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Author(s): Vähätalo, Anssi V.; Xiao, Yihua; Salonen, Kalevi

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1 **Biogenic Fenton reaction – a possible mechanism for the mineralization of**
2 **organic carbon in fresh waters**

3 *Anssi V. Vähätalo^{1,2}, Yihua Xiao^{1,3,*}, Kalevi Salonen²*

4 ¹Department of Biological and Environmental Science, University of Jyväskylä, 40014,
5 Jyväskylä, Finland;

6 ²Lammi Biological Station, University of Helsinki, 00014, Lammi, Finland;

7 ³School of Environmental & Municipal Engineering, Qingdao University of Technology,
8 266033, Qingdao, China

9

10 *Corresponding author:

11 yihua.y.xiao@jyu.fi

12 **ABSTRACT:** To explore the mechanisms that mineralize poorly bioavailable natural organic
13 carbon (OC), we measured the mineralization of OC in two lake waters over long-term
14 experiments (up to 623 days) at different pH and iron (Fe) levels. Both microbial and
15 photochemical mineralization was higher at pH acidified to 4 than at the ambient pH 5 or an
16 elevated pH 6. During 244 days, microbes mineralized up to 60% of OC in 10- μ m filtrates of
17 lake water and more than 27% in 1- μ m filtrates indicating that large-sized microbes/grazers
18 enhance the mineralization of OC. A reactivity continuum model indicated that acidification
19 stimulated the microbial mineralization of OC especially in the later (>weeks) phases of
20 experiment when the bioavailability of OC was poor. The reactive oxygen species produced by
21 light or microbial metabolism could have contributed to the mineralization of poorly
22 bioavailable OC through photochemical and biogenic Fenton processes catalyzed by
23 indigenous Fe in lake water. When Fe was introduced to artificial lake water to the
24 concentration found in the study lakes, it increased the densities of bacteria growing on solid
25 phase extracted dissolved organic matter and in a larger extent at low pH 4 than at pH 5. Our
26 results suggest that in addition to the photochemical Fenton process (photo-Fenton), microbes
27 can transfer poorly bioavailable OC into labile forms and CO₂ through extracellular Fe-
28 catalyzed reactions (i.e., biogenic Fenton process).

29

30 *Keywords:*

31 Organic carbon

32 Reactive oxygen species

33 Iron

34 Biogenic Fenton

35 Microbes

36 Reactivity continuum

37 1. Introduction

38 In fresh waters, the mineralization of natural organic carbon (OC) emits 2.1 Pg CO₂-C
39 yr⁻¹ to the atmosphere (Raymond et al., 2013). Solar radiation-induced photochemical reactions
40 can account for one tenth of the CO₂ emission (Aarnos et al., 2018; Koehler et al., 2014).
41 Additional mechanisms are needed for the mineralization of OC in fresh waters with a typical
42 first order decay coefficient of ~0.00076 d⁻¹ corresponding to approximately 2.5 years half-
43 lives (Catalán et al., 2016). The mechanisms responsible for the mineralization of poorly
44 bioavailable OC are mostly unknown and have been seldom addressed with long term
45 experiments (Koehler et al., 2012).

46 Reactive oxygen species (ROS) may contribute to the slow mineralization of poorly
47 bioavailable OC (Waggoner et al., 2017). Three major processes produce ROS in the
48 environment. (i) ROS are produced at redoxclines when reduced forms of dissolved organic
49 matter (DOM), iron (Fe) or other metals enter from anoxic to oxic strata and react with O₂
50 (Liao et al., 2019; Minella et al., 2015; Page et al., 2012, 2013; Trusiak et al., 2018; Waggoner
51 et al., 2017). (ii) Photochemistry produces ROS at narrow surface strata during daytime
52 (Micinski et al., 1993; Wolf et al., 2018; Zepp et al., 1992). (iii) Microbes produce ROS over
53 the entire oxic water column (Diaz & Plummer, 2018; Dixon et al., 2013; Zhang et al., 2016).
54 When integrated over the water column and the 24 hours of day, the production rate of
55 superoxide (O₂^{•-}) and hydrogen peroxide (H₂O₂) has been an order of magnitude larger through
56 biology than photochemistry (Vermilyea et al., 2010). Thus, microbes have a high potential to
57 mineralize OC through ROS reactions. O₂^{•-} and H₂O₂ mineralize OC poorly, but they can be
58 reduced to hydroxyl radical (•OH) through the Fenton process (Gligorovski et al., 2015). In
59 fresh waters, •OH reacts primarily with DOC and can transform it through organic
60 intermediates into CO₂ (Goldstone et al., 2002; Vione et al., 2014).

61

62 Photochemical and biogenic Fenton processes generate $\bullet\text{OH}$. Solar radiation-induced
63 photochemical production of the Fenton reactants, Fe(II) and H_2O_2 , initiates the photochemical
64 Fenton process (photo-Fenton; Faust & Zepp, 1993; Vione et al., 2014; Voelker et al., 1997).
65 Bacteria can degrade OC through biogenic Fenton process (bio-Fenton) (Gu et al., 2016, 2018;
66 Ma et al., 2016; Sekar & DiChristina, 2014; Xiao et al., 2016). For example, bacteria reduce
67 Fe(III) to Fe(II) and the oxidation of Fe(II) generates $\bullet\text{OH}$ (Sekar & DiChristina, 2014).
68 Alternatively, bacteria produce extracellular $\text{O}_2^{\bullet-}$, a precursor for H_2O_2 and siderophores that
69 reduce Fe(III) to Fe(II) (Gu et al., 2018). In this study, the bio-Fenton process refers to the
70 metabolic pathways that lead to extracellular Fe(II) and H_2O_2 followed by abiotic Fenton
71 process that produces $\bullet\text{OH}$.

