1   # $\delta^{13}C$ and $\delta^{37}Cl$ isotope fractionation to characterize

2   # aerobic vs. anaerobic degradation of

3   # trichloroethylene

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20   ABSTRACT

21   Trichloroethylene (TCE) is a carcinogenic organic chemical impacting water resources

22   worldwide. Its breakdown by reductive vs. oxidative degradation involves different types of

23   chemical bonds. Hence, if distinct isotope effects are reflected in dual element (carbon and

24   chlorine) isotope values, such trends could help distinguishing both processes in the environment.

25   This work explored dual element isotope trends associated with TCE oxidation by two pure

26   bacterial cultures: *Pseudomonas putida* F1 and *Methylosinus trichosporium* OB3b, where the latter

27   expresses either soluble methane-monooxygenase (sMMO) or particulate methane-

28   monooxygenase (pMMO). Carbon and chlorine isotope enrichment factors of TCE ($\varepsilon^{13}C$ = -11.5,-

29   2.4 and -4.2‰; $\varepsilon^{37}Cl$ = 0.3, -1.3 and -2.4‰ respectively) differed strongly between the strains. The

30   dual element isotope trend for strain F1 ($\varepsilon^{13}C/\varepsilon^{37}Cl$ = -38) reflected, as expected, primary carbon

31   and negligible chlorine isotope effects, whereas unexpectedly large chlorine isotope effects

32   became apparent in the trend obtained with strain OB3b ($\varepsilon^{13}C/\varepsilon^{37}Cl$ = +1.7 for sMMO and

33   pMMO). Therefore, although dual element isotope analysis partly reflects predicted differences in

34   oxidative vs. reductive ($\varepsilon^{13}C/\varepsilon^{37}Cl$ = 3.4 to 5.7) degradation, the unexpected OB3b fractionation

35   data may challenge field interpretation.

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37

38   **Introduction**

39   Aliphatic chloro-organic compounds of anthropogenic origin, among them trichloroethylene

40   (TCE), threaten the quality of water resources worldwide [1,2]. TCE is a volatile organic compound

41   used primarily as an industrial solvent in metal and textile industry. Due to its high density and

42   low solubility, TCE spills may move downwards in the subsurface to lower permeability layers,

2

43   forming pool(s) of dense non-aqueous phase liquids [3] which can serve as a source of contamination

44   spread [4]. TCE is toxic and carcinogenic to humans [5], and its drinking water concentration limit is

45   set by the EPA to 5 μg/l [6].

46   Biodegradation is regarded as an essential component of plume remediation, where two main

47   processes may be responsible at TCE-contaminated sites: anaerobic reductive dechlorination and

48   aerobic co-metabolism [7]. Anaerobic reductive dechlorination has been extensively investigated [7–

49   11]. In this process TCE serves as an electron acceptor in the respiratory chain and hydrogen as an

50   electron donor. The hydrogen atoms sequentially replace chlorine atoms ("hydrogenolysis") from

51   tetrachloroethylene (PCE), through TCE, *cis*-dichloroethylene (cDCE) and vinyl chloride (VC)

52   down to ethylene, where the lower chlorinated transformation products tend to be of greater

53   toxicity than TCE [12]. The oxidative pathway, in contrast, leads to degradation products of lower

54   toxicity than VC until full mineralization (CO_2, Cl^-) is achieved. Thus, the two differ in their

55   environmental toxicities [13]. The oxidative pathway can proceed co-metabolically, where TCE is

56   being degraded fortuitously by monooxygenase or dioxygenase enzymes that are induced for

57   oxidation of bacterial growth substrates ("auxiliary substrates") including, e.g., methane, ethane,

58   ammonium or aromatic hydrocarbons [14]. Aerobic metabolic degradation, on the other hand, was

59   rarely shown for TCE [15]. Since TCE oxidation normally does not lead to easily monitored

60   byproducts, it is difficult to assess this process importance at polluted sites.

