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Article:

Flynn, TM, O'Loughlin, EJ, Mishra, B et al. (2014) Sulfur-mediated electron shuttling during bacterial iron reduction. *Science*, 344 (6187). pp. 1039-1042. ISSN: 0036-8075

<https://doi.org/10.1126/science.1252066>

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1 **Geochemical Explanation for the Prevalence of S⁰ Reduction Among Many** 2 **Fe(III)-Reducing Bacteria**

3 **Authors:** Theodore M. Flynn^{1,2}, Edward J. O'Loughlin¹, Bhoopesh Mishra^{1,3}, Thomas J.
4 DiChristina³, Kenneth M. Kemner^{1*}

5 **Affiliations:**

6 ¹Biosciences Division, Argonne National Laboratory, 9700 S. Cass Ave., Argonne, IL 60439.

7 ²Computation Institute, The University of Chicago, Chicago, IL 60637.

8 ³Physics Department, Illinois Institute of Technology, Chicago, IL 60616.

9 ⁴School of Biology, Georgia Institute of Technology, Atlanta, GA 30332.

10 *Correspondence to: kemner@anl.gov.

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 (S⁰). 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 S⁰ 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 S⁰-
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 (Fe²⁺), 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 (S⁰) as an electron
33 acceptor. The ecological significance of S⁰ 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 S⁰ 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 S⁰, we inoculated pH-buffered suspensions of Fe(III)- and S⁰-bearing minerals
61 (goethite and rhombic S⁰) 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 S⁰ (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 S⁰ 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 S⁰, 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 Fe²⁺ 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 Fe²⁺ was produced when formate was added versus the no-donor control; at pH 9.0, Fe²⁺
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 Fe²⁺ 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 S⁰, the overall production of Fe²⁺ 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 Fe²⁺
98 when given formate compared to no-donor controls (Figure 2C). The rate at which Fe²⁺
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 Fe²⁺ produced by PSRA1 at pH
101 9.0 differs little with or without S⁰ (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 S⁰. 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 S⁰ 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 S⁰-
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, Fe²⁺ 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 S⁰ 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 S⁰ 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 CO₂ 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 CO₂ (28). The reductive dissolution of Fe(III)
146 minerals to aqueous Fe²⁺—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
154 **Acknowledgments:** This research is part of the Subsurface Science Scientific Focus Area (SFA)
155 at Argonne National Laboratory supported by the Subsurface Biogeochemical Research Program,
156 DOE Office of Science, Office of Biological and Environmental Research under DOE
157 contract DE-AC02-06CH11357. We appreciate the technical assistance of M. Newville and A.
158 Lanzarotti. K. Neelson and J. Fredrickson provided helpful comments which improved the
159 manuscript. X-ray analyses were conducted at Argonne National Laboratory's Advanced Photon
160 Source (APS), GeoSoilEnviroCARS (Sector 13), supported by the National Science Foundation-
161 Earth Sciences (EAR-1128799) and the U.S. Department of Energy (DOE)-GeoSciences (DE-
162 FG02-94ER14466). Use of the APS was supported by the DOE Office of Science, Office of Basic
163 Energy Sciences. T.F. was supported in part by an Argonne Director's Fellowship and the National
164 Institute of Allergy and Infectious Diseases, National Institutes of Health, Department of Health
165 and Human Service (Contract No. HHSN272200900040C). T.D. was supported by the National
166 Science Foundation (Molecular and Cellular Biosciences Grant No. 1021735). All additional
167 information has been archived in the Supplementary Online Material.

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 $\Delta G_U = 0 \text{ kJ mol}^{-1}$ 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 \pm 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

