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The influence of iron-binding ligands in the corrosion of carbon steel driven by iron-reducing bacteria

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Iron reducing bacteria (IRB) are thought to accelerate the corrosion of steel by removing the Fe(III)-oxide passivating layer through iron respiration. We investigated the effect of the iron-binding ligands oxalate, malonate, and succinate on the corrosion of carbon steel driven by the IRB *Shewanella oneidensis*. These dicarboxylates were found to accelerate the corrosion of carbon steel driven by IRB up to 2.6 times more than the abiotic experiment without dicarboxylates. Iron dissolution was enhanced by dicarboxylates, and this influenced the ability of planktonic cells to engage in iron respiration. The strong iron-binding ligands oxalate and malonate supported iron reduction by planktonic cells, whereas in the experiments with succinate or without dicarboxylate, a direct contact mechanism with the solid Fe(III)-oxide was observed. Faster microbial respiration rates were found in experiments with succinate than with oxalate or malonate, suggesting a competition for iron between the microbial cells and the strong iron-binding ligands.

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INTRODUCTION

Corrosion of metallic infrastructure facilitated by microorganisms is a costly phenomenon (estimated as 0.68% of the global gross domestic product each year^{1,2}) that affects a wide range of industries, including aviation³, water distribution⁴, and oil and gas^{5–7}. There is a mounting amount of literature that suggests that carbon steel, the preferred construction material in structural components and pipes across industries⁸, is highly susceptible to corrosion facilitated by microorganisms^{9–12}.

Some of the organisms frequently linked to corrosion of steel belong to the genus *Shewanella*^{13–15}. However, no consensus exists on the role of *Shewanella* spp. on the corrosion of steel. *Shewanella oneidensis* MR-1 has been previously shown to accelerate the corrosion of carbon steel up to 1.3–1.8 times via the dissolution of the magnetite passivating layer^{16,17}. However, some authors have argued that *S. oneidensis* MR-1 would decelerate rather than accelerate the corrosion of carbon steel by decreasing the dissolved oxygen in the system¹⁸ (through the production of Fe(II) and the ability of *Shewanella* species to respire oxygen) or by blocking the steel surface with a dense biofilm¹⁹. In both cases, the Fe(III) reducing metabolism of *Shewanella* spp. underlies the involvement of *Shewanella* spp. on the corrosion of carbon steel.

Iron is the fourth most abundant element in the earth's crust and occurs in two main redox states in the environment: oxidized ferric iron (Fe(III)) and reduced ferrous iron (Fe(II)). When steel is oxidized, an iron (hydr)oxide passivating layer forms. Fe(III) is poorly soluble at neutral pH and cannot easily enter the cell's interior, challenging the metabolic utilization of this redox-active substrate. *Shewanella* spp. have overcome this challenge by exhibiting Fe(III)-reductases on their outer membrane that complex and reduce the bioavailable Fe(III) by direct and indirect contact mechanisms²⁰. Consequently, the rates of microbial iron reduction are dictated by the thermodynamics and kinetics of iron coordination and solubility.

The dissolution of Fe(III) oxides is very slow at circumneutral pH, but the mobility of iron in aquatic environments could be enhanced by the formation of soluble Fe(III) complexes with chelating ligands. Short-chain dicarboxylic acids are effective iron-binding ligands that play a key role in mobilizing iron in the environment by dissolving ferric iron from naturally occurring iron oxy-hydroxides^{21–25}. At neutral pH, the widespread short-chain dicarboxylic acids oxalic acid ($pK_{a1} = 1.23$, $pK_{a2} = 4.19$), malonic acid ($pK_{a1} = 2.83$, $pK_{a2} = 5.69$), and succinic acid ($pK_{a1} = 4.19$, and $pK_{a2} = 5.48$)²⁶ occur at the deprotonated state, which forms the strong soluble complexes with Fe(III) (Table 1).

