (Fig. 1c). DT<sub>50</sub> in sub-soils increased from  
267 30.8 d at 40-50 cm depth to 83.6 d at 70-80 cm depth. Soil depth had no significant  
268 effect on the maximum degradation rate, which averaged at 0.59 µg mecoprop-p g<sup>-1</sup> soil  
269 d<sup>-1</sup>. K<sub>d</sub> averaged 0.15 g<sup>-1</sup> ml<sup>-1</sup> and was not significantly affected by depth (data not  
270 shown).

271

272 3.2. Number of Mecoprop-p degraders

273 Prior to mecoprop-p application the most probable number (MPN) of mecoprop-  
274 p degrading organisms was lower than the detection limit of 100 degraders g<sup>-1</sup> soil in all  
275 samples. At the point of 100% degradation numbers of mecoprop-p degrading  
276 organisms had increased in all samples to between 4.0 to 5.9 log cells g<sup>-1</sup> soil, although  
277 there were no significant differences in the number proliferating at the different soil  
278 depths (Table 1), and no relationship between the number of degraders and DT<sub>50</sub>.

279

280 3.3. Diversity and relative abundance of *tfdAα* and *tfdA* genes

281 Using the Itoh et al. (2002) primers, which amplify both *tfdAα* and *tfdA* genes,  
282 products of the correct size (356 bp) could be amplified from the pooled 0-10 cm  
283 sample prior to mecoprop-p addition and at the point of 100% mecoprop-p degradation  
284 and these were cloned. Phylogenetic analysis of cloned sequences is shown in Fig. 2.  
285 The data indicates that the soil supported diverse *tfdAα* and *tfdA* sequences, although  
286 some of the branches of the phylogenetic tree were not well supported using the  
287 neighbour joining method with bootstrap percentages less than 50%. Clones with high  
288 homology to the *tfdAα* gene were found with the same abundance prior to and after the  
289 degradation of mecoprop-p. The tree shows fairly strong support (92%) for 27 of the  
290 soil clones from both mecoprop-p treated and untreated soil clustering with between  
291 69% (clone U20) and 91% (clone M22) identity to known *tfdAα* sequences from  
292 bradyrhizobial isolates (e.g. *Bradyrhizobium* strain RD5-C2) and also with sequences  
293 amplified from enrichment cultures from other UK soils. There was also strong support  
294 (100%) for one clone sequence from mecoprop-p treated soil (clone M1) clustering with  
295 99% identity to the *tfdA* of *Achromobacter xylosoxidans* EST4002, a known class III  
296 *tfdA*, and, with 78% identity to *tfdA* from *C. necator* JMP134 pJP4 (class I). The  
297 analysis also identified that the remainder of the clones (both mecoprop-p-treated and  
298 untreated; M20, U15, U26, U14, M23, U18, U1, M15, U17, M9, M24) did not cluster  
299 with *tfdA* or *tfdAα* from cultured strains.

300

301 3.4. Quantitative PCR of *tfdA* genes

302 Prior to mecoprop-p treatment, the number of *tfdA* genes in the soils was below  
303 400 g<sup>-1</sup> soil. Even though it was possible to detect *tfdA* genes in the soils prior to  
304 mecoprop-p application (Fig. 3), the reliability of the PCR decreases below 400 *tfdA*  
305 genes g<sup>-1</sup> soil so that quantification was not possible. Subsequent to mecoprop-p