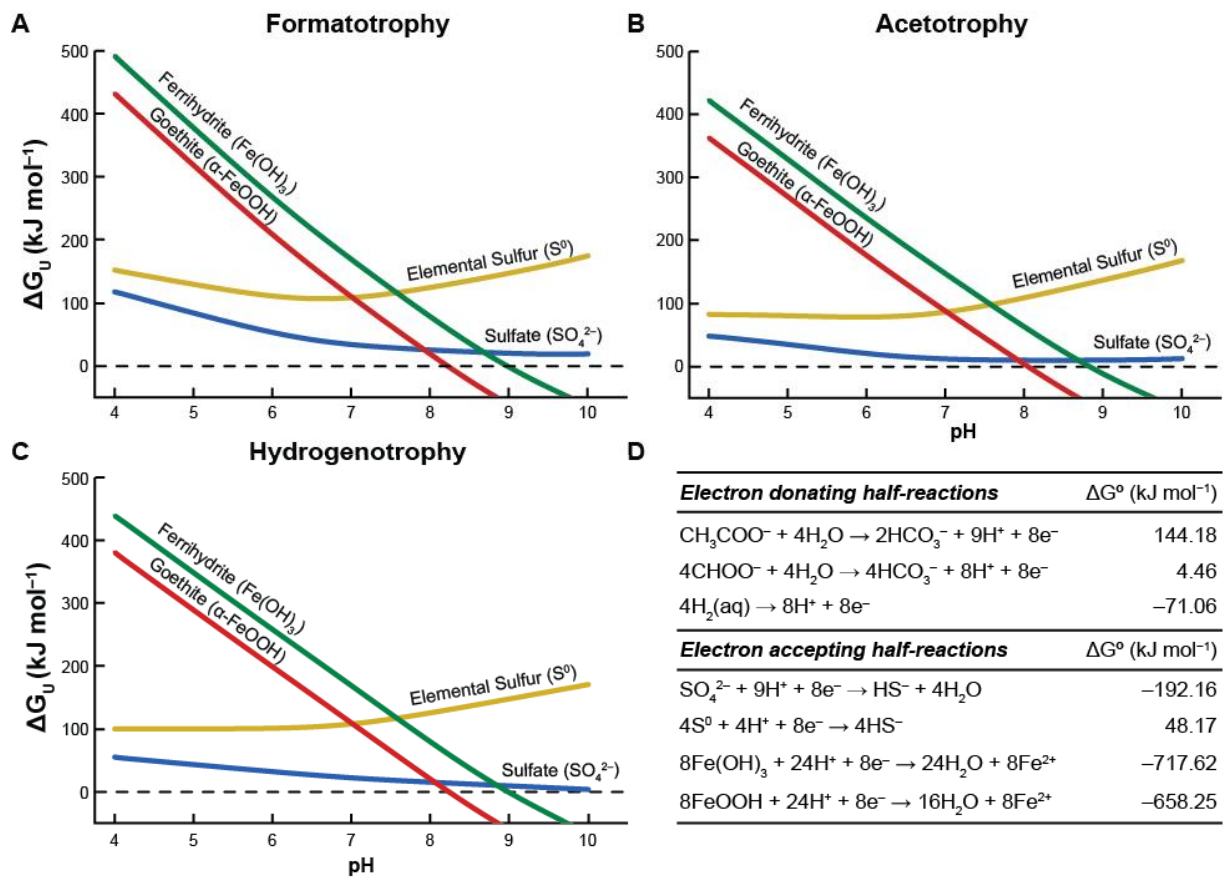
187 Figure S1

188 References 31–44.

