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

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ABSTRACT: To explore the mechanisms that mineralize poorly bioavailable natural organic carbon (OC), we measured the mineralization of OC in two lake waters over long-term experiments (up to 623 days) at different pH and iron (Fe) levels. Both microbial and photochemical mineralization was higher at pH acidified to 4 than at the ambient pH 5 or an elevated pH 6. During 244 days, microbes mineralized up to 60% of OC in 10-µm filtrates of lake water and more than 27% in 1-µm filtrates indicating that large-sized microbes/grazers enhance the mineralization of OC. A reactivity continuum model indicated that acidification stimulated the microbial mineralization of OC especially in the later (>weeks) phases of experiment when the bioavailability of OC was poor. The reactive oxygen species produced by light or microbial metabolism could have contributed to the mineralization of poorly bioavailable OC through photochemical and biogenic Fenton processes catalyzed by indigenous Fe in lake water. When Fe was introduced to artificial lake water to the concentration found in the study lakes, it increased the densities of bacteria growing on solid phase extracted dissolved organic matter and in a larger extent at low pH 4 than at pH 5. Our results suggest that in addition to the photochemical Fenton process (photo-Fenton), microbes can transfer poorly bioavailable OC into labile forms and CO_2 through extracellular Fe-catalyzed reactions (i.e., biogenic Fenton process).

Keywords:

Organic carbon

Reactive oxygen species

Iron

Biogenic Fenton

Microbes

Reactivity continuum

1. Introduction

In fresh waters, the mineralization of natural organic carbon (OC) emits 2.1 Pg CO_2-C yr^{-1} to the atmosphere (Raymond et al., 2013). Solar radiation-induced photochemical reactions can account for one tenth of the CO_2 emission (Aarnos et al., 2018; Koehler et al., 2014). Additional mechanisms are needed for the mineralization of OC in fresh waters with a typical first order decay coefficient of ~0.00076 d^{-1} corresponding to approximately 2.5 years half-lives (Catalán et al., 2016). The mechanisms responsible for the mineralization of poorly bioavailable OC are mostly unknown and have been seldom addressed with long term experiments (Koehler et al., 2012).

Reactive oxygen species (ROS) may contribute to the slow mineralization of poorly bioavailable OC (Waggoner et al., 2017). Three major processes produce ROS in the environment. (i) ROS are produced at redoxclines when reduced forms of dissolved organic matter (DOM), iron (Fe) or other metals enter from anoxic to oxic strata and react with O_2 (Liao et al., 2019; Minella et al., 2015; Page et al., 2012, 2013; Trusiak et al., 2018; Waggoner et al., 2017). (ii) Photochemistry produces ROS at narrow surface strata during daytime (Micinski et al., 1993; Wolf et al., 2018; Zepp et al., 1992). (iii) Microbes produce ROS over the entire oxic water column (Diaz & Plummer, 2018; Dixon et al., 2013; Zhang et al., 2016). When integrated over the water column and the 24 hours of day, the production rate of superoxide ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2) has been an order of magnitude larger through biology than photochemistry (Vermilyea et al., 2010). Thus, microbes have a high potential to mineralize OC through ROS reactions. $O_2^{\bullet-}$ and H_2O_2 mineralize OC poorly, but they can be reduced to hydroxyl radical ($^{\bullet}OH$) through the Fenton process (Gligorovski et al., 2015). In fresh waters, $^{\bullet}OH$ reacts primarily with DOC and can transform it through organic intermediates into CO_2 (Goldstone et al., 2002; Vione et al., 2014).

Photochemical and biogenic Fenton processes generate $^{\bullet}OH$. Solar radiation-induced photochemical production of the Fenton reactants, Fe(II) and H_2O_2, initiates the photochemical Fenton process (photo-Fenton; Faust & Zepp, 1993; Vione et al., 2014; Voelker et al., 1997). Bacteria can degrade OC through biogenic Fenton process (bio-Fenton) (Gu et al., 2016, 2018; Ma et al., 2016; Sekar & DiChristina, 2014; Xiao et al., 2016). For example, bacteria reduce Fe(III) to Fe(II) and the oxidation of Fe(II) generates $^{\bullet}OH$ (Sekar & DiChristina, 2014). Alternatively, bacteria produce extracellular $O_2^{\bullet-}$, a precursor for H_2O_2 and siderophores that reduce Fe(III) to Fe(II) (Gu et al., 2018). In this study, the bio-Fenton process refers to the metabolic pathways that lead to extracellular Fe(II) and H_2O_2 followed by abiotic Fenton process that produces $^{\bullet}OH$.