Although dicarboxylates are frequently considered corrosion inhibitors due to the formation of dicarboxylate salts that weakly adsorb on the steel surface^{27–29}, it has been shown that not all dicarboxylates act as corrosion inhibitors in deaerated conditions³⁰. This highlights the need to research the corrosion effects of dicarboxylates under anoxic conditions. Oxalic acid has been shown to inhibit corrosion of iron alloys in aerated solution at pH < 6.0 by forming a passive ferrous oxalate layer^{27,28,31}, whereas succinic acid was found to inhibit corrosion of carbon steel at pH < 3 but the inhibition becomes insignificant at pH > 4³². To the best of our knowledge, the effect of short-chain dicarboxylates on the corrosion of carbon steel under anoxic and circumneutral pH conditions has not been studied.

The complexity of the corrosion chemistry further increases when considering threefold interactions between microbes, ligands, and steel surfaces. Therefore, this research aimed to determine the role of short-chain dicarboxylates on the corrosion of carbon steel driven by *Shewanella oneidensis* MR-1. It was hypothesized that short-chain dicarboxylates would dissolve the iron oxide passivating layer from an oxidized carbon steel surface, increasing the bioavailability of Fe(III) and facilitating the microbial iron reduction in *S. oneidensis* MR-1. The enhanced dissolution of the iron oxide passivating layer would ultimately render the carbon steel surface susceptible to increased corrosion.

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Table 1. Stability constants of ferric iron-ligand (Fe–L) complexes.

Ligand	Most stable species	Log β_{ab}
Oxalate	FeL ₃ ^{3−} ($a = 1$; $b = 3$)	18.6 ⁴²
Malonate	FeL ₃ ^{3−} ($a = 1$; $b = 3$)	16.6 ⁴²
Succinate	FeL ₂ [−] ($a = 1$; $b = 2$)	13.3 ⁴²
Lactate	FeL ($a = 1$; $b = 1$)	6.4

RESULTS

Short-chain dicarboxylates solubilize Fe from oxidized steel

The effect of short-chain dicarboxylates (oxalate, malonate, and succinate) on the dissolution of the iron oxide passivating layer of carbon steel was tested by conducting abiotic microcosm experiments with oxidized carbon steel and 50 mM amendments of dicarboxylates under anoxic conditions. Figure 1 shows the concentration of total dissolved iron in abiotic microcosm experiments throughout time. The addition of 50 mM dicarboxylates resulted in the higher dissolution of iron in comparison to the control experiment without dicarboxylate. Dicarboxylates solubilized iron from the oxidized steel coupon following the trend: oxalate > malonate > succinate. This trend correlates well (Pearson correlation coefficient, $r = 0.997$) with the stability constant of the most stable species of the corresponding iron-ligand complex (Table 1). The zero-order iron dissolution rates with the different dicarboxylates are shown in Table 2. Up to 2.5 times faster iron dissolution rates were observed when short-chain dicarboxylates were present.

Enhanced Fe dissolution does not trigger faster respiration

It was hypothesized that the high concentrations of total dissolved iron driven by the presence of dicarboxylates would facilitate the microbial iron reduction by *Shewanella oneidensis* MR-1. Figure 2 shows a timecourse of dissolved iron speciation in microcosm experiments under both biotic and abiotic conditions. In the abiotic experiments, the total dissolved iron is predominantly (>90%) in the form of Fe(III), but in biotic experiments, the dissolved Fe(II) concentration increases throughout time due to microbial iron reduction by *S. oneidensis* MR-1. Experiments amended with 50 mM oxalate or malonate showed a shorter lag phase for the microbial reduction of Fe(III) (Fig. 2a, b) when compared to the experiments with 50 mM succinate (Fig. 2c) or the control experiment without any dicarboxylate (Fig. 2d). In the biotic experiments with oxalate and malonate, the dissolved Fe(II) concentration increases linearly throughout time after a lag phase of 4 h at a rate of $0.86 \mu\text{mol h}^{-1}$ and $1.40 \mu\text{mol h}^{-1}$, respectively. These rates are lower than the total iron dissolution rates in the abiotic experiments with oxalate ($2.20 \mu\text{mol h}^{-1}$) and malonate ($2.05 \mu\text{mol h}^{-1}$; Fig. 1). In the biotic experiments with succinate and without any ligand, the dissolved Fe(II) concentration increases after a lag phase of 15 h at a rate of $1.85 \mu\text{mol h}^{-1}$ and $1.78 \mu\text{mol h}^{-1}$, respectively. These rates are higher than the total iron dissolution rates in the abiotic experiments with succinate ($1.73 \mu\text{mol h}^{-1}$) and without any dicarboxylate ($0.90 \mu\text{mol h}^{-1}$; Fig. 1).