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

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

320

### 321 3.5. Denaturing gradient gel electrophoresis of *tfdA* genes

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

332

## 333 4. Discussion

### 334 4.1. Biodegradation kinetics

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

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

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

361

#### 362 *4.2. Dynamics of *tfdA* genes and most probable number of mecoprop degraders*

363 Degradation of mecoprop-p was shown to be associated with a significant increase in  
364 numbers of *tfdA* genes in the soils. Increasing numbers of *tfdA* genes as a response to  
365 phenoxyalkanoic acid degradation has been shown for the related herbicides MCPA  
366 (Bælum et al., 2006; Bælum et al., 2008) and 2,4-D (Lee et al., 2005). The number of  
367 *tfdA* genes at 100% degradation reported in the present work is very similar to numbers  
368 reported by Bælum et al. (2006), where  $\sim 3 \times 10^4$  *tfdA* genes  $g^{-1}$  soil were reported after  
369 mineralization of 2.3 mg MCPA  $kg^{-1}$  soil. In the present work we report numbers of  
370  $4.74 \times 10^4$ - $7.66 \times 10^4$  for 5 mg mecoprop-p  $kg^{-1}$  soil. The MPN of degraders at 100%  
371 degradation was not related to  $DT_{50}$  values, and showed far greater variability than the  
372 number of *tfdA* genes. Furthermore, it can be seen that ratio of MPN mecoprop-p  
373 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<sub>50</sub> 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

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

416 The melting curve analysis which linked mecoprop-p degradation to class III  
417 *tfdA* genes was supported by the DGGE analysis. All bands were excised and all  
418 sequences obtained had 99-100% homology to class III *tfdA* genes, supporting the  
419 findings obtained by melting curve analysis. In particular, the *tfdA* genes associated  
420 with mecoprop-p degradation in the current study showed >99 % homology to *tfdA*  
421 genes associated with MCPA degradation in a Danish agricultural field (Bælum et al.,  
422 2006), suggesting conservation of genes involved in degradation of related compounds  
423 in different geographical locations.

424 Interestingly, when we used primers that targeted both *tfdA* and *tfdAα*, we only  
425 recovered one clone (M1), from soil treated with mecoprop-p, which was closely related  
426 (99% sequence identity) to class III *tfdA*. That the only *tfdA* sequence to be detected was  
427 class III is in agreement with the melting profile analysis which indicated that the class  
428 III *tfdA* became enriched in response to mecoprop-p addition. However, the low  
429 recovery ratios of *tfdA*-to-*tfdAα* in the clone libraries suggests that *tfdAα* appears to be  
430 more abundant than *tfdA* both before and after mecoprop-p addition. The finding here of  
431 considerable *tfdAα* abundance in soil, even before mecoprop-p addition, is in agreement  
432 with the conclusions of other research which suggests that *tfdAα* is present in  
433 *Bradyrhizobia* and possibly other genera in the α-proteobacteria within the soil  
434 community independently of phenoxyacetic acid herbicide exposure (Itoh et al. 2002,  
435 2004; Parker and Kennedy, 2006).

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

442

443

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451

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557



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 <sup>-1</sup> soil)	DT <sub>50</sub> (days) <sup>a</sup>	Lag phase (days)	log MPN (g <sup>-1</sup> dw soil) <sup>b,c</sup>	<i>tfdA</i> copy no. (g <sup>-1</sup> dw soil) <sup>b,d</sup>	Ratio degraders: <i>tfdA</i> copy no.	MPN <i>tfdA</i>
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 ( <i>P</i> <0.05)	0.29	16.0	(0.51)	14.2	1.5	129513	21.0	
Significance of effect of depth <sup>e</sup>	***	***	*	***	NS	NS	NS	

562 <sup>a</sup> figures in brackets represent log transformed data to which LSD relates

563 <sup>b</sup> At the point of 100% mecoprop-p degradation

564 <sup>c</sup> Number of degraders at time 0 were below detection limits

565 <sup>d</sup> *tfdA* copy number at time 0 < 400 g<sup>-1</sup> dw soil

566 <sup>e</sup> NS, not significant; \*\*\* significant *P*<0.001; \* significant *P*<0.05



## 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/ $\alpha$ -KG dioxygenase. Clusters representing Type I *tfdA*, Type II *tfdA* and Type III *tfdA* (McGowan et al. 1998) and *tfdA $\alpha$*  (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)  $[-d(\text{RFU})/dT]$  plotted against temperature ( $^{\circ}\text{C}$ ). 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

