Humic Substances and Extracellular Electron Transfer

Annette Piepenbrock and Andreas Kappler

Abstract Humic substances (HS) are redox-active organic molecules that are present in virtually all environments. A wide variety of bacteria including Fe(III)-reducers, sulfate reducers, methanogens, and fermenting bacteria can reduce HS and in a second, abiotic step, the reduced HS can transfer their electrons to terminal electron acceptors such as poorly soluble Fe(III) minerals, in summary a process called humic substance electron shuttling. Electron shuttling between HS-reducing bacteria and Fe(III) minerals can increase the rate of Fe(III) reduction compared to direct Fe(III) reduction and, furthermore, enables the indirect reduction of Fe(III) minerals by some bacterial groups that are not able to reduce the Fe(III) minerals directly. This chapter will first summarize the knowledge about the redox properties of humic substances including a discussion of their redox-active functional groups. We then focus on the mechanism of electron shuttling and evaluate the advantages and disadvantages of electron shuttling versus direct contact Fe(III) mineral reduction. The role of solid-phase humics and other extracellular electron shuttles is discussed as well as the environmental consequences for long-range electron transfer via humic substances. The chapter concludes by illustrating some remaining open questions and by providing suggestions for future research.

A. Piepenbrock · A. Kappler (✉)
Geomicrobiology, Center for Applied Geosciences, University of Tübingen,
Sigwartstrasse 10, D-72076 Tübingen, Germany
e-mail: andreas.kappler@uni-tuebingen.de

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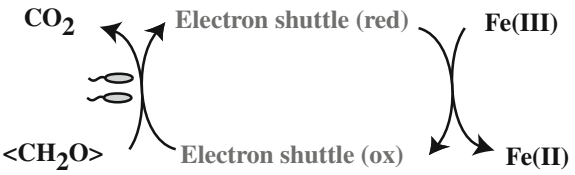

Fig. 1 Schematic illustration of electron shuttling between microbial cells and Fe(III) by dissolved electron shuttles, e.g., humic substances

1 Electron Shuttling in Metal Respiration

Fe(III) is an important electron acceptor for microbial respiration in natural soils and sediments (chapter "Energetic and Molecular Constraints on the Mechanism of Environmental Fe(III) Reduction by *Geobacter*", and "Biochemistry of Extracellular Respiration in *Shewanella oneidensis*", this book; Kappler and Straub 2005; Konhauser et al. 2011; Weber et al. 2006) due to its abundance in these environments (see chapter "Minerals and Aqueous Species of Iron and Manganese as Reactants and Products of Microbial Metal Respiration"). At circumneutral pH the solubility of Fe(III) is very low and it is therefore mainly present as poorly crystalline and crystalline Fe(III) (oxyhydr)oxides. Unlike other electron acceptors such as O_2, particulate Fe(III) is more difficult for microbes to access since it cannot easily be taken up into the cells. Instead, bacteria must transfer electrons out of the cells and onto a solid Fe(III) mineral using outer membrane cytochromes. However, this electron transfer requires direct contact between the cells and the Fe(III) mineral. Therefore, microbial Fe(III) reduction is often limited by available mineral surface area (Kappler and Straub 2005; Weber et al. 2006). There are several strategies bacteria can use to overcome the limitations of a non-dissolved electron acceptor and restricted access to a mineral surface. Reguera et al. (2005) suggested that conductive cell appendages, so-called "nano-wires", can facilitate electron transfer to non-dissolved electron acceptors. Bacteria can also excrete chelators that complex and thereby dissolve the Fe(III), which is subsequently taken up into the cell and reduced (Lovley et al. 1994). Finally, it has been shown that microbial Fe(III) reduction can be facilitated by electron shuttling (Fig. 1) (Lovley et al. 1996). Dissolved, redox-active molecules serve as electron shuttles, which are reduced by the bacteria and, in a second step, transfer electrons to the Fe(III) mineral. The electron shuttle is re-oxidized by the Fe(III) mineral in the process and can again accept electrons from the cells (Fig. 1). Thus, the same electron shuttle molecule can be recycled many times, transferring multiple generations of electrons from bacteria to the Fe(III) mineral.

