

1 Critical evaluation of the 2D-CSIA scheme for
2 distinguishing fuel oxygenate degradation reaction
3 mechanisms

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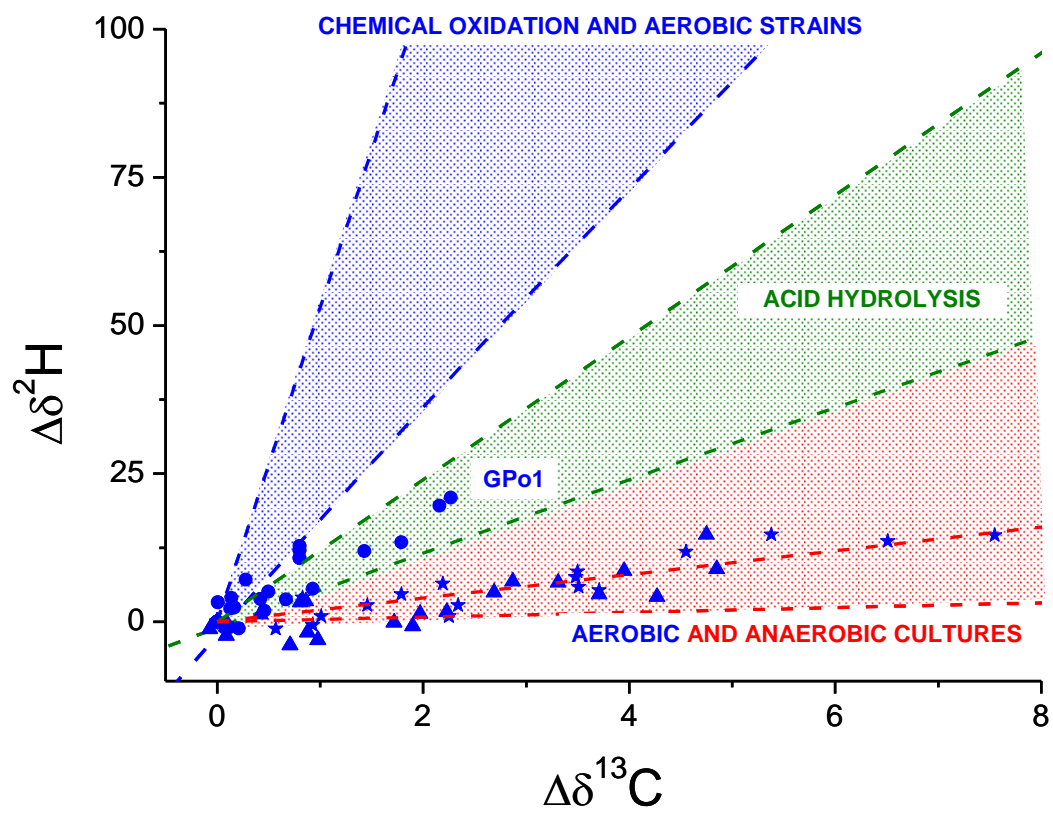
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21 Manuscript for submission in *Environmental Science and Technology*



25 **Abstract**

26 Although the uniform initial hydroxylation of methyl *tert*-butyl ether (MTBE) and other
27 oxygenates during aerobic biodegradation has already been proven by molecular tools, variations
28 in carbon and hydrogen enrichment factors (ϵ_C and ϵ_H) have still been associated with different
29 reaction mechanisms (McKelvie et al. *Environ. Sci. Technol.* **2009**, *43*, 2793-2799). Here, we
30 present new laboratory-derived ϵ_C and ϵ_H data on the initial degradation mechanisms of MTBE,
31 ethyl *tert*-butyl ether (ETBE) and *tert*-amyl methyl ether (TAME) by chemical oxidation
32 (permanganate, Fenton reagents), acid hydrolysis and aerobic bacteria cultures (species of
33 *Aquicola*, *Methylibium*, *Gordonia*, *Mycobacterium*, *Pseudomonas* and *Rhodococcus*). Plotting
34 of $\Delta\delta^2H/ \Delta\delta^{13}C$ data from chemical oxidation and hydrolysis of ethers resulted in slopes (A
35 values) of 22 ± 4 and between 6 and 12, respectively. With *A. tertiaricarbonis* L108, *R. zopfii* IFP
36 2005 and *Gordonia* sp. IFP 2009, ϵ_C was low ($<|-1|\text{‰}$) and ϵ_H insignificant. Fractionation
37 obtained with *P. putida* GPo1 was similar to acid hydrolysis and *M. austroafricanum* JOB5 and
38 *R. ruber* DSM 7511 displayed A values previously only ascribed to anaerobic attack. The
39 fractionation patterns rather correlate with the employment of different P450, AlkB and other
40 monooxygenases, likely catalyzing ether hydroxylation via different transition states. Our data
41 questions the value of 2D-CSIA for a simple distinguishing of oxygenate biotransformation
42 mechanisms, therefore caution and complementary tools are needed for proper interpretation of
43 groundwater plumes at field sites.

44
45 **Keywords:** MTBE; ETBE; TAME; CSIA (compound-specific stable isotope analysis); aerobic
46 biodegradation; permanganate oxidation, Fenton reaction.

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50 INTRODUCTION

51 Fuel oxygenates have been providing added value to the petrol industry for decades. Methyl *tert*-
52 butyl ether (MTBE) has been used since the 1970's as gasoline additive due to its high octane
53 number and its oxygen content which led to a reduction in emissions of exhaust pollutants.
54 Today, in the era of climate change concerns and renewable energy ambitions, fuel oxygenates
55 still play a major role. Since 2003, due to the promotion of the use of biofuels through tax
56 incentives (Directive 2003/30/EC), some European MTBE refiners converted their plants to
57 synthesize ethyl *tert*-butyl ether (ETBE) using bio-ethanol from agricultural feedstocks.
58 Moreover, the new EU Renewable Energy Directive (2009/28/EC) contemplates the feasibility of
59 bio-MTBE production using bio-methanol based on crude glycerin, a waste product from
60 biodiesel production ¹. Overall, the estimated current annual production capacity of fuel ethers in
61 Europe is approximately 6 million tones, split between almost 70 production plants and these
62 compounds make up about 4% of the gasoline supply ². Despite the promotion of best practices
63 for handling fuel ethers, their widespread use in gasoline caused contamination of groundwater
64 tables worldwide ^{3, 4}. Similar to MTBE, ETBE and *tert*-amyl methyl ether (TAME) showed
65 recently to cause odor and flavor problems making water sources unpalatable at very low
66 concentrations (ETBE with 1–2 µg/L and MTBE and TAME from 7 to 16 µg/L) ⁵. Mainly due to
67 these public concerns, fuel oxygenate ethers are now partially or absolutely banned in some states
68 of USA and substituted by ethanol ⁶. However, drinking water resources will be threatened for a
69 long time, as fuel oxygenates are recalcitrant to microbial attack particularly by the limited
70 concentrations of oxygen typically found in fuel-contaminated aquifers ⁷.

