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1 **Geochemical Explanation for the Prevalence of S^0 Reduction Among Many**
2 **Fe(III)-Reducing Bacteria**

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11 **Abstract: Microbial reduction of ferric iron [Fe(III)] is an important biogeochemical process**
12 **in anoxic aquifers. Depending upon groundwater pH, dissimilatory metal-reducing bacteria**
13 **(DMRB) can also respire alternative electron acceptors to survive, including elemental sulfur**
14 **(S^0). To understand the interplay of Fe/S cycling under alkaline conditions, we combined**
15 **thermodynamic geochemical modeling with bioreactor experiments using Shewanella**
16 **oneidensis MR-1. Under these conditions, S. oneidensis can enzymatically reduce S^0 but not**
17 **goethite (α-FeOOH). The HS^- produced subsequently reduces goethite abiotically. Due to the**
18 **prevalence of alkaline conditions in many aquifers, Fe(III) reduction may thus proceed via S^0–**
19 **mediated electron-shuttling pathways whereby DMRB may require an active sulfate-reducing**
20 **bacterial partner to respire.**

21 **One Sentence Summary:** Under alkaline conditions, metal-reducing bacteria are shown to
22 respire elemental sulfur rather than ferric iron.

23 **Main Text:**

24   Dissimilatory metal-reducing bacteria (DMRB) are diverse microorganisms that can use

25 insoluble, extracellular substrates as electron acceptors for respiration (1, 2). Although DMRB can

26 reduce a variety of chemical compounds, their ability to reduce ferric iron [Fe(III)] is their most

27 studied trait. Fe(III) is common in the environment as insoluble (oxyhydr)oxide minerals such as

28 ferrihydrite ($Fe(OH)_3$) or goethite (α-FeOOH) . The reductive dissolution of these minerals by

29 DMRB produces highly reactive ferrous ions (Fe^{2+}), making Fe(III) reduction important to water

30 quality (3), contaminant fate and transport (4), the biogeochemical cycling of carbon (5), and the

31 geochemical evolution of the early Earth (6).

32   In addition to Fe(III), many DMRB strains can use elemental sulfur (S^0) as an electron

33 acceptor. The ecological significance of S^0 reduction in aquifers, however, is poorly understood.

34 Although Fe(III) minerals are abundant in these environments, the steady-state concentration of

35 S^0 is frequently below detection (7). Nevertheless, S^0 may still serve as a transient but important

36 electron sink there (8). S^0 is also abundant in marine sediments where steep redox gradients allow

37 the direct mixing of sulfidic waters with dissolved O_2, but it can be created in anoxic, freshwater

38 systems by the reaction of dissolved sulfide with ferric minerals such as goethite (9). Many

39 common DMRB in these environments (e.g. several Shewanella, Desulfuromonas, Geothrix,

40 Pelobacter, and Geobacter spp.) can respire S^0 directly. Genetic evidence suggests that this ability

41 is derived from an enzymatic mechanism distinct from the pathway used to reduce Fe(III) (10) and

42 is therefore unlikely to be simply an incidental consequence of these microorganisms' ability to

43 reduce transition metals. Rather, the common co-occurrence in metal reducers of the ability to

44 reduce Fe(III) and S^0 suggests an evolutionary explanation linked to the ecology of the terrestrial

45 subsurface, where metal-reducing microorganisms are frequently abundant (2).

46 Most microorganisms can respire using a variety of substrates, but their ability to use any

47 one respiratory pathway depends on the amount of thermodynamic energy available from that

48 reaction (11). The available energy can be calculated directly from the chemical activity of

49 reactants and products in the metabolic reaction being catalyzed (12). For example, some

50 geomicrobial reactions such as Fe(III) reduction are strongly proton-consuming and therefore

51 much less energetically-favorable in alkaline environments (11).

52 Alkaline aquifers are common and serve as critical water resources, especially in arid

53 regions where water-rock interactions drive the pH up to 8–10 (13). Furthermore, alkaline

54 groundwater is often associated with high levels of arsenic, a toxic metal whose mobility in

55 groundwater has been tied to the activity of Fe(III) and sulfate-reducing bacteria (SRB) (14).