61   During the last ≈15 years, compound specific isotope analysis (CSIA) has been established as

62   an important tool to demonstrate the occurrence of contaminant degradation at contaminated sites

63   [16]. The CSIA approach is based on the fact that bonds with light isotopes are normally cleaved

64   slightly faster than bonds with heavy isotopes, resulting in a kinetic isotope effect (KIE). Thus, as

65   degradation proceeds, the remaining pool of contaminant becomes gradually enriched with heavy-

66 isotope bearing molecules [16–18]. Consequently, a direct indication of degradation is obtained, and

67 its extent may be assessed even without the need for concentration-based mass balances [16,19,20].

68 Additionally, it was demonstrated that CSIA can be used for elucidating degradation mechanisms

69 [21]. Multi-element isotope analysis may point to specific bonds involved in the rate-limiting step of

70 a reaction and different degradation mechanisms can further be distinguished [22–27]. Recent

71 analytical developments in Cl-CSIA, either by continuous flow gas chromatography isotope ratio

72 mass spectrometry (GC/IRMS) [28] or by GC-quadrupole mass spectrometry (GC/qMS) [26,27,29–32]

73 have facilitated the measurement of chlorine isotope ratios in chlorinated ethylenes. It was shown

74 that reductive dechlorination can be distinguished from abiotic permanganate oxidation of TCE

75 using both carbon and chlorine isotope enrichment factors [33]. Extending this capability also for

76 microbial oxidation of TCE is desired, yet only carbon isotope effects were formerly studied,

77 whereas chlorine isotope effects are still lacking.

78 Carbon isotope enrichment factors have been reported for TCE oxidation, ranging from εC = -

79 1.1 to -20.7‰ [35–37] and overlapping with the range of its reductive dechlorination (εC =-2.5 to -

80 15.3‰ [25,33,34,38–41]). While mechanistic details of TCE oxidative biodegradation have been

81 investigated [42–44] (Scheme 1), reasons for the differences in carbon isotope enrichment factors are

82 still not understood.

83 Interpretation of CSIA could become challenging due to several different reasons, all driven

84 from the fact that the apparent isotope effect cumulates various different factors that may diverge

85 the observable isotope effect from the intrinsic isotope effect [45–47]. These include membrane

86 induced equilibrium isotope effect [48], equilibrium isotope effects on enzyme binding [49,50] or

87 following product branching [51]. In these cases distinction e.g. between anaerobic and aerobic

88   microbial degradation processes for TCE would be more complex, but in exchange the differences

89   in dual isotope trends may provide additional mechanistic insight.

90   TCE oxidation by a monooxygenase enzyme is represented in *Methylosinus trichosporium*

91   OB3b. This widely studied strain synthesizes either soluble methane monooxygenase (sMMO)

92   soluble in the cytoplasm at low copper-to-biomass ratios [52,53] or membrane bound particulate

93   methane monooxygenase (pMMO) when copper-to-biomass ratio increases[54,55]. Both enzymes can

94   fortuitously mineralize TCE to CO_2 [55]. Fox et al [43], (Scheme 1) proposed that TCE degradation by

95   sMMO can occur through generation of either an epoxide or (2,2,2-Trichloroethane-1,1-diol)

96   chloral hydrate (either C=C bond epoxidation or intramolecular Cl^- migration). Experimentally,

97   however, chloral hydrate was shown to be a minor product [57]. Furthermore, Fox et al. [43] did not

98   observe chloral hydrate formation by hydrolysis of authentic TCE epoxide, supporting the

99   assumption that chloral hydrate is not a degradation product of TCE epoxide.

100   TCE oxidation induced by pMMO enzyme has been studied with pMMO extracted from

101   *Methylococcus capsulatus* and whole cells of *Methylomicrobium album* Bath BG8 [42]. This

102   proposed mechanism suggests TCE degradation occurs through an epoxide intermediate, similar

103   to the sMMO pathway, but with no formation of chloral hydrate.

