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Microbes Pumping Iron:

Anaerobic Microbial Iron Oxidation and Reduction

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Abstract

Iron (Fe) has long been a recognized physiological requirement for life, yet its role for a broad diversity of microorganisms persisting in water, soils, and sediments extends well-beyond a nutritional necessity. Microorganisms from within both the domain Archaea and Bacteria are capable of metabolically exploiting the favorable redox potential between the Fe(III)/Fe(II) couple. Identification of these microbial metabolisms has recognized that reduced Fe, can serve as an energy source for lithotrophic Fe-oxidizing microorganisms (FOM) under both oxic and anoxic conditions. Alternatively, oxidized Fe can serve as a terminal electron acceptor under anaerobic conditions for lithotrophic and heterotrophic Fe-reducing microorganisms (FRM). Given that Fe is the fourth most abundant element in the Earth's crust, iron redox reactions have the potential to support a significant fraction of microbial biomass. The broad diversity of environments supporting FOM and FRM display an extreme range of chemical and physical conditions. As such, biological iron apportionment has been described as one of the most ancient forms of microbial metabolisms on earth as well as a conceivable extraterrestrial metabolism on other iron mineral rich planets such as Mars. The contribution of microbially-catalyzed iron redox and phase transformation to soils and sedimentary environments can profoundly affect the geochemistry and mineralogy of these environments. Furthermore the metabolic versatility of FOM and FRM has resulted in the application of several biological strategies in order to remediate organic and metal/radionuclide contaminated environments as well as harvest energy.

Iron Cycling: The microbial redox workout

At circumneutral (~pH 7) and greater pH values iron primarily exists as insoluble, solid-phase minerals in the divalent ferrous or trivalent ferric oxidation states (denoted as Fe(II) and Fe(III) respectively)[1]. The solubility of Fe(III) increases with decreasing pH values[2]. Below pH 4.0, Fe(II) primarily exists as an aqueous species even in the presence of oxygen. The biogeochemical role of FOM in acidic environments has been well established (refer to REF. 3 for review) (Box 1). Given the reactivity of iron oxide mineral surfaces, microbial Fe redox cycling can significantly affect the geochemistry of hydromorphic soils and sediments leading to the mineralization of organic matter, mineral dissolution and weathering, the formation of geologically significant minerals, mobilization or immobilization of various anions and cations including contaminants[1,4-7]. Thus the redox transition between the Fe(II) and Fe(III) valence states plays a fundamental role in modern environmental biogeochemistry and was likely an important biogeochemical process on early earth.

In neoteric environmental systems in the anoxic zone at pH >4.0, Fe(III) oxides provide an electron sink and are reduced either chemically or biologically. Prior to the recognition of microbially mediated iron redox reactions, abiotic mechanisms were thought to control iron redox chemistry[8]. Despite their insoluble nature, Fe(III) oxide minerals can still serve as a terminal electron acceptor for the microbial oxidation of organic matter and/or H_2 (FIG. 1). It is now established that microbial catalysis primarily controls Fe(III) reduction in non-sulfidogenic sedimentary environments[9-11]. In environments where active microbial sulfate reduction is occurring, biogenic H_2S can result in the abiotic reduction of a proportion of the Fe(III). However the potential for enzymatic Fe(III) reduction persists, as studies demonstrated that as much as 75% of the organic matter oxidized in marine sediments was coupled to enzymatic Fe(III) reduction[12,13]. Ammonium as an electron donor may also play a role in Fe(III)

reduction[14-16], however direct pure culture evidence is required to support this observation. The activity of FRM results in the generation of aqueous Fe(II) ($Fe(II)_{aq}$) as well as solid-phase Fe(II) ($Fe(II)_s$) bearing minerals (i.e. siderite and vivianite) including geologically significant mixed valence Fe(II)-Fe(III) minerals such as magnetite and green rust[17-19]. The ubiquity of FRM combined with the elemental abundance of iron in the earth's crust establishes the global significance of microbial iron reduction[11].

Microbially-mediated Fe(II) oxidation is also known to contribute to a dynamic iron biogeochemical cycle at circumneutral pH in both oxic and anoxic environments (FIG. 1). With the identification of FOM, the fate of Fe(II) in environmental systems is now recognized to undergo both biological and abiotic reactions. Abiotic oxidation of $Fe(II)_{aq}$ may be mediated through a reaction with oxidized manganese (Mn(IV)) species or by the diffusion of Fe(II) into an oxic environment subsequently reacting with molecular oxygen (O_2). Benthic bioturbation by macrophytes and macrofauna can induce particle mixing and aeration resulting in the subsequent oxidation of $Fe(II)_{aq}$ and $Fe(II)_s$[20-25]. However, microbially-mediated oxidative processes in direct association with bioturbation have not been studied to any significant degree with studies limited to the rhizosphere[26-28] . Microaerophilic FOM capable of competing with the abiotic oxidation kinetics between O_2 and Fe(II) have been shown to contribute to Fe cycling in the oxic environment coupling this metabolism to growth[29-33].

While aerobic microbial oxidation of Fe(II) has been recognized for decades, the recent identification of anaerobic Fe(II) bio-oxidation closed a missing gap in the iron redox cycle[34,35]. Recent evidence indicates that anaerobic Fe(II) oxidation can contribute to a dynamic anaerobic iron redox cycle[36-38] in addition to soil and sediment biogeochemistry, mineralogy, and heavy metal and radionuclide immobilization[6,7,38,39]. In environments devoid of oxygen, microbial

Fe(II) oxidation coupled to the reduction of nitrate, perchlorate, and chlorate (FIG. 1) has been demonstrated[35,38,40]. These FOM are capable of oxidizing solid-phase Fe(II)[6,39,41,42] as well as Fe(II) associated with structural Fe in minerals such as almandine, an iron aluminum silicate[6,43,44]. In zones of sufficient light penetration, Fe(II) oxidizing phototrophic bacteria are capable of oxidizing Fe(II) generating Fe(III) (FIG. 1). FOM are ubiquitous and have been identified in numerous environments. Anaerobic, biogenically formed Fe(III) oxides potentially serve as a terminal electron acceptor for FRM perpetuating a dynamic microbially-mediated Fe iron redox cycle (FIG. 1).

Microbial Iron(II) Oxidation

Aerobic

The microbial oxidation of Fe(II) coupled to the reduction of O_2 in environments of acidic and circumneutral pH values has been recognized for more than a century (REF. 29,45 and references therein). Yet, despite the historical knowledge of FOM , the geological significance of aerobic Fe(II) oxidation had been discounted based on the rapid abiotic Fe(II) oxidation rate coupled to the reduction of O_2[5]. The enrichment of microaerophilic FOM using opposing diffusion Fe(II)-O_2 gradient systems has expanded our knowledge of aerobic, neutrophilic FOM beyond *Gallionella* sp. and *Leptothrix* sp. isolating Alpha-, Beta-, and Gammaproteobacteria as FOM[30,33,46,47]. Environments potentially supporting aerobic, neutrophilic Fe(II) oxidation are stream sediments, groundwater Fe seeps, wetland surface sediments, sediments associated with the rhizosphere, cave walls, irrigation ditches, subsurface bore holes, municipal and industrial water distribution systems, deep ocean basalt, as well as hydrothermal vents[26,33,48,49]. These microaerophilic FOM are able to successfully compete with the kinetics of abiotic Fe(II) oxidation in these environments. Although the quantitative significance of this microbial metabolic process in terms of accelerating Fe(II) oxidation rates is subject to interpretation, unequivocal evidence demonstrates that FOM are capable of conserving energy from this process and converting inorganic carbon, CO_2, into biomass[30,33,46]. Aerobic Fe(II) oxidation not only plays a role in Fe redox reactions at the oxic/anoxic interface but also influences mineral weathering in the environment[33,50].

Anaerobic

Before O_2 was available as an oxidant in the Fe(II)-rich Precambrian environment, anaerobic microbial oxidation of Fe(II) potentially provided an early respiratory metabolism contributing to the precipitation of iron oxide minerals including magnetite found in Archean banded iron formations (BIF)[6,34,51,52]. The reduction potential of the Fe(III)/Fe(II) couple is sufficient to provide reducing power between bacterial photosystems or alternative terminal electron acceptors involved in respiratory processes (FIG. 2) in order to sustain microbial growth. Within the last decade Fe(II) oxidation mediated by Archaea and Bacteria has been described in environments devoid of oxygen at circumneutral pH[34,35,51].

