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Authors:	M Sonia Rodríguez-Cruz, Jacob Bælum, Liz J. Shaw, Sebastian R. Sørensen, Shengjing Shi, Thomas Aspray, Carsten S. Jacobsen and Gary D. Bending
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3
4 **Names of authors:** M^a Sonia Rodríguez-Cruz^{1,5}, Jacob Bælum², Liz J. Shaw³,
5 Sebastian R. Sørensen², Shengjing Shi¹, Thomas Aspray^{1,6}, Carsten S.
6 Jacobsen^{2&4} and Gary D. Bending^{1*}

7
8 **Postal addresses and affiliations:**

9 ¹Warwick HRI, University of Warwick, Wellesbourne, Warwick CV 35 9EF, UK

10 ²Department of Geochemistry, Geological Survey of Denmark and Greenland (GEUS),
11 Øster Voldgade 10, DK-1350 Copenhagen K, Denmark

12 ³School of Human and Environmental Sciences, Department of Soil Science, The
13 University of Reading, RG6 6DW, UK

14 ⁴Department of Basic Science and Environment, University of Copenhagen,
15 Thorvaldsensvej 40, DK-1871 Frederiksberg C

16
17 **Present addresses of authors:**

18 ⁵Institute of Natural Resources and Agrobiolgy (IRNASA-CSIC), P.O. Box 257,
19 37071 Salamanca, Spain

20 ⁶Environmental Reclamation Services Ltd, Westerhill Road, Bishopbriggs, Glasgow,
21 G64 2QH

22
23 **Author for correspondence and proofs:** Gary D. Bending, Warwick HRI, University
24 of Warwick, Wellesbourne, Warwick CV 35 9EF, UK

25 *Email:* gary.bending@warwick.ac.uk

26 *Telephone (direct line):* +44 (0) 24 76575057

27 *Fax:* +44 (0) 24 76574500

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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_{50}) 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 *tfdA α* 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 $0.1\mu\text{g l}^{-1}$ 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 α -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 β - and γ - proteobacteria.

109 *tfdA*-like genes whose product accepts 2,4-D as a substrate have also been
110 detected in oligotrophic α -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 α* . Also implicated in
113 the first step of 2,4-D catabolism in the α -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 α -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 α* 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 β - and γ - 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⁻¹ 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 *tfdA α* (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 μ M 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 μ l of 10-fold diluted DNA extract. In order to
221 ensure a highly specific reaction 25.5 μ g 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 (DT₅₀) 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 (DT₅₀) 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 (7th 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 DT₅₀ 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). DT₅₀ 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⁻¹ soil
269 d⁻¹. K_d averaged 0.15 g⁻¹ ml⁻¹ 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⁻¹ 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⁻¹ 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 DT₅₀.

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⁻¹ 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⁻¹ 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 4.74×10^4 - 7.66×10^4 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 4.74×10^4 - 7.66×10^4 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 DT₅₀ 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

452 **References**

- 453 Alexander, M. 1994. Biodegradation and Bioremediation. Academic Press, San Diego.
- 454 [Bælum, J.](#), [Henriksen, T.](#), [Hansen, H.C.B.](#), [Jacobsen, C.S.](#) 2006. Degradation of 4-
455 chloro-2-methylphenoxyacetic acid in top- and sub-soil is quantitatively linked to
456 the class III *tfdA* gene. Applied and Environmental Microbiology 72, 1476-1486.
- 457 Bælum, J., Nicolaisen, M.H., Holben, W.E., Strobel, B.W., Sørensen, J., Jacobsen, C.S.
458 2008. Direct analysis of *tfdA* gene expression by indigenous bacteria in phenoxy
459 acid amended agricultural soil. ISME Journal 2, 677-687.
- 460 Bending, G.D., Lincoln, S.D., Sørensen, S.R., Morgan, J.A.W., Aamand, J., Walker, A.
461 2003. In-Field spatial variability in the degradation of the phenyl-urea herbicide
462 isoproturon is the result of interactions between degradative *Sphingomonas* spp. and
463 soil pH. Applied and Environmental Microbiology 69, 827-834.
- 464 Bending, G.D. & Rodriguez-Cruz, M.S. 2007. Microbial aspects of the interaction
465 between soil depth and biodegradation of the herbicide isoproturon. Chemosphere
466 66, 664-671.
- 467 Buss, S.R., Thrasher, J., Morgan, P., Smith, J.W.N. 2006. A review of mecoprop
468 attenuation in the subsurface. Quarterly Journal of Engineering Geology and
469 Hydrogeology 39, 283-292.
- 470 Environment Agency. 2003. Pesticide use in England and Wales. Pesticides 2002. The
471 annual report of the Environment Agency pesticide monitoring programme, Oxon,
472 UK. (www.environment-agency.gov.uk)
- 473 European Environment Agency. 1999. Groundwater quality and quantity in Europe.
474 Environmental assesment report no 3, European Environmental Agency,
475 Copenhagen, Denmark.