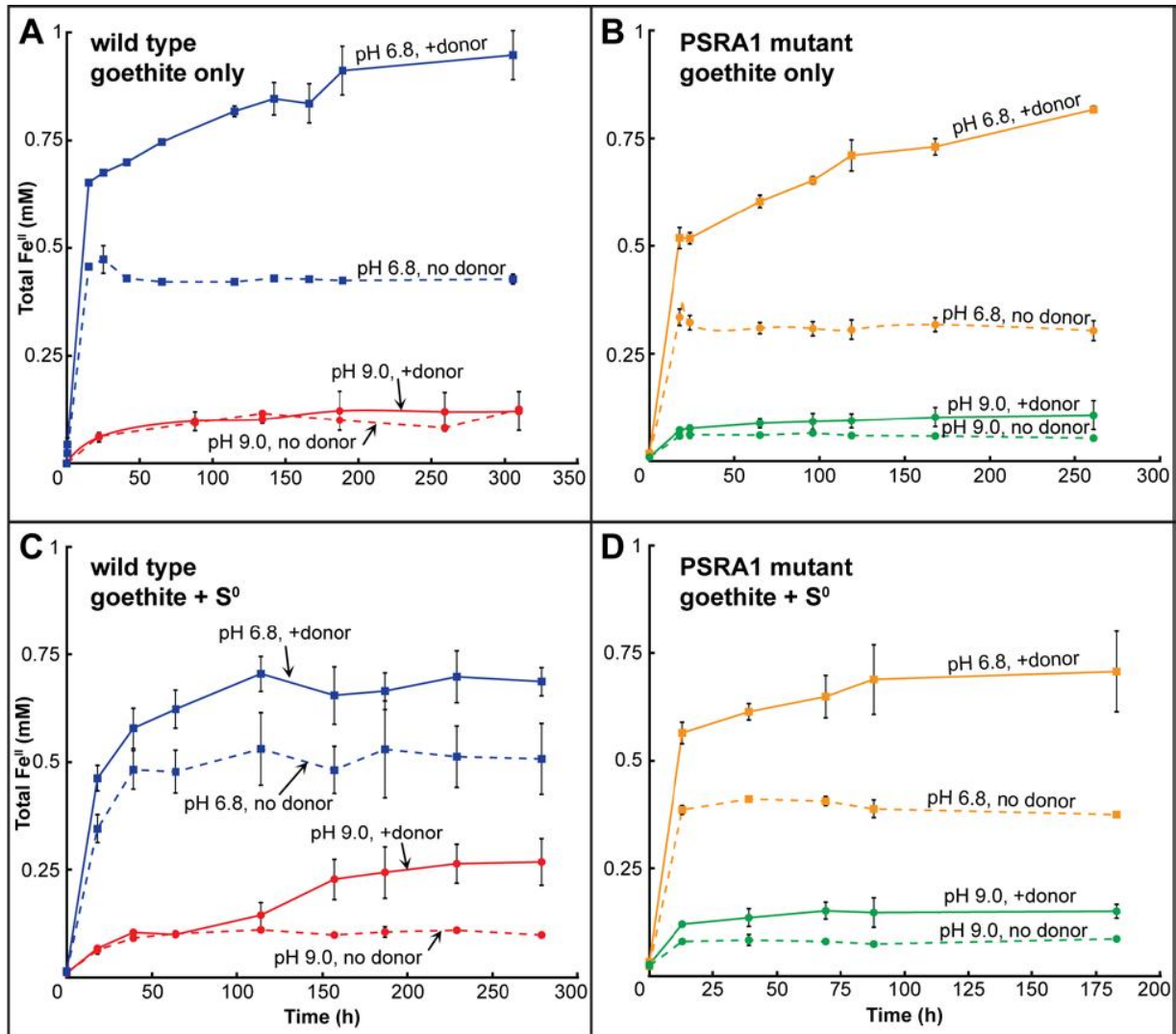
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191 **Figures**

