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# Contribution of Microaerophilic Iron(II)-Oxidizers to Iron(III) Mineral Formation

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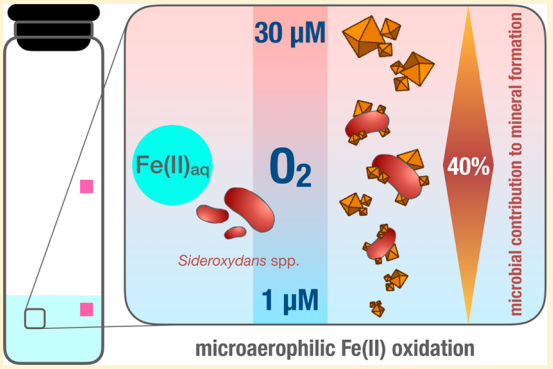
Supporting Information

**ABSTRACT:** Neutrophilic microbial aerobic oxidation of ferrous iron (Fe(II)) is restricted to pH-circumneutral environments characterized by low oxygen where microaerophilic Fe(II)-oxidizing microorganisms successfully compete with abiotic Fe(II) oxidation. However, accumulation of ferric (bio)minerals increases competition by stimulating abiotic surface-catalyzed heterogeneous Fe(II) oxidation. Here, we present an experimental approach that allows quantification of microbial and abiotic contribution to Fe(II) oxidation in the presence or initial absence of ferric (bio)minerals. We found that at 20 $\mu$M $O_2$ and the initial absence of Fe(III) minerals, an iron(II)-oxidizing enrichment culture (99.6% similarity to *Sideroxydans* spp.) contributed 40% to the overall Fe(II) oxidation within approximately 26 h and oxidized up to 3.6 × $10^{-15}$ mol Fe(II) $cell^{-1}$ $h^{-1}$. Optimum $O_2$ concentrations at which enzymatic Fe(II) oxidation can compete with abiotic Fe(II) oxidation ranged from 5 to 20 $\mu$M. Lower $O_2$ levels limited biotic Fe(II) oxidation, while at higher $O_2$ levels abiotic Fe(II) oxidation dominated. The presence of ferric (bio)minerals induced surface-catalytic heterogeneous abiotic Fe(II) oxidation and reduced the microbial contribution to Fe(II) oxidation from 40% to 10% at 10 $\mu$M $O_2$. The obtained results will help to better assess the impact of microaerophilic Fe(II) oxidation on the biogeochemical iron cycle in a variety of environmental natural and anthropogenic settings.






## INTRODUCTION

Microaerophilic Fe(II) oxidation represents a biological process contributing to iron redox cycling in many environments such as lacustrine and marine sediments,[1,2] groundwater seeps,[3] the rhizosphere,[4,5] deep sea vents,[6] and on the rusty surface of shipwrecks.[7,8] Under circumneutral pH and atmospheric $O_2$ concentrations, the abiotic oxidation of dissolved Fe(II) proceeds rapidly, forming poorly soluble Fe(III) (oxyhydr)oxides.[9,10] Under such conditions, microbial Fe(II) oxidation is kinetically outcompeted by fast abiotic oxidation. Optimum conditions for microbial Fe(II) oxidation are thus shifted toward niches where $O_2$ concentrations are sufficiently low to slow down the abiotic oxidation reaction and thus increase the bioavailability of dissolved Fe(II).[11−13] The range in oxygen concentrations where microaerophilic Fe(II) oxidation has been observed was determined in a variety of experimental setups such as in classical cultivation gradient setups (gradient tubes),[14] bioreactors,[5] or in microbial mats[15] to be in the range of 5−50 $\mu$M.[16,17]

However, the need of continuously low oxygen concentrations complicates the cultivation of microaerophilic Fe(II)-oxidizing bacteria in classical liquid microcosm culture setups.[18] Moreover, ferric iron minerals that get produced during the biotic and abiotic oxidation of Fe(II) serve as surface catalyst for rapid abiotic heterogeneous Fe(II) oxidation.[9,15] Even under low oxygen concentrations, heterogeneous Fe(II) oxidation kinetically outcompetes microbial Fe(II) oxidation as soon as sufficient reactive ferric mineral surface is produced. This surface-catalytic effect drastically enhances abiotic Fe(II) oxidation and subsequently decreases Fe(II) availability for microaerophilic Fe(II) oxidation.[19] The contribution of microaerophilic Fe(II)-oxidizing bacteria to the Fe(II) turnover was estimated to be 50% to 80% over a wide range of micro-oxic conditions.[13,20,21] Nevertheless, most studies lack an accurate quantification of microbial cells at constantly low $O_2$ concentrations, the possibility to follow Fe(II) oxidation and to derive microaerophilic Fe(II) turnover rates in the presence of abiotic homogeneous and autocatalytic abiotic heterogeneous Fe(II) oxidation.

The goal of our study was to fill this research gap and to establish an experimental approach that allows to quantify (i) the contribution of neutrophilic microaerophilic Fe(II)-oxidizing bacteria to the overall Fe(II) oxidation and

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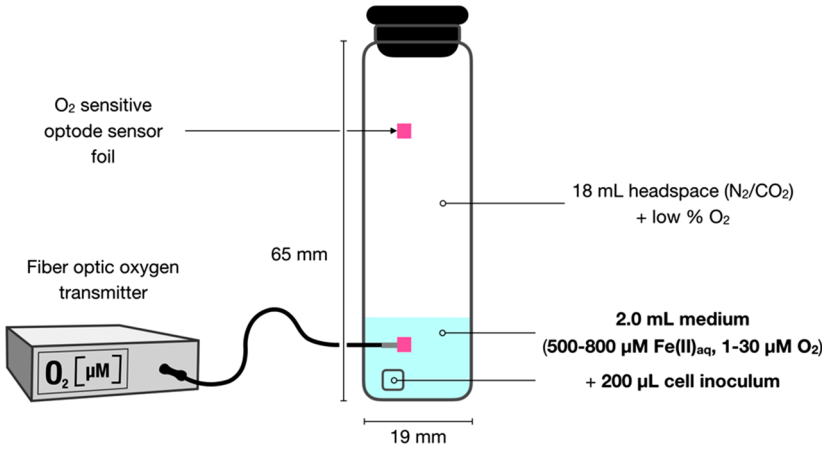