72 Hydroxyl radicals have short life times ($\sim\mu\text{s}$), extremely low concentrations ($\leq 10^{-15}$ – 10^{-18}
73 mol L^{-1}) and their detection is difficult at the time scale of OC turnover (Burns et al.,
74 2012). Therefore alternative approaches are needed to evaluate a possible role of $\bullet\text{OH}$ on the
75 long-term mineralization of poorly bioavailable OC. For example, long-term experiments can
76 be designed either to favor or hinder the production of $\bullet\text{OH}$ radicals. The rate of $\bullet\text{OH}$ radical
77 production by the Fenton process is negligible in the absence of dissolved Fe (which would
78 function as a catalyst), but increases with the concentration of Fe (Christoforidis et al., 2015;
79 Rush & Bielski, 2005). Although the Fenton process produces $\bullet\text{OH}$ radicals at neutral pH, low
80 pH increases the rates of $\bullet\text{OH}$ production (Georgi et al., 2007; Pignatello et al., 2006; Zepp et
81 al., 1992). The rates of bio-Fenton should increase with the increasing size of microbial
82 community, because the number of ROS producers increases and may include eukaryotic
83 microbes (Diaz & Plummer, 2018), which possibly explain why $>5\text{-}\mu\text{m}$ size fraction were
84 responsible for $>85\%$ of ROS production in an earlier study (Zhang et al., 2016).

85 We hypothesize that the bio-Fenton process along with the photo-Fenton contribute to
86 the long-term mineralization of poorly bioavailable OC. The hypothesis was tested with long-

87 term (up to 623 days) experiments that measured microbial and photochemical mineralization
88 of OC in 1- μm and 10- μm filtrates of two lake waters, and assessed bacterial growth on solid
89 phase extracted dissolved organic matter (SPE-DOM) at different Fe and pH levels. Gamma
90 reactivity continuum model (Vähätalo et al., 2010; Arndt et al. 2013) described the rate
91 constants for the microbial mineralization of OC separately in the early and the late phases of
92 bioassays corresponding to the labile and poorly bioavailable fractions of OC, respectively. If
93 the latter and the total amount of mineralized OC associates positively with experimental
94 acidification, the concentration of Fe, and large-sized microbial community, the associations
95 indicate the mineralization of poorly bioavailable OC through the bio-Fenton process. If
96 acidification enhances photochemical mineralization, it indicates that part of OC is mineralized
97 through the photo-Fenton process.

98 **2. Materials and methods**

99 *2.1 Water sampling, DOM extraction, DOM-Fe, artificial lake water and microbial isolate*

100 Surface water samples (0–1 m) were collected between July and October from Lake
101 Vakea-Kotinen and Iso Valkjärvi in Finland (Table 1, lake characteristics given in Table S1
102 and Text S-III). For the experiment with different levels of introduced Fe(III), the SPE-
103 extractable part of DOM (typically >60% of total DOC) was isolated from Lake Valkea-
104 Kotinen (“Fe” experiment, Table 1). The SPE followed the method of Dittmar et al. (2008)
105 except we introduced 0.01 M sodium fluoride (NaF, Sigma-Aldrich) to filtered (<0.2 μm) lake
106 water to exchange Fe(III) from DOM to fluoride ligands. SPE removed 96.6% of Fe from lake
107 water but the SPE-DOM nevertheless contained 8.5 nmol Fe per milligram DOM to satisfy the
108 microbial requirement of Fe in the “Fe” experiment.

109 DOM-Fe(III) complexes were prepared from $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ (Sigma-Aldrich) and SPE-
110 DOM. Acidified (pH 2, HCl) SPE-DOM solution (50 mg L^{-1} in ultrapure water) received 1
111 mM Fe(III) and was titrated with NaOH to pH 4 or 5. During the titration, the binding sites of

112 DOM suppressed the hydrolysis of Fe(III) and DOM-Fe was formed (Karlsson & Persson,
113 2012). Finally, the solution of DOM-Fe received a stock solution of inorganic ions (Table S2)
114 to simulate the composition of lake water in Valkea-Kotinen (“Fe” experiment, Table 1).

115 A grazer-free microbial community for “Fe” experiment was isolated from the same
116 water sample as SPE-DOM as described in Xiao et al. (2016).