(i) Seeing the light – Anaerobic, phototrophic oxidation of Fe(II):

Photoautotrophic, anaerobic Fe(II) oxidation was the first demonstration of microbially-mediated oxidation of Fe(II) in anoxic environments[34]. These FOM oxidize Fe(II), utilizing light energy to fix CO_2 into biomass (Eq. 1)

$$HCO_3^- + 4Fe(II) + 10H_2O \rightarrow < CH_2O > + 4Fe(OH)_3 + 7H^+ \text{ (Eq. 1)}$$

Although phototrophic FOM described within the domain Bacteria are phylogenetically diverse (FIG. 3), to date an archeon capable of this metabolism has not been identified. Several purple and green anoxygenic, photosynthetic Fe(II) oxidizing bacteria have been isolated from freshwater and marine environments and described in pure culture[34,52-56]. With the exception of *Rhodomicrobium vannielii*, these phototrophic FOM are capable of completely oxidizing soluble $Fe(II)_{aq}$ to Fe(III). Incomplete Fe(II) oxidation by *R. vannielii* has been attributed to encrustation of the cell with biogenic Fe(III) oxides inhibiting further metabolic activity[34,54]. The production of low molecular weight compounds solubilizing biogenic Fe(III) has been suggested as a mechanism for preventing cell encrustation in cultures of other phototrophic FOM including *Rhodovulum robiginosum* and *Chlorobium ferrooxidans*[53,57]. In contrast to observations made

with *R. vannielii*[57], concentrations of soluble Fe(II) and Fe(III) in spent culture media of *R. robiginosum* and *C. ferrooxidans* far exceeded the amount predicted by solubility constants and an uninoculated control[57]. However, a subsequent study failed to identify significant evidence supporting the direct role of organic compounds or chelators complexing Fe when *R. robiginosum*, *C. ferrooxidans*, and *Thiodictyon* sp. strain F4 were grown on $Fe(II)_{aq}$ or solid-phase ferrous sulfide (FeS)[58]. As such, it was proposed that Fe(III) was released from the active cell as an inorganic aqueous complex or colloidal aggregate[58]. Nonetheless, a fundamental difference remains to be explained as to why *R. vannielii* is subject to cell encrustation while other phototrophic FOM are not affected by the formation of insoluble Fe(III) oxides. The production of compounds capable of solubilizing Fe(III) has profound implications not only for FOM cellular metabolism but also the dissolution of solid-phase minerals and the release of soluble Fe as a terminal electron acceptor or as a micronutrient for other aquatic and terrestrial living organisms.

Phototrophic Fe(II) oxidation results in the formation of poorly crystalline Fe(III) oxides[59] which subsequently transform into more crystalline Fe(III) oxide minerals, goethite and lepidocrocite, in the presence of metabolically active FOM[58]. However, the significance of phototrophic Fe(II) oxidation processes in natural terrestrial environments is limited by the maximum penetration of light to a depth of 200 μm into soil and sediment[60]. Furthermore, recent studies indicate that phototrophic Fe(II) oxidizing bacteria are unable to promote Fe(II) mineral dissolution and are limited by the mineral solubility[58]. Therefore the impact of this microbial process to Fe redox cycling may be locally significant but may play a minor global role in contemporary iron biogeochemical cycling in terrestrial environments.

(ii) Working in the dark - Anaerobic nitrate-dependent Fe(II) oxidation:

Anaerobic Fe(II) oxidation is not limited to environments exposed to light. At circumneutral pH, light independent microbially-mediated oxidation of both soluble and insoluble Fe(II) coupled to nitrate reduction has been demonstrated in a variety of freshwater and saline environmental systems, including paddy soil, pond, stream, ditch, brackish lagoon, lake, wetland, aquifer, hydrothermal, and deep sea sediments[6,33,35,38,39,43,51,61-68]. These environmental systems support abundant nitrate-dependent Fe(II) oxidizing microbial communities in the order of 1 x 10^3 to 5 x 10^8 cells g^{-1} sediment[38,65,66,68,69], potentially contributing to Fe redox cycling. A variety of microorganisms in the domain Archaea and Bacteria (FIG. 3), representing an extreme range of optimal thermal growth conditions (psycrohphilic, mesophilic, to hyperthermophilic) have been identified. The ubiquity and diversity of these anaerobic FOM suggests that metabolic, light-independent reactions such as nitrate-dependent Fe(II) oxidation have the potential to contribute to anoxic Fe(II) oxidative processes on a global scale provided adequate concentrations of a suitable electron acceptor are readily available.

FOM are capable of exploiting the favorable thermodynamics between $Fe(OH_3)$/Fe(II) (Eh = +0.014) and nitrate reduction redox pairs ($NO_3^-/½N_2$, Eh = + 0.713; NO_3^-/NO_2^-, Eh = + 0.431; NO_3^-/NH_4^+, Eh = + 0.36) as well as other potential terminal electron acceptors such as perchlorate and chlorate (ClO_4^-/Cl^-, Eh = + 0.873; ClO_3^-/Cl^-, Eh = + 0.616; respectively) (FIG. 2)[6,35,38,40,51,67,68]. Several phylogentically diverse mesophilic bacteria have been described as capable of nitrate-dependent Fe(II) oxidation (FIG. 3), however in most cases growth was either not associated with this metabolism or was not demonstrated in the absence of an additional electron donor or organic carbon as an energy source at circumneutral pH[6,35,40,43,67,70].

The oxidation of Fe(II), including solid-phase Fe(II) bearing minerals, coupled to nitrate reduction is energetically favorable at neutral pH and should yield enough energy to support

carbon fixation and microbial growth. However, to date autotrophic growth under nitrate-dependent Fe(II) oxidizing conditions has only been demonstrated in two pure culture isolates; a hyperthermophilic archeon[51], and a mesophilic Betaproteobacterium, *"Candidatus* Cosmobacter millennium" strain 2002[68]. Even in the case of a known Fe(II)-oxidizing chemolithoautotrophic bacterium, *Thiobacillus denitrificans*, energy conservation directly coupled to nitrate-dependent metal oxidation, Fe(II) or U(IV), could not be determined as an additional electron donor was required for metabolic activity to occur[71]. Until the recent demonstration of nitrate-dependent Fe(II) oxidation by *Geobacter metallireducens* resulting in the production of ammonium[38], NO_2^- or N_2 were identified as the sole nitrate reduction end-products[6,35,51,70]. Growth of FOM capable of autotrophy or mixotrophy associated with perchlorate/chlorate-dependent Fe(II) oxidation has not been identified to date. However, perchlorate/chlorate-dependent Fe(II) oxidizing metabolic activity is observed under stationary growth phase conditions in cultures of *Azospira suillum* strain PS (formerly *Dechlorosoma*)[6,41]. Although perchlorate and chlorate are not considered to be naturally abundant compounds, their potential to serve as an electron acceptor in environmental systems cannot be discounted[72]. Legal discharge of perchlorate into natural waters has led to widespread anthropogenic contamination throughout the United States. Given the ubiquity of perchlorate reducing bacteria[72] and the ability of some of these microorganisms, specifically *Azospira* sp. and *Dechloromonas* sp., to oxidize Fe(II), anaerobic perchlorate/chlorate-dependent Fe(II) oxidation may impact iron biogeochemical cycling in environments exposed to contaminated waters. Further studies are needed to quantify the potential influence of perchlorate/chlorate-dependent Fe(II) oxidation.

Doing hard time: Solid-Phase Microbial Fe(II) Oxidation

In contrast to phototrophic FOM, solid-phase Fe(II) bearing minerals, including surface bound Fe(II)[38,39], crystalline Fe(II) minerals (siderite, magnetite, pyrite, aresnopyrite, and chromite)[6,39], and structural Fe(II) in nesosilicate (almandine and staurolite)[6] and phyllosicate (nontronite)[43] minerals are known to be subject to direct nitrate-dependent microbial oxidation. Although we know that nitrate-dependent FOM play a role in the oxidation of Fe(II) structurally incorporated into silicate minerals as well as contribute to Fe(II) mineral dissolution, virtually nothing is known about the mineral structure and stability of the residual material. The oxidative dissolution of solid-phase Fe(II) minerals in an anoxic environment presents an additional mechanism for rock weathering and inducing Fe(III) oxide mineral precipitation in anoxic soils and sedimentary environments. To date bio-oxidation products of amorphous solid-phase Fe(II) minerals have been characterized. A variety of biogenic Fe(III) oxide minerals, 2-line ferrihydrite[41,61], goethite[38], lepidocrocite[73], hematite[6], as well as mixed phase Fe(II)-Fe(III) minerals, magnetite, maghemite, and green rust[6](K. Weber unpublished data) were identified as oxidation products. As a result of this biogenic formation of magnetite, nitrate-dependent Fe(II) oxidation has been implicated as playing a direct role in the genesis of banded iron formations in Precambrian Earth[6,51].