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

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

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

2. Materials and methods

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

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

DOM-Fe(III) complexes were prepared from $FeCl_3 \cdot 6H_2O$ (Sigma-Aldrich) and SPE-DOM. Acidified (pH 2, HCl) SPE-DOM solution (50 mg L^{-1} in ultrapure water) received 1 mM Fe(III) and was titrated with NaOH to pH 4 or 5. During the titration, the binding sites of

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

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

2.2 Experimental procedures

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

To examine the mineralization of OC by small- or large-size microbes, lake waters were filtered either through 1-µm or 10-µm (Nuclepore) filters, respectively (the experiments "1-µm dark" and "1-µm or 10-µm dark"; Table 1). For assessing the effect of pH on the mineralization of OC, the ambient pH of filtrates (5.2–5.5) was adjusted with H_2SO_4 or NaOH to pH 4, 5 or 6 (Table 1). Eventually within a day of sample collection, 5 mL of the pH adjusted filtrates were sealed in pre-combusted (450°C for 2 h) clear borosilicate glass ampoules with an approximately 7.5 mL headspace of air (Text S-I; McDowell et al., 1987; Salonen & Kononen, 1984) and incubated at room temperature (approximately 23°C) in the dark up to 584 days. Three ampoules were periodically sacrificed for the determination of inorganic carbon (IC) content to calculate the mineralization of OC along the incubation as described in *2.3.1*.

2.2.2 Photochemical mineralization of OC alone or together with microbes

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

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

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

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

2.3 Analytical methods

2.3.1 Mineralization of OC

The content of IC in the ampoules was periodically measured as CO_2 after purging dissolved IC in lake water together with CO_2 in an air headspace to a carbon analyzer (Text S-I; Figure S2; Salonen, 1981). An increase in IC during the experiments described the amount of mineralized OC. The concentrations of OC along the experiment in Figure 1 were calculated by subtracting the mineralized OC from the concentration of OC determined from GF/C-filtered (nominal pore size 1.2 µm, Whatman) lake water prior to experiments by high-temperature combustion (Salonen, 1979).

2.3.2 Bacterial densities

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

2.3.3 Reactivity continuum modeling of OC mineralization

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

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

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

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

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

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

2.4 Statistical analyses

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

3. Results

3.1 Microbial mineralization of OC in 1-µm filtered lake waters adjusted to different pHs

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

We further hypothesized that microbes mineralize first the labile OC through direct uptake or enzymatic hydrolysis independently of the bio-Fenton, but after the depletion of labile OC the contribution of bio-Fenton process to the mineralization of OC increases in the late phase of biodegradation. The gamma model can assess the impact of pH on the first order rate constants for mineralization of OC, $k(t)$, separately in the initial, the early and the late phase of biodegradation (Figure 2a&b, Table 2). In the 1-µm filtrate of Lake Valkea-Kotinen, 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 was larger at pH 4 (64.5 d) than at pH 5 (38.1 d) indicating that the average lifetime of more reactive OC components was longer in the acidified treatment (Table 2). In both lake waters, the values of $k(t)$ remained higher at pH 4 than at pH 5 or 6 in the late phase of experiment (Figure 2a&b). The values of ν were higher at pH 4 than at 5 or 6 (Table 2) indicating that acidification promoted mineralization of OC in the late phase of biodegradation. Thus along