104   TCE oxidation by a dioxygenase enzyme, finally, is represented by the toluene degrader

105   *Pseudomonas putida* F1. This strain fortuitously mineralizes TCE to CO_2 by toluene dioxygenase

106   (TDO). It was previously postulated that the rate-determining step of the reaction is a cleavage of

107   the carbon double bond, creating a dihydroxy-TCE intermediate [44].

108   In the present study we aimed to explore dual ^{37}Cl and ^{13}C isotopic effects associated with

109   degradation of TCE by the methanotroph OB3b (expressing either pMMO or sMMO enzymes)

110   and the toluene degrader *Pseudomonas putida* F1. An additional objective of the study was to

111   evaluate to what extent dual carbon – chlorine isotope data can be used for distinguishing anaerobic

112   and aerobic biodegradation of TCE in the field.
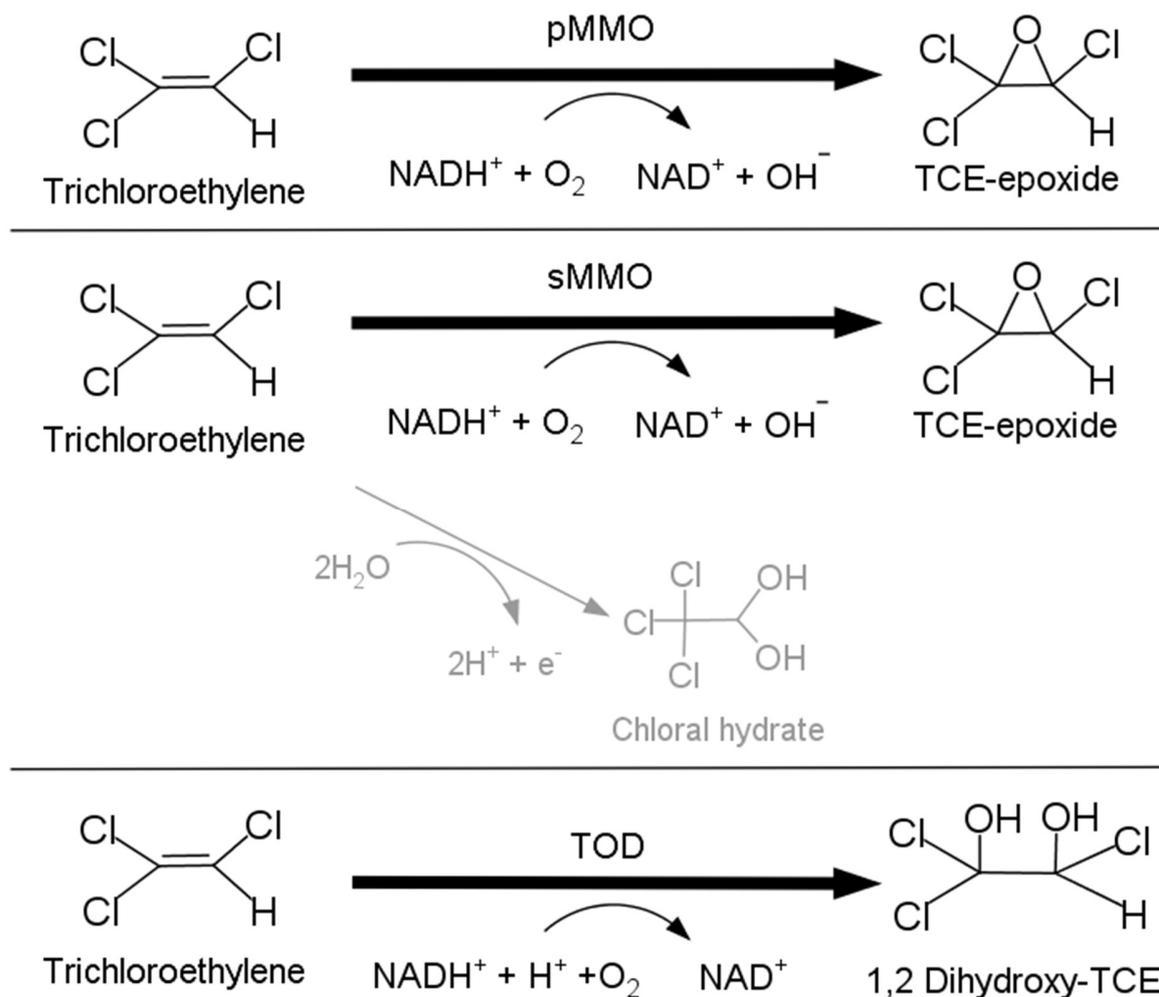
113   **Scheme 1.** Proposed rate limiting step at TCE oxidation by (upper) pMMO enzyme resulting in