71

72 In that respect, reliable methods for detecting *in situ* fuel oxygenates biodegradation and insights
73 into the functioning of the responsible microorganisms are crucial for improving remediation
74 technologies based on the activities of such degrading microorganisms. In recent years,
75 compound-specific stable isotope analysis (CSIA) has been used as an important tool for
76 monitoring biodegradation of organic contaminants in environmental systems ^{8, 9} and for
77 characterizing the initial reaction mechanisms ¹⁰. The reaction-dependent compound-specific
78 isotope enrichment factors (ϵ) can be quantified using a modified form of the Rayleigh distillation
79 equation under defined laboratory conditions ¹¹ and converted to the apparent kinetic isotope
80 effect (AKIE) to obtain information on the transition state of the bond cleavage reaction. The
81 kinetic isotope effect (KIE) allows obtaining information on rate limitation and bond change of
82 enzymatic reactions ¹². In environmental studies, isotope fractionation is used to characterize
83 environmental processes and to predict reaction mechanisms, however, recent results on the
84 diversity of isotopic fractionation during bacterial fuel oxygenate degradation question whether
85 the extent of fractionation can be used to identify specific metabolic reactions ¹³.

86
87 The aerobic microbial MTBE degradation is initiated by a hydroxylation of the methyl group of
88 the ether by a monooxygenase catalyzed reaction ¹⁴ and the breaking of the C-H bond would be
89 expected to lead to similar carbon and hydrogen isotope effects. However, there are three
90 different clusters of isotopic fractionation patterns found for aerobic MTBE degradation: (i) high
91 fractionation of carbon and hydrogen was observed for *Methylibium* strains PM1 and R8 with ϵ_C
92 and ϵ_H ranging from -2.0 to -2.4‰ and -33 to -40‰, respectively; (ii) similar carbon but higher
93 hydrogen fractionation ($\epsilon_H = -100‰$) was discovered for *Pseudonocardia*
94 *tetrahydrofuranoxydans* K1; (iii) whereas much lower carbon fractionation ($\epsilon_C \leq |-0.5|‰$) and
95 practically non-detectable hydrogen fractionation was observed for *Aquicola tertiaricarbonis*

96 L108 and *Rhodococcus ruber* IFP 2001^{13, 15, 16}. Although this diversity in observed isotope
97 effects questions the uniform hydroxylation step, studies on knockout mutants have clearly
98 proven the involvement of monooxygenases in the initial attack of MTBE in the high-
99 fractionating strain PM1¹⁷ and in the low-fractionating strains L108 and IFP 2001^{18, 19}.
100 Therefore, low fractionation is likely associated with more efficient C-H bond breaking combined
101 with rate determining non-fractionating steps involved in typically multistep processes of
102 enzymatically catalyzed hydroxylation, thus masking the intrinsic isotope effect. However, as this
103 suppression should equally affect isotopic fractionation of both elements, two-dimensional (2D)-
104 CSIA graphs are thought to cancel these effects (when a reaction is committed to catalysis and
105 become irreversible) and the ratio of hydrogen to carbon isotope fractionation should be able to
106 distinguish between hydroxylation and other enzymatic reactions¹⁰. Considering analytical errors
107 for determining low ϵ_H values, the fractionation observed with strains L108 and IFP 2001 may
108 indeed give a similar slope as observed with strains PM1 and R8. However, it is clear that the
109 two-dimensional scheme is not really applicable for very low isotopic fractionation. The different
110 slopes (A) found for strain K1 ($A = 48$) vs. *Methylibium* spp. ($A = 18$) would lead to question the
111 generally accepted assumption of a uniform hydroxylation mechanism for aerobic bacteria.
112 Although the authors considered the high hydrogen enrichment factor of strain K1 consistent with
113 the one they previously measured for abiotic oxidation of MTBE by permanganate ($\epsilon_H = -109\%$),
114 no information about ϵ_C or A was provided²⁰. It is not clear, therefore, how a mechanism can be
115 predicted for aerobic ether-degrading bacteria against alternative reaction mechanisms, such as
116 the acid hydrolysis S_N1 ($A = 11$) and hydrolysis by (enzymatic) nucleophilic attack S_N2 suggested
117 for the anaerobic degradation pathway ($A = 1.2$)²¹. In addition, the wide variability of enrichment
118 factors ϵ_C from -0.37 to -2.29‰ and ϵ_H from $<|-5|$ to -66‰ as well as A values in the range from
119 12 to 45 found in several aerobic mixed cultures from contaminated sites suggest the coexistence

120 and activity of all the described degradation mechanisms within environmental microbial
121 communities^{15, 22-26}. These findings seriously complicate evaluation of *in situ* biodegradation of
122 MTBE and other fuel oxygenates by 2D-CSIA. Therefore, additional studies are needed to
123 elucidate how CSIA could be used for assessing fuel oxygenate biodegradation at groundwater
124 contaminated sites.

125 Although there are isotope fractionation data for MTBE, there are few studies with ETBE and
126 TAME. Therefore knowledge on the variability of isotopic fractionation among abiotic and biotic
127 processes is needed for a proper evaluation of fuel oxygenate biodegradation employing CSIA for
128 characterizing potentially contaminated sites in the future. For that purpose, we performed
129 systematic work with a total of 10 pure strains (six of them were never tested before for isotopic
130 fractionation studies) including *Aquicola*, *Methylibium*, *Gordonia*, *Mycobacterium*,
131 *Pseudomonas* and *Rhodococcus* species which are able to grow aerobically on or cometabolically
132 degrade MTBE, ETBE or TAME and compared their isotopic patterns with the results obtained
133 in abiotic chemical oxidation experiments (permanganate vs. Fenton with $\text{Fe}^{2+/3+}$ and H_2O_2) as
134 well as acid hydrolysis (HCl) in order to better understand their enzymatic mechanisms.
135 Moreover, the present results could be useful to evaluate whether Fenton-like transformation of
136 fuel oxygenates produces a significant isotopic fractionation pattern that can be used to monitor
137 *in situ* chemical oxidation (ISCO), and to determine whether this pattern can be used to
138 differentiate between biotic and abiotic transformation at a field site.

139

140 **MATERIALS AND METHODS**

141 **Chemicals.** All the chemicals and organic solvents were either purchased from Sigma-Aldrich
142 (Munich, Germany) or Merck (Darmstadt, Germany) at analytical grade quality.