Some microorganisms produce and excrete electron shuttles, e.g., *Shewanella* species (Marsili et al. 2008; von Canstein et al. 2008), and endogenous electron shuttles are discussed in the chapter "On the Role of Endogenous Electron Shuttles in Extracellular Electron Transfer". There are, however, redox-active compounds naturally present in the environment that can function as electron shuttles for bacteria. This chapter will focus on these exogenous electron shuttles, especially on humic substances.

2 Humic Substances: Composition and Properties

Humic substances (HS) are chemically heterogeneous polyfunctional organic molecules and constitute operationally defined fractions of organic matter (OM) that is present at varying concentrations in almost all natural environments (Stevenson 1994). Concentrations of OM in the environment are typically quantified as organic carbon (OC), which represents approximately half of the OM. Soils contain up to 5–10 % (w/w) OC (Stevenson 1994), whereas natural surface waters and ground waters commonly contain up to 30 and 10 mg dissolved OC/L, respectively (Aiken et al. 1985). However, OM values much higher than these are found in selected natural waters and soils (D. Macalady, "personal communication"). Depending on the environment, HS originate primarily from the degradation of senescent plant organic matter, and can also contain various amounts of animal and microbial remains. Consequently, HS can contain fragments of aromatic lignin derivatives, peptides, carbohydrates, and aliphatic molecules. HS are thought to be largely recalcitrant and rather inert to chemical and microbial degradation (Stevenson 1994), contain a wide variety of reactive functional groups, absorb strongly to mineral surfaces, have the ability to complex metal ions, and have long been known to be redox-active (Aiken et al. 1985; Stevenson 1994; Visser 1964). Natural HS are isolated first by extraction from soils and waters using alkaline solvents, then partial precipitation with a strong acid followed by separation over columns filled with resins that have different affinity to different HS fractions (different XAD materials, i.e., hydrophobic cross-linked polystyrene copolymer resins). In particular, the treatment with strong base and acid is known to cause some chemical changes in the molecules.

Due to differences in origin and diagenesis, the chemical composition and structure of HS in nature vary significantly among different HS samples and are impossible to describe accurately by chemical formulas. For a long time, HS were thought to be large macromolecules consisting of complex aromatic and aliphatic structures containing functional groups such as hydroxyl, carboxyl, amino, and phenolic groups (Stevenson 1994). Due to its varying chemical structure, natural OM has been operationally classified according to solubility and molecular weight criteria. HS are classified as fulvic acids, which have lower molecular weights (0.5–5 kDa) and are soluble at all pH values, or humic acids, which are bigger (20–100 kDa) and are soluble at alkaline pH only. Humins, the largest OM fraction in soils, are insoluble at all pH values (Aiken et al. 1985; Stevenson 1994).

In contrast to attempts to characterize the macromolecular structure of HS, Piccolo (2001) and Sutton and Sposito (2005) described a new model for the structure of HS. According to this model, HS do not consist of single macromolecules but rather of associations of smaller organic molecules of different kinds held together by hydrophobic effects and hydrogen bonds. Upon changes in geochemical conditions, these bonds are strengthened or loosened, causing structural changes, that may even lead to the separation of single small organic molecules from the associations (Sutton and Sposito 2005). The molecules contributing to HS can be all kinds of organic