114   epoxide [42]. (middle) sMMO enzyme resulting in epoxide and chloral hydrate formation (minor

115   product marked gray), with the later as a minor product [43] (lower) TDO enzyme [44] resulting in

116   1,2-Dihydroxy-TCE.

117

Materials and Methods

Experimental setup

Two pure strains were used in batch experiments: *Pseudomonas putida* F1 and *Methylosinus trichosporium* OB3b. Strains were kindly provided by Prof. Lawrence P. Wackett, University of Minnesota, and Dr. Jeremy Semrau, University of Michigan. The growth media for F1 was prepared as described previously [35] and amended with trace element [58]. OB3b was cultivated in liquid media as described previously [59] with or without $CuSO_4$ for either pMMO or sMMO expression, respectively. More details on the growth conditions are provided in the Supporting Information.

Pure cultures were harvested by centrifugation and re-suspended in fresh growth media. Fresh media was amended with resazurin as redox indicator (1 mg/l), TCE (5.8 mg/l), and either ethanol (158 mg/l) or potassium formate (0.02 M) as NADH source for F1 or OB3b, respectively. Growth media was led to equilibrate overnight on a magnetic stirrer prior inoculation. For initiating the experiments, one milliliter of harvested bacteria was transferred into 60 ml autoclaved serum bottles. Growth media was then added (1:5 liquid to air) maintaining a large headspace for sufficient oxygen in the system and bottles were immediately crimped with a Viton septa. All experiments were conducted in triplicates, and accompanied by abiotic and biotic controls (see SI). Halting the degradation process was done by adding phosphoric acid (98%) to each bottle, to reach pH≤ 2. Once the experiment was completed, final TCE concentrations were measured in each bottle by GC/MS (Trace 1310 coupled to a ISQ LT, Thermo Fisher Scientific). The growth media was divided into glass vials, sealed with Teflon lined septa, and preserved frozen [60]. Frozen samples from F1 and sMMO experiments were distributed between the Geological Survey of Israel and the Helmholtz Zentrum München for comparison of chlorine isotope measurements. Chloral

141 hydrate formation was monitored on a separate experiment following MTBE liquid-liquid

142 extraction modified from Nikolaou et al. [61] and GC/MS analysis (see SI for more detailed

143 description).

Isotope analysis

145 Chlorine isotope analysis was done by two different methods: (i) GC/IRMS at the Helmholtz

146 Zentrum München [30] and (ii) GC/MS at the Geological Survey of Israel. For the later, six ions

147 were monitored (m/z 60, 62, 95, 97, 130 and 132), and data was evaluated following Sakaguchi-

148 Söder et al [32]. Calibrating the $\delta^{37}Cl$ measurements by GC/MS to the SMOC scale was achieved

149 using three $\delta^{37}Cl$ differing in-house standards (see SI) that were isotopically characterized by GC-

150 IRMS relative to known EIL-1 and EIL-2 [30] standards .

151 Carbon isotope analysis was done by GC-IRMS (either Delta-V or MAT-253, Thermo Fisher

152 Scientific). More details on the isotope analysis methods are found in the SI.

Calculations

154 TCE isotope ratios measured by either GC/IRMS or GC/qMS are reported using the delta

155 notation (eq 1):

156 (1) $\delta\ ^{h}E_{sample} = \dfrac{R_{sample}}{R_{standard}} - 1$

158 where R is the isotope ratio of carbon ($^{13}C/^{12}C$) or chlorine ($^{37}Cl/^{35}Cl$), respectively. Since

159 variations of isotope ratios are often small, the δ-values are expressed on a per mill scale.