**Figure 1.** Schematic representation of the miniaturized microcosm setup, 18 mL $N_2/CO_2$ (v/v, 90/10) atmosphere in headspace and low % $O_2$. 2.2 mL MWMM medium amended with $Fe(II)_{aq}$ (500−800 $\mu$M) and constant $O_2$ concentrations ranging from 1 to 30 $\mu$M $O_2$. In the biotic setups, 2 × $10^6$ cells/mL of a microaerophilic Fe(II)-oxidizing enrichment culture were added and inhibited with sodium azide (15 mM) for abiotic control setups. $O_2$ concentrations in headspace and medium were adjusted and monitored noninvasively measuring with a fiber optic oxygen transmitter.

consecutive Fe(III) mineral formation at various (1−30 $\mu$M) $O_2$ concentrations in a laboratory-controlled classical liquid culture. Moreover, we envisaged to (ii) quantify the impact of Fe(III) mineral particles on the acceleration of the abiotic Fe(II) oxidation. For this, we incubated a microaerophilic Fe(II)-oxidizing enrichment culture (99.6% similarity to *Sideroxydans* spp., isolated from a rice paddy field (Vercelli, Italy)) in miniaturized microcosms and followed the oxidation of dissolved ferrous iron, as well as cell numbers at a range of low oxygen concentrations (1−30 $\mu$M $O_2$). We quantified minimum and maximum threshold $O_2$ concentrations for optimum microbial Fe(II) oxidation for this enrichment culture and determined the theoretical Fe(II) turnover by abiotic (homogeneous and heterogeneous) oxidation reactions in biotically incubated and abiotic control setups. With a set of experiments, we were able to decipher the extent in Fe(II) oxidation for this microaerophilic enrichment in the presence and absence of surface-reactive minerals. Moreover, the presented approach and gathered data offers the possibility to compare Fe(II) turnover rates of various microaerophilic strains and enrichment cultures[22] and allows one to estimate the impact these environmentally abundant microaerophilic communities can have on the Fe(II) oxidation in the respective habitat, e.g., acid-mine drainage,[23] marine sediments,[24] or wetlands.[4]

## MATERIALS AND METHODS

**Experimental Setup.** Miniaturized microcosms were prepared in 20 mL glass vials (with a flat bottom), filled with 2 mL of anoxic Modified Wolfe's Mineral Medium (MWMM) containing 550−800 $\mu$M dissolved ferrous iron ($Fe(II)_{aq}$) (preparation details see Supporting Information, SI) and sealed with butyl rubber stoppers. The headspace was exchanged with $N_2/CO_2$ (v/v; 90/10) prior to inoculation and adjustment of $O_2$ concentrations. The large headspace volume allowed to maintain constantly low and stable $O_2$ concentrations in the medium over the course of the incubations. For abiotic control incubations, sodium azide ($NaN_3$, 15 mM) was added to individual microcosms.[20,25] Setups were prepared in triplicate unless otherwise stated.

**Inoculum.** A microaerophilic Fe(II)-oxidizing enrichment culture (99.6% similarity to *Sideroxydans* spp., 97% similarity to *S. lithotrophicus* ES-1 (based on 16S rRNA)), isolated from a rice paddy field (Vercelli, Italy), was precultivated on zerovalent iron (ZVI) plates[26] and harvested (SI, Culture preparation). Prior to inoculation and to minimize the effect of surface reactive minerals and abiotic heterogeneous Fe(II) oxidation, biomineral residues (SI, Mössbauer spectroscopy, Figure S3) from the preculture were removed by dissolution: cell suspensions were washed with anoxic sterile 0.1% oxalate solution for 2 min before washing with a bicarbonate buffer solution (10 mM, pH 6.8). Shorter oxalate washing procedures did not dissolve all mineral precipitates, while longer washing steps resulted in partial cell death. Cell viability after washing was verified by fluorescence microscopy and D/L staining (SI, Figure S1). For each setup 0.2 mL of cell suspension were transferred into each miniaturized microcosm by needle injection through the butyl rubber stopper.

**Geochemical Analyses and Cell Quantification.** Vials were equipped with optode foil sensors (4 × 4 $mm^2$) (PSt3, PreSens, Regensburg, Germany) glued (Silicone rubber compound RS692−542, RS Components, Northants, U.K.) to the inner side of the glass wall (one located at the bottom where it was covered with medium, a second one located in the headspace). Oxygen was then quantified noninvasively reading from outside the vial using a fiber optic oxygen meter (FiBox3, PreSens, Regensburg, Germany) as described in Maisch et al. (2016)[27] (Figure 1).

For $Fe(II)_{aq}$ quantification, 150 $\mu$L sample were taken and centrifuged for 10 min at 3.600 rpm under anoxic conditions (glovebox, 100% $N_2$). The supernatant was acidified in 1 M HCl to prevent Fe(II) oxidation outside the glovebox and was consecutively analyzed by the Ferrozine assay.[28] Due to small total sample volume, a quantification of total Fe(II) and Fe(III) was not possible and only dissolved $Fe(II)_{aq}$ was quantified. The pellet that remained after centrifugation was broken up and shaken for 10 s on a vortexer. A subsample was fixed in paraformaldehyde (10%; PFA) for cell quantification using constant-sheath flow cytometry (see SI for sample preparation). Doubling times ($T_d$) for cell growth were calculated for the initial incubation period of 45 h when >10% of the initially present Fe(II) was still bioavailable for energy generation and optimum growth yield conditions were expected.