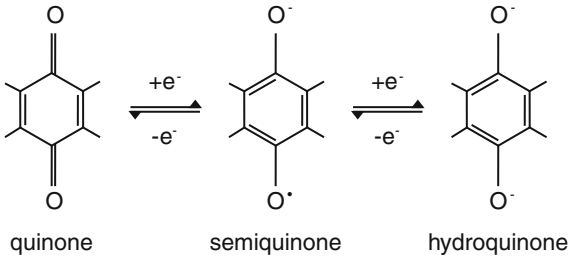

Fig. 2 Two-step reduction and oxidation of quinones and quinoid functional groups via the semiquinone radical. Note that for simplification protonation equlibria of the reduced species were disregarded. (For details of protonation and deprotonation equilibria of reduced quinones see Jiang et al. (2009)).

molecules including biomolecules (e.g., fatty acids, carboxylic acids, alcohols, lignins, sugars), which, by the old macromolecular definition of HS, were not even part of HS (Stevenson 1994). However, the new model considers these molecules as part of HS (Sutton and Sposito 2005).

Different functional groups in HS (see below) are known to accept and donate electrons, i.e., to participate in redox reactions (Aiken et al. 1985; Visser 1964). Dunnivant et al. (1992) showed that natural OM can mediate the reduction of substituted nitrobenzenes by the bulk electron donor hydrogen sulfide. They proposed for the first time that this effect could be due to an electron shuttling activity of the OM, which was proposed to be reduced by the bulk electron donor and subsequently transfer its electrons to the organic pollutant. This model was further developed by Lovley et al. (1996), who were the first to show that HS can be reduced microbially and that the reduction of HS can support microbial growth. They also proposed that reduced HS can then transfer electrons abiotically to Fe(III) minerals and thus shuttle electrons between the microorganisms and Fe(III) minerals (Fig. 1) (Lovley et al. 1996). This process of electron shuttling by HS greatly enhances the rate of microbial Fe(III) reduction (see Sect. 4) (Jiang and Kappler 2008; Lovley et al. 1996).

When it was proposed for the first time that HS can function as electron shuttles, quinones were suggested to be the redox-active functional groups in HS (Dunnivant et al. 1992). Quinones are aromatic molecules that can accept electrons according to the formula shown in Fig. 2. As indicated, the reduction of quinones is a two-step process leading to the formation of a semiquinone radical and, upon transfer of a second electron, to the formation of a hydroquinone. Both electron transfer processes are reversible (Uchimiya and Stone 2009).

The hypothesis that quinones are the most important electron accepting functional groups also in microbial HS reduction (Lovley et al. 1996) was further supported by the work of Scott et al. (1998), who used electron spin resonance (ESR) measurements to quantify organic radicals in microbially reduced HS. They found that microbial reduction of HS results in an increase in radical content that is proportional to the number of electrons taken up by the respective HS samples (electron accepting capacity, see below). Furthermore, the ESR spectra obtained

for the HS samples were consistent with semiquinones being the main organic radicals (Scott et al. 1998). Furthermore, Chen et al. (2003) demonstrated that the electron accepting capacities of different OM samples was correlated to the aromaticity of the samples. These data indicate that quinones or quinone-like functional groups are the major functional groups responsible for the electron accepting capacity of HS samples.

Indications that quinones are the most important redox-active functional groups in HS at environmentally relevant conditions also come from the observation that electron transfer to HS is reversible (Aeschbacher et al. 2011; Bauer and Kappler 2009; Ratasuk and Nanny 2007) and from the comparison of electrochemical and spectroscopic properties of HS with those of selected model quinones (Fimmen et al. 2007; Nurmi and Tratnyek 2002; Ratasuk and Nanny 2007). Additionally, electrons and protons were consumed in a ratio close to 1:1 during electrochemical reduction of different HS samples, which also points toward the reduction of quinoid functional groups as the active electron acceptors (Aeschbacher et al. 2010, 2011; Maurer et al. 2010).

Besides quinoid functional groups, complexed metal ions such as iron were suggested to be responsible for part of the redox-active behavior of HS (Struyk and Sposito 2001). However, Chen et al. (2003) and Peretyazhko and Sposito (2006) found that the amount of electrons transferred by their HS samples (reducing capacities, see below) could not be explained by complexed iron alone. Furthermore, Lovley and Blunt-Harris (1999) showed that although the iron in HS was indeed being reduced during microbial HS reduction, the iron content of several commercially available HS [International humic substances society (IHSS)] was too small to contribute significantly to the reducing capacities measured.