Function over Form: Physiology of Fe(II) Oxidation at Circumneutral pH

Virtually nothing is known regarding the biochemistry or genetic regulation of anaerobic Fe(II) oxidation at circumneutral pH. The reduction potential of the myriad of Fe(III)/Fe(II) redox pairs (range of Eh = -0.314 to +0.014) indicates that Type *b, c, or a* cytochromes may be involved in electron transport (FIG. 2). Reduced minus oxidized difference spectra[72] of *A. suillum* whole cells anaerobically incubated in the presence of Fe(II) demonstrated the direct

reduction of the type-*c* cytochrome content[6], implicating the involvement of a type-*c* cytochrome in the the transfer of electrons to nitrate under Fe(II) oxidizing conditions. The demonstrated capability of some FOM to utilize CO_2 as the sole source of carbon requires a CO_2 fixation pathway. In the case of the archeon, *F. placidus*, grown on CO_2 the reductive acetyl coenzyme A pathway is expressed, implicating its involvement in in carbon assimilation[74]. Interestingly, genes associated with the reductive pentose phosphate cycle, RuBisCo, were identified in the finished genome sequence of *Dechloromonas aromatica*, an Fe(II) oxidizing bacterium capable of utilizing nitrate, chlorate, or perchlorate as alternative electron acceptors[68], however autotrophic growth associated with Fe(II) oxidation could not be demonstrated and the conditions under which these genes are expressed remains unidentified (J.D. Coates, unpublished). In contrast, PCR amplification of the genomic DNA from "*C. millennium"* strain 2002, the mesophilic autotrophic nitrate-dependent Fe(II) oxidizing bacterium, with degenerative RuBisCo primers did not yield a PCR product[68]. The CO_2 fixation pathway expressed in "*C. millennium"* under nitrate-dependent Fe(II) oxidizing conditions is currently unknown. Regardless, the availability of genomic sequence information of FOM, *D. aromatica*, *Marinobacter aquaeolei*, *G. metallireducens*, and *T. denitrificans*, provides the first opportunity to aggressively address functional genes and regulatory pathways associated with this metabolism. However, other phylogentically and physiologically distinct microorganisms such as *F. placidus*, "*C. millennium*", and *A. suillum* which also potentially play a key role in anaerobic nitrate-dependent Fe(II) oxidation have yet to be genome sequenced.

The Other Half – Microbial Iron Reduction

Antiquity of Iron Reduction

It has been proposed that life emerged on a hot (possibly as high as 140°C -150°C), Fe(II)-rich early Earth 3.8 billions years ago[75,76]. The photochemical generation of Fe(III) and H_2 would have provided an electron acceptor and energy source, respectively, for ancient life (Eq. 2) (REF. 11,77,78 and references therein).

$$2\ Fe(II) + 2\ H^+ \xrightarrow{h\nu} 2\ Fe(III) + H_2 \quad (2)$$

As such iron respiration has been proposed as one of the first forms of microbial metabolism to have evolved preceding the development of oxygen, nitrate, and sulfate respiration[79-82]. In support of this, Fe(III) respiration has been identified in a broad diversity of extant microorganisms including those most closely related to the last common ancestor[81,83]. Extracellular electron transfer to insoluble Fe(III) oxide minerals has been conserved throughout the hyperthermophilic *Archaea*[81,83-85] and is widely distributed among the *Bacteria* (FIG. 3)[11,82] further suggesting that this is an early metabolism which spread through the microbial domains throughout evolution.

A hard rock chord: crystalline Fe(III) oxide reduction

Poorly crystalline Fe(III) oxide minerals, such as ferrihydrite, readily serve as an electron acceptor for FRM[86]. This is not surprising given the relative energetic favorability of the reduction of amorphous ferric iron oxides (FIG. 2). However, iron oxides predominantly exist in a crystalline phase or as a structural component of clays in modern soils and sedimentary environments[1]. Although the thermodynamic favorability of crystalline Fe(III) oxide mineral reduction (goethite, hematite, and magnetite) is decreased (FIG. 2), previous studies have suggested that FRM are capable of living on the energetic edge utilizing the structural[43,44,87] or

crystalline solid-phase Fe(III) as an electron acceptor[88-91]. Considerable debate has been put forward arguing the direct environmental relevance of laboratory studies which have examined reduction of crystalline Fe minerals under artificially organic and nutrient rich conditions in order to optimize the growth of FRM[11,92]. Glasauer and colleagues (2003) did not observe the reduction of goethite and hematite in defined minimal media by *Shewanella putrefaciens* strain CN32 which is in direct contrast to previous reduction studies conducted with this organism in nutrient rich media[19,90,91]. However the generalization that FRM are unable to reduce crystalline Fe(III) oxide minerals under nutrient limited conditions is not universal. Under oligotrophic culture conditions both a culture of *G. metallireducens* and a freshwater enrichment culture (1% vol:vol inoculum) reduced goethite beyond the amorphous Fe(III) oxide content (determined by 0.5 N HCl extraction)[38] indicating the possibility for microbial reduction of crystalline Fe(III) oxides in the environment.

A means to an end: Microbial strategies for reduction of an insoluble electron acceptor

The insoluble nature of Fe(III) oxide minerals at values above pH 4 creates a metabolic dilemma for microorganisms utilizing Fe(III) oxides as a respiratory terminal electron acceptor. A variety of mechanisms have been proposed describing possible strategies utilized by the microorganisms in order to transfer electrons to the extracellular Fe(III) oxide mineral. Direct contact between the cell and the solid-phase Fe(III) oxide mineral was determined as a requirement for the reduction of insoluble Fe(III) oxides in *Geobacter* sp.[93] (FIG. 4A). The formation of flagella and pili had been proposed as the mechanism in which *Geobacter* sp. directly attached to the Fe(III) oxide surface[94]. Recent evidence indicates that pili are not required for attachment of *Geobacter* sp. to the solid-phase Fe(III) oxide surface but rather serve

as an electrical conduit for the transfer of electrons to insoluble Fe(III) oxides and potentially other solid-phase terminal electron acceptors[95]. The formation of these conductive cellular "nanowires" expands the accessible spatial area available beyond the cell membrane allowing the penetration of nanometer pore spaces in soils and sedimentary environments previously thought physically unavailable to the cell. The potential for these "nanowires" to create a bridge between individual cells introduces the possibility of cell to cell communication[95], as well as for the cell attached to the Fe(III) oxide or other electron acceptor to function as an electron shuttle.

The identification of conductive "nanowires" was apparently exclusive to *Geobacter* sp. as pili produced by other metal reducing organisms tested including *Shewanella oneidensis* were not conductive[95]. However, FRM may not necessarily need to establish direct contact with the solid-phase surface to reduce Fe(III) oxides as other alternative active mechanisms could be employed. Exogenous[96] and endogenously produced[97-99] soluble external electron shuttles can be exploited as mediators to complete the transfer of electrons to the solid-phase terminal electron acceptor (FIG. 4B). The electron shuttle alleviates the need for the FRM to directly contact the Fe(III) oxide functioning in a combination of a microbially-catalyzed and abiotic process where (*i*) the FRM oxidizes an electron donor coupled to the reduction of the soluble electron shuttle and (*ii*) the reduced electron shuttle diffuses and subsequently abiotically donates electrons to the solid-phase Fe(III) oxide[96] (FIG. 4B). The abiotic regeneration of the oxidized electron acceptor in part (*ii*) restarts the cycle (FIG. 4B).

Redox reactive organic compounds common in soils and sedimentary environments such as humic acids[96], plant exudates[100], and antibiotics[101] have been identified as electron shuttles. Although the true significance of these compounds to serve as electron shuttles for microbially-mediated Fe(III) reduction in eutrophic environments is still unknown, their utility in

oligotrophic environments may be limited due to the low availability of suitable redox reactive refractory organic substances. However, the endogenous production of an electron shuttle recognized in two FRM genera, *Shewanella* sp.[97,99,102] and *Geothrix fermentans*[98], may further mitigate the reliance of these FRM on exogenous electron shuttles. Although the energetic expense of producing and excreting the redox reactive compounds to the environment would not yield a competitive advantage in situations of low cell mass, it has been speculated that the release of electron shuttles in a biofilm community would allocate electron transfer to cells distant from the substrate surface[102]. In addition, the recently identified ubiquitous biological reoxidation of these diffusing electron shuttles coupled to carbon assimilation in the presence of a suitable electron acceptor such as nitrate offers the potential for a previously unidentified symbiotic relationship at the microbial level[103,104].

The iron maidens of rock: Diversity of FRM

A wide phylogenetic diversity of microorganisms capable of conserving energy coupled to growth by the dissimilatory reduction of Fe(III) have been identified throughout the domains *Archaea* and *Bacteria* (FIG. 3). These FRM have been isolated across a vast range of chemical and physical conditions demonstrating the ubiquity of this microbial metabolism. From among these isolated microorganisms, Fe(III) reducing extremophiles such as hyperthermophilic, thermophilic, psychrophilic, acidophilic, and alkaliphilic *Archaea* and/or *Bacteria* have been described in pure culture[83,105-109] (see Lovley et al. 2004 for review[11]). One such isolate surviving in hydrothermal vents has pushed the upper temperature limit for life above 121°C[83]. In modern terrestrial and subsurface environments at circumneutral pH microorganisms identified within the family Geobacteraceae are among the most common and most

comprehensibly studied FRM (REF. 11 and references therein). This group of FRM is considered to play a significant role in dissimilatory Fe(III) reduction and organic matter oxidation in soils and sedimentary environments. Outside of the Deltaproteobacteria another well characterized group of FRM, the *Shewanella* sp has been identified within the Gammaproteobacteria. Although, *Shewanella* sp have been isolated from diverse metal reducing sedimentary environments[91,110,111], several studies focused on the recovery of 16S rDNA sequences representing these FRM in natural Fe(III) reducing environments have not yielded a positive result[38,112-116] suggesting that these microorganisms may only play a minor role in the reduction of Fe(III) oxides *in situ*.