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

3.2 Microbial mineralization of OC in 1-µm and 10-µm filtrates at different pHs

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

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

3.3 Photochemical mineralization of lake water DOC at different pHs

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

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

3.4 Combined photochemical and microbial mineralization of OC at different pHs

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

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

3.5 Effects of Fe on microbial growth on lake water DOC

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

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

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

4. Discussion

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

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

4.1 Photochemical Fenton process

The photo-Fenton process provides an explanation for an increase in the photochemical mineralization of DOC with decreasing pH observed in this and earlier studies (Gu et al., 2017 and references therein). In the photo-Fenton process, irradiation generates the Fenton reactants through a series of reactions that start from the light absorption by CDOM or DOM-Fe(III) complexes illustrated as the processes [10] and [11], respectively (Figure 4). The ligand-to-metal charge transfer in DOM-Fe(III) complexes ([11] in Figure 4) can mineralize a part of DOC to CO_2 and produce Fe(II). The photochemical oxidation of CDOM can reduce O_2 to $O_2^{\bullet-}$ ([10] and [2] in Figure 4). Superoxide may reduce Fe(III) to Fe(II) ([4] in Figure 4) or lead to the production of H_2O_2 ([3] in Figure 4). Finally, the Fenton reaction ([5] in Figure 4) produces $^{\bullet}OH$ that transforms DOC into labile forms or CO_2 ([6] in Figure 4). The microbial consumption of labile forms can explain the enhanced microbial mineralization of OC in the irradiated waters of present study ([7] in Figure 4, Table 3).

In the present and many earlier studies, acidity increases the photochemical mineralization of DOC and this pH dependence associates with the photo-Fenton process (Gu et al., 2017 and references therein). Low pH promotes (i) the protonation of $O_2^{\bullet-}$ to its conjugate acid ($HO_2^{\bullet}$ [2] in Figure 4), (ii) the dismutation of $O_2^{\bullet-}/HO_2^{\bullet}$ to H_2O_2 and (iii) the turnover of Fe(II)-Fe(III) ([3] and [11] in Figure 4; Garg et al., 2015; Rush & Bielski, 1985). The turnover of Fe(II)-Fe(III) is high at low pH, because acidity increases the binding of Fe(III) on DOM into soluble reactive forms ([9] in Figure 4; Neubauer et al., 2013). Additionally, the Fenton process produces $^{\bullet}OH$ at low pH ([5] in Figure 4), but ferryl iron (Fe(IV) or Fe(V)) with a lower oxidation capacity at higher pH (Vione et al., 2014). According to an earlier study

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

4.2 Biogenic Fenton process

If photochemically produced ROS can initiate the photo-Fenton process in our lake waters, ROS produced by microbes should be able to initiate the bio-Fenton process. The bio-Fenton process can provide a mechanistic explanation for the enhanced microbial mineralization of poorly bioavailable OC in the late phases of biodegradation at low pH and the enhanced growth of bacteria on our SPE-DOM-Fe(III) with increasing concentration of Fe. In this study and in oxic surface waters with DOM-Fe(III) in general, a plausible start for the bio-Fenton process is a transport of electron from the cellular metabolism to O_2 for the production of $O_2^{\bullet-}$ ([1–2] in Figure 4; Diaz et al., 2013). The produced $O_2^{\bullet-}$ can initiate a series of abiotic reactions that eventually lead to the Fenton reaction. Several mechanisms can reduce $O_2^{\bullet-}$ to H_2O_2 ([3] in Figure 4; Garg et al., 2011; Petasne & Zika, 1987) and $O_2^{\bullet-}$ can reduce Fe(III) bound on DOM to Fe(II) ([4] in Figure 4; Halliwell, 1978; Yuan et al., 2016). H_2O_2 and Fe(II) undergo the Fenton reaction and produce $^{\bullet}OH$, which breaks down OC into CO_2 and labile forms ([5]–[6] in Figure 4; Goldstone et al., 2002; Zazo et al., 2005). Biology gets involved again when microbes take up labile OC, respire it to CO_2 and produce reducing equivalents (e.g., NADH) for oxidoreductases that generate extracellular $O_2^{\bullet-}$ ([7]–[8], [1] in Figure 4). An introduction of $O_2^{\bullet-}$ to the same 20 µM SPE-DOM-Fe(III) from Lake Valkea-

Kotinen dissolved in the same artificial lake water used in this study produces $^{\bullet}OH$ in autocatalytic manner and breaks down DOM (Xiao et al., 2020). The earlier study provides further evidence for the proposed bio-Fenton process, where microbially produced $O_2^{\bullet-}$ reacts with DOM-Fe(III) and generates $^{\bullet}OH$, which eventually breaks down OC.