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Iron minerals before and after incubation were identified by Mössbauer spectroscopy (SI, Mössbauer spectroscopy, Table S1). Statistical analysis was performed as described in the SI (SI, Statistical treatment).

**$O_2$ Adjustment in Miniaturized Microcosms.** In order to quantify optimum $O_2$ conditions at which microaerophilic Fe(II)-oxidizing bacteria successfully compete for Fe(II) with the abiotic Fe(II) oxidation, the enrichment culture was grown in FeS gradient tubes.[14] Oxygen and $Fe(II)_{aq}$ concentrations were quantified along the vertical gradient in the tube using microsensors (methods described in Lueder et al., 2018[17]). The recorded $O_2$ concentrations in the characteristic growth band represent the basis for the choice of the $O_2$ range used for the incubation in our miniaturized microcosm (SI, Growth conditions for microaerophilic Fe(II)-oxidizing enrichment culture, Figure S2). Ambient air was injected into the headspace of the miniaturized microcosms by a gastight syringe through a sterile filter (0.22 $\mu$m) to reach dissolved oxygen concentrations in the medium of 1, 5, 10, 20, and 30 $\mu$M $O_2$. Subsequently, the microcosms were gently shaken to equilibrate $O_2$ between headspace and medium and to equally distribute the cells in the medium.

**Quantification of Fe(II) Oxidation Kinetics.** Abiotic (homogeneous and heterogeneous) $Fe(II)_{aq}$ oxidation rates and half-life for $Fe(II)_{aq}$ ($^{Fe(II)}t_{1/2}$) were calculated for every sampling time point in each setup. This allows one to follow changes in rates and Fe(II) half-lives throughout the entire incubation at designated sampling points and to find the time frame in which microbial Fe(II) oxidation can compete with the abiotic Fe(II) oxidation. Given that the overall Fe(II) oxidation is a combination of homogeneous and heterogeneous iron oxidation, the individual oxidation rates for each reaction pathway were calculated individually following the approach as presented in Lueder et al. (2018[17]):

The homogeneous oxidation of $Fe(II)_{aq}$ by dissolved $O_2$ to Fe(III) (as Fe(III) hydroxide precipitation) is as follows:[29]

$$Fe(II)_{aq} + 0.25O_2 + 2.5H_2O \rightarrow Fe(OH)_3 + 2H^+ \quad (1)$$

Accounting only for homogeneous $Fe(II)_{aq}$ oxidation ($FeOx_{hom}$) in the setups that contain oxalate-washed cells (no initial Fe(III) precipitates as residues from precultivation), the kinetic rate law ($r(FeOx_{hom})$):

$$-\frac{d[Fe(II)_{aq}]_{hom}}{dt} = k \times [Fe(II)_{aq}] \quad (2)$$

with $k = k_0\ [O_2]\ [OH^-]^2$, universal rate constant for homogeneous $Fe(II)_{aq}$ oxidation by $O_2$, $k_0 = 2.3 \times 10^{14}$ mol$^3$ L$^{-3}$ s$^{-1}$ at 25 °C,[9] and $[Fe(II)_{aq}]$ as the measured $Fe(II)_{aq}$ concentration at time point $t$.

The heterogeneous oxidation of $Fe(II)_{aq}$ ($FeOx_{het}$) is described by by Fe(III) minerals that accelerate Fe(II) oxidation via the catalyzing effect of mineral surfaces.[30] In the setups with oxalate-washed cells, the amount of Fe(III) minerals is considered to be equal to the concentration of oxidized $Fe(II)_{aq}$:[9]

$$[Fe(III)] = [Fe(II)_{aq}]_{t0} - [Fe(II)_{aq}] \quad (3)$$

The rate law of heterogeneous $Fe(II)_{aq}$ ($r(FeOx_{het})$) oxidation can be described as follows:

$$-\frac{[Fe(II)_{aq}]_{het}}{dt} = k' \times [Fe(III)] \times [Fe(II)_{aq}] \quad (4)$$

with $k' = \frac{k_{s,0} \times [O_2] \times K}{[H^+]}$, specific rate constant for the heterogeneous reaction, $k_{s,0}$ being 73 mol L$^{-1}$ s$^{-1}$[9] and the dimensionless adsorption constant of ferrous iron on ferric hydroxide $K$ being $10^{-4.85}$.[31] Although this adsorption constant was empirically determined for abiogenic minerals with ideal crystal lattice properties, we consider it as an appropriate approximation for the iron mineral surfaces formed during incubation.

The combined rate equation of the total $Fe(II)_{aq}$ oxidation ($r(FeOx_{total})$) includes both, homogeneous ($FeOx_{hom}$) and heterogeneous ($FeOx_{het}$) oxidation, is described as follows:[30]

$$-\frac{d[Fe(II)_{aq}]}{dt} = (k + k' \times [Fe(III)]) \times [Fe(II)_{aq}] \quad (5)$$

On the basis of this equation, the half-life for $Fe(II)_{aq}$ was calculated to be the following:

$$t_{1/2,het} = \frac{\ln(2)}{k' \times [Fe(III)]} \quad (6)$$

The total Fe(II) oxidation rates ($r(FeOx_{total})$) for abiotic and biotic incubation setups were calculated as follows:

$$\text{abiotic setups: } r(FeOx_{total}) = r(FeOx_{hom}) + r(FeOx_{het}) \quad (7)$$

$$\text{biotic setups: } r(FeOx_{total}) = r(FeOx_{hom}) + r(FeOx_{het}) + r(FeOx_{bio}) \quad (8)$$

Microaerophilic Fe(II) oxidation rates ($r(FeOx_{bio})$ with $Fe(II)_{aq}$ cell$^{-1}$ hour$^{-1}$) within the initial incubation phase were calculated via the following equation:

$$\text{microb. Fe(II) ox. per cell} = \{[Fe(II)_{aq}]_{tx,abio} - [Fe(II)_{aq}]_{tx,bio}\} \times t^{-1} \times n^{-1} \quad (9)$$

At time point $x$, with $t$ as elapsed incubation time in hours and $n$ as the total cell number.