117 *2.2 Experimental procedures*

118 *2.2.1 Microbial mineralization of OC – microbial size-fractions and different pH levels*

119 To examine the mineralization of OC by small- or large-size microbes, lake waters were
120 filtered either through 1- μ m or 10- μ m (Nuclepore) filters, respectively (the experiments “1- μ m
121 dark” and “1- μ m or 10- μ m dark”; Table 1). For assessing the effect of pH on the mineralization
122 of OC, the ambient pH of filtrates (5.24–5.45) was adjusted with H₂SO₄ or NaOH to pH 4, 5
123 or 6 (Table 1). Eventually within a day of sample collection, 5 mL of the pH adjusted filtrates
124 were sealed in pre-combusted (450°C for 2 h) clear borosilicate glass ampoules with an
125 approximately 7.5 mL headspace of air (Text S-I; McDowell et al., 1987; Salonen & Kononen,
126 1984) and incubated at room temperature (approximately 23°C) in the dark up to 584 days.
127 Three ampoules were periodically sacrificed for the determination of inorganic carbon (IC)
128 content to calculate the mineralization of OC along the incubation as described in 2.3.1.

129 *2.2.2 Photochemical mineralization of OC alone or together with microbes*

130 The ampoule experiments explained above were modified to assess the mineralization
131 of OC through abiotic photochemistry alone or combined with microbial metabolism at pH 4
132 or 5 (the experiments “0.1- μ m photochemistry” and “1- μ m light or dark”, respectively; Table
133 1, details in Text S-I). For the “1- μ m light or dark”-experiment, lake water was filtered through
134 1- μ m, adjusted to pH 5 or 4 and sealed in the ampoules. For the “0.1- μ m photochemistry”-
135 experiment, the 1- μ m filtrates were filtrated further through 0.1- μ m, adjusted to pH 5 or 4,
136 sealed in ampoules and autoclaved. Half of the ampoules received irradiance from fluorescent

137 lamps at 15°C (Figure S1) and the remaining half (the dark controls) were kept in the dark at
138 the same temperature up to 623 days (Table 1).

139 *2.2.3 Microbial growth on DOC at different levels of Fe and pH*

140 “Fe” experiment examined the effect of both pH and Fe on microbial growth on DOC
141 (“Fe” experiment in Table 1). DOM-Fe(III) (0, 5 or 20 µM Fe) was dissolved to artificial lake
142 water (the final concentration of DOC, 9.8 mg L⁻¹, Table S2) and adjusted to pH 4 or 5. The
143 isolated grazer-free bacterial community from Lake Valkea Kotinen (Xiao et al. (2016) was
144 inoculated (7% vol/vol) in the artificial lake waters with DOM-Fe(III) and incubated in the
145 dark at 23°C for 28 days. During the incubation, microbial growth was periodically assessed
146 as bacterial densities.

147 *2.3 Analytical methods*

148 *2.3.1 Mineralization of OC*

149 The content of IC in the ampoules was periodically measured as CO₂ after purging
150 dissolved IC in lake water together with CO₂ in an air headspace to a carbon analyzer (Text S-
151 I; Figure S2; Salonen, 1981). An increase in IC during the experiments described the amount
152 of mineralized OC. The concentrations of OC along the experiment in Figure 1 were calculated
153 by subtracting the mineralized OC from the concentration of OC determined from GF/C-
154 filtered (nominal pore size 1.2 µm, Whatman) lake water prior to experiments by high-
155 temperature combustion (Salonen, 1979).

156 *2.3.2 Bacterial densities*

157 Bacterial samples were periodically fixed (final concentration of 1% paraformaldehyde
158 with 0.05% glutaraldehyde) from the Fe-experiment and counted with a BD FACSCalibur™
159 flow cytometer (BD Biosciences, USA) using SYBR Green I (Sigma-Aldrich) as nucleic acid
160 stain (Gasol & Del Giorgio, 2000).

161 2.3.3 Reactivity continuum modeling of OC mineralization

162 The mineralization of OC was described by the gamma reactivity continuum model,
163 which expresses mathematically the conceptual decomposition processes that remove
164 preferentially the most labile parts of OC and shift the reactivity continuum of OC toward poor
165 bioavailability with time (Arndt et al., 2013; Catalán et al., 2016; Vähätalo et al., 2010):

$$166 \text{OC}(t) = \text{OC}(t_0) (a (a + t)^{-1})^v \quad (1),$$

167 where $\text{OC}(t)$ is the concentration of OC (mg C L^{-1}) at time t (d), $\text{OC}(t_0)$ is the initial
168 concentration of OC, a (d) and v (dimensionless) are fitting parameters (Koehler et al., 2012).
169 The values of parameters were determined by the curve fitting toolbox version 3.5.2 of Matlab
170 R2015b (The MathWorks Inc.) using non-linear least squares method and trust-region
171 algorithm (Vähätalo et al., 2010).

172 The first order decay coefficient of OC at time t , $k(t)$, was described as:

$$173 k(t) = v (a + t)^{-1} \quad (2).$$

174 At time $t = 0$, $k(t_0) = v/a$ (d^{-1}) expresses the initial first order decay coefficient. Although the
175 value of $k(t)$ depends on both v and a , the value of a has the largest impact on the value of $k(t)$
176 in the early phase of mineralization process (Arndt et al., 2013). In the early phase of
177 mineralization, the value of a describes the average lifetime of more reactive OC components
178 and small values of a refer to high values of $k(t)$ (Arndt et al., 2013). In the late phases of
179 decomposition when the value of $t \gg a$, the value of $k(t)$ depends mainly on v and high values
180 of v refer to high values of $k(t)$ for poorly bioavailable OC (Arndt et al., 2013).

181 2.4 Statistical analyses

182 All experiments had three replicates at each time point. The differences between
183 treatments were tested with paired t test with two-tailed distributions.