143

144 **Abiotic oxidation reactions.** Stoichiometric Fenton (with Fe^{2+}) and catalytic Fenton-like (with
145 Fe^{3+}) reactions were carried out at $22 \pm 2^\circ\text{C}$ in 100-mL reactors, covered with foil paper in order
146 to avoid photo-reactions, at pH 3 using solutions of $\text{FeSO}_4 \cdot 7 \text{H}_2\text{O}$ or $\text{Fe}(\text{NO}_3)_3$, respectively, to
147 oxidize 100 mg/L of each fuel oxygenate. In the stoichiometric Fenton reaction, equimolar
148 concentrations of Fe^{2+} and H_2O_2 were used (10 mM of each). In order to avoid quenching of
149 hydroxyl radicals by excess H_2O_2 , H_2O_2 was added stepwise (1 mM each 5 min). In the catalytic
150 Fenton-like reaction, an excess of H_2O_2 with respect to Fe^{3+} was used (0.03 mM Fe^{3+} ; 100 mM
151 H_2O_2). Permanganate oxidation reaction was also carried out at $22 \pm 2^\circ\text{C}$ in 100-mL reactors
152 using a solution of 0.11 M of KMnO_4 to oxidize 250 mg/L of each fuel oxygenate. All the
153 oxidation experiments were carried out with an individual fuel oxygenate compound, except one
154 experiment with permanganate which was done with a mixture of 100 mg/L of MTBE and
155 ETBE. For sampling, always 2-mL aliquots were taken in 5-mL vials over time. Sodium
156 thiosulfate was added in order to stop the reaction (200 mM). Afterwards, the concentration of
157 MTBE and ETBE was determined (see below). Samples were kept in the freezer at -20°C before
158 analysis of isotopic fractionation. In addition, heterogeneous Fenton-like reactions with
159 orthoferrites such as BiFeO_3 and LaFeO_3 in the presence of H_2O_2 at neutral pH and $22 \pm 2^\circ\text{C}$
160 were tested for MTBE and ETBE and can be found in the Supporting Information (SI).

161

162 **Acid hydrolysis experiments.** Hydrolysis was conducted in 100-mL serum bottles sealed with
163 butyl rubber stoppers and incubated on a shaker at 30°C . The reaction was started by adding 100
164 μL of each pure fuel oxygenate to 100 mL of different dilutions of HCl (1, 3 or 5 M prepared
165 from a Combi-Titrisol® 5M HCl, Merck, Darmstadt, Germany). At each sampling event, 3-mL

166 aliquots were removed with a syringe and each 1-mL subsample was transferred into 20-mL
167 headspace vials for duplicate headspace GC analysis²⁷ or one 10-mL vial for the stable isotope
168 analysis, all of them containing 1 mL of the corresponding molarity of NaOH and cooled on ice
169 to avoid losses during neutralization. Such a procedure served to quench the reaction and ensured
170 that samples were free of volatile corrosive compounds.

171

172 **Bacterial strains and cultivation conditions.** *Aquicola tertiaricarbonis* L108^{28, 29} and
173 *Methylibium* sp. R8¹⁶ were obtained from the strain collection of the UFZ department of
174 Environmental Microbiology. *Methylibium petroleiphilum* PM1^{30, 31} were purchased from the
175 American Type Culture Collection (ATCC BAA-1232). *Mycobacterium austroafricanum* IFP
176 2012, *Rhodococcus zopfii* IFP 2005, *Gordonia* sp. IFP 2009 and *Rhodococcus ruber* IFP 2001
177 were provided by F. Fayolle-Guichard (IFP Energies nouvelles, France). *Rhodococcus ruber*
178 DSM 7511 was obtained from the German Collection of Microorganisms and Cell Cultures
179 (DSMZ) and *Mycobacterium austroafricanum* JOB5 and *Pseudomonas putida* GPo1 from the
180 collection of Institute Pasteur (CIP 105723 and CIP 105816, respectively). In general, the
181 bacteria were grown in 1-L glass bottles filled with about 25% (v/v) mineral medium as described
182 elsewhere³² ensuring oxic conditions, 10% of inoculum (v/v) and appropriate growth substrate/s
183 (see details in SI Table S1) on a rotary shaker at 30°C. After 5 days, cells were harvested by
184 centrifugation (8500 rpm, 10 min at 4°C), washed with mineral salt solution and suspended at a
185 high density. For resting-cell experiments, 60 mL of cell suspension were placed in 240-mL
186 serum bottles, supplemented with vitamins and cobalt as for growing, 10-20 µL of each fuel
187 oxygenate (MTBE, ETBE or TAME) and if necessary additional substrates for cometabolic
188 degradation or enzyme inducers (see SI Table S1), closed immediately with butyl rubber stoppers
189 and incubated as above. Control bottles were always prepared in parallel and were monitored for

190 substrate concentrations and isotopic composition in order to evaluate abiotic losses or cross-
191 contamination. Samples for substrate concentration, isotopic composition and pH were taken
192 periodically according to Rosell et al. ²⁷ until complete degradation (when possible).

193
194 **Analytical methods.** Monitoring of concentrations (fuel oxygenates and corresponding main
195 degradation products) was performed using a headspace-gas chromatography system with flame
196 ionization detector (HS-GC-FID) as described elsewhere ²⁷ except for the chemical oxidation
197 experiments where a mass spectrometer was used as detector ³³. Carbon and hydrogen stable
198 isotopic composition of the fuel oxygenates were measured in all cases using gas
199 chromatography-combustion-isotope ratio monitoring mass spectrometry systems (GC-C-IRM-
200 MS) described in our previous work ¹⁶. A Zebron ZB1 column (60 m length x 0.32 mm ID x 1
201 μm film thickness; Phenomenex, Aschaffenburg, Germany) was used for separation. Each sample
202 was analyzed via headspace sampling in triplicate.

203
204 **Stable isotope definitions and calculations.** Carbon and hydrogen isotopic compositions are
205 reported as $\delta^{13}\text{C}$ and $\delta^2\text{H}$ values in parts per thousand (‰) relative to Vienna Pee Dee Belemnite
206 standard (V-PDB) and Vienna Standard Mean Ocean Water (V-SMOW), respectively

207
$$\delta[\text{‰}] = \left(\frac{R_{\text{sample}}}{R_{\text{reference}}} - 1 \right) \times 1000 \quad (1)$$

208 where R_{sample} and $R_{\text{reference}}$ are the atomic ratios of the heavy isotope to the light isotope ($^{13}\text{C}/^{12}\text{C}$
209 or $^2\text{H}/^1\text{H}$) in the sample and the international standard, respectively. A simplified Rayleigh
210 equation for a closed system ¹¹ was used to quantify the isotopic fractionation,

211
$$\ln\left(\frac{R_t}{R_0}\right) = \frac{\varepsilon}{1000} \cdot \ln\left(\frac{C_t}{C_0}\right) \quad (2)$$

212 where the isotopic enrichment factor (ϵ) describes the relationship between changes in isotopic
213 composition $R_t/R_0 = (\delta_t + 1000)/(\delta_0 + 1000)$ and the concentrations during the course of the
214 experiment. When possible, 2D-CSIA was applied to the data sets. To correct for differences in
215 the initial isotopic composition ($\delta^{13}\text{C}_0$ and $\delta^2\text{H}_0$) of MTBE, isotopic shifts for hydrogen ($\Delta\delta^2\text{H}$)
216 and carbon ($\Delta\delta^{13}\text{C}$) were calculated by subtracting the isotopic signature at time t from the initial
217 value ($\Delta\delta = \delta_t - \delta_0$). The slope of a linear regression of $\Delta\delta^2\text{H}$ vs. $\Delta\delta^{13}\text{C}$ describes the relationship
218 between carbon and hydrogen fractionation (Λ).

$$219 \quad \Lambda = \frac{\Delta\delta^2\text{H}}{\Delta\delta^{13}\text{C}} \approx \frac{\epsilon_{\text{H}}}{\epsilon_{\text{C}}} \quad (3)$$

220 Each sample was measured at least in triplicate and all linear regression parameters including the
221 95% confidence intervals (CI) were obtained by the function “Linear Fit” using errors (standard
222 deviation of measured data sets or corresponding propagated errors in both axes) as weight in
223 OriginPro® 7.5.