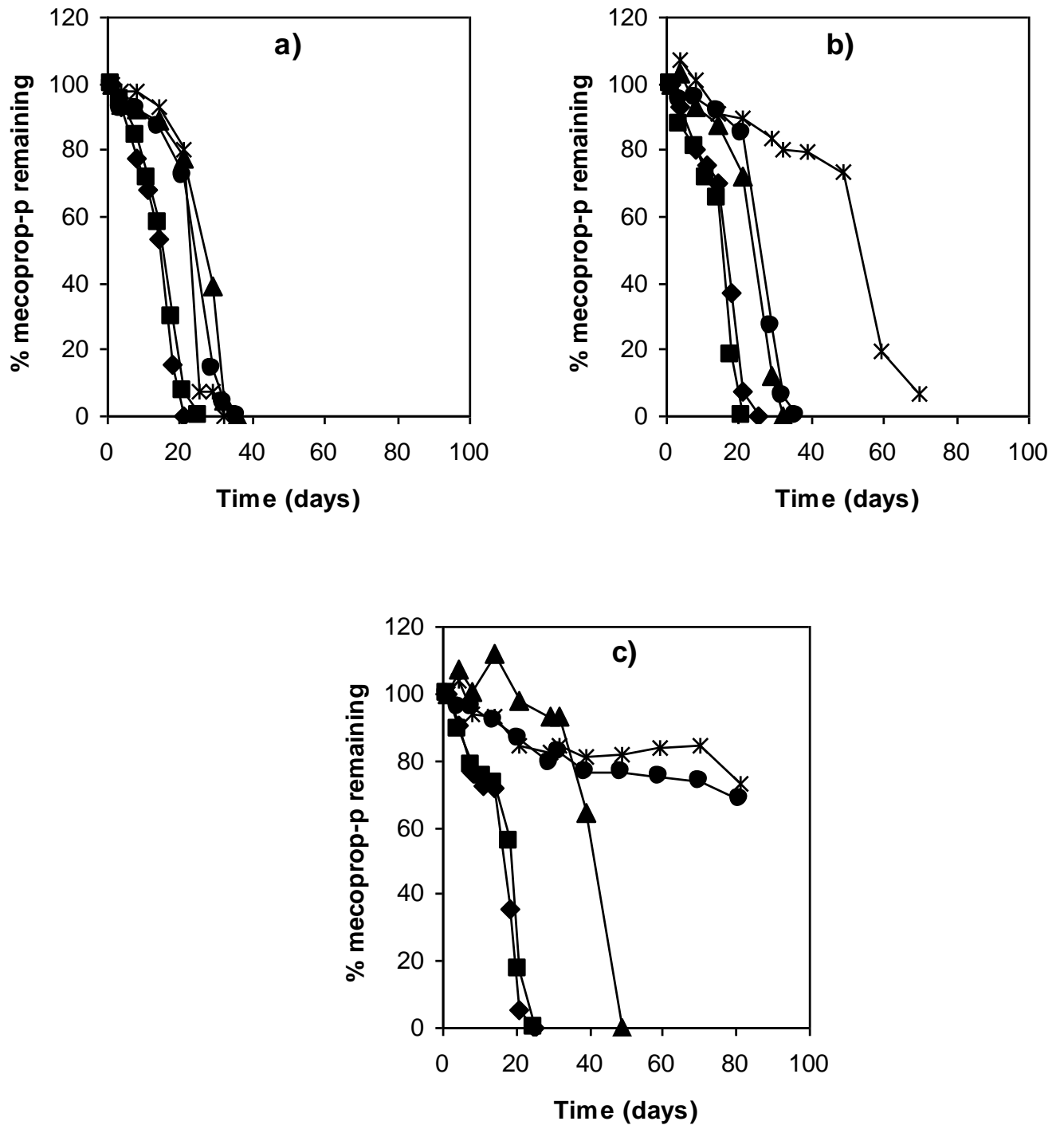


Fig 2