184 3. Results

185 3.1 Microbial mineralization of OC in 1- μ m filtered lake waters adjusted to different pHs

186 In the first experiment, we tested a hypothesis that the ambient concentration of Fe in
187 our lake waters (3.2–4.5 μ M, Table S1) is sufficient to induce the biogenic Fenton process,
188 which due to its pH dependence increases mineralization of OC at low pH. When 1- μ m filtered
189 lake water with small-sized microbes was enclosed in ampoules and incubated in the dark at
190 23°C, microbes mineralized up to $26.9 \pm 0.4\%$ and $24.2 \pm 0.3\%$ of OC in water from Lake
191 Valkea-Kotinen and Lake Iso Valkjärvi, respectively, during 584 days (Figure 1a&b). The
192 markers of Figure 1 show the experimental data and the curves illustrate the concentration of
193 OC calculated according to the gamma model (Eq. 1) using the values of a and v reported in
194 Table 2. An acidification of lake water to pH 4 increased the microbial mineralization of OC
195 compared to the treatments adjusted at higher pH 5 or 6 (t -test, $P < 0.05$, $n = 3$; Figure 1a&b)
196 and supported our hypothesis.

197 We further hypothesized that microbes mineralize first the labile OC through direct
198 uptake or enzymatic hydrolysis independently of the bio-Fenton, but after the depletion of
199 labile OC the contribution of bio-Fenton process to the mineralization of OC increases in the
200 late phase of biodegradation. The gamma model can assess the impact of pH on the first order
201 rate constants for mineralization of OC, $k(t)$, separately in the initial, the early and the late
202 phase of biodegradation (Figure 2a&b, Table 2). In the 1- μ m filtrate of Lake Valkea-Kotinen,
203 the initial $k(t_0)$ was similar at pH 4 and 5 ($k(t_0) = 0.0021$ – 0.0022 d^{-1} ; Table 2). The value of a
204 was larger at pH 4 (64.5 d) than at pH 5 (38.1 d) indicating that the average lifetime of more
205 reactive OC components was longer in the acidified treatment (Table 2). In both lake waters,
206 the values of $k(t)$ remained higher at pH 4 than at pH 5 or 6 in the late phase of experiment
207 (Figure 2a&b). The values of v were higher at pH 4 than at 5 or 6 (Table 2) indicating that
208 acidification promoted mineralization of OC in the late phase of biodegradation. Thus along

209 with our hypothesis, the acidification of lake waters to pH 4 did not necessary change the initial
210 microbial mineralization rates and even slowed down the consumption of more reactive OC
211 components in the early phase (high values of a) but caused elevated rates of mineralization in
212 late phase of biodegradation (high values of v ; Figure 2a&b, Table 2).

213 *3.2 Microbial mineralization of OC in 1- μ m and 10- μ m filtrates at different pHs*

214 Based on an earlier observation that large-sized microbes are primarily responsible for
215 the production of ROS (Zhang et al., 2016) and we hypothesized higher rates of bio-Fenton
216 reactions in 10- μ m than 1- μ m filtrates of lake water. When the microbial mineralization of OC
217 in two size fractions is compared, microbes mineralized more OC in the 10- μ m than in the 1-
218 μ m filtrates and more at pH 4 than at pH 5 (Figure 1c&d). For example, at the end of experiment
219 (day 244) in the 10- μ m filtrate of Lake Valkea-Kotinen, microbes had mineralized $60.1 \pm 3.0\%$
220 of OC at pH 4 and more than $26.6 \pm 0.8\%$ at pH 5, which is close to the ambient pH of lake
221 water (Figure 1c).

222 The a -values were lower but the values of v typically were higher in the 10- μ m than in
223 the 1- μ m filtrate within each pH-treatment (Table 2). These kinetic parameters indicate that in
224 the early phase of biodegradation, the large-sized microbes consumed quickly the labile OC
225 most likely without a notable contribution from the bio-Fenton process. In the late phases of
226 experiment, high values of v and the extensive amount of mineralized OC indicate that large-
227 sized microbes were able to mineralize poorly bioavailable OC extensively possibly through
228 the bio-Fenton process because the acidification to pH 4 again increased the mineralization
229 (Figure 2c&d, Table 2).

230 *3.3 Photochemical mineralization of lake water DOC at different pHs*

231 If the photo-Fenton process contributes to the photochemical mineralization of DOC,
232 the mineralization of DOC should increase in irradiated acidified waters because low pH
233 promotes the photo-Fenton process. Irradiation mineralized up to $14.8 \pm 0.5\%$ and $12.4 \pm 0.3\%$

234 of DOC in the autoclaved 0.1- μm filtrates of Lake Valkea-Kotinen and Lake Iso Valkjärvi,
235 respectively, by the end of the 623 d experiment (Figure 1e&f). In the dark controls, the
236 mineralization of DOC remained negligible (Figure 1e&f). The amount of photochemically
237 mineralized DOC and the values of $k(t)$ for the photochemical mineralization were larger at pH
238 4 than at pH 5 (Figure 2e&f; Table 3), which agrees with an elevated rate of photo-Fenton
239 process at acidic conditions.