476 Fomsgaard, S., Kristensen, K. 1999. Influence of microbial activity, organic carbon
477 content, soil texture and soil depth on mineralisation rates of low concentrations of
478 ¹⁴C-mecoprop-development of a predictive model. *Ecological Modelling* 122, 45-
479 68.

480 Fredslund, L., Vinter, F.P., Brinch, U.C., Elsgaard, L., Rosenberg, P., Jacobsen, C.S.
481 2008. Spatial variation in 2-methyl-4-chlorophenoxyacetic acid mineralization and
482 sorption in a sandy soil at field level. *Journal of Environmental Quality* 37, 1918-
483 1928.

484 Fulthorpe, R.R., McGowan, C., Maltseva, O.V., Holben, W.E., Tiedje, J.M. 1995. 2,4-
485 dichlorophenoxyacetic acid-degrading bacteria contain mosaics of catabolic genes.
486 *Applied and Environmental Microbiology* 61:3274–3281.

487 Helweg, A. 1993. Degradation and adsorption of ¹⁴C-mecoprop (MCP) in surface
488 soils and in subsoil. Influence of temperature, moisture content, sterilization and
489 concentration on degradation. *Science of The Total Environment* 132, 229-241.

490 Huong, N.L., Itoh, K., Suyama, K. 2007. Diversity of 2,4-dichlorophenoxyacetic acid
491 (2,4-D) and 2,4,5-trichlorophenoxyacetic acid (2,4,5-T)-degrading bacteria in
492 Vietnamese soils. *Microbes and Environments* 22, 243-256.

493 Itoh, K., Kanda, R., Sumita, Y., Kim, H., Kamagata, Y., Suyama, K., Yamamoto, H.,
494 Hausinger, R.P., Tiedje, J.M. 2002. tfdA-like genes in 2,4-dichlorophenoxyacetic
495 acid degrading bacteria belonging to the *Bradyrhizobium-Agronomas-Nitrobacter-*
496 *Afipia* cluster in α -proteobacteria. *Applied and Environmental Microbiology* 68,
497 3449-3454.

498 Itoh, K., Tashiro, Y., Uobe, K., Kamagata, Y., Suyama, K., Yamamoto, H. 2004. Root
499 nodule *Bradyrhizobium* spp. harbour tfdA α and cadA, homologous with genes
500 encoding 2,4-dichlorophenoxyacetic acid-degrading proteins. *Applied and*
501 *Environmental Microbiology* 70, 2110-2118.

502 Johnson, A.C., White, C., Bhardwaj, C.L., Dixon, A. 2003. The ability of indigenous
503 microorganisms to degrade isoproturon, atrazine and mecoprop within aerobic UK
504 aquifer systems. *Pest Management Science* 59, 1291-1302.

505 Johannesen, H., Aamand, J. 2003. Mineralization of aged atrazine, terbuthylazine, 2,4-
506 D, and mecoprop in soil and aquifer sediment. *Environmental Toxicology and*
507 *Chemistry* 22, 722-729,

508 Kamagata Y., Fulthorpe R.R., Tamura K., Takami H., Forney L.J., Tiedje J.M. 1998.
509 Pristine environments harbour a new group of oligotrophic 2,4-dichlorophenoxy
510 acid-degrading bacteria. *Applied and Environmental Microbiology* 63, 2266-2272.