160 To determine carbon or chlorine isotope enrichment factors (ε) along TCE oxidation, a modified

161 Rayleigh equation was used (eq 2):

162 (2) $\ln\dfrac{R_t}{R_0} = \varepsilon \times \ln(f)$

164   where R_0 and R_t are isotope ratios of the beginning and during degradation, respectively, and f is

165   the remaining fraction of non-degraded TCE.

166

167   **Results and Discussions**

168   **Isotope enrichment factors and degradation mechanism**

169   Chlorine isotope analysis along TCE oxidation was carried out by GC/IRMS and by GC/qMS.

170   Both techniques resulted in $\varepsilon^{37}Cl$ values that are not significantly different (Table S1). Oxidation

171   of TCE by strain F1 resulted in a negligible chlorine isotope enrichment factor ($\varepsilon^{37}Cl=0.3\pm0.2$‰)

172   along with a strong carbon isotope enrichment factor ($\varepsilon^{13}C=-11.5\pm2.4$‰) (Figure 1). The $\varepsilon^{13}C$

173   value is in good agreement with earlier reported values for this strain ($\varepsilon^{13}C=-13.8\pm1.6$‰) [37], as

174   well as with theoretical expectations from similar experiments ($\varepsilon^{13}C=-11$‰ as average over both

175   C atoms) [62].

176   The negligible isotope enrichment along TCE oxidation by strain F1 suggest that carbon-

177   chlorine bonds are not involved in the rate-limiting step of the reaction, in line with the accepted

178   mechanistic pathway (Scheme 1). This is also in agreement with previously reported values along