240 *3.4 Combined photochemical and microbial mineralization of OC at different pHs*

241 When microbes were present in irradiated waters, the irradiation and lower pH
242 increased the mineralization of OC (Figure 1g&h). In water from Lake Valkea-Kotinen, the
243 irradiation stimulated the biological mineralization of OC, because the difference in the amount
244 of mineralized OC between the irradiated and the dark control treatments was larger in the 1-
245 μm filtrates than in the autoclaved 0.1 μm filtrates (Table 3).

246 The values of $k(t)$ and v were larger at the low pH and in the irradiated 1- μm filtrates
247 than in the corresponding dark controls (Figure 2g&h, Table 2). The high values of v in the
248 irradiated 1- μm filtrates and at low pH indicate a contribution of Fenton process to the
249 mineralization of poorly bioavailable OC in the late phases of experiment.

250 *3.5 Effects of Fe on microbial growth on lake water DOC*

251 Based on the experiments with filtered lake waters (Figure 1), it is clear that low pH
252 increased the mineralization of OC (Table 4) and in particular at the late phases of
253 biodegradation (Figure 2; higher values of v at pH 4 than pH 5, Table 2). These observations
254 suggest that the ambient concentration of Fe was high enough to support the Fenton process in
255 our lake waters. The concentration of Fe was higher in Lake Valkea-Kotinen than in Lake Iso
256 Valkjärvi (Table S1) and accordingly the values of v were typically higher for microbial
257 mineralization of OC in the water from Lake Valkea-Kotinen than from Lake Iso Valkjärvi
258 (Table 2). These results indicate that a higher concentration of Fe increased the contribution of

259 bio-Fenton process to the mineralization of poorly bioavailable DOC in the late phases of
260 biodegradation.

261 To further study whether microbial growth on DOC depends on the concentration of Fe
262 in addition to pH, we examined how microbes from Lake Valkea-Kotinen grow on SPE-DOM
263 from Lake Valkea-Kotinen with or without introduced Fe at pH 4 or 5 (Figure 3). In the SPE-
264 DOM dissolved in artificial lake water with 0.17 μM Fe, microbes grew similarly at both pH
265 levels (Figure 3a). When the SPE-DOM was complexed with 5 μM Fe(III) approximating the
266 ambient concentration of Fe in Lake Valkea Kotinen (Table S1), microbial growth increased
267 compared to SPE-DOM without introduced Fe (compare Figure 3a and 3b). In the presence of
268 5 μM SPE-DOM-Fe, bacteria reached higher densities at pH 4 than at pH 5 during 28-day
269 experiment (*t*-test, $P < 0.05$, $n = 3$; Figure 3b). Microbes reached highest densities when they
270 grew on SPE-DOM with 20 μM Fe (compare Figure 3c to 3a&b) and the final densities were
271 higher at pH 4 than at pH 5 (*t*-test, $P < 0.05$, $n = 3$; Figure 3c). Thus, the combination of Fe and
272 low pH increased the growth of microbes on DOC. The final density of microbes in the end of
273 experiment increased with the concentration of Fe (compare the panels a, b and c in Figure 3)
274 indicating that the bio-Fenton process supported higher bacterial density with increasing
275 concentration of Fe.

276 4. Discussion

277 Our experiment with SPE-DOM shows that Fe enhances microbial growth on DOC and
278 in particular in acidic water (Figure 3). This study further shows that a decrease in pH from 5
279 to 4 increases the biological mineralization of OC in lake waters containing the ambient
280 concentration of Fe (Figure 1), but does not enhance the growth of microbes on SPE-DOM
281 extract without introduced Fe (Figure 3a). Acidification increases also the photochemical
282 mineralization of DOC in this and many earlier studies, but not in waters with low
283 concentrations of Fe (Gu et al., 2017 and references therein). In this study, the combination of

284 low pH and Fe enhances both photochemical and biological mineralization of OC. A plausible
285 explanation for the enhancement is the Fenton reaction either driven by microbial metabolism
286 (bio-Fenton) or light (photo-Fenton).

287 *4.1 Photochemical Fenton process*

288 The photo-Fenton process provides an explanation for an increase in the photochemical
289 mineralization of DOC with decreasing pH observed in this and earlier studies (Gu et al., 2017
290 and references therein). In the photo-Fenton process, irradiation generates the Fenton reactants
291 through a series of reactions that start from the light absorption by CDOM or DOM-Fe(III)
292 complexes illustrated as the processes [10] and [11], respectively (Figure 4). The ligand-to-
293 metal charge transfer in DOM-Fe(III) complexes ([11] in Figure 4) can mineralize a part of
294 DOC to CO₂ and produce Fe(II). The photochemical oxidation of CDOM can reduce O₂ to
295 O₂^{•-} ([10] and [2] in Figure 4). Superoxide may reduce Fe(III) to Fe(II) ([4] in Figure 4) or
296 lead to the production of H₂O₂ ([3] in Figure 4). Finally, the Fenton reaction ([5] in Figure 4)
297 produces •OH that transforms DOC into labile forms or CO₂ ([6] in Figure 4). The microbial
298 consumption of labile forms can explain the enhanced microbial mineralization of OC in the
299 irradiated waters of present study ([7] in Figure 4, Table 3).