224

225 **RESULTS**

226 **Chemical oxidation of MTBE and ETBE.** By means of the stoichiometric Fenton reaction (10
227 mM Fe^{2+} and stepwise addition of 10 mM of H_2O_2) $\geq 99\%$ removal of MTBE and ETBE was
228 achieved in less than 1 h (See SI Figure S1). The catalytic Fenton-like reaction (initiated with
229 0.03 mM Fe^{3+} and 100 mM H_2O_2) resulted in slower degradation of MTBE and ETBE (> 20 h
230 required for $\geq 95\%$ removal). ETBE reacts slightly faster than MTBE in both reaction systems.
231 Fitting of the fuel oxygenate oxidation by pseudo-first order kinetics gives a ratio of the rate
232 constants between MTBE and ETBE of 0.68 and 0.56 for the Fenton and Fenton-like reactions,
233 respectively. This is in good agreement with the ratio of 0.59 previously reported with OH-

234 radicals³⁴. In the reactions with permanganate, the removal of fuel oxygenates followed pseudo-
235 first order kinetics (for 3 experiments $R^2=0.93-0.995$). The half-lives for MTBE and ETBE were
236 17.8 h and 0.81 h, respectively (see SI Figure S1). The ratio of the rate constants is 0.046.
237 Permanganate selectively oxidizes the carbon atom adjacent to the ether bond under formation of
238 an ester, whereby H-abstraction is the first step. In general, MnO_4^- has a higher reactivity with
239 primary alkyl than with methyl carbon atoms in ethers^{35, 36}. Thus ETBE is expected to degrade
240 faster than MTBE, which is confirmed by our results.

241 The Rayleigh approach (equation 2) was applied to quantify the isotopic fractionation. The
242 highest MTBE carbon and hydrogen isotopic fractionation during chemical oxidation of $\Delta\delta^{13}C =$
243 $(15.6 \pm 0.1)\text{‰}$ and $\Delta\delta^2H = (387 \pm 7)\text{‰}$ was observed after 94% conversion by permanganate
244 corresponding to ϵ_C and ϵ_H values of $(-5.53 \pm 0.04)\text{‰}$ and $(-109 \pm 4)\text{‰}$, respectively (see Table
245 1). In contrast, lower MTBE carbon and hydrogen isotopic fractionation was detected during the
246 Fenton and Fenton-like oxidation reactions. Low values for the kinetic deuterium isotope effect
247 (KDIE) in the range of 1.06 to 1.08 have been also reported for other reactions where H-
248 abstraction from aliphatic carbon by hydroxyl radicals is considered as the predominant
249 degradation pathway^{37, 38}. In our study, no significant differences were found between ϵ_C $(-1.2 \pm$
250 $0.2)\text{‰}$ vs. $(-1.4 \pm 0.1)\text{‰}$ and ϵ_H $(-29 \pm 6)\text{‰}$ vs. $(-31 \pm 9)\text{‰}$ values obtained when using Fe^{2+} or
251 Fe^{3+} , respectively. Moreover, when plotting the respective $\Delta\delta^2H$ vs. $\Delta\delta^{13}C$ values (equation 3) for
252 the three tested oxidation reactions, comparable 2D-CSIA slopes, λ , were obtained fitting into a
253 range between 20 and 25 (mean λ of 22 ± 4 for MTBE oxidation, $R^2 = 0.94$, $n = 43$, see Figure
254 1). Independently of the different reaction rates, analogous isotopic enrichment factors were
255 found for MTBE and ETBE during each type of oxidation. A similar mean λ of 23 ± 2 was also
256 calculated for ETBE oxidation with a good correlation ($R^2 = 0.9$) when plotting all the values

257 together ($n = 11$, Figure S2 in SI). Comparable results were also obtained when using
258 orthoferrites as solid catalysts (see Table S2 in SI).

259

260 **Acid hydrolysis of fuel oxygenates.** Dissolved fuel oxygenate concentrations decreased
261 exponentially with time in the eight batch experiments ($R^2 = 0.82-0.9997$) obtaining in all cases
262 concomitant accumulation of main degradation products *tert*-butyl alcohol (TBA) and *tert*-amyl
263 alcohol (TAA) from 46 to 94% of its parental compound (see SI Figure S2). At 30°C and in the
264 presence of 5 M HCl, MTBE was hydrolyzed with a half-life of 0.64 h, whereas ETBE and
265 TAME reacted even faster (0.35 and 0.03 h, respectively). In order to lower the hydrolysis rates
266 for better monitoring of the reactions, further experiments were performed with lower HCl
267 concentrations (3M and 1M). In this way, the half-life of MTBE at 1M HCl increased to 34 h,
268 while ETBE and TAME required 17 and 8.5 h, respectively. For the three compounds,
269 degradation >99% was achieved (see Table 1). Pronounced MTBE carbon and hydrogen isotopic
270 fractionation of $\Delta\delta^{13}\text{C} = (28.58 \pm 0.05)\text{‰}$ and $\Delta\delta^2\text{H} = (106 \pm 12)\text{‰}$ was observed after 99.3%
271 and 92% conversion, respectively, which corresponded to ε_{C} value of $(-6.05 \pm 0.03)\text{‰}$ and ε_{H}
272 value of $(-43 \pm 4)\text{‰}$, when combining all data points (see Table 1). The data fitted the Rayleigh-
273 type linear fractionation model with R^2 of 0.997 and 0.99, respectively, indicating that no
274 significant difference in isotopic fractionation was detected at different acid concentrations. The
275 corresponding 2D-CSIA slope, A , was 6.2 ± 0.5 ($R^2 = 0.997$) (equation 3). However, values
276 closer to those previously published for MTBE were obtained for ETBE and TAME (see more
277 details in Table 1) with A of 9.0 ± 0.3 ($R^2 = 0.99$) and 11.5 ± 0.2 ($R^2 = 0.999$), respectively.

278

279 **Aerobic biodegradation of fuel oxygenates.** In growing and resting-cell experiments with *A.*
280 *tertiaricarbonis* L108, TAME was completely degraded without TAA accumulation and with low

281 carbon fractionation of $\epsilon_C = (-0.4 \pm 0.1)\text{‰}$. Hydrogen fractionation was too low to be detectable
282 with our methods. With *R. zopfii* IFP 2005 and *Gordonia* sp. IFP 2009, cometabolic MTBE and
283 TAME degradation did not reach more than 30% after 2 weeks which was insufficient for
284 determining isotopic fractionation. In contrast, ETBE degradation required only some hours.
285 Both strains accumulated the ether metabolites TBA and TAA stoichiometrically. The ETBE
286 carbon fractionation was low with $\epsilon_C = (-0.4 \pm 0.1)\text{‰}$ and $(-0.62 \pm 0.03)\text{‰}$ for strains IFP 2005
287 and IFP 2009, respectively, and no significant ^2H enrichment was detected. The two *Methylibium*
288 strains PM1 and R8 could grow on TAME and displayed identical carbon fractionation of $\epsilon_C = (-$
289 $1.9 \pm 0.1)\text{‰}$. For two of the four batch experiments with strain PM1, the hydrogen fractionation
290 was measured resulting in ϵ_H of $(-52 \pm 2)\text{‰}$ and A of 25 ± 1 ($R^2 = 0.86$).