511 Kitagawa, W., Takami, S., Miyauchi, K., Masai, E., Kamagata, Y., Tiedje, J.M.,
512 Fukuda, M 2002. Novel 2,4-dichlorophenoxyacetic acid degradation genes from
513 oligotrophic *Bradyrhizobium* sp. strain HW13 isolated from a pristine environment.
514 *Journal of Bacteriology* 184, 509-518.

515 Kristensen, G.B., Johannesen, H., Aamand, J. 2001. Mineralization of aged atrazine and
516 mecoprop in soil and aquifer chalk. *Chemosphere* 45, 927-934.

517 [Lee, T.H.](#), [Kurata, S.](#), [Nakatsu, C.H.](#), [Kamagata, Y.](#) 2005. Molecular analysis of bacterial
518 community based on 16S rDNA and functional genes in activated sludge enriched
519 with 2,4-dichlorophenoxyacetic acid (2,4-D) under different cultural conditions.
520 *Microbial Ecology* 49, 151-162.

521 [McGowan, C.](#), [Fulthorpe, R.](#), [Wright, A.](#), [Tiedje, J.M.](#) 1998. Evidence for interspecies
522 gene transfer in the evolution of 2,4-dichlorophenoxyacetic acid degraders. *Applied*
523 *and Environmental Microbiology* 64, 4089-4092.

524 Parker, M.A., Kennedy, D.A. 2006. Diversity and relationships of bradyrhizobia from
525 legumes native to eastern North America. *Canadian Journal of Microbiology* 52,
526 1148-1157.

527 Pemberton, J.M., Corney, B., Don, R.H. 1979. Evolution and spread of pesticide
528 degrading ability among soil microorganisms. In *Plasmids of medical,*
529 *environmental and commercial importance.* Eds K.N. Timmis and A. Puhler.
530 Elsevier, Amsterdam, The Netherlands pp287-299.

531 Reffstrup, T.K.J., Sørensen, H., Helweg, A. 1998. Degradation of mecoprop at different
532 concentrations in surface and subsurface soil. *Pesticide Science* 52, 126-132.

533 Rodriguez-Cruz, M.S., Jones, J.E., Bending, G.D. 2006. Field-scale study of the
534 variability in pesticide biodegradation with soil depth and its relationship with soil
535 characteristics. *Soil Biology and Biochemistry* 38, 2910-2918.

536 Sakai, Y., Ogawa, N., Fujii, T., Sugahara, K., Miyashita, K., Hasebe, A. 2007. 2,4-
537 dichlorophenoxyacetic acid-degrading genes from bacteria isolated from soil in
538 Japan: Spread of *Burkholderia cepacia* RASC-type degrading genes harbored on
539 large plasmids. *Microbes and Environments* 22, 145-156.

540 Shaw, L.J., Burns, R.G. 2005. Rhizodeposits of *Trifolium pratense* and *Lolium perenne*:
541 their comparative effects on 2,4-D mineralization in two contrasting soils. *Soil*
542 *Biology and Biochemistry* 37, 995-1002.

543 Stanier, R.Y., Palleroni, J.J., Doudoroff, M. 1966. The aerobic pseudomonads: a
544 taxonomic study. *Journal of General Microbiology* 43, 159–271.

545 Suwa, Y., Wright, A.D., Fukimori, F., Nummy, K.A., Hausinger, R.P., Holben W.E.,
546 Forney L.J. 1996. Characterization of a chromosomally encoded 2,4-
547 dichlorophenoxyacetic acid/alpha-ketoglutarate dioxygenase from *Burkholderia* sp.
548 strain RASC. *Applied and Environmental Microbiology* 62, 2464-2469.

549 Tonso, N.L., Matheson, V.G., Holben, W.E 1995 Polyphasic characterisation of a suite
550 of bacterial isolates capable of degrading 2,4-D. *Microbial Ecology* 30, 3-24.

551 Whitfield, W.A.D. 1974. The soils of the national vegetable research station.
552 Wellesbourne. In: Report of the national vegetable research station for 1973, pp. 21-
553 30.