Although microbial Fe(II) oxidation rates were determined for each sampling interval, the microbial contribution to the total Fe(II) oxidation ($FeOx_{total}$) was then quantified for the initial incubation phase of 0−26 h, in which approximately 50% of the initially available Fe(II) was oxidized and differences between biotic and abiotic setups were most prominent. Within this initial incubation phase, fastest cell doubling times and highest extent in microbial Fe(II) oxidation were expected. The microbial contribution to the total Fe(II) oxidation was calculated as follows

$$\text{biol. contribution } (\%)_t = \frac{[Fe(II)_{aq}]_{tx,abio} - [Fe(II)_{aq}]_{tx,bio}}{[Fe(II)_{aq}]_{t0,bio} - [Fe(II)_{aq}]_{tx,bio}} \times 100 \quad (10)$$

## RESULTS AND DISCUSSION

**Effect of $O_2$ Concentrations on Microaerophilic Fe(II) Oxidation Kinetics.** Microaerophilic Fe(II)-oxidizing bacteria can be found where Fe(II) is bioavailable and $O_2$ is sufficiently low that microorganisms can kinetically outcompete abiotic Fe(II) oxidation. Although a few studies performed estimations on microbial microaerophilic Fe(II) oxidation rates,[16,32−34] little is known about the microbial contribution to the overall Fe(II) oxidation at constantly low $O_2$ concentrations in the presence of abiotic homogeneous and autocatalytic abiotic heterogeneous Fe(II) oxidation. We therefore quantified the microbial contribution to Fe(II) oxidation and estimated biological Fe(II) turnover rates at



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different microoxic $O_2$ concentrations (10, 20, and 30 $\mu$M $O_2$). The cell inoculum used for this experiment was prewashed with oxalate solution to remove any mineral residues from precultivation and to suppress initially autocatalytic and rapid abiotic heterogeneous Fe(II) oxidation.

While in all setups, preadjusted oxygen concentrations remained constant throughout incubation (Figure S5), $Fe(II)_{aq}$ decreased rapidly by more than 50% in all biotic setups within the initial incubation phase of 26 h. In the abiotic control, significantly less Fe(II) oxidation was observed within this initial incubation phase at 10 and 20 $\mu$M $O_2$ (Figure 2A)

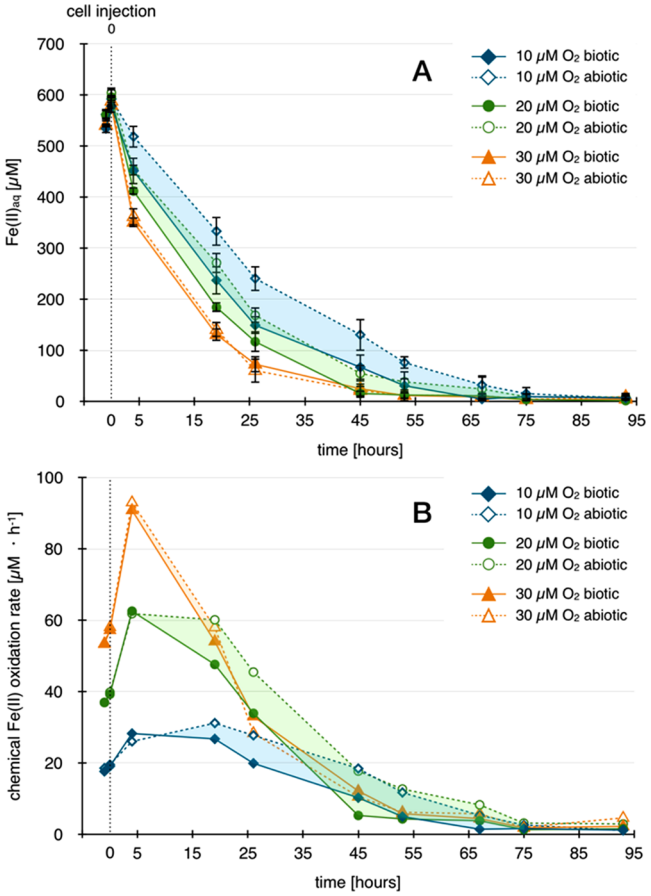


**Figure 2.** (A) $Fe(II)_{aq}$ concentrations in biotic and abiotic incubations with oxalate-washed cells from a microaerophilic Fe(II)-oxidizing enrichment culture at different $O_2$ concentrations; error bars represent standard deviations from experimental triplicates; shaded areas represent the difference in Fe(II) oxidation between abiotic and biotic incubations at similar $O_2$ concentrations that can be attributed to the impact of microaerophilic Fe(II)-oxidizing bacteria. (B) Calculated abiotic $Fe(II)_{aq}$ oxidation rates (homogeneous + heterogeneous Fe(II) oxidation).

compared to biotic incubations. At 30 $\mu$M $O_2$, the total Fe(II) oxidation rate (incl. both biotic and abiotic Fe(II) oxidation) was highest, with a nonsignificant difference between $Fe(II)_{aq}$ oxidation in biotic vs abiotic setups, which suggested that the microaerophilic Fe(II)-oxidizing bacteria did not impact nor enhance the overall Fe(II) turnover.