291
292 With *P. putida* GPO1, it was difficult to reach high degrees of degradation for MTBE and TAME
293 (ETBE was not tested due to known low rates³⁹). The best results were obtained after (i) growing
294 the cells on glucose with dicyclopropylketone (DCPK) (the alternative growing substrate n-
295 octane was difficult to wash out and inhibited temporarily fuel oxygenate degradation), (ii)
296 several additions of glucose during the resting-cell experiments and (iii) the use of larger bottles
297 (500 mL) for increasing the headspace volume and thus the oxygen supply. Under these
298 conditions, the maximum degradation for MTBE and TAME reached 80 and 59%, respectively,
299 along with the corresponding stoichiometric accumulation of TBA and TAA (*tert*-butyl formate,
300 TBF, was not detected). A new MTBE fractionation pattern was discovered with intermediate
301 carbon fractionation of $\epsilon_C = (-1.4 \pm 0.1)\text{‰}$ and low hydrogen fractionation of $\epsilon_H = (-11 \pm 2)\text{‰}$
302 resulting in a distinct A of 8 ± 1 ($R^2 = 0.85$). For TAME, the values were found in the same order
303 of magnitude (see Table 2 for details).

304

305 With *M. austroafricanum* IFP 2012, degradation of the three fuel oxygenates was performed with
306 TBA-grown resting cells. Although this strain is able to grow on MTBE, an inhibition of
307 degradation at high concentration was observed by Francois et al.⁴⁰. At an initial concentration of
308 480 μM (approx. 42 mg/L) when the degradation products (TBF and TBA) reached high
309 concentrations, the MTBE degradation rate slowed down and stopped. Due to limited sensitivity
310 of the isotope analysis, our resting-cells experiments were performed initially at MTBE
311 concentrations 5 times higher (200 mg/L) compared to previous experiments⁴⁰. We obtained a
312 similar behavior and after 8 h when MTBE conversion was around 50%, TBF and TBA
313 accumulated at about 4 and 44%, respectively, of initial parental concentration. At this point, the
314 MTBE degradation slowed down and stopped at 66% after 9 days. Higher degradation levels
315 were obtained in subsequent batch experiments by (i) reducing MTBE initial concentration to 100
316 mg/L (92% after the same incubation time) or/and (ii) adding acetate or glucose at 0.5 g/L at the
317 beginning of the experiment (substantial reduction of experimental time and lower TBF
318 concentrations). Comparable TAME degradation rates were observed under the same conditions
319 whereas ETBE was always weakly degraded to a maximum 27% of the initial concentration. In
320 all cases, the metabolites did not become degraded until the parental compound was almost
321 depleted. In the case of the related strain *M. austroafricanum* JOB5, the fuel oxygenate
322 degradation was strictly cometabolic in the presence of 0.5 g/L glucose. Although the main
323 degradation products (TBA, TBF or TAA) were accumulated similarly to the experiment with
324 strain IFP 2012; the degradation of TAME was around 5 times faster than of MTBE by strain
325 JOB5. As with strains GPo1 and IFP 2012, ETBE was poorly degraded (4% in 50 days) and
326 accompanied by TBA formation. Whereas the carbon isotopic fractionation of ϵ_{C} (-2.5 ± 0.1)‰
327 was similar to the values obtained with the *Methylibium* strains, the hydrogen isotopic
328 fractionation was extremely low ($\epsilon_{\text{H}} < |-5|$ ‰) leading to a very small λ value when it was possible

329 to calculate it ($A = 1.7 \pm 0.3$ for strain JOB5, $R^2 = 0.6$). The same tendency was obtained for
330 TAME degradation (see Table 2). Similar isotopic values were observed for *R. ruber* DSM 7511
331 during MTBE and TAME degradation. Strain DSM 7511 could not grow on ETBE, but it was
332 able to degrade it completely in the presence of 0.5 g/L glucose.

333

334 **DISCUSSION**

335 **Chemical model reactions.** MTBE hydrogen fractionation was observed for all oxidation
336 reactions tested (by permanganate, Fenton and Fenton-like reagents). However, the highest ϵ_H
337 values were obtained by permanganate, fitting exactly the fractionation reported by Elsner et al.
338 ²⁰ for the same reaction. Interestingly, we observed also significant carbon fractionation for the
339 latter process, while ϵ_C values for the Fenton reactions were much lower. Although individual
340 enrichment factors for permanganate vs. Fenton reactions were different, they all gave the same A
341 value. It was suggested that permanganate performs selective oxidation of C-H bonds next to an
342 ether group, whereas Fenton's reagent is known to oxidize C-H bonds in a less selective fashion
343 ^{20, 35}. The ratio of the rate constants for OH-radical attack at the methyl and *tert*-butyl groups of
344 MTBE was reported to be 3:2 ⁴¹. However, by means of CSIA, the exact mechanism can not be
345 distinguished. The similar fractionation pattern with ETBE may point to a similar mechanism of
346 C-H bond cleavage upon these reactions. Moreover, the use of iron in different oxidation states
347 and species ($Fe^{2+/3+}$ and solid Fe(III)-oxide) has an insignificant effect on the bulk isotope
348 fractionation of the ethers and, therefore, it is not possible to distinguish between these reactions .
349 This might be explained by the fact that hydroxyl radicals are the dominant reactive species in all
350 of the tested Fenton reactions (see further discussion on ferryl species in the SI).

351

352 It is not evident why our results of the acid hydrolysis as a model for S_N1 reactions with various
353 concentrations of HCl at 30°C were slightly different from those obtained by Elsner et al.²⁰ when
354 using 2 M HCl at room temperature. However, the enrichment factors are in the same order of
355 magnitude and differences of less than 1‰ (taking into account the 95% CI) might be attributed
356 to lower precision obtained at a lower ether removal of 88%. Additional experiments are needed
357 to understand the extent of variability associated with this reaction.