554 [Zakaria, D.](#), [Lappin-Scott, H.](#), [Burton, S.](#), [Whitby, C.](#) 2007. Bacterial diversity in soil
555 enrichment cultures amended with 2 (2-methyl-4-chlorophenoxy) propionic acid
556 (mecoprop). *Environmental Microbiology* 9, 2575-2587.

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 ⁻¹ soil)	DT ₅₀ (days) ^a	Lag phase (days)	log MPN (g ⁻¹ dw soil) ^{b,c}	<i>tfdA</i> copy no. (g ⁻¹ dw soil) ^{b,d}	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 ^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

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 *tfdA α* (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

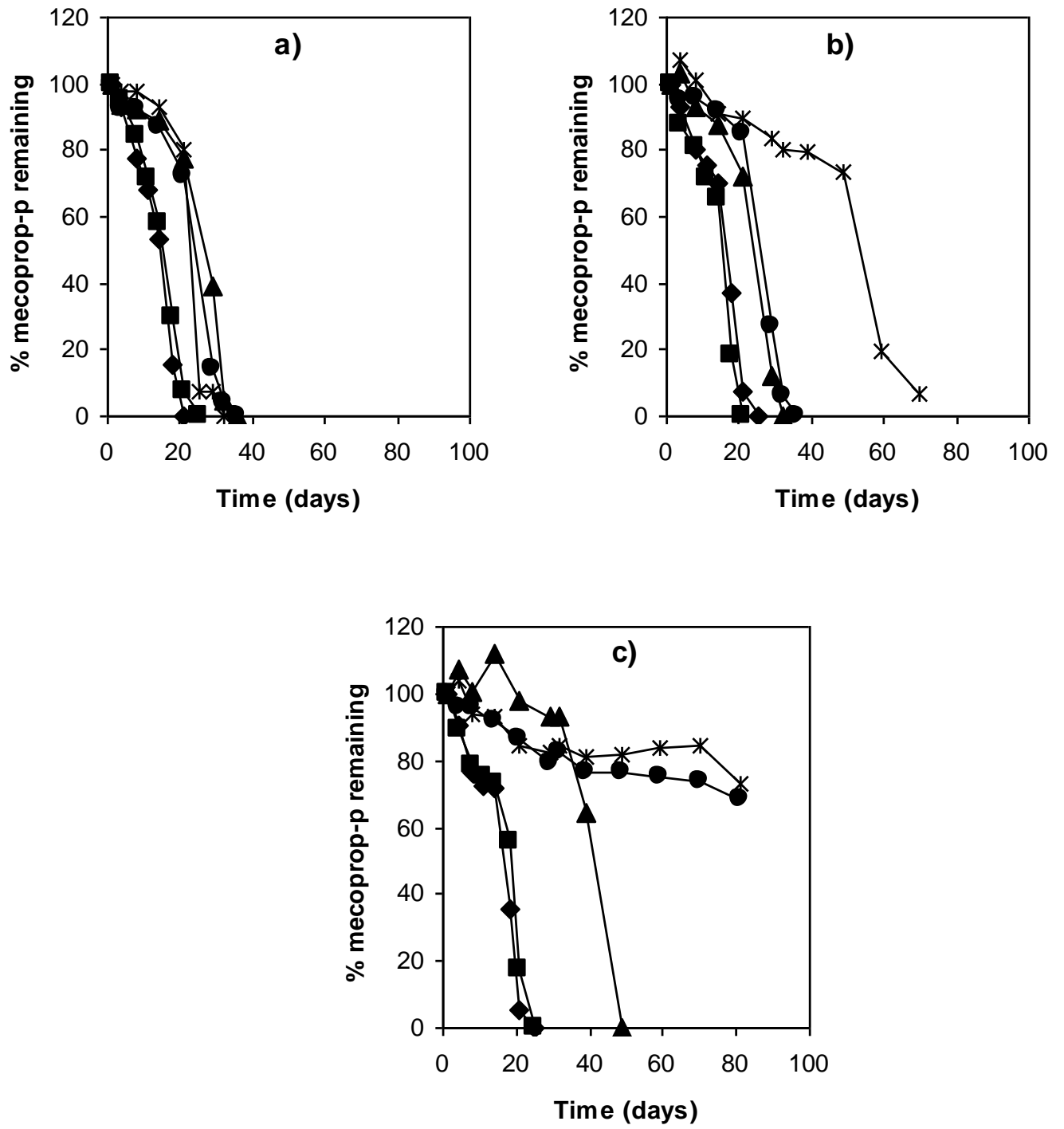


Fig 2

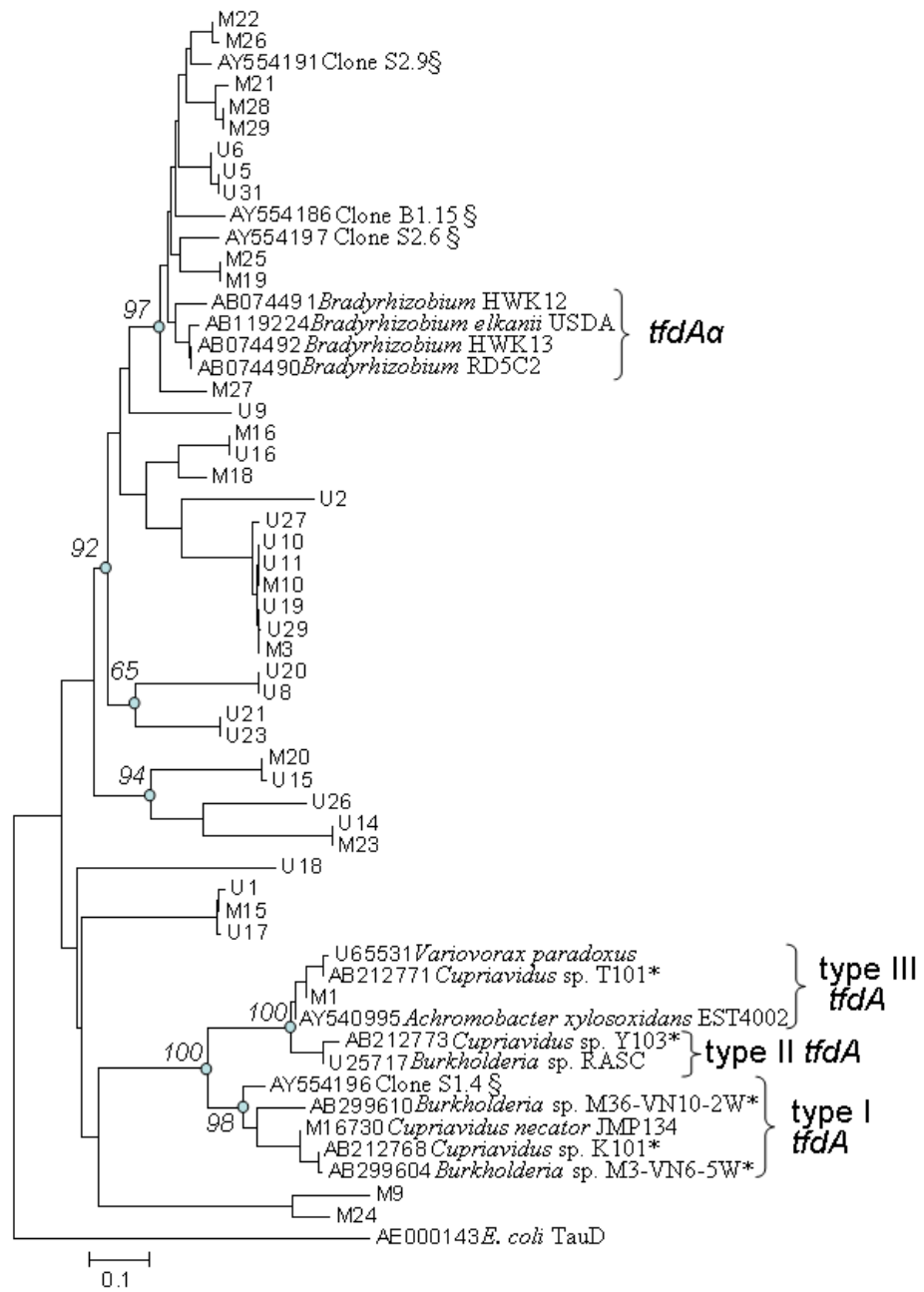


Fig 3