56   To better understand the biogeochemistry of Fe and S in alkaline environments, we

57   calculated the energy available to microorganisms from the reduction of Fe(III) and S^0 versus

58   sulfate by creating a thermodynamic model of a pristine, anoxic, electron-donor-limited aquifer

59   (Table S1). To test the model predictions regarding the effect of pH on the microbial reduction of

60   Fe(III) and S^0, we inoculated pH-buffered suspensions of Fe(III)- and S^0-bearing minerals

61   (goethite and rhombic S^0) with Shewanella oneidensis MR-1, a DMRB capable of reducing both.

62   We chose strain MR-1 because a genetic mutant, PSRA1, contains an in-frame deletion of the gene

63   psrA and is unable to respire S^0 (10). Additional information on methodology is available as

64   Supplementary Online Materials.

65   Our thermodynamic models show that under these hypothetical groundwater conditions,

66   the reduction of Fe(III)-containing minerals is favored much more strongly at acidic pH than

67   alkaline (Figure 1). With all three electron donors tested, goethite reduction yields as much energy

68   as sulfate reduction at pH 8 but considerably less than S^0 reduction above pH 7. The reduction of

69   ferrihydrite provides more energy per mole of substrate than reduction of goethite (Table S1), but

70   this pathway also ceases to provide sufficient energy for respiration at roughly pH 9 for the

71   conditions tested. Although the amount of energy available from these reactions also depends on

72   the concentration of the electron donor being utilized, the strong correlation of pH with the amount

73   of energy available from reducing ferric minerals shows that these means of respiration are likely

74   to be much less favorable at the near-neutral to slightly basic pH of aquifers like the Columbia

75   River Basalt Group (15) or the Continental Intercalaire aquifer (13). The reduction of S^0, in

76   contrast, is energetically favorable at any pH and becomes more favorable with increasing pH.

77   Under the modeled conditions, the reduction of Fe(III) provides insufficient

78   thermodynamic energy to support the respiration of DMRB at alkaline pH. Still, DMRB might

79	respire and grow under these conditions. Indeed, under laboratory conditions with abundant

80	nutrients and large concentrations of electron donor and acceptor, microbial reduction of Fe(III)

81	has been shown to occur at pH > 11 via microorganisms such as Geoalkalibacter and

82	Anaerobranca (16). However, these idealized conditions differ markedly from those in most

83	aquifers, where concentrations of organic acids such as acetate and formate are typically found in

84	micromolar concentrations or less and the thermodynamic driving force is small (17).

85	 In goethite-only bioreactors inoculated with wild-type S. oneidensis, considerably more

86	Fe^{2+} was produced at pH 6.8 than pH 9.0 (Figure 2A). We attribute some reduction without added

87	donor to the accumulation of residual reducing power in S. oneidensis cells during their initial

88	growth in rich medium (see Supplementary Materials). At pH 6.8, however, more than twice as

89	much Fe^{2+} was produced when formate was added versus the no-donor control; at pH 9.0, Fe^{2+}

90	production was the same in control and donor-containing experiments. This result suggests that

91	under the alkaline conditions tested, no respiratory reduction of goethite coupled to formate

92	oxidation occurred, where our model predicts it to be thermodynamically unfavorable (Figure S1).

93	As previously reported (10), the production of Fe^{2+} via goethite reduction did not differ between

94	the PSRA1 mutant or the wild type (Figure 2A and 2B).

95	 In bioreactors containing both goethite and S^0, the overall production of Fe^{2+} at pH 6.8 was

96	nearly equivalent to that of goethite-only experiments at pH 6.8 for both the wild-type and PSRA1

97	(Figures 2C and 2D). At pH 9.0, however, the wild type produced nearly three times more Fe^{2+}

98	when given formate compared to no-donor controls (Figure 2C). The rate at which Fe^{2+}

99	accumulated was slower at pH 9.0, which is likely due to the slower reaction kinetics between

100	sulfide and goethite at alkaline pH (18). In contrast, the amount of Fe^{2+} produced by PSRA1 at pH

101	9.0 differs little with or without S^0 (Figures 2B and 2D). Synchrotron-based measurement of sulfur

102 speciation by x-ray absorption spectroscopy confirmed that at pH 9.0, S^0 was reduced to sulfide

103 by the wild type but not by PSRA1 (Figure 3), leading to the formation of mackinawite (FeS).