These papers establish that functional groups other than complexed metal ions, e.g., quinone moieties, are the more important electron accepting functional groups. However, two important caveats must be mentioned. First, although they may represent only a small fraction of the electron accepting capacity, very reactive Fe species, could still be important for situations such as reduction of pollutants, e.g., chlorinated compounds or toxic metals such as Cr(VI), U(VI), or As(V). Second, the low Fe content in HS might be due to the harsh chemical extraction and purification treatment of the samples during HS isolation that will remove most of the Fe in the HS. It is therefore possible that HS in the environment contain much higher amounts of complexed metals than these purified IHSS samples and, in the environment, Fe could play a more important role than implied by these laboratory experiments with highly purified HS.

Besides quinones and complexed metal ions, non-quinone aromatic constituents (Chen et al. 2003) and sulfur-containing functional groups (Einsiedl et al. 2008) have also been suggested to be relevant as possible redox-active sites in humic substances. However, detailed investigations of the contributions of these functional groups to HS redox activity are missing until now.

Since HS consist of a variety of different redox-active functional groups with different redox potentials (see below), they can be present in a wide range of redox states. In order to describe the redox state of HS on a quantitative basis, the terms

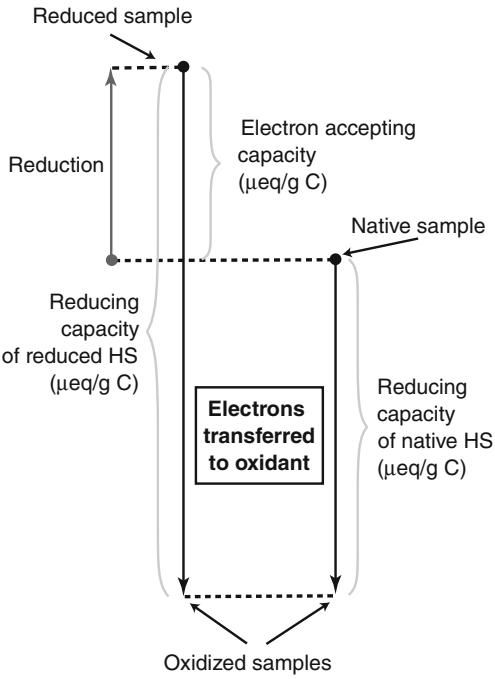

Fig. 3 Scheme illustrating the determination of the reducing capacity and electron accepting capacity of native and reduced HS samples

reducing capacity and electron accepting capacity are used (Sposito 2011). The redox state of HS is usually determined by oxidizing the HS with different oxidizing agents (e.g., ferric citrate, potassium ferricyanide, I_2 or electrochemically) and measuring the number of electrons that are transferred. This value is called the reducing capacity of the HS sample (Fig. 3) and is usually given in µeq (microequivalents, i.e., micromoles electrons transferred) per g HS or per g C.

The reducing capacity can be determined for native and for chemically as well as for microbially reduced HS. Upon reduction, the reducing capacity toward the same oxidizing agent increases (Fig. 3). The difference between the reducing capacity of the native and the reduced HS sample reflects the amount of electrons that are transferred to the HS during reduction, i.e., the electron accepting capacity of the HS (Fig. 3). As this approach is based on the assumption that all electrons that are transferred onto the HS during reduction can be recovered during oxidation, it is advisable also to determine the electron accepting capacity directly, e.g., by reduction with Zn (Blodau et al. 2009), in order to obtain a systematic characterization of the redox state of HS.