358
359 **Bacterial isotopic fractionation challenges the simple use of the 2D-CSIA concept.** The
360 variability of carbon and hydrogen isotopic patterns observed during bacterial fuel ether
361 degradation can only partially be explained by the chemical model reactions such as oxidation
362 and hydrolysis (see Figure 1 as well as S2 and S3 in the SI). The MTBE pattern observed for the
363 two *Methylibium* spp. (strains PM1 and R8) correlates with the assumed oxidation of the methyl
364 group. In addition, the low carbon and insignificant hydrogen fractionation obtained with strains
365 IFP 2005 and 2009 may be interpreted by masking effects due to more efficient hydroxylation
366 catalysis, as has already been assumed for MTBE and ETBE attack by strains L108 and IFP 2001
367^{26, 27}. In contrast, the isotopic pattern discovered for strain K1 with such a high λ value cannot be
368 explained by oxidation reactions such as by permanganate, as it was suggested previously by
369 comparing only the hydrogen fractionation²⁰, or by any other chemical reaction tested. In
370 addition, from a mechanistic point of view, the λ value (8 ± 1) found for strain GPo1 would be
371 interpreted as S_N1 hydrolysis reaction mechanism (λ from 5.7 to 12)²⁰. This tendency was also
372 observed for TAME degradation (see Figure S3 in SI). However, by testing OCT plasmid-
373 deficient variants, it has unambiguously been proven that strain GPo1 employs a non-heme diiron
374 alkane hydroxylase of the AlkB-type for attacking ether oxygenates³⁹. More serious, MTBE
375 isotopic patterns obtained with the aerobic strains IFP 2012 and JOB5 exhibited a λ value fitting

376 to the obtained values of an anaerobic enrichment culture ($\lambda = 1.2 \pm 0.8$) which was suggested to
377 be an initial hydrolysis step via an S_N2 mechanism at the H_3C-O group^{20, 21}. However, the
378 formation of TBF during MTBE degradation by these strains clearly indicates the activity of
379 hydroxylases in the initial fuel ether attack. Further, the involvement of another AlkB-type
380 monooxygenase has been suggested for strains IFP 2012 and JOB5 as well as in other
381 mycobacterial strains^{40, 42, 43}. Likewise puzzling is the fractionation observed with strain DSM
382 7511. While the MTBE λ value fitted to the anaerobic pathway slope (but also to the aerobic
383 strain JOB5, see Figure 1), for ETBE a behavior closer to the acid hydrolysis (see Figure S2 in
384 SI) was observed, suggesting different enzymatic mechanisms for the initial attack of each ether
385 by this strain.

386
387 **Biochemistry of C-H bond cleavage and transition state considerations.** Considering the
388 substantial evidence collected for the uniform hydroxylation step in the initial attack of fuel
389 ethers by aerobic bacterial strains¹⁴, hydrolysis reactions or other oxygen-independent processes
390 can be ruled out. Hence, the interpretation of isotopic fractionation patterns only by the 2D-CSIA
391 scheme without taking the extent of isotope fractionation into account is obviously misleading.
392 For example, the significant diversity of fuel ether-attacking hydroxylase enzymes documented
393 thus far cannot be ignored^{14, 17, 44}. Consequently, accepting the well-known fact that different
394 enzymes can catalyze identical reactions via different transition states (see recent review⁴⁵) is a
395 more straightforward explanation for the observed variability in isotopic fractionation.

396
397 The almost absence of isotopic fractionation observed for MTBE, ETBE and TAME degradation
398 by strains L108, IFP 2001, IFP 2005 and IFP 2009 correlates well with the finding that in all
399 these strains the P450 monooxygenase EthB is involved in the initial C-H bond breaking^{19, 26, 46}.

400 All four strains share EthB enzymes with nearly identical amino acid sequences, showing 98 to
401 99% identity. This high degree of similarity suggests nearly identical catalysis and substrate
402 specificities allowing degradation of all ethers tested. Nevertheless, different transition states may
403 still occur, as the interaction with the amino acid residues in the active site of the P450 enzymes
404 likely vary for the different ether substrates. However, as C-H bond breaking is obviously not
405 rate-limiting, the intrinsic isotope effects are nearly completely suppressed in all cases.
406 Consequently, only slight variations in isotopic fractionation were observed.

407
408 Interpretation of the isotopic fractionation observed with strain K1 is more difficult. This strain ⁴⁷
409 and the closely related strain *Pseudonocardia* sp. ENV478 ⁴⁸ attack MTBE most likely by a four-
410 component monooxygenase involved in degradation of tetrahydrofuran ^{13, 49}, as only cells pre-
411 grown on this cyclic ether compound show significant MTBE conversion to TBA. In addition, it
412 has already been argued that the extremely high hydrogen fractionation found with strain K1 is
413 consistent with the effects found for abiotic oxidation ¹³, supporting the involvement of a
414 hydroxylation step. However, the published A value of about 50 is not reached by any of the
415 tested oxidation reactions (Table 1). Hence, an effect simulating an “asymmetric masking”
416 significantly affecting only the carbon isotopic fractionation must be postulated. Similar
417 observations have been made with C-H bond breaking in the course of dehydrogenase reactions
418 ⁵⁰⁻⁵² and may suggest that chemical reactions should be taken with caution as a reference to
419 predict mechanisms of enzymatic reactions. High deuterium fractionation was related to a
420 transition state early in the reaction coordinate resembling more the substrate, while ¹³C isotope
421 effects were stronger in later transition states where the bond breaking is more advanced ⁵⁰.
422 Therefore, an early transition state (maximum hydrogen but reduced carbon fractionation) might
423 be the case for the catalysis of the hydroxylating enzymes involved in ether attack in strain K1.

424 Interestingly, Δ values obtained for ETBE and TAME with strain K1 were also close to 50,
425 suggesting very similar transition states for all substrates.

426
427 Finally, we need a better understanding of the fractionation patterns observed with bacterial
428 strains employing non-heme diiron alkane monooxygenases of the AlkB-type and possibly other
429 types of hydroxylases. Strain PM1 uses the AlkB-type enzyme, MdpA, in the initial attack on
430 MTBE¹⁷. This enzyme is also present in the closely related strain R8 and both strains show
431 identical MdpA sequences²⁶. In addition, both strains degrade MTBE with similar efficiency but
432 are not able to attack ETBE⁵³. Therefore, it is likely that MdpA is also involved in ether
433 degradation in strain R8. Accordingly, the identical MTBE and TAME isotopic fractionation
434 obtained for these strains would indicate similar transition states in MdpA catalysis in both
435 strains. Potential masking of isotopic effects, if present, seem to affect equally carbon and
436 hydrogen as Δ value fit the expected symmetric intrinsic isotopic effects of the C-H bond
437 breaking. In contrast, totally different Δ values were obtained with the other strains where
438 employment of AlkB-like monooxygenases has been suggested. This finding might be surprising
439 at first glance, as all these enzymes belong to the same enzyme family and show significant
440 sequence similarity including the four conserved motifs of iron-complexing histidine residues
441 (see SI Figure S4). However, phylogenetic analysis has already revealed that MdpA, the AlkB
442 from strain GPo1 and the mycobacterial enzymes form distinct groups^{17, 54}. Particularly, amino
443 acid residues of the second transmembrane domain directly interacting with the substrate deviate
444 among the different AlkB enzymes^{17, 55} (see TM helix 2 in SI Figure S4). Consequently,
445 variations in substrate specificity are observed, as MdpA and the AlkB from strain GPo1 can only
446 attack ethers, while the mycobacterial strains which likely also employ AlkB enzymes have a
447 broader substrate spectrum including also the alcohols TBA and TAA. Highly likely, all these