The calculated half-life for $Fe(II)_{aq}$ at circumneutral pH conditions (in our experiments pH 6.8) negatively correlated with $O_2$ concentrations. While the half-life of Fe(II) in fully oxygenated water is <40 min, it increased to 4 and 20 h (depending on $O_2$ and Fe(II)) under low oxygen concentrations (Table 1). Incubations at 30 $\mu$M $O_2$ suggest that the

**Table 1. Half-life of $Fe(II)_{aq}$ [$^{Fe(II)}t_{1/2}$ in Hours] at Different $O_2$ Concentrations in Biotic and Abiotic Incubations with Initial Absence of Fe(III) Minerals**[a]

| | $^{Fe(II)}t_{1/2}$ 10 µM $O_2$ | | $^{Fe(II)}t_{1/2}$ 20 µM $O_2$ | | $^{Fe(II)}t_{1/2}$ 30 µM $O_2$ | |
|---|---|---|---|---|---|---|
| time | abiotic | biotic | abiotic | biotic | abiotic | biotic |
| 4 | 40.2 | **23.4** | 9.8 | **8.1** | 4.3 | **4.3** |
| 19 | 11.4 | **8.6** | 4.5 | **3.6** | 2.2 | **2.2** |
| 26 | 8.4 | **6.9** | 3.4 | **3.1** | 1.8 | **2.0** |
| 45 | 6.4 | **5.8** | 2.7 | **2.6** | 1.7 | **1.8** |
| 53 | 5.7 | **5.4** | 2.6 | **2.5** | 1.7 | **1.7** |
| 67 | 5.3 | **5.1** | 2.5 | **2.4** | 1.7 | **1.7** |

[a]Uncertainties for calculated Fe(II) half-lives are $\leq \pm$ 0.08.

shorter the Fe(II) half-lives are, the less bioavailable Fe(II) was over time for microaerophilic Fe(II)-oxidizing bacteria. In contrast, incubations at 10 and 20 $\mu$M $O_2$ quantitatively confirmed that 2- and 5-fold longer Fe(II) half-lives prolonged the persistence of Fe(II) and therefore increased the bioavailability for microaerophilic Fe(II)-oxidizing bacteria and the ability to compete with abiotic Fe(II) oxidation.[16]

Incubations at 30 $\mu$M $O_2$ showed a slight increase in cell numbers within the initial 45-h incubation period. However, the initial cell doubling times were significantly longer compared to incubations at 10 and 20 $\mu$M $O_2$ (Table 2). The increase in cell numbers within the first 45 h is reflected in the fastest doubling times at all $O_2$ concentrations (Table 2) that positively correlate to the highest extent in Fe(II) oxidation with only 10% of the initial Fe(II) left in all treatments. After 45 h, cell doubling times increased around 3-fold to more than 150 h until the end of the incubation which suggests that cells were potentially limited in Fe(II) bioavailability. At 10 and 20 $\mu$M $O_2$, Fe(II) half-lives were longer and total Fe(II) oxidation rates low enough to detect a biotic impact on total Fe(II) oxidation. The difference in the extent of Fe(II) consumption between abiotic and biotic setups within the initial incubation phase is attributed to microbial Fe(II) turnover.[17] Microbial contribution to Fe(II) oxidation and microbial Fe(II) turnover rates reached a maximum within the initial incubation phase of 26 h (10 $\mu$M and 20 $\mu$M $O_2$: 1.1–8.5 × $10^{-15}$ and 1.7–3.0 × $10^{-16}$ mol $Fe(II)_{aq}$·cell$^{-1}$·h$^{-1}$, respectively) (Table 2) and suggest a contribution of up to 40% by microaerophilic bacteria to the overall Fe(III) mineral formation. In agreement with these data, we measured highest cell growth and fastest doubling times ($T_d$ 40–42 h) in setups that were incubated at 20 and 10 $\mu$M $O_2$ (Table 2).

The setup with the oxalate-washed cells had the benefit of initially decreasing the abiotic heterogeneous Fe(II) oxidation and thus prolonging the bioavailability for the microaerophilic Fe(II)-oxidizing enrichment. However, over time also in these setups Fe(III) minerals formed which initiated the surface-catalyzed reaction and enhanced the autocatalytic heterogeneous Fe(II) oxidation (Figure S6). The increase in abiotic Fe(II) oxidation rates consequently decreased Fe(II) bioavailability and thus increased the pressure on microbial Fe(II) oxidation to compete with abiotic Fe(II) oxidation reactions. These calculated abiotic (heterogeneous and homogeneous) Fe(II) oxidation rates considerably differed between the different $O_2$ treatments in both, the biotic and abiotic control


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**Table 2. Mean Cell Numbers (and Standard Deviation) from Experimental Triplicates, Doubling Times [$T_d$] within the Initial 45 h and Mean $Fe(II)_{aq}$ Oxidation Rates Per Cell [$10^{-15}$ mol cell$^{-1}$ h$^{-1}$] over the Course of Incubations at 10, 20, and 30 $\mu$M $O_2$ with the Initial Absence of Fe(III) Minerals**

| | 10 $\mu$M $O_2$ | | | 20 $\mu$M $O_2$ | | | 30 $\mu$M $O_2$ | | |
|---|---|---|---|---|---|---|---|---|---|
| time | cell number [$10^6$ mL$^{-1}$] | $T_d$ | microb. $Fe(II)_{aq}$ ox. rate | cell number [$10^6$ mL$^{-1}$] | $T_d$ | microb. $Fe(II)_{aq}$ ox. rate | cell number [$10^6$ mL$^{-1}$] | $T_d$ | microb. $Fe(II)_{aq}$ ox. rate |
| 0 | 1.96 (±0.06) | 41.8 | | 2.03 (±0.11) | 39.6 | | 2.17 (±0.08) | 52.1 | 0.12 |
| 19 | 2.38 (±0.13) | | 8.53 | 2.56 (±0.08) | | 0.31 | 2.87 (±0.20) | | 0.04 |
| 26 | 3.12 (±0.18) | | 2.12 | 3.46 (±0.26) | | 0.35 | 3.12 (±0.23) | | [a] |
| 45 | 4.13 (±0.48) | | 1.13 | 4.46 (±0.52) | | 0.17 | 3.95 (±0.42) | | [a] |
| 53 | 3.92 (±0.21) | | 0.34 | 5.01 (±0.41) | | 0.14 | 3.62 (±0.36) | | 0.07 |
| 67 | 4.50 (±0.26) | | 0.22 | 4.83 (±0.24) | | 0.11 | 4.43 (±0.32) | | 0.04 |
| 75 | 4.26 (±0.32) | | 0.09 | 5.87 (±0.18) | | 0.09 | 5.13 (±0.56) | | 0.02 |
| 93 | 4.48 (±0.14) | | [a] | 5.21 (±0.34) | | 0.15 | 4.88 (±0.35) | | 0.01 |