104 Sulfide was detected in S^0-containing bioreactors of both wild-type and PSRA1 cells at pH 6.8,

105 although for the mutant this likely resulted from the abiotic reaction of Fe^{2+} with S^0 to form

106 mackinawite through a polysulfide intermediate (19). Our results indicate that, as predicted by the

107 model (Figure 1), under alkaline conditions S. oneidensis can enzymatically reduce S^0 but not

108 goethite. The production of Fe^{2+} at pH 9 is instead due to the abiotic reduction of goethite by

109 sulfide produced through the enzymatic reduction of S^0, suggesting that Fe(III) reduction at

110 alkaline pH proceeds via an indirect, sulfur-dependent electron shuttling pathway similar to those

111 previously known to occur via flavins or humic substances (20).

112   The primary source of dissolved sulfide in the subsurface is microbial sulfate reduction

113 (21), a process where the available energy is affected little by changes in pH (Figure 1). By

114 reducing sulfate to HS^- in the presence of Fe(III) minerals in an alkaline aquifer, the respiration of

115 SRB would create S^0 and allow DMRB like Shewanella spp. to respire (Figure 4). Many studies

116 indicate that Fe(III) reduction and sulfate reduction co-occur frequently in the subsurface (22).

117 Therefore, under alkaline conditions DMRB would depend on the activity of SRB to respire in a

118 commensal or even mutualistic relationship (23). In addition to modern aquifers, such an

119 interaction could have been important on the early Earth, where alkaline conditions are thought to

120 have predominated in large areas of the ocean (24), and may have contributed to the formation of

121 sedimentary pyrite during the Archean and early Proterozoic (25). The extreme alkalinity of the

122 early oceans (pH >10) makes the direct, enzymatic reduction of Fe(III) even less likely to have

123 been energetically favorable, and dissimilatory iron reduction alone probably would not be

124 responsible for the production of Fe^{2+} there.

125   This ecological connection explains why many DMRB would maintain separate genetic

126   pathways to respire Fe(III) and S^0. In the presence of active sulfate reduction and faced with an

127   inability to respire Fe(III) due to energetic limitations, a microbe able to respire both S^0 and Fe(III)

128   would have a competitive advantage. For example, the microbial reduction of the Fe(III) minerals

129   ferrihydrite and goethite coupled to formate or acetate oxidation results in significant increases in

130   pH due to H^+ consumption during the corresponding catabolic half reactions (Table S1). The

131   ability to transition from enzymatic reduction of Fe(III) minerals at circumneutral pH to a S^0-

132   reducing pathway at alkaline pH where Fe(III) minerals are thermodynamically unavailable for

133   use as electron acceptors thus provides DMRB with a mechanism to sustain energy-generating

134   electron transport processes over a much wider pH range (approaching nearly 4 orders of

135   magnitude) than direct enzymatic Fe(III) reduction alone. Furthermore, at alkaline pH, Fe^{2+} ions

136   are thought to sorb more strongly to the surfaces of iron oxides and thereby inhibit direct enzymatic

137   reduction (26). Sulfide production through the reduction of sulfate and S^0 would strip these sorbed

138   ions away and thereby circumvent the passivation of Fe(III) oxide surfaces, providing further

139   evidence for the important of sulfate reduction to the reduction of Fe(III) oxides at alkaline pH.