The iron speciation follows a similar trend in experiments with oxalate or malonate, and this is distinct from the trends exhibited in experiments with succinate and without any dicarboxylate. In experiments with oxalate and malonate, the total dissolved iron concentration is the same irrespective of the presence of bacteria (Fig. 2a, b), whereas, in the experiments with succinate, the total dissolved iron concentration after 15 h in abiotic experiments is higher than in the biotic experiments (Fig. 2c). When no dicarboxylate is present, the total dissolved iron concentration

Fig. 1 Total dissolved iron concentration in abiotic corrosion experiments amended with dicarboxylates. Error bars represent standard deviations (too small to be noticeable in the figure) of triplicate measurements. Experiments were carried out under anoxic and dark conditions, at circumneutral pH, and 30 °C.

Table 2. Zero-order Fe dissolution rates in experiments with oxidized carbon steel and dicarboxylates.

Dicarboxylate	Fe dissolution rate ($\text{mol h}^{-1} \text{m}^{-2}$)	95% CI	R^2
50 mM oxalate	0.0128	± 0.0022	0.9785
50 mM malonate	0.0119	± 0.0017	0.9840
50 mM succinate	0.0100	± 0.0019	0.9726
No ligand	0.0052	± 0.0014	0.9518

CI confidence intervals (CI) at 95%, R^2 Linear coefficients of determination.

after 15 h is slightly higher in biotic experiments than in abiotic ones (Fig. 2d).

Dicarboxylates distinctly change the surface of carbon steel

The surface of the oxidized carbon steel coupon from biotic experiments was examined under the scanning electron microscope (SEM) after 26 h of experimentation. Figure 3 shows the micrographs. The surface of the coupons from experiments with oxalate and malonate (Fig. 3a, b) looks smoother than the surface of the coupons from experiments with succinate and without ligand (Fig. 3c, d). Flakes of iron oxide, as determined by SEM-EDS (Fig. 3e, f), are readily visible on the surface of the coupon treated with succinate and the coupon from the control experiment (without any dicarboxylate). Higher density of sessile cells is observed on the surface of coupons from experiments with succinate (Fig. 3c; 1.91×10^6 cells/cm²) and without ligand (Fig. 3d; 1.56×10^6 cells/cm²) than on coupons from experiments with oxalate (Fig. 3a; 7.69×10^5 cells/cm²) and malonate (Fig. 3b; 7.51×10^5 cells/cm²). A crystalline precipitate was evidenced on the surface of the carbon steel coupon from the experiment with oxalate. This precipitate was identified as likely ferrous oxalate (Humboldtine) by X-ray diffraction (Fig. 4).

Total iron is higher in biotic experiments with ligands

The total (dissolved and solid-phase) iron concentration was measured at the end of the experiments after removing and collecting the corrosion products from the carbon steel surface and acidifying the entire contents of the aqueous phase of the different microcosm experiments. Figure 5 shows the total iron concentration for the different treatments after 26 h of experimentation. The total iron concentration is 1.97–2.60 times higher in biotic experiments with ligands than in experiments without ligands. Higher total iron concentration was observed in biotic

Fig. 2 Time course of iron speciation in experiments with oxidized carbon steel and 50 mM dicarboxylates. Panels show the iron speciation for the experiments with **a)** oxalate, **b)** malonate, **c)** succinate, and **d)** no ligand. Pale color squares represent abiotic experiments whereas dark color circles represent biotic experiments. The solid lines (–) denote total dissolved iron concentration, whereas dashed lines denote Fe(II) (–) and Fe (III) (····). Error bars represent standard deviations of triplicate measurements. Experiments were carried out under anoxic and dark conditions, at circumneutral pH, and 30 °C.

experiments with malonate (3.10 mM), followed by oxalate (2.94 mM) and succinate (2.35 mM), relative to abiotic experiments (1.36 mM for all abiotic experiments with the different ligands). Experiments without ligands showed the lowest total iron concentration (1.02 mM and 1.19 mM for the abiotic and biotic experiments, respectively). In all cases, the total iron concentration was higher in biotic experiments than in the abiotic counterparts.