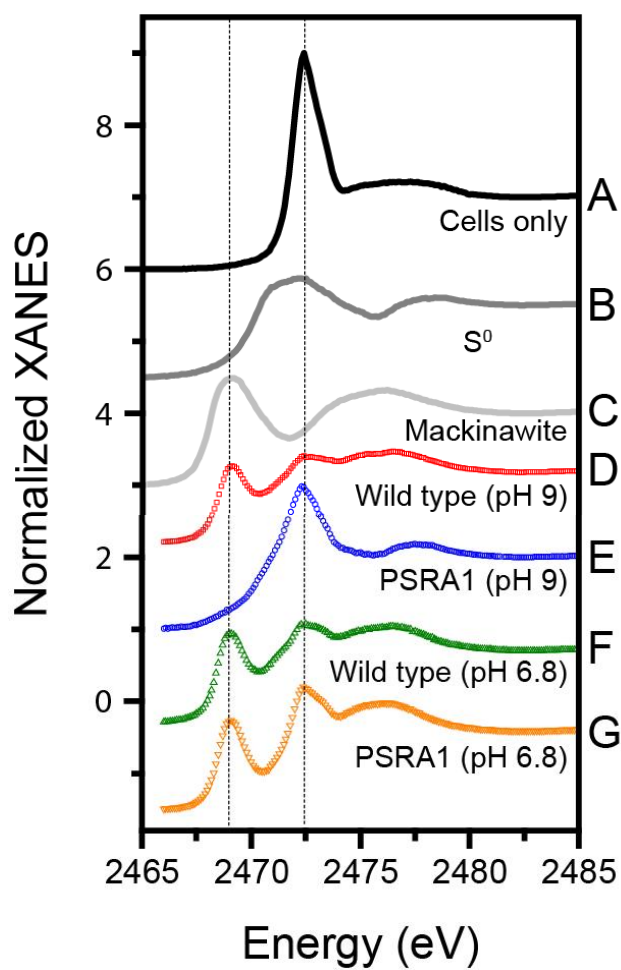


192
 193 **Figure 1.** Free energy change of microbial metabolisms in a hypothetical pristine aquifer. The
 194 amount of usable energy (ΔG_U) available to microorganisms from the reduction of S^0 , Fe(III)
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 196 hydrogen as an electron donor changes with pH. The dotted line at $\Delta G_U = 0 \text{ kJ mol}^{-1}$ represents
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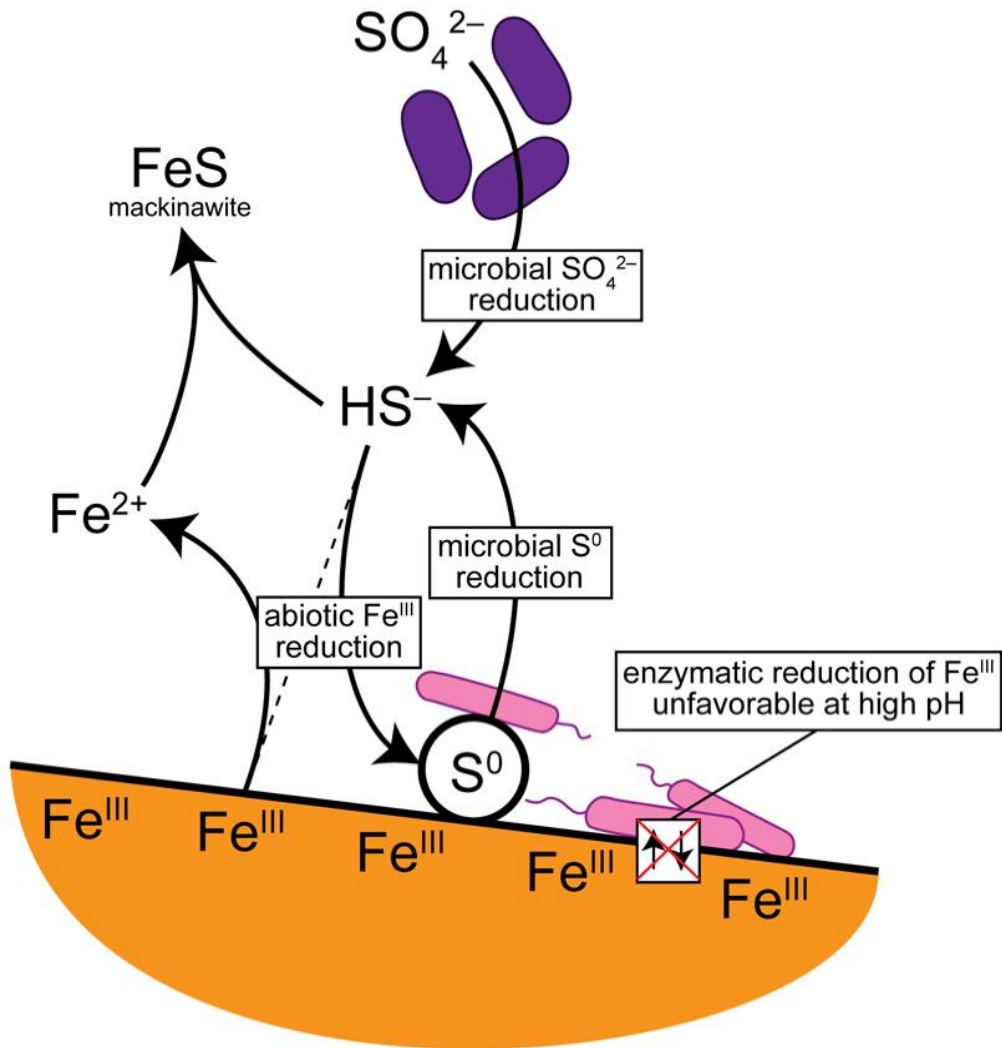


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206
 207 **Figure 3.** Sulfur K-edge XANES spectra of S-containing bioreactors. Standards shown are (A)
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 209 shown from bioreactors containing both goethite and S⁰ at pH 9.0 (D, E) or pH 6.8 (F, G) that
 210 were inoculated with cells of either the wild type (D, F) or PSRA1 mutant (E, G).
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215

Figure 4. Illustration of S^0 -mediated $\text{Fe}(\text{III})$ reduction under alkaline conditions.

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