140   Indirect Fe(III) reduction via a S^0 reduction pathway under alkaline conditions could be

141   highly relevant to geologic carbon sequestration. In addition to their critical role as water resources,

142   alkaline aquifers are primary targets for carbon capture and sequestration in the deep subsurface

143   because they can mineralize injected supercritical CO_2 as carbonate minerals (27). This ability is

144   derived from the superior pH buffering of alkali minerals in the aquifer, where groundwater

145   becomes more acidic after injection of supercritical CO_2 (28). The reductive dissolution of Fe(III)

146   minerals to aqueous Fe^{2+}—regardless of whether it is mediated biotically or abiotically—is critical

147   to this process because these ions react with bicarbonate and precipitate as the mineral siderite,

148 thus trapping carbon in solid form (29). Assuming direct enzymatic reduction of ferric minerals is

149 unlikely to occur in alkaline, oligotrophic environments (Figure 1), microbial sulfate reduction and

150 the subsequent reaction of sulfide with ferric minerals to produce Fe^{2+} and S^0 (which itself could

151 be re-reduced by DMRB to form additional sulfide) would be the primary mechanism responsible

152 for producing Fe^{2+} and sequestering carbon.

153

168 **Fig. 1**. Free energy change of microbial metabolisms in a hypothetical pristine aquifer. The amount
169 of usable energy (ΔG_U) available to microorganisms from the reduction of S^0, Fe(III) minerals
170 (ferrihydrite and goethite), and sulfate with either (A) formate, (B) acetate, or (C) hydrogen as an
171 electron donor changes with pH. The dotted line at $\Delta G_U = 0$ kJ mol^{-1} represents the theoretical
172 minimum energy required to support microbial respiration. Electron donating and accepting
173 processes modeled are shown in D.

174 **Fig. 2.** Total Fe^{2+} production in bioreactor experiments. Experiments were conducted at pH 6.8
175 and 9.0 using S. oneidensis MR-1 wild type (A,C) and psrA-deficient mutant PSRA1 (B,D) as an
176 inoculum. Bioreactors contained either 10 mM goethite alone (A,B) or 10 mM each of goethite
177 and S^0 (C,D). Data points represent the average of triplicate bioreactors with error bars ± standard
178 deviation.

179 **Fig. 3.** Sulfur K-edge XANES spectra of S-containing bioreactors. Standards shown are (A)
180 unreacted S. oneidensis MR-1 cells, (B) rhombic S^0, and (C) mackinawite (FeS). Samples are

181   shown from bioreactors containing both goethite and S^0 at pH 9.0 (D, E) or pH 6.8 (F, G) that were
182   inoculated with cells of either the wild type (D, F) or PSRA1 mutant (E, G).

183   **Fig. 4.** Illustration of S^0-mediated Fe(III) reduction under alkaline conditions.

184   **Supplementary Materials:**
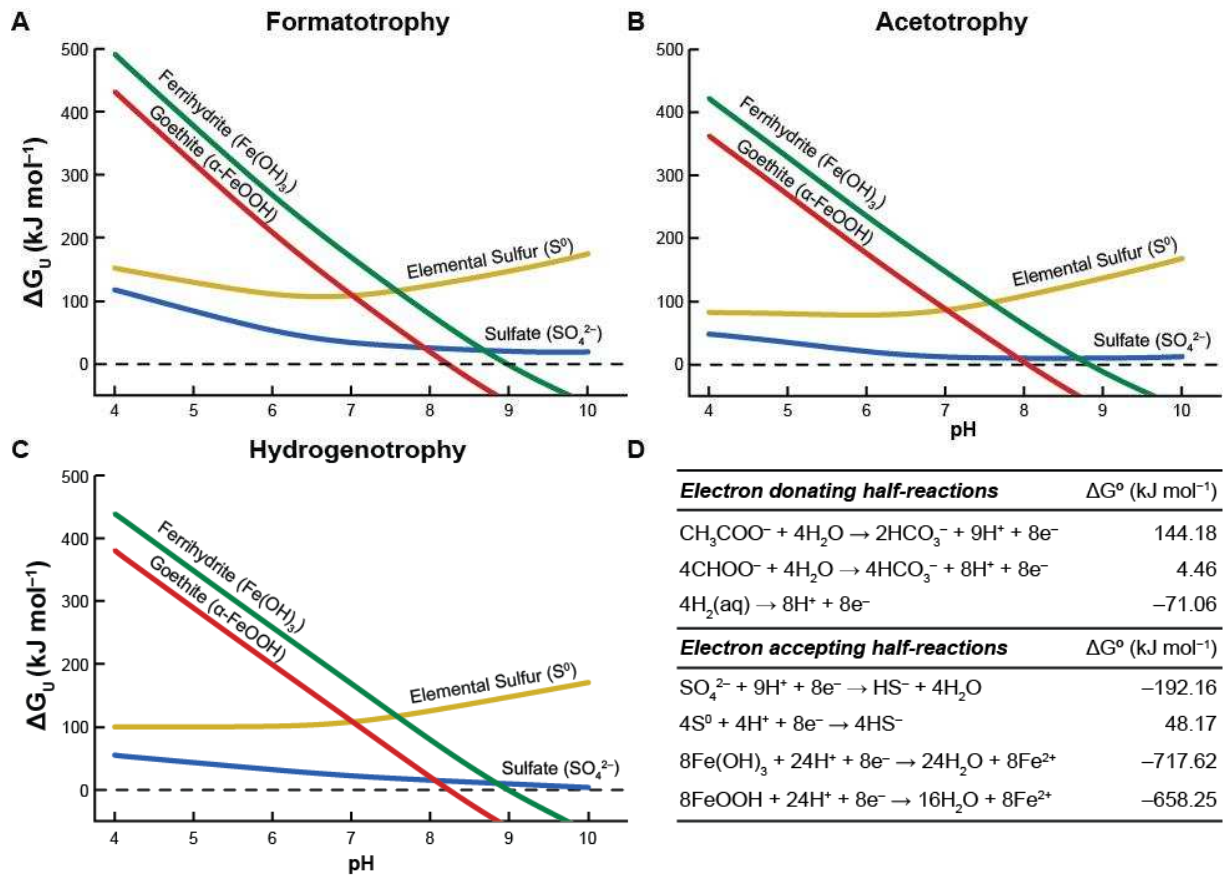
185   Materials and Methods