448 factors will influence transition state structure and, consequently, isotopic fractionation patterns.
449 Significant differences between transition states have already been found for homologous
450 enzymes with 87% sequence identity and 100% conserved catalytic sites ⁴⁵. In the case of fuel
451 oxygenate degradation, with strains belonging to the same genus, i. e. *Methylibium*, *Pseudomonas*
452 or *Mycobacterium*, similar fractionation patterns were obtained, indicating a correlation between
453 functional and phylogenetic relationships. In this respect, it can be speculated whether the
454 *Mycobacterium*-related Gram-positive strain DSM 7511 also employs the mycobacterial AlkB
455 enzyme for ether degradation, as isotopic fractionation for MTBE corresponds well with the
456 values obtained with strains IFP 2012 and JOB5. The higher ETBE $A = 7 \pm 1$ for the
457 *Rhodococcus* strain may indicate the presence of a P450 hydroxylase, as this fractionation is in
458 the same range of magnitude than the one of strain IFP 2001 ($A = 10 \pm 3$). Indeed, a common
459 feature of many alkane degraders is that they contain multiple alkane hydroxylases with
460 overlapping substrate ranges ⁵⁶. In particular, a study with 27 alkyl ether utilizing rhodococci
461 strains proved that 26 of them contained multiple alkB genes encoding non-heme iron alkane
462 monooxygenases as well as diverse P450 systems ⁵⁷. Since involvement of other hydroxylating
463 enzymes (including AlkB monooxygenases) cannot be ruled out for strains IFP 2012 and JOB5
464 ⁴³, isotopic fractionation patterns could also be the result of catalysis by multiple hydroxylases.
465 However, identical 2D-CSIA slopes obtained with the latter strains correlate with the exclusive
466 use of a single AlkB enzyme. The slightly different A values observed with strain DSM 7511 may
467 also correspond to minor changes in transition states of the same enzyme due to molecular size
468 differences of MTBE and ETBE.

469
470 **Recommendations for biodegradation evaluation.** Our study clearly shows that the 2D-CSIA
471 scheme on its own cannot distinguish between aerobic and anaerobic fuel oxygenate degradation

472 pathways (as it was previously assumed by Zwank et al. ⁵⁸) or between biotic and abiotic
473 degradation in the case of ISCO applications at contaminated field sites. Our finding rather
474 questions whether previous studies reporting low λ values were really caused by anaerobic
475 degradation processes. For example, Van Breukelen et al. ⁵⁹ has criticized the conclusions of
476 Zwank et al. ⁵⁸ showing that biodegradation at the target field site was unlikely exclusively
477 associated with anaerobic degradation based on his improved 2D-CSIA interpretation. Kuder et
478 al. ²¹ plotted together isotopic data from nine different contaminated sites close to gasoline
479 stations in the USA ($\lambda = 1.3$), but anoxic conditions were only proved by microcosm experiments
480 from one of them. Recently, Kujawinski et al. ⁶⁰ classified as aerobic and anaerobic different
481 sections of the same MTBE/ TAME contamination plume based on (i) the dissolved oxygen
482 values (above or under 1 mg/L respectively) and (ii) the slope of the 2D-CSIA plot. However,
483 some oxic samples fell clearly into the previously assumed anaerobic trend and some bacteria can
484 effectively degrade MTBE below 0.5 mg/L of oxygen ²⁷. Although the detection of high carbon
485 fractionation with ϵ_C values $\geq -6\text{‰}$ may still be indicative for anaerobic ether attack (see
486 summary in Youngster et al. ²⁵), geochemical information e.g. redox conditions and availability
487 of other electron acceptors now becomes crucial to validate interpretation of 2D-CSIA analysis.
488 Further insights may be obtained in future studies when a third element (oxygen fractionation,
489 $^{18}\text{O}/^{16}\text{O}$) is included in the CSIA concept. However, a routine methodology for the analysis of
490 oxygen enrichment factors in bacterial degradation experiments and environmental aqueous
491 samples by GC-C-IRMS has not been developed. More conveniently, 2D-CSIA should be
492 applied together with molecular biological tools such as monitoring the expression of key
493 enzymes ^{26, 61} or stable isotope probing approaches ^{24, 62} for a more reliable characterization of
494 fuel oxygenate degradation. In addition, detection of key metabolites may be helpful. In this
495 respect, it was recently discovered that strains PM1, R8 and L108 emit highly volatile alkenes

496 during fuel oxygenate degradation⁵³. As these compounds can easily be measured by simple GC
497 devices, a further line of evidence on fuel ether biodegradation activities may be derived by
498 analyzing gas samples from contaminated sites.

499

500 **ACKNOWLEDGMENTS**

501 We thank Carsten Vogt for helping in the obtaining of some strains and the initial cultivation
502 performance; Françoise Fayolle-Guichard (IFP Energies nouvelles, Rueil-Malmaison, France) for
503 providing us four IFP strains; U. Günther, M. Gehre, F. Bratfisch and S. Hinke for technical
504 support in our isotope and cultivation laboratories.

505 M. Rosell was supported by a Beatriu de Pinós postdoctoral grant (2008 BP-A 00054) from the
506 Autonomous Government of Catalonia (Agència de Gestió d'Ajuts Universitaris i de Recerca,
507 AGAUR) and R. González-Olmos by a Marie Curie Intra-European Fellowship (PIEF-GA-2009-
508 236583) within Marie Curie Mobility Actions of the European Commission 7th Framework
509 Program.

510

511 **Supporting Information Available**

512 Details for bacterial cultivation (Table S1); plots for the degradation rate of the studied
513 compounds in the chemical model reactions (permanganate, Fenton, Fenton-like and acid
514 hydrolysis) (Figure S1); ETBE and TAME 2D-CSIA plots for the so far discovered initial
515 reaction mechanisms (Figures S2 and S3); comparison of clustalW2 alignment of amino acid
516 sequences of AlkB enzymes (Figure S4) as well as additional MTBE and ETBE isotopic results
517 by heterogeneous Fenton-like reactions with orthoferrites BiFeO₃ and LaFeO₃ (Table S2) are

518 provided in the Supporting Information. This information is available free of charge via the
519 Internet at <http://pubs.acs.org>.

520

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714 **Table captions**

715 **Table 1.** Comparison of fuel oxygenates carbon and hydrogen isotopic enrichment factors (ϵ) and
716 2-D CSIA slopes (A) caused by different chemical oxidation reactions and acid hydrolysis
717 experiments. When possible the respective 95% confidence intervals (\pm 95% CI) are provided.

718

719 **Table 2.** Carbon and hydrogen isotopic enrichment factors (ϵ) of fuel oxygenates and 2-D CSIA
720 slopes (A) during biodegradation by aerobic pure strains grouped due to similar isotopic
721 fractionation pattern. When possible the respective 95% confidence intervals (\pm 95% CI) are
722 provided.