179   VC and *cis*-DCE oxidation ($\varepsilon^{37}Cl=\sim-0.3$‰) [63]. Likewise, negligible chlorine isotope enrichment

180   values were reported for TCE oxidation by permanganate ($\varepsilon^{37}Cl=0.1\pm0.1$‰) [33].
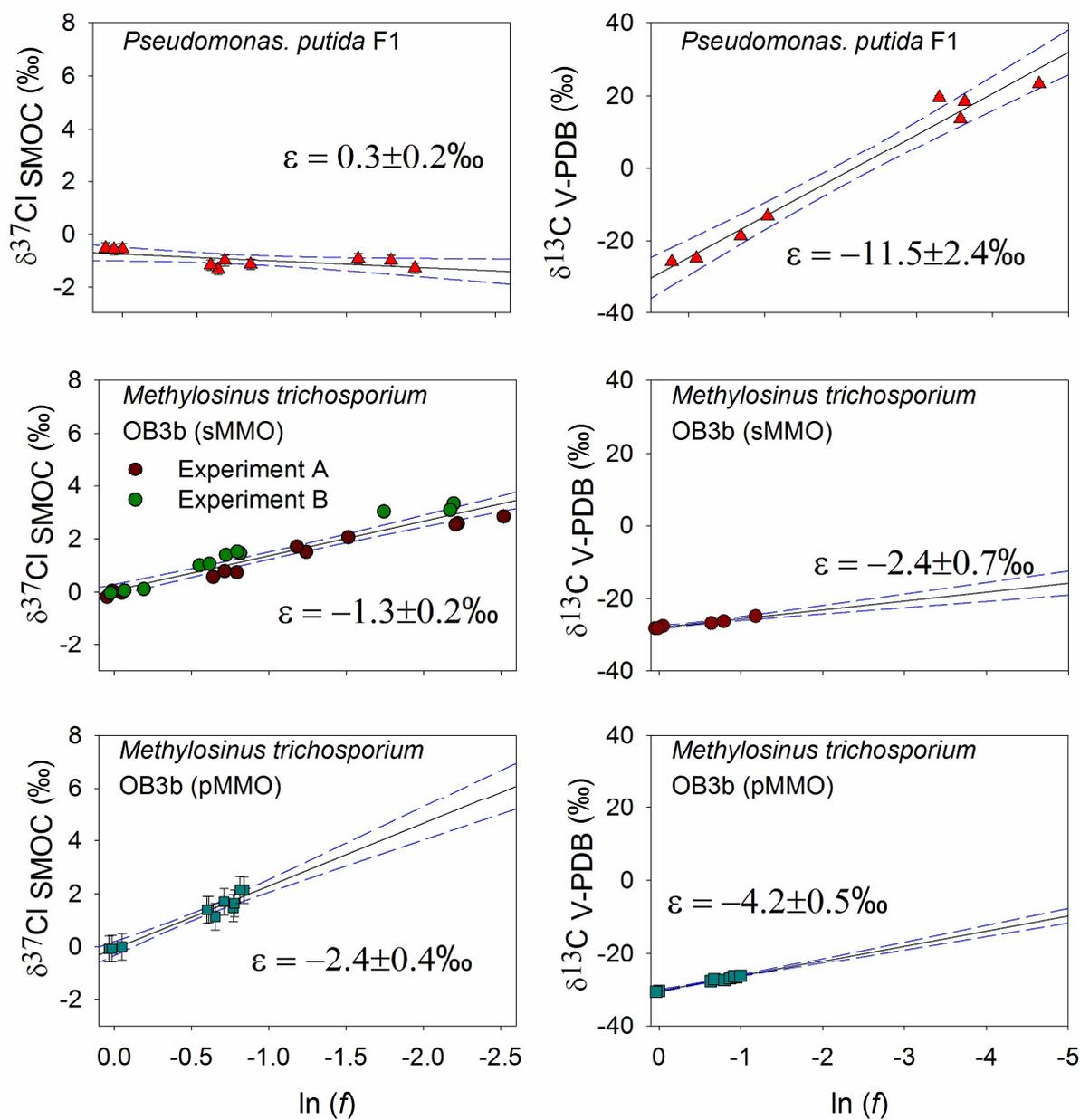
Figure 1. Chlorine (left) and carbon (right) and isotope composition of TCE oxidized by TDO (strain F1), pMMO (strain OB3b), or sMMO (strain OB3b). Dashed lines represent 95% confidence intervals and the error bars represent the uncertainty of the method (±0.5‰ for ^{13}C-IRMS and [37]Cl-GC/MS and ±0.2 for [37]Cl-IRMS).

187   Carbon isotope enrichment along TCE oxidation by strain OB3b was significantly different from

188   strain F1, in consistence with previous studies [36,37]. Oxidation by sMMO, presented a lower carbon

189   isotope enrichment ($\varepsilon^{13}C$=-2.4±0.7‰) than F1, together with a comparatively pronounced chlorine

190   isotope enrichment ($\varepsilon^{37}Cl$=1.3±0.2‰). This pattern was reinforced by results of TCE oxidation by

191   pMMO, where both carbon ($\varepsilon^{13}C$=-4.2±0.5‰) and chlorine ($\varepsilon^{37}Cl$=-2.4±0.4‰) isotope enrichment

192   factors were twice as high compared to sMMO (Figure 1), so that the dual element isotope trend

193   remained the same.

194   Chloral hydrate was detected as a minor intermediate along TCE oxidation by sMMO, and was

195   not detected for pMMO. Since this intermediate did not accumulate in the growth medium, we

196   were unable to assess the overall magnitude of this chlorine migration route for sMMO.