DISCUSSION

Short-chain dicarboxylic acids are effective iron-binding ligands that play a key role in mobilizing iron in the environment by dissolving ferric iron from naturally occurring iron oxyhydroxides^{21–25}. At neutral pH, the short-chain dicarboxylic acids oxalic, malonic acid, and succinic, occur at the deprotonated state, which forms strong soluble complexes with Fe(III) (Table 1). Because the low solubility of Fe(III) at circumneutral pH imposes significant challenges to the microbial iron respiratory metabolism, it was hypothesized that the addition of dicarboxylates would result in the dissolution of the iron oxide passivating layer of carbon steel, facilitating the microbial Fe (III) reduction to Fe(II) and rendering the steel surface susceptible to enhanced corrosion.

Figure 1 shows that the addition of short-chain dicarboxylates results in higher dissolution of iron from oxidized steel when compared to the control experiment without any dicarboxylate (Fig. 1). The iron dissolution rates of the abiotic experiments amended with the 50 mM short-chain dicarboxylates (Table 2) follow the trend: oxalate > malonate > succinate > no dicarboxylate. The strong correlation (Pearson correlation coefficient, $r = 0.997$) of the ligand-promoted dissolution rates with the stability constant of the most stable species of the corresponding iron-ligand complex (Table 1) suggests that the formation of a

thermodynamically stable product drives, at least in part, the ligand-promoted dissolution of Fe(III) from the iron oxide passivating layer of carbon steel.

Mechanistically, the ligand-promoted dissolution of iron oxides is proposed to start with an adsorption step of the dicarboxylate to the iron oxide surface, followed by the formation of a coordination complex with the iron atom on the surface that breaks the Fe–O bonds of the crystal lattice, facilitating the detachment of the iron-ligand complex and resulting in the solubilization of iron²¹. Furrer and Stumm proposed that the detachment step is the rate-determining step of the mechanism and that five-membered chelate rings are the most readily detachable groups, followed by six- and seven-membered rings³³. This explains the higher dissolution rate of iron by oxalate (five-membered chelate ring), followed by malonate (six-membered chelate ring), and succinate (seven-membered chelate ring).

In the abiotic experiments, the total dissolved iron was predominantly in the redox state Fe(III) (Fig. 2). Although it is likely that the oxide layer on the carbon steel is comprised of mixed-valence iron oxides³⁴, the stability constants of the Fe(II)-dicarboxylate complexes are at least 10 orders of magnitude lower than those of the Fe(III)-dicarboxylate complexes³⁵, explaining why the Fe(III) is preferentially dissolved over Fe(II). Despite the reports of many organic acids being capable of reducing Fe(III) to Fe(II), no significant iron reduction to Fe(II) was observed in our abiotic experiments. This is in agreement with the need for such reductions to be activated by the presence of oxygen or light^{21,23}. Since our microcosm experiments were carried out under anoxic and dark conditions, the ligand-facilitated photochemical iron reduction was not activated.

In the biotic experiments, the concentration of dissolved Fe(II) increased after a lag phase as the result of microbial iron reduction

Fig. 3 SEM micrographs of the surface of oxidized carbon steel coupons after 26 h of experimentation with *S. oneidensis* MR-1 and dicarboxylates. **a** 50 mM oxalate, **b** 50 mM malonate, **c** 50 mM succinate, and **d** no ligand. Panel **e** and **f** show the SEM micrograph of an oxidized carbon steel coupon before experimentation and its corresponding EDS spectrum.