300 In the present and many earlier studies, acidity increases the photochemical
301 mineralization of DOC and this pH dependence associates with the photo-Fenton process (Gu
302 et al., 2017 and references therein). Low pH promotes (i) the protonation of O₂^{•-} to its
303 conjugate acid (HO₂[•] [2] in Figure 4), (ii) the dismutation of O₂^{•-}/HO₂[•] to H₂O₂ and (iii) the
304 turnover of Fe(II)-Fe(III) ([3] and [11] in Figure 4; Garg et al., 2015; Rush & Bielski, 1985).
305 The turnover of Fe(II)-Fe(III) is high at low pH, because acidity increases the binding of Fe(III)
306 on DOM into soluble reactive forms ([9] in Figure 4; Neubauer et al., 2013). Additionally, the
307 Fenton process produces •OH at low pH ([5] in Figure 4), but ferryl iron (Fe(IV) or Fe(V))
308 with a lower oxidation capacity at higher pH (Vione et al., 2014). According to an earlier study

309 with different pH and Fe levels, an acidification from pH 5 to pH 4 increases the photochemical
310 mineralization of DOC by 32% in 10 mg L⁻¹ DOM associated with 3 μM Fe approximating the
311 conditions in the lakes of present study (Table 4; Gu et al., 2017). The corresponding acidity-
312 induced increase in photochemical mineralization in this study is similar (27–35%) to the
313 earlier estimate (32%; Table 4; Gu et al. 2017). In the absence of Fe, acidification does not
314 change the rate of photochemical mineralization (Gu et al., 2017) indicating that the impact of
315 acidification found in this study is connected to the photo-Fenton process catalyzed by the
316 ambient concentrations of Fe in the examined lake waters.

317 4.2 Biogenic Fenton process

318 If photochemically produced ROS can initiate the photo-Fenton process in our lake
319 waters, ROS produced by microbes should be able to initiate the bio-Fenton process. The bio-
320 Fenton process can provide a mechanistic explanation for the enhanced microbial
321 mineralization of poorly bioavailable OC in the late phases of biodegradation at low pH and
322 the enhanced growth of bacteria on our SPE-DOM-Fe(III) with increasing concentration of Fe.
323 In this study ~~and~~ in oxic surface waters with DOM-Fe(III) in general, a plausible start for the
324 bio-Fenton process is a transport of electron from the cellular metabolism to O₂ for the
325 production of O₂^{•-} ([1–2] in Figure 4; Diaz et al., 2013). The produced O₂^{•-} can initiate a series
326 of abiotic reactions that eventually lead to the Fenton reaction. Several mechanisms can reduce
327 O₂^{•-} to H₂O₂ ([3] in Figure 4; Garg et al., 2011; Petasne & Zika, 1987) and O₂^{•-} can reduce
328 Fe(III) bound on DOM to Fe(II) ([4] in Figure 4; Halliwell, 1978; Yuan et al., 2016). H₂O₂ and
329 Fe(II) undergo the Fenton reaction and produce •OH, which breaks down OC into CO₂ and
330 labile forms ([5]–[6] in Figure 4; Goldstone et al., 2002; Zazo et al., 2005). Biology gets
331 involved again when microbes take up labile OC, respire it to CO₂ and produce reducing
332 equivalents (e.g., NADH) for oxidoreductases that generate extracellular O₂^{•-} ([7]–[8], [1] in
333 Figure 4). An introduction of O₂^{•-} to the same 20 μM SPE-DOM-Fe(III) from Lake Valkea-

334 Kotinen dissolved in the same artificial lake water used in this study produces $\bullet\text{OH}$ in
335 autocatalytic manner and breaks down DOM (Xiao et al., 2020). The earlier study provides
336 further evidence for the proposed bio-Fenton process, where microbially produced $\text{O}_2^{\bullet-}$ reacts
337 with DOM-Fe(III) and generates $\bullet\text{OH}$, which eventually breaks down OC.

338 *4.3 Microbial size fractions*

339 In this study, microbes mineralize up to 5 times more OC in the 10- μm than in the 1-
340 μm filtrates and the mineralization rates remain high up to 244 days, thus concerning also
341 poorly bioavailable OC (Figure 1c&d). We attribute the elevated mineralization rates of poorly
342 bioavailable OC to a more extensive production of ROS and bio-Fenton process in the 10- μm
343 than 1- μm filtrate (more details in Text S-IV). In an earlier study, the biological production of
344 $\text{O}_2^{\bullet-}$ and H_2O_2 decreased remarkably when water was filtered through a 5- μm filter (Zhang et
345 al., 2016). This finding together with the present study suggests that large-sized microbes in
346 particular contribute to reactive species for degradation of recalcitrant OC.

347 *4.4 Bio-Fenton process as a possible adaptation for the utilization of poorly bioavailable OC*

348 In this study, an acidification of lake water increases the microbial mineralization of
349 OC in particular in the late phases of microbial succession (Figure 2; high values of v in Table
350 2) and enhances bacterial growth on DOM-Fe(III) but only after a two–three weeks lag period
351 (Figure 3). A similar lag time took place for bacteria growing on DOM-Fe(III) in an earlier
352 study and involved drastic changes in the composition of bacterial community (Xiao et al.,
353 2016). Our results and those of Xiao et al. (2016) suggest that microbes can adapt to the
354 depletion of labile OC by promoting the bio-Fenton process to break down poorly bioavailable
355 OC to labile forms that support microbial growth and mineralize OC.