197   Nevertheless, throughout the experiment chloral hydrate concentrations did not exceed 6% of the

198   initial TCE concentration (Figure S1), similar to earlier reports [43]. If this route is significant, one

199   might speculate that the pronounced chlorine isotope enrichment may be attributable to chlorine

200   migration. However, this would not explain the pronounced ε value along TCE oxidation by

201   pMMO, where chloral hydrate was not detected (Scheme 1). Hence, we infer that the pronounced

202   chlorine isotope enrichment for both sMMO and pMMO is not related to chlorine migration.

203   Recently published studies reported unexpectedly large chlorine isotope enrichment values

204   along different transformation processes of chlorinated hydrocarbons. These were reported for

205   both primary [64] as well as secondary chlorine isotope effects [34,64–66], providing analogous

206   observations that relatively high chlorine isotope effects can be observed – like with sMMO and

207   pMMO - even though a C-Cl bond may not be cleaved in all circumstances. Future computational

208   work is needed in order to understand the underlining cause of the unexpectedly large chlorine

209   enrichment values of OB3b, in similarity to former studies on dechlorination [67].

210   Dual element isotope trends were plotted for the different enzymes reflecting similar trends for

211   sMMO and pMMO and a different trend for strain F1 (Figure 2). The sMMO and pMMO enzymes

212   differ in their metal centers [66], degradation products (formation of chloral hydrate) [42] and

213   degradation rates [53]. Their similar dual element isotope trends (1.8±0.6 and 1.7±0.4‰

214   respectively), may suggest a similar reaction mechanism [22–27]. The different dual element isotope

215   trends observed for the strains OB3b and F1 (1.7±0.4 and -38±27‰ respectively), may either be

216   influenced by equilibrium isotope effects on binding to enzyme or a different biochemical reaction

217   mechanism. Recent publications [49,50] suggests that dual-element isotope slope may not necessarily

218   reflect a chemical bond conversion, but instead preceding steps prior to catalysis. However we

219   cannot exclude that the different dual element isotope slopes are due to different biochemical

220   reaction mechanisms (i.e., a different manner of bond changes).

221   **Environmental significance**

222   An attractive feature of the dual isotope approach is the possibility to distinguish between

223   transformation mechanisms in the environment. Our results show that for strain F1 as a model

224   organism, a distinction between aerobic and anaerobic degradation may indeed be possible if both

225    $\delta^{13}C$ and $\delta^{37}Cl$ values are measured (Figure 2). The observed $\varepsilon^{13}C$ vs. $\varepsilon^{37}Cl$ trend for TCE

226   oxidation by F1 strain is similar to permanganate oxidation [33] and it differs significantly from

227   reported trends for biotic [25,33,34] and abiotic [25,68] reduction. This observation meets the intuitive

228   assumption that chlorine isotope enrichment along TCE oxidation should be negligible, facilitating

229   the distinction between both pathways [33,63].

230   On a more refined level, the surprising differences in the dual element isotope results between

231   strain F1 and strain OB3b (-38±27‰ vs. 1.7±0.4‰) show that this overarching mechanistic picture

232   warrants further investigation. Although both strains OB3b and F1 are thought to oxidize TCE via

233 oxidation of the double bond, they do not present similar dual element isotope trends. Moreover,

234 while the F1 dual element isotope trend is greater than the anaerobic trend, that of OB3b is smaller.

235 Thus, at first sight the dual element isotope trend observed with OB3b seems to interfere with

236 pathway distinction. However, it is important to note that the isotope enrichment is rather small

237 for strain OB3b (Figure 2) and may not necessarily lead to large misinterpretations at field sites.

238 From the environmental perspective, OB3b results indicate a surprising chlorine involvement in

239 what until now has been considered as a classical epoxidation mechanism. Further research is

240 therefore needed to determine whether the OB3b results are generally representative of

241 monooxygenase enzymes and what the relevance of this monooxygenase enzyme-catalyzed

242 pathway is for TCE oxidation at polluted sites.