Fig. 4 X-ray diffractogram of the surface of the coupon from the biotic experiment with oxalate. X-ray diffractogram of the surface of the coupon is indicated by the red top line. The X-ray diffractograms of humboldtine (FeC₂O₄·2H₂O) (black middle line) and C1018 steel (yellow bottom line) are provided for reference.

by *S. oneidensis* MR-1. The shorter lag phase in experiments amended with 50 mM oxalate or 50 mM malonate (Fig. 2) seems to be driven by the increased availability of Fe(III) in solution, which would support microbial iron reduction by planktonic cells. Conversely, in the experiments amended with 50 mM succinate or not amended with any dicarboxylate, the dissolved Fe(III) concentration is hypothesized to be too low to support microbial iron reduction by planktonic cells in the first 15 h of experimentation. Most likely, the microbial iron reduction in the latter cases is initiated once the cells reach the surface of the iron oxide layer and establish a biofilm. This is in agreement with the higher cell density observed on the surface of the steel coupons coming from the experiments with 50 mM succinate (1.91×10^6 cells/cm²) or without dicarboxylate (1.56×10^6 cells/cm²) in comparison to the

Fig. 5 Total (dissolved and solid phase) iron at the end of experiments with oxidized carbon steel and 50 mM dicarboxylates. Experiments were carried out under anoxic and dark conditions, at circumneutral pH, and 30 °C.

coupons from the experiments with 50 mM oxalate (7.69×10^5 cells/cm²) or malonate (7.51×10^5 cells/cm²) as well as with the low availability of the iron oxide layer on the coupons exposed to the strong iron-binding ligands oxalate and malonate (Fig. 3). Because these were resting cell experiments that were originally amended with the same number of cells, the observed difference in cell density on the steel surface represents a difference in the microbial distribution between planktonic and surface-attached states.

The distinct mechanisms for microbial iron reduction when different dicarboxylates are present are also evidenced by the differences in iron reduction rates and how they compare to the ligand-promoted iron dissolution rates. In the case of the experiments with oxalate and malonate, the ligand-promoted iron dissolution rates are faster than the microbial iron reduction rates, suggesting that the microbial iron reduction is not limited by the availability of Fe(III) in solution. Furthermore, the difference in iron reduction rates in experiments with oxalate ($0.86 \mu\text{mol h}^{-1}$) in comparison to the experiments with malonate ($1.40 \mu\text{mol h}^{-1}$) suggests a distinct nature of the substrate being reduced (Fe(III)-oxalate $_3^{3-}$ vs Fe(III)-malonate $_3^{3-}$). In the experiments with 50 mM succinate or without any dicarboxylate, once the microbial iron reduction starts, the reduction rates are not only faster than the corresponding iron dissolution rates, but they are similar ($\sim 1.80 \mu\text{mol h}^{-1}$) for the two treatments despite the big difference in the dissolution rates (1.73 vs $0.90 \mu\text{mol h}^{-1}$). This suggests that a similar iron reduction mechanism is used by cells exposed to succinate and the treatment without dicarboxylate, and that this mechanism is independent of the ligand-promoted iron dissolution dynamics. The iron reduction rate for the experiments with succinate and without dicarboxylate is half of the reported reduction rate of Fe(III) (hydr)oxide by *S. oneidensis* MR-1 through a direct contact mechanism²⁰. However, the same authors reported a 46% reduction in the iron reduction rate when chloramphenicol was present due to the impeded synthesis of new proteins. Since our experiments had chloramphenicol, the iron reduction rates observed in our experiments without any dicarboxylate and with succinate support the proposed biofilm-mediated reduction process for experiments with weak iron-binding ligands.