Recent evidence indicates that other organisms belonging to the Betaproteobacteria may also play a role in the reduction of Fe(III) in sedimentary environments although the extent of this role has yet to be quantified[38,114,117-119]. Similarly, enzymatic Fe(III) reduction is not limited to the Proteobacteria. The potential of the poorly described Acidobacteria to contribute to Fe(III) reduction was demonstrated with the isolation of *Geothrix fermentans*[120] and supported by the identification of 16S rDNA most similar to this microorganism in the highest dilution of an Fe(III) reducing most probable number enumeration series from subsurface sediments[121]. To date the exact role of members of this phylum in the environment remains elusive because of the limited availability of pure cultures; however, given that Acidobacteria are ubiquitous in the environment[122-124], the contribution of these microorganisms to Fe biogeochemical cycling in soils and sediments could be globally significant.

In addition to the FRM described above which rely on energy conservation for growth from the dissimilatory reduction of Fe(III), a variety of fermentative microorganisms have also been recognized to enzymatically catalyze the reduction of Fe(III) (REF. 125-127 and references

therein). These fermentative microorganisms transfer only a minor fraction, ca. 5%, of the available reducing equivalents to Fe(III)[126], and Fe(III) is not required for growth. The diversion of reducing equivalents to Fe(III) may provide an energetic advantage utilizing the oxidation of NAD(P)H coupled to Fe(III) reduction to yield ATP[128]. As such, Fe(III) reduction coupled to a fermentative metabolism is considered to play a minor role in iron geochemical cycling relative to respiratory Fe(III) reduction. In addition to the fermentative microorganisms that have been identified to reduce Fe(III), some sulfate reducing[129,130] and methanogenic[131] microorganisms are also capable of Fe(III) reduction without the demonstrated benefit of growth. Competition for available electron donors between microbial communities supporting these metabolisms has been implicated in the suppression of sulfate reduction and methanogenesis in sedimentary environments[132,133]. However, the direct enzymatic reduction of Fe(III) coupled to the oxidation of H_2 by sulfate reducing and methanogenic microorganisms would directly inhibit sulfate reducing and methanogenic metabolisms at the cellular level. The physiological advantage created by oxidizing H_2 coupled to Fe(III) reduction is unknown. In fact, this metabolism would scavenge reducing equivalents impairing the ability of these microorganisms to grow with the transfer of electrons to sulfate or carbon dioxide. The true ecological implications of this alternative lifestyle stills remains a mystery for these organisms.

Go with the flow: Electron transport to Fe(III) oxides

Iron(III) oxide minerals are insoluble and thus unable to diffuse inside the cell. Therefore electron transport to the terminal electron acceptor cannot occur within the periplasm as it does for soluble electron acceptors such as nitrate or fumarate[134] and would require the reduction of Fe(III) to occur outside of the cell with a protein localized in the a outer membrane, presumably a terminal iron reductase. Just as the strategies utilizing insoluble Fe(III) oxides as a terminal

electron acceptor differ between the two model FRM, *Shewanella* sp. and *Geobacter sp.*, the proteins involved in electron transport are also dissimilar. The only general similarity between the electron transport mechanisms commences with the transfer of electrons from the dehydrogenase to a quinone pool consisting of ubiquinones and menaquinones in the cytoplasmic membrane to *c*-type cytochromes and finally to a reductase in the outer membrane[97,135-139]. While an iron reductase has eluded identification to date, significant developments have advanced our knowledge of Fe(III) reduction biochemistry in two model FRM, *Shewanella* sp. and *Geobacter sp*. Development of electron transport models are in the formative years as the number of *c*-type cytochromes directly implicated in Fe(III) reduction is far less than has been identified within completed genome sequences. The completed genome sequence of *S. oneidensis* and *G. sulfurreducens* revealed 39 and 111 putative *c*-type cytochromes respectively[140,141]. The high number of *c*-type cytochromes, specifically in *G. sulfurreducens*, has led to the suggestion of multiple pathways of electron transport[142]. The models depicted in Figure 5 illustrate the components known to be directly involved in electron transport to Fe(III) in both *Shewanella* sp. and *Geobacter* sp. as described to date.

In *Shewanella* sp. the electrons are transferred from the menaquinone to a tetraheme *c*-type cytochrome, CymA, located in the cytoplasmic membrane[136,143-145]. The electrons are then passed to electron carriers within the periplasm. To date, two *c*-type cytochromes in the periplasm have been identified as potential electron carriers necessary for Fe(III) reduction within *Shewanella* sp., MtrA and Cyt C3[146-148]. A small tetraheme c-type cytochrome, Cyt C3, within the periplasm known to be associated with Fe(III) reduction [149,150] may serve as an electron shuttle between electron carriers[151]. MtrA is a decaheme c-type cytochrome which is similarly thought to accept electrons from the cytoplasmic membrane electron carrier CymA, and

transfer these electrons to an outer membrane protein[147]. It has also been proposed that MtrA alternatively serves as a terminal reductase for soluble Fe(III) within the periplasm[148] (FIG. 5). An outer membrane cytochrome partially exposed on the cell surface[147,152], OmcB (formerly denoted as mtrC[147]), accepts electrons from periplasmic proteins and has the potential to directly reduce extracellular Fe(III)[145,152] (FIG. 5). This is supported by studies involving a mutation in *omcB* which resulted in a decreased ability to reduce Fe(III), however some Fe(III) reduction did still occur[145,153] demonstrating that Fe(III) reduction is not absolutely dependent on a functioning *omcB* (FIG. 5).

Given the limited information available to date, the relationship between the periplasmic cytochromes and cytoplasmic outer membrane proteins has not been firmly established in *Geobacter* sp.[154]. A key periplasmic cytochrome, MacA, localized in the cytoplasmic membrane was identified and shown to play a central role in the transfer of electrons to Fe(III). It is predicted to serve as an intermediate carrier similarly to MtrA in *Shewanella* sp.[154]. MacA, may then pass electrons to other periplasmic proteins such as PpcA, a tri-heme periplasmic *c*-type cytochrome involved in electron transport to proteins located in the outer membrane[155] (FIG. 5). One such outer membrane protein, OmcB, was determined to play a significant role in Fe(III) reduction[142,156]. Disruptions in *omcB* by gene replacement impaired the ability of *G. sulfurreducens* to reduce Fe(III) by ca. 94-97%[156]. However, the *omcB* deficient mutant adapted to growth on soluble Fe(III) over time with similar reduction rates to the wild type, although growth was only ca 60% of the wild type[142]. Interestingly the adapted mutant was unable to reduce insoluble Fe(III) oxides indicating that different electron transport mechanisms are employed to reduce insoluble and soluble Fe(III) sources[142]. The recent identification of conductive "nanowires" implicated the involvement of other outer membrane proteins in electron

transport to Fe(III) oxides. Reguera et al. (2005)[95] have proposed that pili directly accept electrons from intermediary electron transfer proteins located in the periplasm and/or outer membrane for transfer to the solid-phase Fe(III) oxide surface (FIG. 5). The concept of electron tunneling is not a foreign concept in biology in which nanowires can be formed with redox cofactors within close proximity (14Å) of each other[157], yet the identification of redox reactive nanowires introduces an exciting new twist to cell biology.

Concluding Remarks

Over the last two decades, recognition of the microbial reduction and oxidation of the fourth most abundant element in the Earth's crust has identified a globally significant biogeochemical process. While it is recognized that these microorganisms are ubiquitous, the physiology of Fe(III) reduction and Fe(II) oxidation remains an enigma, as a terminal Fe(III) reductase has yet to be identified and only *c*-type cytochrome(s) have been implicated in Fe(II) oxidation at circumneutral pH. Genome sequencing and the subsequent development of *in silico* physiological models yield a tool to predict microbial metabolisms in response to the environmental conditions[158,159] and can potentially provide greater insight into Fe oxidation and reduction reactions in environmental systems. These advances can also be applied to enhance and predict the behavior of microorganisms exploited for their metabolism associated with biotechnological applications (Box 3). While significant advances have been made, we are continuously learning of previously unidentified microbially-mediated redox reactions and initiating the quest for microorganisms responsible for catalyzing these unique metabolisms, i.e. ammonium oxidation coupled to Fe(III) reduction[14-16], that can perpetuate a dynamic anaerobic iron redox cycle.

Box 1. Acidogenic Fe(II) Oxidation

The dissolution of metal-sulfide rich minerals (i.e. pyrite, FeS_2) via chemical weathering when exposed to fluids containing O_2 results in the generation of protons creating an acidic environment enriched with Fe(II) and SO_4^{2-} according to equation B1.

$$FeS_2 + 3.5O_2 + H_2O \rightarrow Fe(II) + 2SO_4^{2-} + 2H^+ \textit{ abiotic} \quad \text{(B1)}$$

Unlike environments at circumneutral pH, the abiotic reaction between dissolved Fe(II) and O_2 is insignificant at low pH values such as those in these acidic environments (pH<4.0). Microbial catalysis (Eq. B2) by lithotrophic Archaea (*Ferroplasma acidiphilum, F. acidarmanus, Metallosphaera sedula*) and Bacteria (*Acidithiobacillus ferrooxidans, Acidimicrobium ferrooxidans*) accelerate the Fe(II) oxidation rate forming $Fe(III)_{aq}$ and a variety of Fe(III) minerals, jarosite, schwertmannite, goethite, and ferrihydrite[1].

$$14Fe(II) + 3.5O_2 + 14H^+ \rightarrow Fe(III) + 7H_2O \textit{ biotic} \quad \text{(B2)}$$

The generation of Fe(III) perpetuates pyritic dissolution via abiotic oxidation of sulfide as noted in equation B3.

$$FeS_2 + 14Fe(III) + 8H_2O \rightarrow 15Fe(II) + 2SO_4^{2-} + 16H^+ \textit{ abiotic} \quad \text{(B3)}$$

The combined interaction between microbial catalysis and mineral dissolution can result in a sustainable cycle potentially driving the pH of the environment to below values of 2 (REF. 3).