186   Table S1

187   Figure S1

188   References 31–44.

189

190

Figures

Figure 1. Free energy change of microbial metabolisms in a hypothetical pristine aquifer. The amount of usable energy (ΔG_U) available to microorganisms from the reduction of S^0, Fe(III) minerals (ferrihydrite and goethite), and sulfate with either (A) formate, (B) acetate, or (C) hydrogen as an electron donor changes with pH. The dotted line at $\Delta G_U = 0$ kJ mol^{-1} represents the theoretical minimum energy required to support microbial respiration. Electron donating and accepting processes modeled are shown in D.
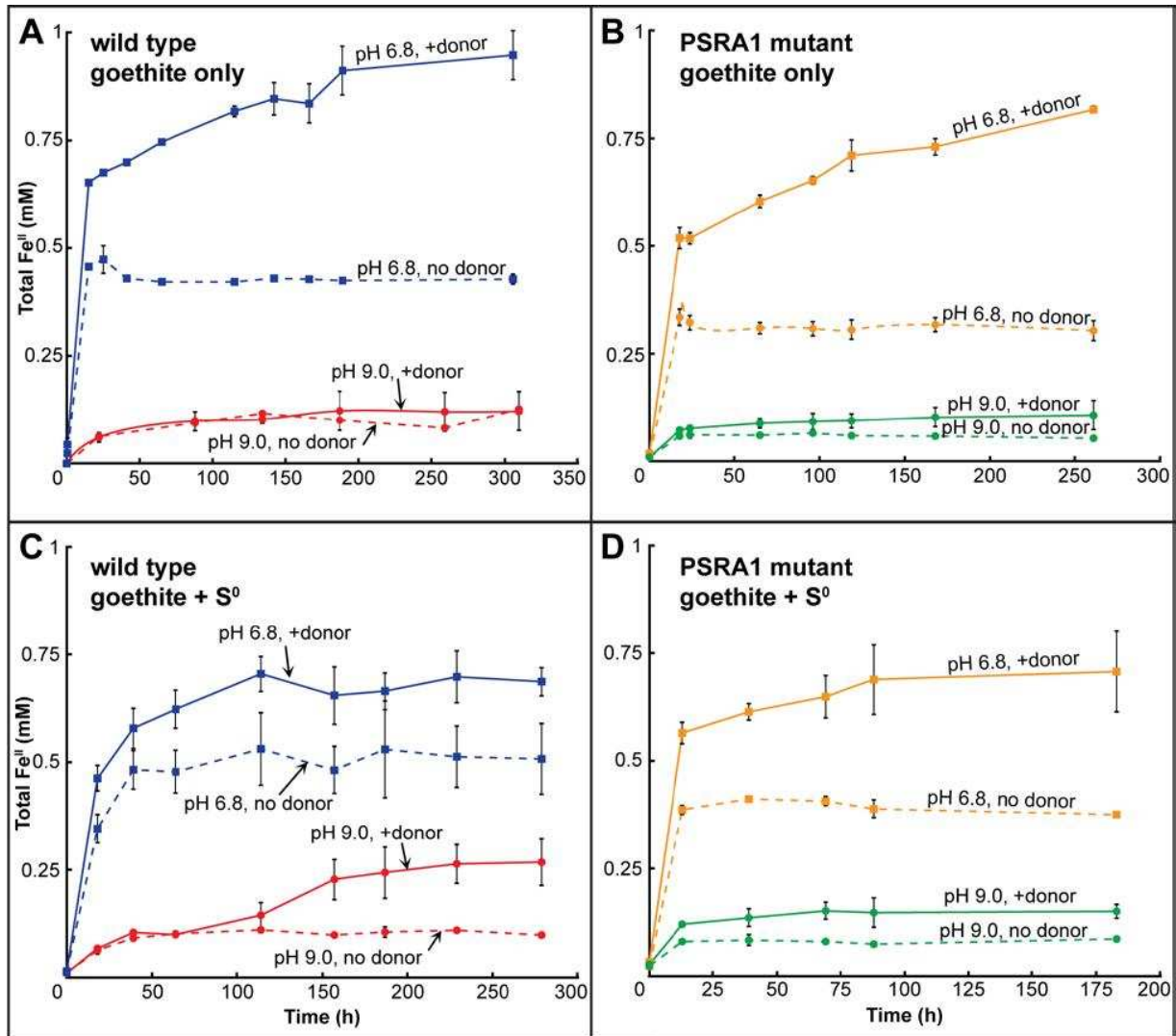
199
200 **Figure 2.** Total Fe^{2+} production in bioreactor experiments. Experiments were conducted at pH
201 6.8 and 9.0 using Shewanella oneidensis MR-1 wild type (A,C) and psrA-deficient mutant
202 PSRA1 (B,D) as an inoculum. Bioreactors contained either 10 mM goethite alone (A,B) or 10