Interestingly, cells exposed to 50 mM oxalate did not yield the highest microbial iron reduction (highest dissolved Fe(II) concentrations) despite oxalate being the ligand that promoted the dissolution of Fe(III) to the greatest extent. It is hypothesized that the Fe(III)-oxalate complex is so strong that oxalate acts as a competitor for Fe(III) relative to the *S. oneidensis* outer membrane cytochromes responsible for binding and reducing Fe(III). A similar equilibrium competition for Fe(III) between strong Fe(III)-chelating agents and the Fe(III)-sorbing functional groups on *Shewanella putrefaciens* cell surface was reported by Haas and Dichristina³⁶. Similarly, Taillefert et al. proposed that *S. putrefaciens* produce Fe(III)-ligands that help solubilize Fe(III) oxides, and therefore a competition equilibrium could be established with exogenous ligands that strongly bind iron³⁷. An alternative and perhaps complementary hypothesis is that formation of the low solubility ($K_{sp} = 3.2 \times 10^{-7} \text{ M}^2$)³⁸ product ferrous oxalate occurs when oxalate is present and Fe(III) is reduced to Fe(II). This product was shown to form and adsorb to the carbon steel surface (Figs. 3a and 4), lowering the Fe(II) concentration in solution, and possibly blocking at some extent the further dissolution of Fe(III).

The total dissolved iron concentration is the same irrespective of the presence of bacteria when oxalate and malonate are present. However, when succinate is present, the total dissolved iron concentration in the abiotic treatment is higher than in the biotic one. This is explained by the fast iron dissolution driven by oxalate and malonate, which could result in a quick replenishment of the dissolved Fe(III) consumed by microbial iron reduction. Since the succinate-facilitated iron dissolution rate is slower than that for oxalate and malonate and a greater biofilm coverage is evidenced on the surface of coupons exposed to succinate, it is reasonable that the replenishment of the consumed dissolved Fe(III) does not occur as readily as when oxalate or malonate are present, resulting in a lower total dissolved iron concentration in the experiments with bacteria when compared to the abiotic counterpart.

To determine the combined effect of the iron-binding dicarboxylates and *S. oneidensis* MR-1 on the corrosion of carbon

steel, the total iron at the end of the microcosm experiments was quantified after removing the corrosion products from the carbon steel surface and collecting them in the acidified aqueous phase. The total iron concentration is higher in biotic experiments with ligands, showing that the ligands accelerate the microbiologically influenced corrosion up to 2.6 times compared to the experiments without dicarboxylates. The highest corrosion, as measured by the total iron concentration, was observed in the biotic experiments with malonate, followed by oxalate, and succinate. In the control experiment without dicarboxylate, *S. oneidensis* MR-1 accelerated the corrosion 1.2 times relative to the abiotic counterpart. This is in agreement with the corrosion acceleration rates reported by Schütz¹⁶. The mere presence of dicarboxylates accelerated the corrosion of carbon steel 1.3 times compared to the experiment without dicarboxylates, showing that individually, ligand-promoted dissolution or microbial iron reduction of the iron oxide passivating layer have a mild impact on the corrosion of carbon steel under dark and anoxic conditions. However, when both iron-binding ligands and microbes with iron-reducing capabilities are present together, the corrosion of carbon steel is significantly exacerbated.

In conclusion, we demonstrated that the ligand-facilitated iron dissolution dynamics impacts the microbial iron reduction and intensifies its effect on the corrosion of carbon steel under anoxic conditions. When weak iron-binding ligands such as succinate are present, the microbial iron reduction is mainly driven by cells in close contact with the steel surface, whereas when strong iron-binding ligands are present, planktonic cells appear to be the main responsible for the reduction of Fe(III). We hypothesize that the difference in cell lifestyle is driven by the sustained availability of dissolved Fe(III) when strong iron-binding ligands are present. The readily available dissolved Fe(III) could help maintain the cells metabolically active through sustained respiration, whereas when dissolved Fe(III) is not readily available or quickly replenished after microbial reduction, the cells would face energetic limitations that trigger biofilm formation. Future research will need to address this hypothesis.

METHODS

Chemicals

Sodium oxalate (CAS number: 62-76-0) ACS reagent $\geq 99.5\%$, sodium malonate dibasic (CAS number: 141-95-7) $\geq 97.0\%$, sodium succinate dibasic (CAS number: 150-90-3) $\geq 98.0\%$, and ferric citrate (CAS number: 3522-50-7) were purchased from Sigma Aldrich.