Box 2. Banded Iron Formations

The deposition of alternating iron-rich and silica-rich mineral layers in the late Archean to early Proterozoic resulted in the genesis of the Precambrian banded iron formations (BIFs)[160]. Early models suggested that BIFs were formed via the consequence of abiotic reactions involving the metabolic end-product of oxygenic photosynthesis, O_2[161], or photo-oxidation of Fe(II)[77,162] resulting in the precipitation of insoluble Fe(III) oxides to form the iron-rich laminae, often containing magnetite, a mixed valence phase Fe(II)-Fe(III) bearing mineral. In recent years microbially-mediated reductive and oxidative respiratory metabolisms have been implicated in the deposition of Fe(II) and/or Fe(III) bearing minerals in the iron-rich laminae of BIFs[6,34,163-165]. Microaerophilic Fe(II) oxidizers such as *G. ferruginea* may have oxidized Fe(II) coupled to the reduction of O_2 generated by oxygenic photosynthesis[164,166]. However, this model assumes the evolution of oxygenic photosynthesis generating enough oxygen to serve as the electron acceptor for the precipitation of Fe(III) rich minerals. Much debate has been put forward in contention with the evolution of O_2 in the early Proterozoic and thus the role of O_2 in the deposition of BIFs[167-169]. Other microbially-mediated mechanisms defining the precipitation of Fe(II) and Fe(III) rich minerals in anoxic environments have been proposed as plausible alternatives to the oxic deposition of BIFs, *i*) microbial Fe(III) reduction, *ii*) phototrophic Fe(II) oxidation, and *iii*) nitrate-dependent Fe(II) oxidation.

The precipitation of magnetite associated with microbial Fe(III) reduction linked the deposition of BIFs to FRM[17,82,163]. Yet, the generation of Fe(III) as a terminal electron acceptor from the pool of available Fe(II) would have to precede the metabolic activity of FRM. In addition to photo-oxidation, the deposition of Fe(III) oxides under anoxic conditions during late Archean to early Proterozoic has been proposed to be microbially-catalyzed by photoautotrophic

Fe(II) oxidizing bacteria[34,82,165] or nitrate-dependent Fe(II) oxidizing microorganisms[6]. The bio-oxidation of Fe(II) not only results in the precipitation of Fe(III) oxides but also results in the direct precipitation of magnetite[6,52,56], further linking FOM to BIFs. The availability of light in the Precambrian is inarguable, as such phototrophic Fe(II) oxidation has received much attention as model describing the deposition of BIFs. Light-independent nitrate-dependent Fe(II) oxidation has received much less attention as a model, however it provides an additional mechanism of Fe(III) oxide deposition leading to BIF in the Precambrian. Lightning discharge contributed to the fixation of N_2 into NO (~10^{12} g/yr) further forming N_2O, NO_2^- and NO_3^- through abiotic disproportion reactions[170-172]. In support of this, genetic and isotopic evidence indicates that biological nitrogen fixation arose early in the history of life with the last common ancestor[173-175] which would have then provided a biologically produced oxidized nitrogen species. Such oxidized nitrogen species could then have served as an electron acceptor for early FOM leading to the deposition of BIFs in the anoxic environment. In neoteric environmental systems microbial communities contribute to a dynamic anoxic iron redox cycle, similar communities consisting of phototrophic FOM, nitrate-dependent FOM, and FRM may have existed in the Precambrian to form the BIFs. The precipitation of biomass with the biogenic Fe(III) oxides would contribute to diagensis and mineralization of organic matter, resulting in the heterotrophic reduction of Fe(III) by FRM and the formation of additional magnetite in BIFs[82,165] which is further supported by the isotopically light carbon associated with BIFs[79].

Box 3. Biotechnological applications

Goodwill of FOM—Heavy metal and radionuclide immobilization

The end-products of anaerobic microbial oxidation of Fe(II) can also influence contaminant geochemistry through the formation of a broad variety of environmentally relevant Fe(III) bearing minerals which can regulate contaminant solubility in natural environments[7,36,67]. Previous studies have identified the biogenic formation of minerals such as ferric oxyhydroxide, goethite, hematite, iron hydrogen carbonate, or maghemite[6,38,41,61]. The precipitation of these biogenic Fe(III) oxides provides a mechanism for the immobilization of heavy metals and metalloids through coprecipitation or physical envelopment as well as providing a reactive surface with an adsorptive affinity for anions (i.e. PO_4^{3-}) and cations (i.e. Zn^{2+}, As^{5+}, Co^{2+}, U^{6+})[7,36,73]. Heavy metals and radionuclides including U(VI) are rapidly removed (as much as 80% of the initial 100 μM within 5 days) from solution during anaerobic nitrate-dependent microbial Fe(II) oxidation in association with the biogenic Fe(III) oxides[7]. As such, the anaerobic formation of biogenic Fe(III) oxide-containing minerals has been identified as a plausible bioremediative strategy for permanently immobilizing heavy-metals and radionuclides[7,176].

Goodwill of FRM—Energy generation and contaminant immobilization/degradation

An electrode, yet another solid-phase electron acceptor exploited by FRM to our energetic advantage. The harvest of electrical energy mediated by FRM in sedimentary environments as well as a microbial fuel cell is a technological reality[6,177-181]. The utilization of electrodes as an electron acceptor has not only been proposed to harvest energy from sediments but also to remediate environments contaminated with organic compounds, i.e. aromatic

compounds[180] and potentially harvesting energy in the process. FRM are not only capable of generating electricity, previous studies have indicated an incredible metabolic versatility and these organisms have been demonstrated to transform a variety of organic[121,182-185] and heavy metal/radionuclide contaminants[84,186,187].

Figure Text

Figure 1. Microbially-Mediated Iron Redox Cycle

Microorganisms play a significant role mediating iron oxidation and reduction reactions in soils and sedimentary environments. The reduction of Fe(III) oxidations occurs in the absence of oxygen. Reoxidation of the biogenic Fe(II) may occur via several biological mechanisms and is not simply limited abiotic reactions with molecular oxygen. The regeneration of Fe(III) in the anoxic environment promotes a dynamic iron redox cycle.

Figure 2. Redox Tower

Theoretical Eh (volts) values for reduction-oxidation couples significant to microbially-mediated iron redox cycling calculated at circumneutral pH. The redox tower is an effective way to visualize the potential electron donors and acceptors utilizable by microorganisms measuring the tendency of a compound to interact as an electron donor or acceptor. An electron donor will have a more negative potential than will the electron acceptor. Reduction potentials were obtained from the following sources: a[188], b[134], c[13], calculated based on data collected from refs 72, 2, e189.

Figure 3. Phylogenetic affiliation of Microorganisms contributing to Iron Redox Cycling

Unrooted phylogenetic tree based on nearly complete 16S rDNA sequences from representative iron cycling prokaryotes. Iron reducers are depicted in red, iron oxidizers are depicted in black.

Figure 4. Microbial strategies mediating electron transfer to insoluble Fe(III) oxides

Three primary strategies have been proposed to facilitate the electron transfer between microorganisms and solid Fe(III) oxide surface. In *Geobacter* sp. direct contact with the oxide surface is required. The production "nanowires", conductive extracellular appendages, facilitate electron transfer serving as an electrical conduit to the Fe(III) oxide surface (A). Endogenously or exogenously produced electron shuttle mediates electron transfer to solid-phase Fe(III) oxides (B). The production of complexing ligands as in the case of *Geothrix* sp. aids in the dissolution of the solid-phase Fe(III) oxide providing a soluble Fe(III) form more readily available to the microorganism (C). While these strategies have been demonstrated for Fe(III) reducing microorganisms, similar strategies may be employed by Fe(II) oxidizing microorganisms that are utilizing solid-phase Fe(II) electron donors.

Figure 5. Physiological model of Fe(III) reduction in *Shewanella* sp. and *Geobacter* sp.