356 *4.5 Mineralization of OC through the bio-Fenton process estimated from the dark production*
357 *of H₂O₂ and O₂^{•-} in fresh waters*

358 Here we calculate how much the bio-Fenton process potentially contributed to the
359 mineralization of OC in our experiments based on the dark production rates of ROS reported
360 in the literature. According to the literature (Dixon et al., 2013; Marsico et al., 2015; Vermilyea
361 et al., 2010; Zhang et al., 2016; Table S3 in SI), the dark production rates of extracellular O₂^{•-}
362 and H₂O₂ correspond to an average of 4.0 μM e⁻ d⁻¹ (range 0.7–15.4 μM e⁻ d⁻¹) when expressed
363 as electrons transported from cytoplasm to extracellular milieu. According to a stoichiometry
364 of •OH/ 2e⁻ (Eq. S32 in Text S-VI of SI), this rate translates to 2.0 μM •OH d⁻¹ (range 0.4–7.7
365 μM •OH d⁻¹) or cumulatively to 1,200 μM •OH (range 240–4,620 μM •OH) during 20 months
366 corresponding to a typical length of our experiments. If two •OHs mineralize OC to CO₂ (Eq.
367 S35 in Text S-VII of SI), hydroxyl radicals mineralize cumulatively 600 μM (range 120–2,310
368 μM) OC in 20 months, which is more than the observed microbial mineralization in our
369 experiments (128 μM in 20 months; range 66–593 μM in 20 months; Figure 1). Superoxide
370 dismutase and catalase enzymes as well as other sinks scavenge O₂^{•-} and H₂O₂, and decrease
371 the yield of •OH production per produced O₂^{•-} or H₂O₂ (Bielski et al., 1985). The yields for
372 •OH production have been 1.4%–33% from the stoichiometry of •OH/2 e⁻ (Page et al., 2012,
373 2013). According to these yields, the bio-Fenton process mineralized 8.4–198 μM in our
374 experiments during 20 months assuming a daily production of 4.0 μM extracellular e⁻. The
375 calculations above suggest that the bio-Fenton process was potentially able to explain a
376 remarkable fraction of OC mineralized in our long-term experiments.

377 Here we continue to calculate how much the bio-Fenton process can mineralize DOC
378 in generic fresh water with a typical concentration of DOC (approximately 500 μM) and with
379 a typical DOC half-life of approximately 2.5 years (Catalán et al., 2016; Text S-VIII in SI).

380 When accounting for the range of yields in $\bullet\text{OH}$ production reported earlier (1.4%–33% from
381 $\bullet\text{OH}/2\text{ e}^-$; Page et al., 2012, 2013), the mean production of $4.0\ \mu\text{M e}^- \text{d}^{-1}$ corresponds to
382 $0.027\text{--}0.64\ \mu\text{M } \bullet\text{OH d}^{-1}$ that can mineralize $0.014\text{--}0.32\ \mu\text{M DOC d}^{-1}$. As a non-selective
383 oxidant $\bullet\text{OH}$ likely mineralizes the poorly bioavailable rather than labile DOC because the
384 poorly bioavailable fraction dominates the composition of DOC. The calculated daily rates are
385 beyond the precision of conventional analytical techniques (e.g., for DOC) and masked by the
386 fast turnover of labile DOC (e.g., in the respiration measurements). Therefore in the present
387 study, the slow mineralization of poorly bioavailable became detectable only after the depletion
388 of labile OC in the late phases of biodegradation or with a high precision technique (a bacterial
389 density) under circumstances (high DOM-Fe(III) + acidity) that promoted bio-Fenton process.
390 During the typical half-life of freshwater DOC, the bio-Fenton process however can mineralize
391 $13\text{--}292\ \mu\text{M DOC}$ and account for 5.2–117% for the typical amounts of DOC (approximately
392 $250\ \mu\text{M}$) mineralized in 2.5 years. These calculations indicate that the biogenic Fenton process
393 can remarkably contribute to the turnover of DOC in fresh waters, but the large uncertainties
394 in the calculation call upon further research.

395 **5. Conclusions**

396 The biogenic Fenton process couples the biogenic production of extracellular Fe(II) and
397 H_2O_2 to the abiotic Fenton reaction that produces hydroxyl radicals. In oxic surface waters, the
398 ubiquitous microbial extracellular production of superoxide can translate to H_2O_2 and reduce
399 DOM-Fe(III) to Fe(II). The subsequent Fenton reaction produces hydroxyl radicals that
400 transform the poorly bioavailable OC into labile forms and CO_2 at low rates. These rates are
401 too low to be detected with short-term measurement but high enough to remarkably contribute
402 to the turnover of OC in fresh waters.

403

404 **Author contributions**

405 A.V.V. and K.S. contributed to the design of the ampoule experiments. A.V.V. contributed to
406 the preparation, sample collection and measurements of ampoule experiments. A.V.V. and Y.X.
407 contributed to the design of Fe experiment. Y.X. contributed the preparation, sample collection
408 and measurements of Fe experiment. All authors contributed to the writing and editing the
409 manuscript.

410

411 **Declaration of competing interest**

412 The authors declare that there is no known competing financial interests or personal
413 relationships that could have appear to influence the work reported in this article.