[a]Could not be calculated due to negative rate; for comparability with Table 3: initial (0–26 h) total Fe(II) oxidation rates ($^{ini}\nu$) were calculated for abiotic and biotic setups, respectively (see SI).

setup. The total abiotic Fe(II) oxidation rate increased 3-fold from 10 to 30 $\mu$M $O_2$ treatments (Figure 2B) while $O_2$ concentrations remained constantly low (Figure S5). On the basis of eqs 2, 4, and 5 for homogeneous and heterogeneous Fe(II) oxidation, homogeneous Fe(II) oxidation was calculated to be the main abiotic oxidation reaction within the first 1–5 h, while after approximately 5 h, heterogeneous oxidation dominated (Figure S6). After the initial 26 h, the abiotic oxidation rates slowed down in all $O_2$ treatments, likely caused by the decrease in Fe(II) availability (the varying factor for homogeneous and heterogeneous rate calculations, see eqs 2 and 4) (Figure 2B). The shifts in Fe(II) oxidation rates were also reflected in Fe(II) half-life that decreased drastically when Fe(II) oxidation proceeded and heterogeneous oxidation rates accelerated (Figure S6). This decrease in calculated Fe(II) half-lives correlated to increasing abiotic Fe(II) oxidation rates. On the basis of these calculated abiotic rates, a maximum Fe(II) turnover of approximately 30–90 $\mu$M Fe(II) h$^{-1}$ at $O_2$ concentrations of 10–30 $\mu$M $O_2$ can theoretically be reached, which is approximately 20% more compared to the measured data. The slower abiotic oxidation rates that were determined experimentally might be attributed to altered surface properties of the ferric precipitates formed during oxidation in the presence of microbial biomass. Freshly formed low crystalline minerals have high affinity to attach to cell surfaces and EPS,[35] resulting in the formation of mineral aggregates,[36] lowering the number of active mineral surface sites,[37,38] and thus, decreasing the reaction rate.[9]

Our data on the oxidation of Fe(II) in our biotic and abiotic incubations suggests that the microbial contribution to $Fe(II)_{aq}$ oxidation reaches a minimum at $O_2$ 30 $\mu$M (Figures 3 and 4). However, different to Krepski et al. (2013)[39] who did not observe microbial growth at $O_2$ concentrations >29 $\mu$m $O_2$, we noted an increase in cell numbers (Figure S3) which suggests that cells were still able to grow although contribution to Fe(II) oxidation was lower compared to incubations at 10 and 20 $\mu$M $O_2$. Emerson and Moyer (1997)[14] and Neubauer et al., (2002)[5] roughly estimated optimum $O_2$ conditions at 5–14 $\mu$M $O_2$ for a microaerophilic *Sideroxydans* spp. strain, the closest identity to the enrichment culture used in the current study (99.6% 16S rRNA sequence identity), being in agreement with our measurements that showed the best growth, shortest doubling times, and highest microbial Fe(II) oxidation between 10 and 20 $\mu$M $O_2$.

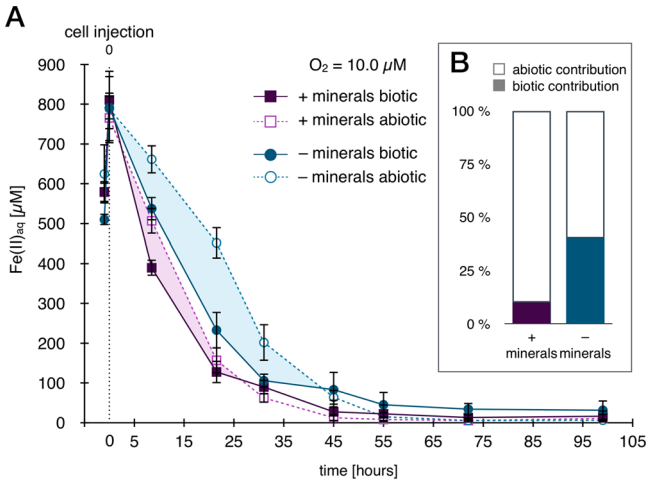


**Figure 3.** (A) $Fe(II)_{aq}$ oxidation in the presence and absence of initial abiogenic/biogenic ferrihydrite minerals in biotic and abiotic incubations of cells by a microaerophilic Fe(II)-oxidizing enrichment culture at 10 $\mu$M $O_2$; error bars represent standard deviations from experimental triplicates. (B) Relative biotic and abiotic contribution to total Fe(II) oxidation during the incubation at 10 $\mu$M $O_2$ in the presence and absence of initial a/biogenic ferrihydrite minerals.

The cell doubling times observed in the current study are relatively long but still comparable with reported values for *Sideroxydans* spp. by Weiss et al., (2007),[40] Druschel et al, (2008)[16] and Haedrich et al. (2019).[41] That indicates that cells were potentially not growing optimally but still metabolically active to enhance Fe(II) oxidation. This in turn suggests, that the reported data on the extent of microbial Fe(II) oxidation might represent only minimal estimates on what the contribution under optimized conditions might be. However, in micro-oxic environments, where habitat parameters are often not at the physiological optimum for microbial communities, the reported Fe(II) turnover rates help to quantitatively estimate the microaerophilic contribution to Fe(II) oxidation for a wide range of micro-oxic (10–30 $\mu$M $O_2$) conditions.