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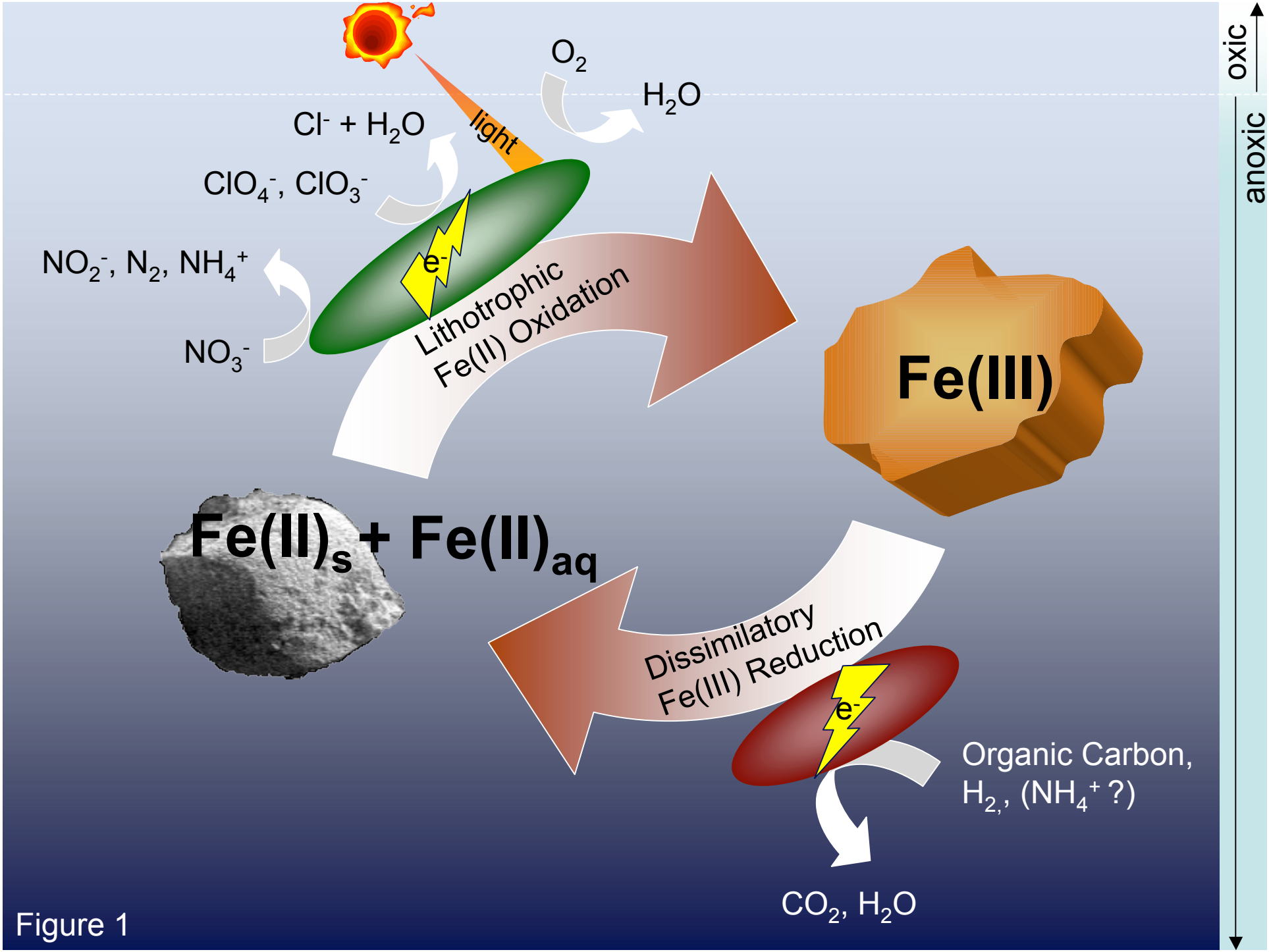
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light
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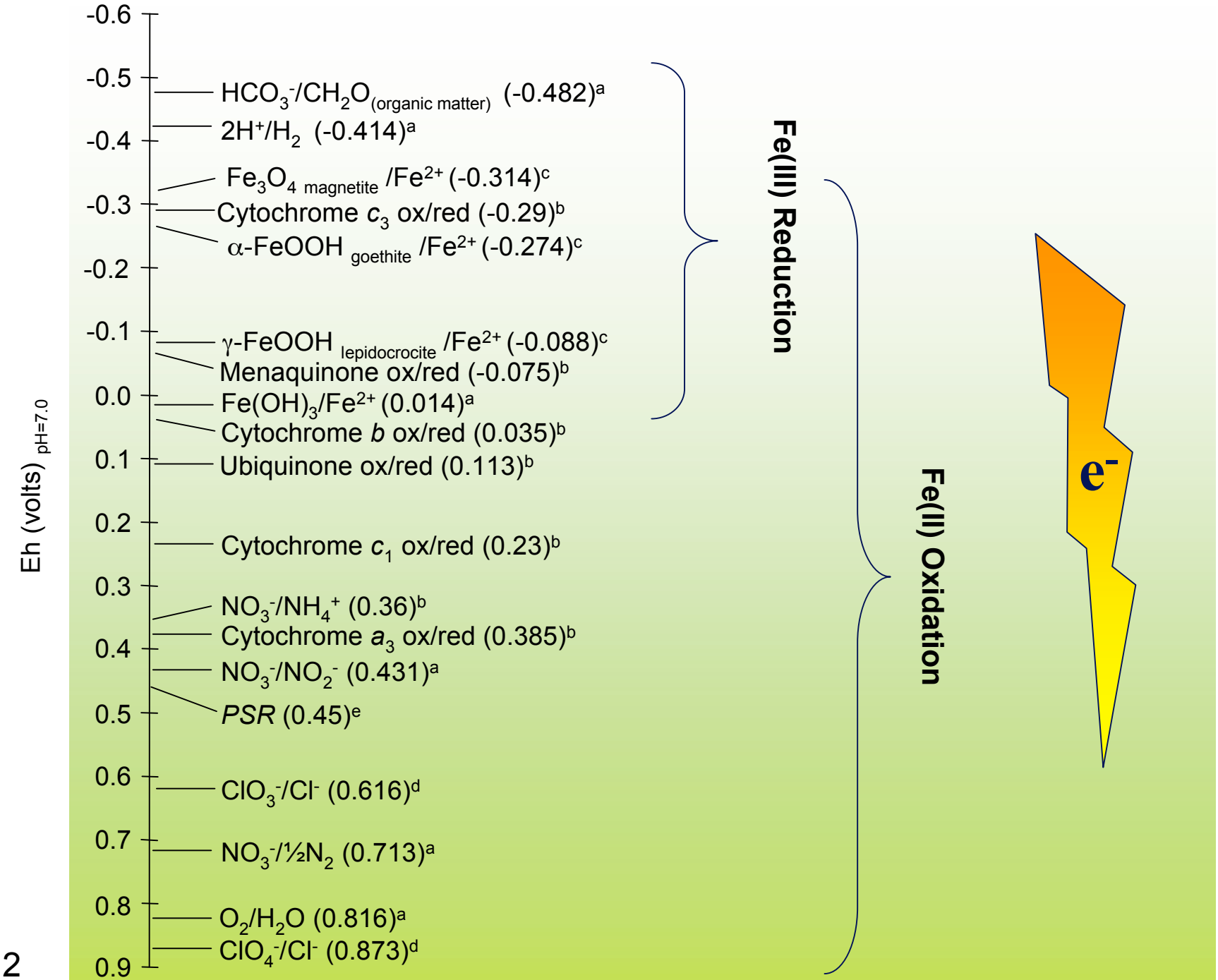
NO_3^-
e-
Lithotrophic
Fe(II) Oxidation
Fe(III)
$Fe(II)_s + Fe(II)_{aq}$
Dissimilatory
Fe(III) Reduction
e-
Organic Carbon,
H_2, (NH_4^+ ?)
CO_2, H_2O