723

Table 1

Reaction	Target	±95%					±95%					Λ ± 95% CI	Reference	
		ε _C [‰]	CI [‰]	R ²	D [%]	N	ε _H [‰]	CI [‰]	R ²	D [%]	N			
Chemical oxidation														
Oxidation by permanganate (n=1)	MTBE	?											?	20
(n=3)	MTBE	-5.53	0.04	0.98	94	24	-109	4	0.92	94	24	20.1 ± 0.5	This study	
(n=2)	ETBE	-5.2	0.1	0.996	99	6	-128	24	0.97	97	5	24 ± 4	This study	
Oxidation Fenton (Fe ²⁺) (n=2)	MTBE	-1.2	0.2	0.90	99.1	11	-29	6	0.92	71	9	22 ± 4	This study	
(n=1)	ETBE	-0.6	0.2	0.996	95	4	-18	-	0.82	66	3	23	This study	
Oxidation Fenton (Fe ³⁺) (n=2)	MTBE	-1.4	0.1	0.95	97	12	-31	9	0.74	85	10	25 ± 5	This study	
(n=1)	ETBE	-1.1	0.1	0.998	98	5	-18	-	1.00	92	3	17	This study	
Acid hydrolysis (S_N1)														
2M HCl	MTBE	-4.9	0.6	0.995	88	6	-55	7	0.99	88	6	11 ± 1	20	
5M (n=1) and 1M HCl (n=1)	MTBE	-6.05	0.03	0.997	99.3	17	-43	4	0.99	92	12	6.2 ± 0.5	This study	
5M (n=1), 3M (n=1) and 1M HCl (n=1)	ETBE	-5.1	0.1	0.99	99	14	-47	2	0.96	99	14	9.0 ± 0.3	This study	
5M (n=1), 3M (n=1) and 1M HCl (n=1)	TAME	-5.48	0.08	0.997	99	12	-66	2	0.8	97	9	11.5 ± 0.2	This study	

n: number of experiments, N: number of data points; na: not analyzed or not applicable; ns: not significant; CI is 95% Confidence Interval; D%: maximum percentage of substrate initial/partial degradation (do not confuse with complete mineralization to CO₂) which could be analyzed for the corresponding isotopic composition taking into account different detection limits for carbon and hydrogen.

Table 2

Strain	Target	ϵ_C [‰]	$\pm 95\%$ CI [‰]	R^2	D [%]	N	ϵ_H [‰]	$\pm 95\%$ CI [‰]	R^2	D [%]	N	$\Lambda \pm 95\%$ CI	Reference
<i>Aquicola tertiarycarbonis</i> L108 [*]	MTBE	-0.48	0.05	0.94	96	34	ns (-0.2)	8	0.00	82	29	na	¹⁶
	ETBE	-0.67	0.04	0.93	98.9	69	-11.4	0.6	0.7	99.7	74	14 ± 1	¹⁶
	(n=1) TAME	-0.4	0.1	0.9	98	11	ns (+5)	11	0.5	94	7	na	This study
<i>Rhodococcus ruber</i> IFP 2001 [*]	MTBE	-0.28	0.06	0.81	97	27	ns (+5)	20	0.14	88	24	na	¹⁶
	ETBE	-0.8	0.2	0.96	98	23	-10	2	0.8	98	23	10 ± 3	¹⁶
<i>Gordonia sp.</i> IFP 2009 (n=3)	ETBE	-0.62	0.03	0.995	98	15	ns (-10)	11	0.2	93	9	na	This study
<i>Rhodococcus zopfii</i> IFP 2005 (n=2)	ETBE	-0.4	0.1	0.98	91	7	na					na	This study
<i>Methylibium petroleiphilum</i> PM1	MTBE	-2.0 to -2.4	0.1 to 0.3	0.88 to 0.98	93	39	-33 to -37	4 to 5	0.90 to 0.99	80 to 90	26	18 ± 3	^{10, 15}
	(n=4) TAME	-1.89	0.06	0.998	99	20	-52	2	0.9	91	10	25 ± 1	This study
<i>Methylibium sp.</i> R8 [*]	MTBE	-2.3	0.1	0.98	98	40	-40	4	0.95	91	36	17 ± 1	^{16, 24}
	(n=2) TAME	-1.9	0.1	0.99	99	12	na					na	This study
<i>Pseudonocardia tetrahydrofuranoxydans</i> K1	MTBE	-2.3	0.2	0.99		12	-100	10	0.99		11	48 ± 5	¹³
	ETBE	-1.7	0.2	0.99		9	-73	7	0.97		5	49 ± 4	¹³
	TAME	-1.7	0.3	0.99		5	-72	2	0.99		5	45 ± 4	¹³
<i>Pseudomonas putida</i> GPo1 (n=3)	MTBE	-1.4	0.1	0.91	80	28	-11	2	0.8	80	23	8 ± 1	This study
	(n=3) TAME	-1.1	0.2	0.9	59	32	-18	5	0.9	59	17	13 ± 3	This study
<i>Mycobacterium austroafricanum</i> IFP 2012 (n=5)	MTBE	-2.64	0.08	0.96	96	52	ns (+1)	2	0.02	92	43	na	This study
	(n=5) TAME	-2.16	0.05	0.995	96	41	ns (-0.2)	6	0.2	95	27	na	This study
<i>Mycobacterium austroafricanum</i> JOB5 (n=3)	MTBE	-2.50	0.04	0.994	96	31	-4.2	0.9	0.6	86	24	1.7 ± 0.3	This study
	(n=3) TAME	-2.12	0.05	0.998	99	20	ns (+3)	2	0.5	97	18	na	This study
<i>Rhodococcus ruber</i> DSM7511 (n=4)	MTBE	-2.48	0.06	0.997	99	27	-7	3	0.86	95	22	2.4 ± 0.8	This study
	(n=2) ETBE	-1.5	0.1	0.98	95	20	-11	2	0.8	95	20	7 ± 1	This study
	(n=3) TAME	-2.01	0.08	0.995	99	27	ns (-3)	3	0.05	85	22	na	This study

n: number of experiments, N: number of data points; na: not analyzed or not applicable; ns: not significant; CI is 95% Confidence Interval; D%: maximum percentage of substrate initial/ partial degradation (do not confuse with complete mineralization to CO₂) which could be analyzed for the corresponding isotopic composition taking into account different detection limits for carbon and hydrogen.

*Original data was recalculated according to Rosell et al. ²⁷ and in the case of several experiments (e.g. growing and resting cells), the data was combined.

Figure captions

Figure 1. MTBE two dimensional plot of hydrogen versus carbon isotopic shifts for the so far discovered initial reaction mechanisms including (i) the mean slope (λ value) for chemical oxidation (by plotting together permanganate and the two Fenton reactions in this study), (ii) the acid hydrolysis (S_N1 -type, λ range according to Elsner et al.²⁰ and our study), (iii) the S_N2 -type hydrolysis suggested for an anaerobic enrichment culture^{20, 21} although a $\lambda = 6$ has been also reported for an anaerobic enrichment culture when adding syringic acid as co-substrate²⁵ (not shown) and (iv) the isotopic pattern discovered for strain K1¹³. Linear regression curves (solid lines) allow comparison within the 95% confidence intervals (CI) (dashed lines). Measured values for strains GPo1 (circles), JOB5 (triangles) and DSM 7511 (stars) are shown. Previously studied strains PM1 and R8 fit to the chemical oxidation pattern (not shown)^{15, 16}. Due to not measurable hydrogen fractionation, strains L108, IFP 2001¹⁶ and IFP 2012 are not shown.

Figure 1