In addition to the obtained information about Fe(II) oxidation rates, the developed experimental setup (Figure 1) offers the possibility to cultivate microaerophilic Fe(II)-oxidizing bacteria in a nongradient based liquid microcosm at constantly low $O_2$ levels. The microaerophilic Fe(II)-oxidizing enrichment culture was successfully transferred for



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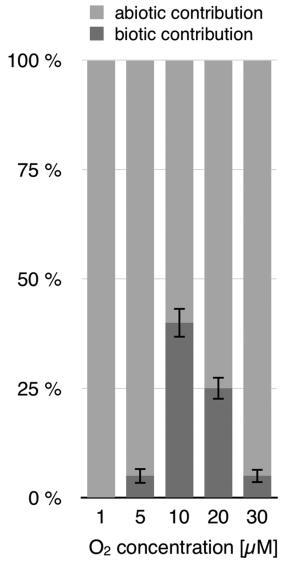


**Figure 4.** Relative abiotic and biotic contribution to total $Fe(II)_{aq}$ oxidation during incubations of a microaerophilic Fe(II)-oxidizing enrichment culture at an initial $Fe(II)_{aq}$ concentration of 600−800 $\mu$M and variable $O_2$ concentrations ranging from 1 to 30 $\mu$M in the absence of initial abiogenic or biogenic ferrihydrite minerals; error bars represent mean absolute deviations from triplicates.

currently more than 38 transfers using the presented approach. The classical cultivation procedure using gradient tubes works perfectly well for isolation of microaerophilic Fe(II)-oxidizing bacteria.[14,40] However, cultivation in liquid culture is essential to quantify microbial turnover rates and to assess their impact on the iron cycle under micro-oxic conditions.

**Effect of Mineral Surfaces on Fe(II) Oxidation Kinetics.** Several studies hypothesized and demonstrated that the surface-catalyzed heterogeneous Fe(II) oxidation can not only accelerate the abiotic Fe(II) oxidation but also decrease the relative amount of Fe(II) available for microaerophilic Fe(II)-oxidizing microorganisms.[5,9,14,33] Thus, far, only a few studies attempted to quantify to which extent microbial Fe(II) oxidation can be outcompeted by the autocatalyzed abiotic heterogeneous Fe(II) oxidation in the presence of mineral surfaces.[15−17,34] The simultaneous occurrence of microbial, abiotic homogeneous and abiotic heterogeneous Fe(II) oxidation calls for a reliable experimental setup in which the individual contributions to Fe(III) mineral formation can be quantified in liquid culture. The method presented here enabled us to confirm the hypothesis that Fe(II) availability for microaerophilic Fe(II)-oxidizing bacteria can be limited due to the acceleration of the abiotic heterogeneous Fe(II) oxidation due to the presence of Fe(III) minerals.[5,15,30] Moreover, we could quantify to which extent the contribution of biotic and abiotic Fe(II) oxidation to mineral formation is controlled by (1) the $O_2$ concentration and (2) the presence of ferric mineral catalytic surfaces. Mineral particles that were produced and associated with cells during precultivation were identified as ferrihydrite (Mössbauer spectroscopy, Figure S3). In order to quantify the effect of these (bio)minerals on the overall Fe(II) oxidation kinetics, the microaerophilic Fe(II)-oxidizing enrichment culture was incubated in the initial absence and presence of ferrihydrite mineral particles at 10 $\mu$M $O_2$ and approximately 800 $\mu$M $Fe(II)_{aq}$. Within the initial 22-h incubation phase, the overall extent of Fe(II) being oxidized increased significantly from less than 75% in biotic incubations with oxalate-washed cells compared to more than 80% when cells were not washed and ferrihydrite minerals from precultivation were still present (Figure 3A).

Differences in Fe(II) concentrations between biotic and abiotic incubations using nonoxalate washed cells showed only a maximum of 10% faster Fe(II) oxidation in biotic incubations within the initial 22 h of incubation (Table 3).

**Table 3. $Fe(II)_{aq}$ Oxidation Kinetics in Biotic and Abiotic Incubations at 10 $\mu$M $O_2$ in the Presence and Absence of Initial Abiogenic/Biogenic Ferrihydrite Minerals[a]**

| $Fe(II)_{aq}$ oxidation [$\mu$M Fe(II)·$h^{-1}$] | + minerals | − minerals |
|---|---|---|
| ${}^{ini}\nu_{abio}$ ($r^2$) | 28.1 (0.83) | 15.7 (0.90) |
| ${}^{ini}\nu_{bio}$ ($r^2$) | 30.8 (0.85) | 25.8 (0.94) |

[a] ${}^{ini}\nu$ − Fe(II) oxidation rate, simple linear fitting results (0-22 h) in abiotic (${}^{ini}\nu_{abio}$) and biotic (${}^{ini}\nu_{bio}$) incubations ($r^2$ − coefficient of determination).

In contrast to that, the microbial contribution to total Fe(II) oxidation was considerably higher by approximately 40% ($p < 0.05$) when no minerals from precultivation were present compared to treatments where iron minerals were not removed prior to incubation, as already shown for the previous 10 $\mu$M $O_2$ setup. This is also reflected in the initial Fe(II) oxidation kinetics (Table 3), where abiotic Fe(II) oxidation rates in mineral-free treatments were slower (compared to unwashed setups) and a significantly faster biotic Fe(II) oxidation rate. This demonstrated that the presence of ferric (oxyhydr)oxide mineral particles, as a metabolic product of microaerophilic Fe(II)-oxidizing bacteria and as a reaction product of the abiotic Fe(II) oxidation, not only most dominantly accelerated the overall (and mainly surface-catalytic) Fe(II) oxidation in biotic and abiotic setups,[31] but also decreased the microbial contribution to Fe(II) oxidation to only 10% on average within the initial 22-h incubation phase (Figure 3B). The rate of total $Fe(II)_{aq}$ oxidation in the abiotic setup was almost 2-fold faster within these initial 22 h in the presence of minerals compared to the abiotic treatment that was incubated with inactivated cells that did not carry any mineral residues from precultivation (Table 3).