Figure 1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0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
HCO3-/CH2O(organic matter) (-0.482)a
2H+/H2 (-0.414)a
Fe3O4 magnetite /Fe2+ (-0.314)c
Cytochrome c3 ox/red (-0.29)b
α-FeOOH goethite /Fe2+ (-0.274)c
γ-FeOOH lepidocrocite /Fe2+ (-0.088)c
Menaquinone ox/red (-0.075)b
Fe(OH)3/Fe2+ (0.014)a
Cytochrome b ox/red (0.035)b
Ubiquinone ox/red (0.113)b
Cytochrome c1 ox/red (0.23)b
NO3-/NH4+ (0.36)b
Cytochrome a3 ox/red (0.385)b
NO3-/NO2- (0.431)a
PSR (0.45)e
ClO3-/Cl- (0.616)d
NO3-/½N2 (0.713)a
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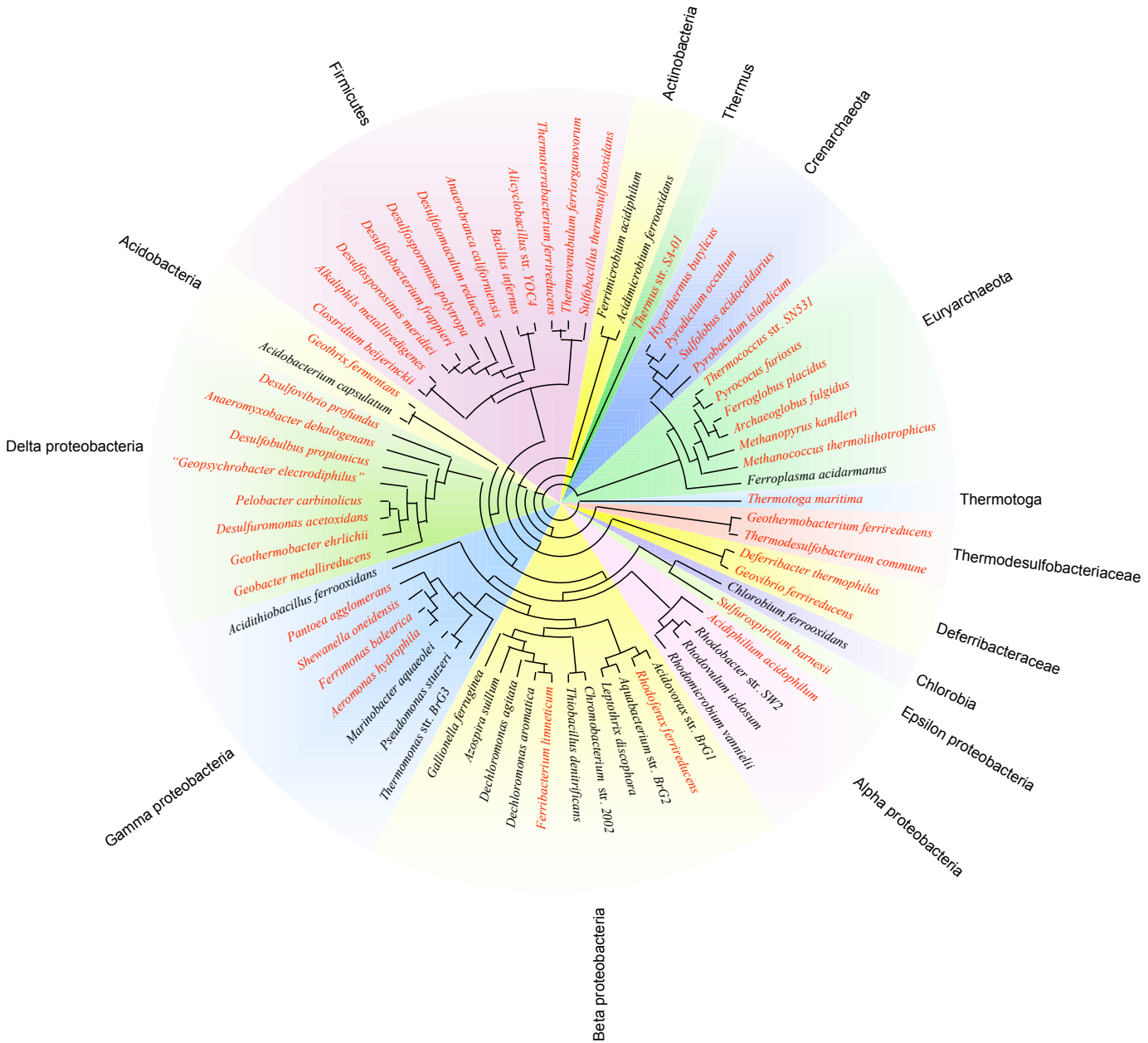
ClO4-/Cl- (0.873)d
Fe(III) Reduction
Fe(II) Oxidation
e-

Figure 2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Alpha proteobacteria
Beta proteobacteria
Gamma proteobacteria
Delta proteobacteria
Acidobacteria
Thermoterrabacterium ferrireducens
Thermovenabulum ferriorganovorum
Sulfobacillus thermosulfidooxidans
Ferrimicrobium acidiphilum
Acidimicrobium ferrooxidans
Thermus str. SA-01
Hyperthermus butylicus
Pyrodictium occultum
Sulfolobus acidocaldarius
Pyrobaculum islandicum
Thermococcus str. SN531
Thermococcus furiosus
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Ferroglobus placidus
Archaeoglobus fulgidus
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Methanococcus thermolithotrophicus
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Thermotoga maritima
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Thermodesulfobacterium commune
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Geovibrio ferrireducens
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Dechloromonas aromatica
Dechloromonas agitata
Azospira suillum
Gallionella ferruginea
Thermomonas str. BrG3
Pseudomonas stutzeri
Marinobacter aquaeolei
Aeromonas hydrophila
Ferrimonas balearica
Shewanella oneidensis
Pantoea agglomerans
Acidithiobacillus ferrooxidans
Geobacter metallireducens
Desulfuromonas acetoxidans
Pelobacter carbinolicus
"Geopsychrobacter electrodiphilus"
Desulfobulbus propionicus
Anaeromyxobacter dehalogenans
Desulfovibrio profundus
Geothrix fermentans
Acidobacterium capsulatum
Clostridium beijerinckii
Alkaliphilus metalliredigenes
Desulfosporosinus meridiei
Desulfitobacterium frappieri
Desulfosporomusa polysaccharovorans
Desulfotomaculum reducens
Anaerobranca californiensis
Bacillus infernus
Alicyclobacillus str. YOC4