Bacterial conditions

Shewanella oneidensis MR-1 was grown in a modified minimal medium (20 mM PIPES buffer pH 7.0, 28 mM NH_4Cl , 1.34 mM KCl, 4.35 mM NaH_2PO_4 , 1.0 mM $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 1.0 mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.02% w/v tryptone, 1X trace minerals solution, and 1X vitamins solution) adjusted to pH 7.0 with NaOH and supplemented with 60 mM sodium lactate. The 100X trace mineral solution contained (per liter): Nitrilotriacetic acid (12.80 g), $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$ (1.0 g), $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ (0.50 g), $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ (0.35 g), ZnCl_2 (0.20 g), $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ (0.044 g), H_3BO_3 (0.02 g), $\text{NiSO}_4 \cdot 6\text{H}_2\text{O}$ (0.10 g), $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ (0.002 g), Na_2SeO_3 (0.006 g), and $\text{Na}_2\text{WO}_4 \cdot 2\text{H}_2\text{O}$ (0.008 g)³⁹. The 1000X vitamin solution contained (per liter): biotin (0.02 g), folic acid (0.02 g), pyridoxine HCl (0.10 g), thiamine HCl (0.05 g), riboflavin (0.05 g), nicotinic acid (0.05 g), DL-pantothenic acid (0.05 g), p-aminobenzoic acid (0.05 g), lipoic acid (0.05 g), choline chloride (2.00 g), and Vitamin B12 (0.01 g)³⁹. Oxygen was removed from the medium by boiling for 1 min and bubbling oxygen-free N_2 gas for 20 min before capping with stoppers and autoclaving at 121 °C for 15 min. After autoclaving, filter-sterilized Fe(III)-citrate was added to the medium at a final concentration of 50 mM. Cultures were incubated overnight at 30 °C and 120 rpm. A liquid inoculum from an anaerobic overnight culture was transferred (1% vol/vol) into 10 mL of fresh modified anaerobic minimal medium supplemented with 60 mM lactate and 50 mM Fe(III)-citrate and incubated at 30 °C and at 120 rpm for 8 h. These cultures were harvested by centrifugation at 1,500 RCF for 10 min under anaerobic conditions and washed and resuspended

twice in the anaerobic minimal medium. This was used as the inoculum for biotic microcosm experiments.

Microcosm corrosion experiments

Round C1018 carbon steel (0.15–0.20% C, 0.6–0.9% Mn, 0.035% maximum S, 0.03% maximum P, and elemental iron as the remainder) coupons (9.53 mm diameter, 1 mm thickness) (Alabama Speciality Products) with a 1.726 cm² surface area were placed in 3 mL of 0.1 M NaNO₃ solution and allowed contact with the atmosphere for 3 days. Upon the development of an iron oxide layer on the steel surface, the coupons were rinsed with nano-pure water, allowed to dry inside a Coy anaerobic chamber (5% H₂, 95% N₂ gas atmosphere), and autoclaved for 30 min at 121 °C under an N₂ atmosphere. Anoxic microcosm experiments were set up in sterile 120-mL serum bottles, each containing an oxidized carbon steel coupon suspended by a PTFE-coated quartz string to a butyl rubber septum, as suggested by Liang and Suflita⁴⁰, and 50 mL of minimal medium MR-1 supplemented with 60 mM lactate. The bottles were crimp-sealed and filled with an N₂ atmosphere by repeating flushing and vacuuming cycles 10 times under sterile conditions. An ethanolic solution of chloramphenicol was added at a final concentration of 20 µg mL⁻¹ to all experiments to prevent bacteria growth in biotic experiments. The corrosion microcosm experiments were conducted under both abiotic and biotic conditions in triplicate. In biotic experiments, 1.0 × 10⁶ cells mL⁻¹ were added to each bottle. Filter-sterilized pH = 7.0 solutions of the dicarboxylates under study (oxalate, malonate, and succinate) were prepared in deaerated water and added at a final concentration of 50 mM. In the control experiments without any dicarboxylate, sterile and deaerated nanopure water was added at the same volume as dicarboxylates to assure the same total volume of experimentation. All the experiments were incubated at 30 °C and 120 rpm for 26 h under dark conditions. Because oxidized carbon steel coupons were used, we could not conduct mass loss measurements at the end of the experiments to determine corrosion rates.