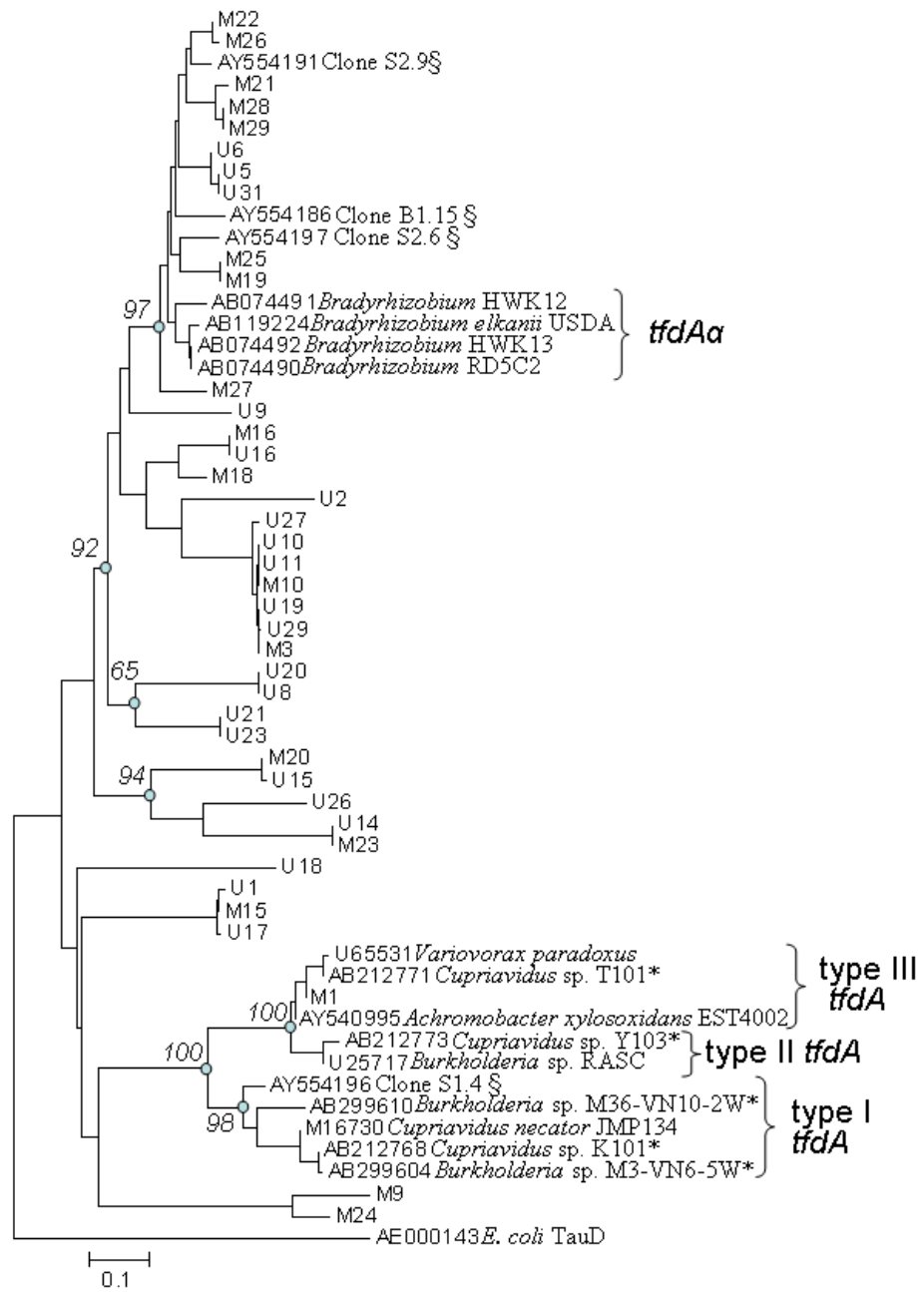




Fig 3