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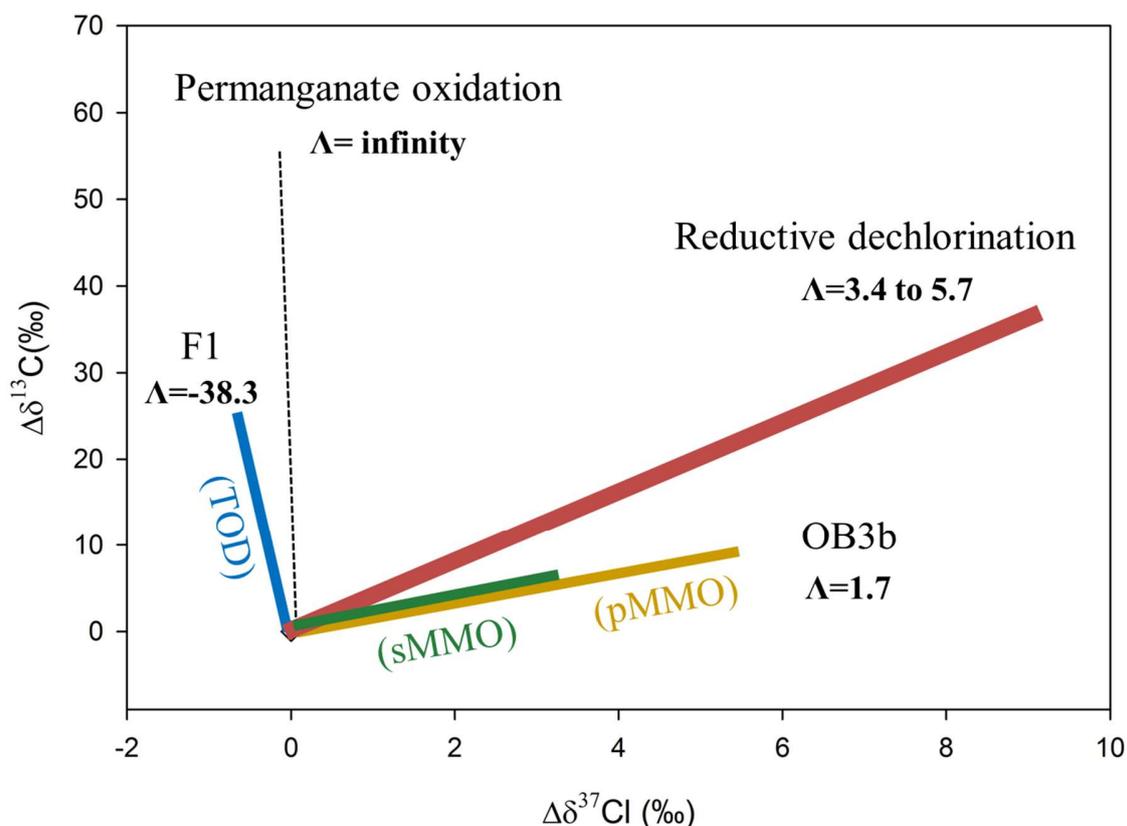
Figure 2. Dual element isotope plot of $\delta^{13}C$ vs. $\delta^{37}Cl$ representing ~ 90% degradation. Trend-lines were shifted to the origin by presenting the change in the isotope composition ($\Delta\delta$) rather than the absolute delta values. The slope of the lines ($\Delta\delta^{13}C/\Delta\delta^{37}Cl$) corresponds approximately to the ratio $\varepsilon^{13}C/\varepsilon^{37}Cl$, represented as Λ. Green: *Methylosinus trichosporium* OB3b expressing sMMO; Orange: *Methylosinus trichosporium* OB3b expressing pMMO; Blue: *Pseudomonas putida* F1 expressing TOD. Black: abiotic permanganate oxidation [33]. Red: biotic reductive dechlorination by *Geobacter lovleyi* [25], *Desulfitobacterium hafniense* Y51 [25] and *Dehalococcoides* [34], abiotic reductive dechlorination by zero valent iron [68] and enzymatic cofactor cobalamin [25].

ACKNOWLEDGMENT

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