203 mM each of goethite and S^0 (C,D). Data points represent the average of triplicate bioreactors
204 with error bars ± standard deviation.
205
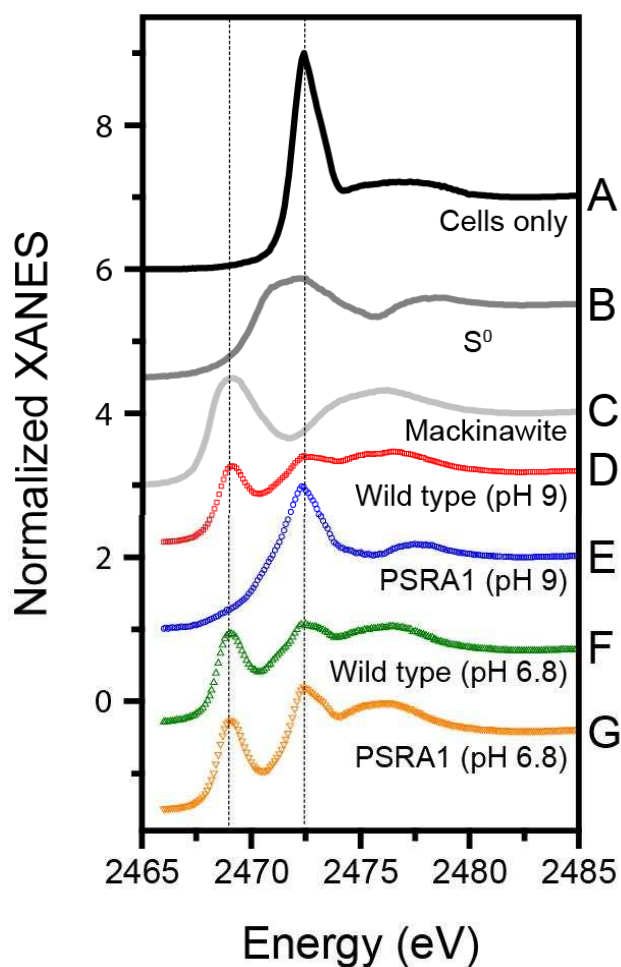
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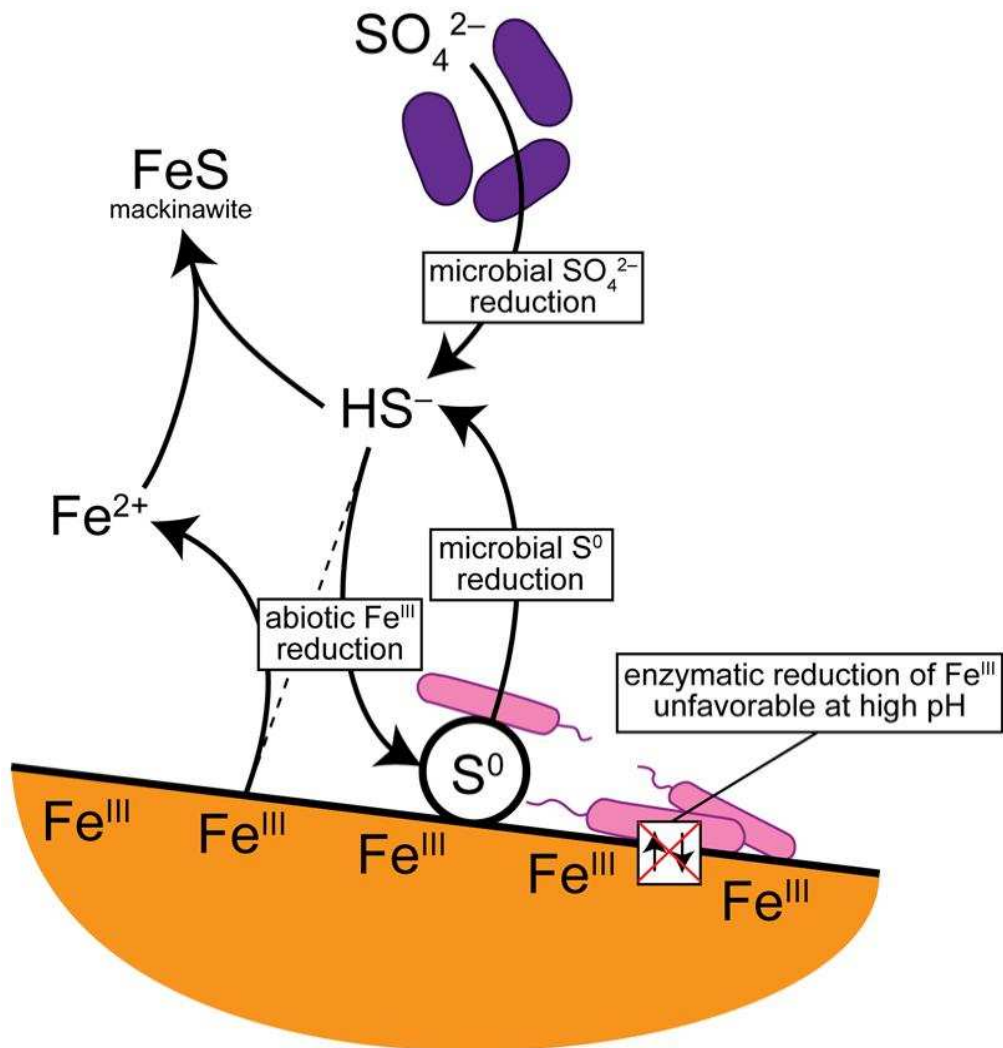
213
214 **Figure 4.** Illustration of S^0-mediated Fe(III) reduction under alkaline conditions.
215

216 **References and Notes**

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