Chemical analyses

Liquid samples were collected under anoxic conditions with N₂-rinsed sterile syringes and acidified with HCl at a final concentration of 0.5 N. Ferrozine assay⁴¹ for the quantification of Fe(II) was performed immediately following sample collection to minimize iron oxidation. Briefly, Ferrozine reagent was prepared by adding 1 g of 3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-p,p'-disulfonic acid monosodium salt in 1 L of 50 mM HEPES pH 7.0 and stored under dark conditions at room temperature. For the assay, 2.5 mL of ferrozine reagent were mixed with 50 µL of the sample or the standard solution, and the extinction of the mixture at 562 nm was measured using a UV-1601 spectrophotometer (Shimadzu) after vigorous shaking for 15 s. Iron reduction rates were calculated over the linear portion of the Fe(II) production curve.

Total dissolved iron was quantified with a NexION 2000 (Perkin Elmer) Inductively Coupled Plasma-Mass Spectrometer (ICP-MS). The isotope ⁵⁶Fe was measured in the dynamic reaction cell (DRC) mode. The plasma gas is 16 L Ar min⁻¹. Ammonia is used as DRC gas at a 0.59 mL min⁻¹ flow rate. A 500 ppb Gallium standard solution (Inorganic Ventures) was used as an internal standard, and a 10,000 µg mL⁻¹ iron standard stock solution (Perkin Elmer) was used to create standard solutions. The samples for total dissolved iron quantification were stored at -20 °C before analysis.

The total iron at the completion of the experiment was quantified after collecting the coupons inside a Coy anaerobic glove box (5% H₂, 95% N₂ gas atmosphere) and submerging them in 3 mL of corrosion cleaning solution (3.5 g L⁻¹ hexamethylenetetramine in 6 N HCl) for 10 min. Then, the coupons were washed with deoxygenated nanopure water and stored under an N₂ atmosphere. The liquid fraction was mixed with the original aqueous phase from each of the microcosm experiments and acidified with HNO₃ at a final concentration of 2.0% (v/v). The total iron concentration in this liquid phase was measured using ICP-MS in a similar way to the dissolved iron measurements.

Scanning electron microscopy

Coupons were collected after experimentation inside a Coy anaerobic glove box (5% H₂, 95% N₂ gas atmosphere), washed with 1 mL of phosphate buffer saline pH 7.2, and submerged in 4 mL of 2.5% glutaraldehyde solution in phosphate buffer saline pH 7.2 and incubated for 10 h at 4 °C. Then, coupons were washed with 1 mL of nanopure water, and ethanol dehydration series (30%, 50%, 60%, 70%, 80%, 90%, 95%, 100%, 100%) was performed by submerging each coupon in 4 mL of each

of the ethanolic solutions for 10 min. Coupons were allowed to dry inside an anaerobic glove box and stored under N₂ atmosphere in crimp-sealed vials until examination with the Zeiss NEON 40 EsB scanning electron microscope at a 5 kV accelerating voltage. SEM micrographs were analyzed with ImageJ and cells on the surface of the steel coupon were counted using the Cell Counter plugin.

X-ray Diffraction

Powder X-ray diffraction (XRD) analyses were performed using a Rigaku Ultima IV diffractometer. Cu-K-alpha radiation (40 kV, 44 mA) was used with a scintillation detector and a curved graphite monochromator. The MDI Jade 2010 software with the ICDD (International Centre for Diffraction Data) PDF4+ database was used for the data analysis.

DATA AVAILABILITY

The authors declare that all data generated or analyzed during this study are included within the paper.

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AUTHOR CONTRIBUTIONS

A.H.S. led the writing of this manuscript, performed microcosm corrosion experiments, ICP-MS and SEM analysis, and contributed to the interpretation of the XRD diffractogram. This work is linked to H.N.K.'s master's thesis. H.N.K. performed initial experiments. M.A.N. contributed to the initial concept and research question, experimental design, the overall data interpretation, and the writing of the manuscript.

COMPETING INTERESTS

The authors declare no competing interests.

ADDITIONAL INFORMATION

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