Direct Contact

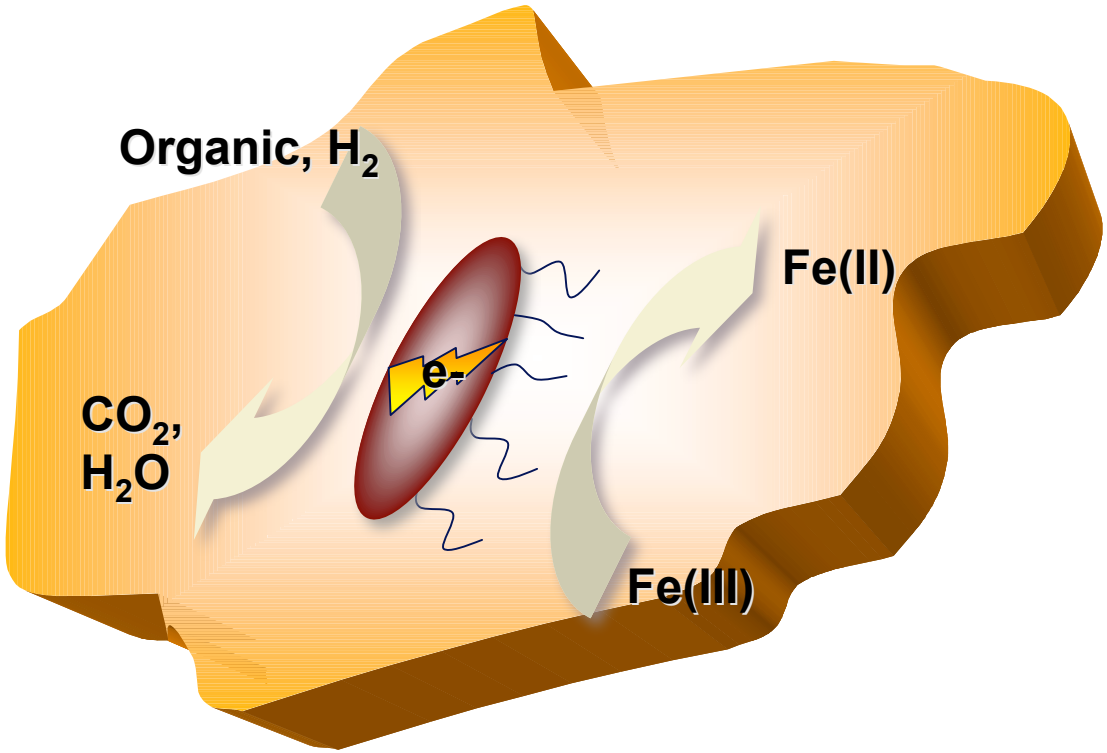


Figure 4a

Extracellular Electron Shuttle

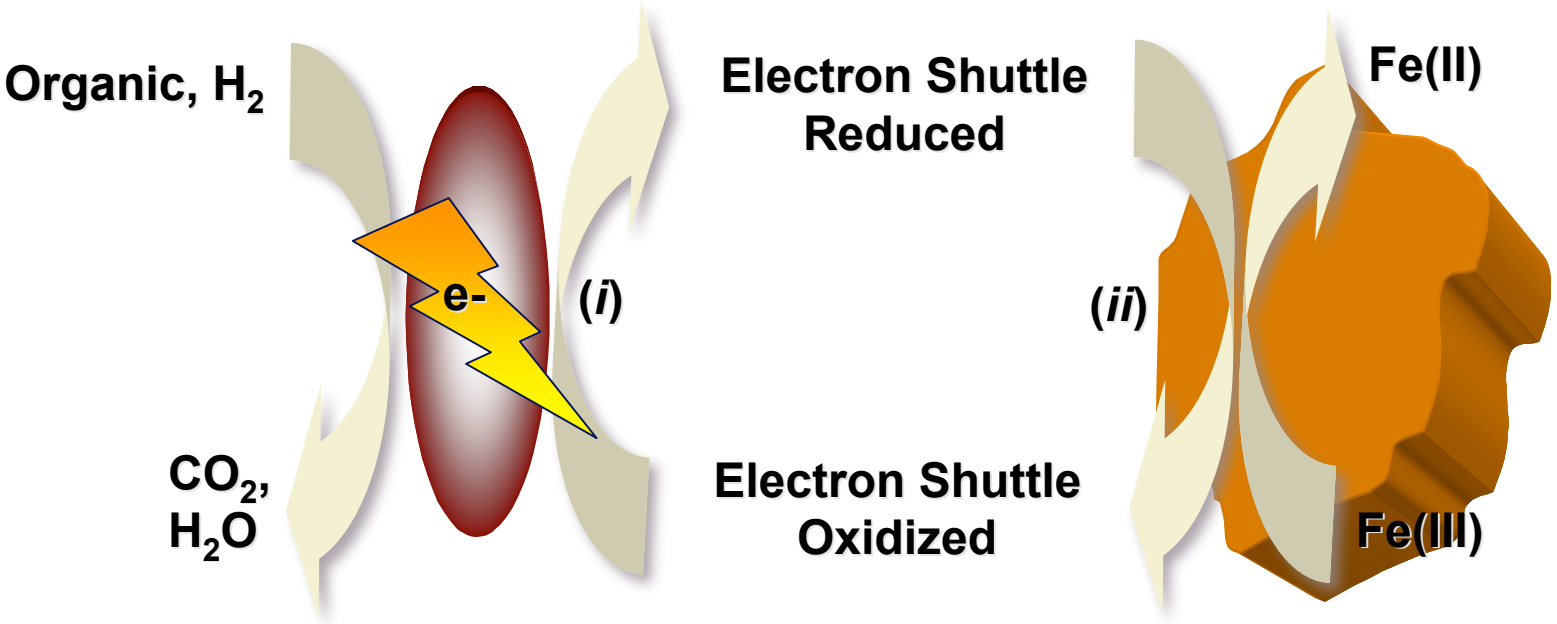


Figure 4b

Fe(III)-complexing Ligand

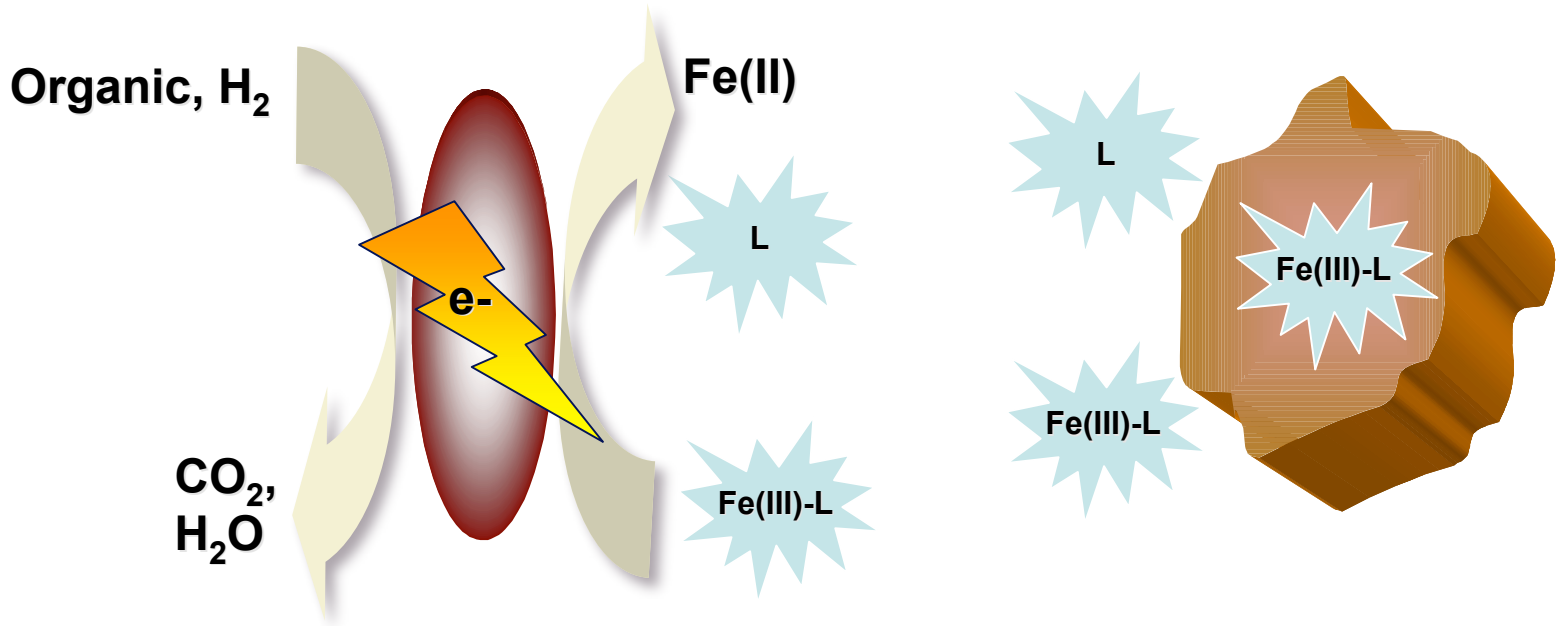
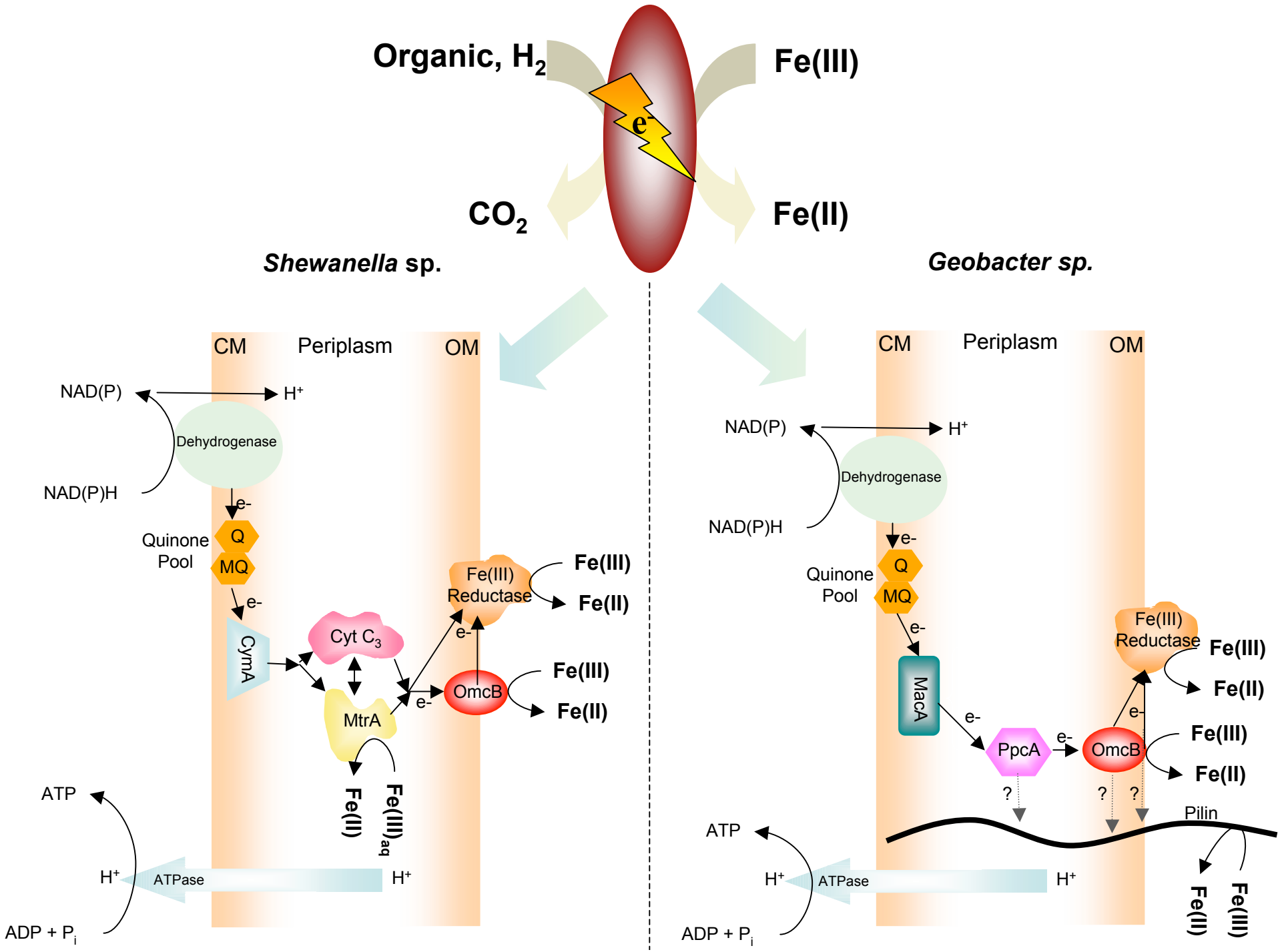


Figure 4c


Organic, H2
Fe(III)
e-
CO2
Fe(II)
Shewanella sp.
Geobacter sp.
CM
Periplasm
OM
NAD(P)
H+
Dehydrogenase
NAD(P)H
e-
Quinone Pool
Q
MQ
CymA
Cyt C3
MtrA
OmcB
Fe(III) Reductase
Fe(III)
Fe(II)
Fe(II)
Fe(III)aq
ATP
H+
ATPase
ADP + Pi
MacA
PpcA
?
Pilin