**Lower Oxygen Threshold Concentrations for Microbial Contribution to Fe(II) Oxidation.** Incubations at 1 and 5 $\mu$M $O_2$ with oxalate-washed cells address an open question raised in Chan et al. (2016):[13] *What is the lower $O_2$ concentration limit of microbial Fe oxidation?* Different from gradient-based cultivation systems, where cells seek for their ideal growth conditions, the presented method allows to adjust $O_2$ concentrations to a minimum that still enables them to metabolically oxidize Fe(II) under $O_2$-limiting conditions. The $O_2$ concentrations during the incubations were at the lower microoxic end, allowing $Fe(II)_{aq}$ generally to persist longer compared to incubations at higher $O_2$. With respect to Fe(II) oxidation, no clear distinction between abiotic and biotic Fe(II) oxidation rates were measured at 1 $\mu$M $O_2$ and 5 $\mu$M $O_2$ (Figure S7). After 30 h mean $Fe(II)_{aq}$ concentrations were approximately 5% lower in biotic active setups incubated at 5 $\mu$M $O_2$ compared to abiotic controls. However, this insignificant difference suggests that microbial contribution to total Fe(II) oxidation was considerably low (<5%) at these low $O_2$ concentrations. Taking into account the increase in cell density within 96 h from initially $1.11 \times 10^6$ to $4.60 \times 10^6$


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$mL^{-1}$ at 5 $\mu$M $O_2$ and from $1.07 \times 10^6$ to $3.97 \times 10^6$ $ml^{-1}$ at 1 $\mu$M $O_2$ (Table S2), respectively indicates some microbial activity. However, cell growth and doubling times were comparatively low as against incubations at 10 and 20 $\mu$M $O_2$ (Tables 2 and S2). These data suggest that cells were potentially limited in $O_2$ availability at such low (1−5 $\mu$M $O_2$) concentrations. However, the observed growth under these conditions and viable transfers from these setups imply that cells were still active and able to reproduce. At 5−10 $\mu$M $O_2$, microbial Fe(II) oxidation of this culture became more dominant compared to abiotic Fe(II) oxidation, allowing bacteria to contribute by up to 40% to total Fe(II) oxidation and mineral formation (Figure 4).

With the set of experiments that is presented in this study, we were able to quantify that the biotic impact on Fe(II) oxidation is less than 5% at 1 and 5 $\mu$M $O_2$ and micro-oxic (30 $\mu$M $O_2$) $O_2$ concentrations, it significantly increased to approximately 40% at 10 and 20 $\mu$M $O_2$ (Figure 4) when surface-catalyzing minerals were absent. When Fe(III) minerals were present and served as a catalyst for heterogeneous, abiotic Fe(II) oxidation the biotic contribution strikingly decreased but still remained detectable by approximately 10% (Figure 3A/B).

**Implications.** The current study provides a description of an experimental setup that allows to quantify microaerophilic Fe(II) oxidation rates and to compare them to abiotic Fe(II) oxidation. The presented setup allows to grow microaerophilic Fe(II)-oxidizing bacteria in liquid culture. So far, quantitative liquid culture microcosm setups have not been successful for microaerophilic Fe(II)-oxidizing bacteria, as constantly low $O_2$ concentrations are difficult to sustain and heterogeneous Fe(II) oxidation dominated due to large cultivation volumes. Due to the relatively small liquid volumes in our setups, the relatively large surface to medium area and an extensive headspace volume, $O_2$ gradients do not establish in a very defined way in the culture medium, while $O_2$ concentrations remained constant over the course of the incubations. Performing inoculation with oxalate-washed cell cultures minimizes initial heterogeneous Fe(II) oxidation and allows to distinguish between abiotic and biotic Fe(II) oxidation rates. The quantification of maximum oxidation rates for microbial Fe(II) turnover under optimized and laboratory-controlled conditions can help to understand the impact microaerophilic bacteria can have on the environmental iron cycle. Moreover, the presented approach can be readily applied to characterize isolated and mutant strains deficient in putative genes that regulate Fe(II) oxidation or to assess the relative contribution of different members of a consortium to total Fe(II) oxidation under varying Fe(II) and $O_2$ concentrations. Although heterogeneous Fe(II) oxidation may be the key driver for Fe(II) oxidation in many natural habitats when $O_2$ is present, microaerophilic Fe(II)-oxidizing bacteria may still be able to compete for Fe(II) oxidation (i) when Fe(II) is continuously formed, e.g., by Fe(III)-reducing organisms that inhabit the same environmental niche[26,42] or (ii) Fe(II) is constantly supplied to the open system, e.g., at hydrothermal vents.[6,43] Providing a method not only to quantify the Fe(II) turnover at variable and constant $O_2$ conditions, our approach represents a quantitative method for a separation of biotic and abiotic Fe(II) oxidation rates, as well as for the accurate characterization of the optimum $O_2$ range for microaerophilic Fe(II)-oxidizing bacteria.

## ■ ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.est.9b01531.

Culture preparation; medium preparation; oxalate washing and cell viability; growth conditions for microaerophilic Fe(II)-oxidizing enrichment culture; Mössbauer spectroscopy; cell quantification; statistical treatment; Oxygen concentrations during incubations at 10, 20, and 30 $\mu$M $O_2$; calculated heterogeneous and homogeneous Fe(II) oxidation rates; and additional references (Figures S1−S7 and Tables S1 and Table S2) (PDF)

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The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

### Notes

The authors declare no competing financial interest.

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