414

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420

421 **Appendix A. Supplementary information**

422 Supplementary information to this article can be found online at xxx.

423

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615

616 **Tables**

617 Table 1. Experimental schemes. VK, Lake Valkea-Kotinen; IV, Lake Iso Valkjärvi.

Name of experiment	Sampling date original pH	Adjusted pH filtration container	Incubation conditions	Measured response
1- μm	26 October pH 5.4 VK, 5.3 IV	pH 4, 5, and 6 1- μm ampoule	23°C 584 d dark	Mineralization
1- μm or 10- μm	2 September pH 5.3 VK, 5.2 IV	pH 4 and 5 1- μm or 10- μm ampoule	23°C 244 d dark	Mineralization
0.1- μm photochemistry	IV: 15 September VK: 17 September pH 5.5 VK, 5.2 IV	pH 4 and 5 0.1- μm (autoclaved) ampoule	15°C 622 d or 623 d light or dark	Mineralization
1- μm light or dark	IV: 15 September VK: 17 September pH 5.5 VK, 5.2 IV	pH 4 and 5 1- μm ampoule	15°C 622 d or 623 d light or dark	Mineralization
Fe	26 October pH 5.4 VK	pH 4 and 5 SPE-DOM + microbial isolate	23°C 28 d dark	Bacterial density

618

619

620 Table 2. Parameters of the reactivity continuum model (Eq. 1 and 2). The values of a and v
 621 were estimated by fitting the Eq. 1 to the measured concentrations of OC shown as markers
 622 in Figure 1.

Experiment	Treatment	Valkea-Kotinen			Iso Valkjärvi		
		a (day)	v	$k_0, v/a$ (day ⁻¹)	a (day)	v	$k_0, v/a$ (day ⁻¹)
1- μm Dark, 23°C, 584 d	pH 4	64.5	0.138	0.0021	29.8	0.089	0.0030
	pH 5	38.1	0.083	0.0022	22.4	0.048	0.0021
	pH 6	53.1	0.088	0.0017	21.5	0.039	0.0018
1- μm or 10- μm Dark, 23°C, 244 d	pH 4 1- μm	94.6	0.109	0.0012	26.9	0.079	0.0029
	pH 5 1- μm	60.2	0.053	0.0009	18.2	0.034	0.0019
	pH 4 10- μm	68.4	0.594	0.0087	-	-	-
	pH 5 10- μm	28.0	0.139	0.0050	20.5	0.157	0.0077
0.1- μm autoclaved Light, 15°C, 622 d or 623 d	pH 4 irradiated	145	0.095	0.0007	308	0.119	0.0004
	pH 5 irradiated	362	0.100	0.0003	204	0.067	0.0003
1- μm Light or Dark, 15°C, 622 d or 623 d	pH 4 irradiated	252	0.382	0.0015	101	0.218	0.0022
	pH 5 irradiated	103	0.136	0.0013	71.2	0.095	0.0013
	pH 4 dark	199	0.113	0.0006	66.6	0.090	0.0014
	pH 5 dark	81.0	0.042	0.0005	128	0.081	0.0006

623 “-” not determined.

624

625 Table 3. Mineralization of OC (% of initial OC) induced by irradiation during 623 days in the
626 0.1- μm autoclaved and in the 1- μm filtered lake water (Table 2).

Lake and pH	Mineralization of OC induced by irradiation (%) [†]	
	0.1- μm autoclaved	1- μm with bacteria
Valkea-Kotinen pH 4	14.8 \pm 0.5	22.6 \pm 3.6
Valkea-Kotinen pH 5	9.6 \pm 0.1	14.5 \pm 0.6
Iso Valkjärvi pH 4	12.4 \pm 0.3	16.0 \pm 4.2
Iso Valkjärvi pH 5	9.1 \pm 1.3	6.1 \pm 2.8

627 [†]calculated as the difference between irradiated waters and their dark controls. Error
628 represents the standard deviations of replicated ($n = 3$) irradiated and dark treatments.

629

630 Table 4. The contribution of acidification (from pH 5 to 4) to the mineralization of OC during
 631 20 months through microbes (1- μm or 10- μm filtrates in the dark), photochemistry and the
 632 combined action of photochemistry and microbes in two lake waters.

Category	Experiment	% mineralized by acidification*			
		Valkea-Kotinen		Iso Valkjärvi	
		$\mu\text{mol L}^{-1}\dagger$	Fraction (%) \ddagger	$\mu\text{mol L}^{-1}\dagger$	Fraction (%) \ddagger
Biological in the dark					
1- μm	1- μm	53	24	58	38
	1- μm or 10- μm	61	39	63	49
10- μm	1- μm or 10- μm	313	53		
Photochemical (abiotic irradiated)					
	0.1 μm autoclaved	41	35	20	27
Biological+Photochemical (biological irradiated)					
	1 μm irradiated	113	38	89	44

633 * Calculated according to the amount of mineralized OC during 20 months using the RC model (Eq. 1)
 634 and values of a and v given in Table 2.

635 \dagger OC mineralized by acidification ($\mu\text{mol L}^{-1}$) = mineralized OC at pH 4 - mineralized OC at pH 5.

636 \ddagger % mineralized by acidification = 100 (mineralized OC at pH 4 – mineralized OC at pH
 637 5)/mineralized OC at pH 4.

638