4.3 Microbial size fractions

In this study, microbes mineralize up to 5 times more OC in the 10-µm than in the 1-µm filtrates and the mineralization rates remain high up to 244 days, thus concerning also poorly bioavailable OC (Figure 1c&d). We attribute the elevated mineralization rates of poorly bioavailable OC to a more extensive production of ROS and bio-Fenton process in the 10-µm than 1-µm filtrate (more details in Text S-IV). In an earlier study, the biological production of $O_2^{\bullet-}$ and H_2O_2 decreased remarkably when water was filtered through a 5-µm filter (Zhang et al., 2016). This finding together with the present study suggests that large-sized microbes in particular contribute to reactive species for degradation of recalcitrant OC.

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

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

4.5 Mineralization of OC through the bio-Fenton process estimated from the dark production of H_2O_2 and $O_2^{\bullet-}$ in fresh waters

Here we calculate how much the bio-Fenton process potentially contributed to the mineralization of OC in our experiments based on the dark production rates of ROS reported in the literature. According to the literature (Dixon et al., 2013; Marsico et al., 2015; Vermilyea et al., 2010; Zhang et al., 2016; Table S3 in SI), the dark production rates of extracellular $O_2^{\bullet-}$ and H_2O_2 correspond to an average of 4.0 μM e^- d^{-1} (range 0.7–15.4 μM e^- d^{-1}) when expressed as electrons transported from cytoplasm to extracellular milieu. According to a stoichiometry of $^{\bullet}OH$/ $2e^-$ (Eq. S32 in Text S-VI of SI), this rate translates to 2.0 μM $^{\bullet}OH$ d^{-1} (range 0.4–7.7 μM $^{\bullet}OH$ d^{-1}) or cumulatively to 1,200 μM $^{\bullet}OH$ (range 240–4,620 μM $^{\bullet}OH$) during 20 months corresponding to a typical length of our experiments. If two $^{\bullet}OH$s mineralize OC to CO_2 (Eq. S35 in Text S-VII of SI), hydroxyl radicals mineralize cumulatively 600 μM (range 120–2,310 μM) OC in 20 months, which is more than the observed microbial mineralization in our experiments (128 μM in 20 months; range 66–593 μM in 20 months; Figure 1). Superoxide dismutase and catalase enzymes as well as other sinks scavenge $O_2^{\bullet-}$ and H_2O_2, and decrease the yield of $^{\bullet}OH$ production per produced $O_2^{\bullet-}$ or H_2O_2 (Bielski et al., 1985). The yields for $^{\bullet}OH$ production have been 1.4%–33% from the stoichiometry of $^{\bullet}OH$/2 e^- (Page et al., 2012, 2013). According to these yields, the bio-Fenton process mineralized 8.4–198 μM in our experiments during 20 months assuming a daily production of 4.0 μM extracellular e^-. The calculations above suggest that the bio-Fenton process was potentially able to explain a remarkable fraction of OC mineralized in our long-term experiments.

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

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

5. Conclusions

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

Author contributions

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

Declaration of competing interest

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

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Appendix A. Supplementary information

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

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Tables

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

Table 2. Parameters of the reactivity continuum model (Eq. 1 and 2). The values of *a* and ν were estimated by fitting the Eq. 1 to the measured concentrations of OC shown as markers 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

"-" not determined.

Table 3. Mineralization of OC (% of initial OC) induced by irradiation during 623 days in the 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 ± 0.5	22.6 ± 3.6
Valkea-Kotinen pH 5	9.6 ± 0.1	14.5 ± 0.6
Iso Valkjärvi pH 4	12.4 ± 0.3	16.0 ± 4.2
Iso Valkjärvi pH 5	9.1 ± 1.3	6.1 ± 2.8

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

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

Category	Experiment	% mineralized by acidification*			
		Valkea-Kotinen		Iso Valkjärvi	
		µmol L^{-1}†	Fraction (%)‡	µmol L^{-1}†	Fraction (%)‡
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

*Calculated according to the amount of mineralized OC during 20 months using the RC model (Eq. 1) and values of *a* and *v* given in Table 2.
†OC mineralized by acidification (µmol L^{-1}) = mineralized OC at pH 4 - mineralized OC at pH 5.
‡% mineralized by acidification = 100 (mineralized OC at pH 4 – mineralized OC at pH 5)/mineralized OC at pH 4.