Reducing capacity values reported in the literature for non-reduced HS samples range from 50 to >10,000 µeq/g HS (Bauer and Kappler 2009; Benz et al. 1998; Jiang and Kappler 2008; Peretyazhko and Sposito 2006; Struyk and Sposito 2001; Wolf et al. 2009). These large variations are partly attributable to the different oxidizing agents used in determining these values, which differ in redox potential. Peretyazhko and Sposito (2006) and Bauer et al. (2007) compared reducing

capacities obtained with different oxidizing agents and found that reducing capacities measured with ferric citrate as oxidant are approximately one order of magnitude smaller than those measured with potassium ferricyanide and about two orders of magnitude smaller than those measured by oxidation with I_2. Peretyazhko and Sposito thus propose to standardize the protocol for determining reducing capacities to the use of ferric citrate, because its standard electrode potential of 309 mV and its chemical structure are most similar to naturally occurring soluble oxidants (Peretyazhko and Sposito 2006). However, although the absolute values of reducing capacities are not comparable if they were obtained with different oxidizing agents, the ratios between electron accepting capacities of different HS samples (e.g. HS of different origin) are still the same within the experimental error, regardless if they were measured toward ferric citrate, potassium ferricyanide, or electrochemically (Aeschbacher et al. 2010; Sposito 2011).

Even if the same oxidant is used, differences in reducing and electron accepting capacities are obtained for different HS samples. This is due to the fact that the composition of HS varies significantly depending on origin and genesis of the HS. These differences commonly affect the magnitude of the reducing and electron accepting capacities. The electron accepting capacity of humic acids is generally higher than that of fulvic acids extracted from the same source material (Aeschbacher et al. 2010; Ratasuk and Nanny 2007) and HS extracted from soils and sediments have higher electron accepting capacities than those from partially aquatic origin due to their higher aromatic content (Aeschbacher et al. 2010; Ratasuk and Nanny 2007; Scott et al. 1998).

As can be expected from the heterogeneity of HS composition and the range in reducing capacities measured, the redox potentials reported for HS at pH 7 span a wide range from −300 to +400 mV versus standard hydrogen electrode (Osterberg and Shirshova 1997; Straub et al. 2001; Visser 1964). Recent studies suggest that the redox potential of a HS sample cannot be expressed by a single value, but rather that HS contain a variety of redox-active functional moieties with a distribution of redox potentials. Thus, the overall redox potential of an HS sample was suggested to be expressed best as a continuum of redox potentials in the range of ca. –300 to +100 mV (Aeschbacher et al. 2011). The actual range of redox potentials and the frequency of redox-active moieties of a specific potential vary between different HS samples (Aeschbacher et al. 2011) depending not only on the origin of the HS but also on its history, i.e., the methods of extraction and pre-treatment of the HS.

3 Electron Shuttling by Humics: A Two-Step Process

Electron shuttling between microorganisms and Fe(III) minerals by HS consists of two steps: (i) The biotic reduction of the dissolved or non-dissolved HS, followed by (ii) an abiotic electron transfer from the reduced HS to the Fe(III) mineral (Fig. 1). The re-oxidation of the reduced HS by the Fe(III) leads once again to oxidized HS, which can again be re-reduced by the bacteria (Fig. 1). The same HS

molecule is thus recycled during this process and can transfer many electrons from the microorganisms to the Fe(III) without being consumed in the process (Ratasuk and Nanny 2007).

The first step of the electron shuttling process (Fig. 1) is the reduction of the HS. This process can be microbially mediated or can occur abiotically. Chemical reduction in laboratory experiments is commonly obtained by incubation of the HS samples under a H_2 atmosphere in the presence of a palladium catalyst (Kappler et al. 2004; Peretyazhko and Sposito 2006; Visser 1964). For microbial HS reduction, HS samples are incubated with HS-reducing bacteria, for example in cell suspensions with cell numbers of 10^8–10^{10} cells/mL or higher (Lovley et al. 1996; Nevin and Lovley 2000). Peretyazhko and Sposito (2006) and Jiang and Kappler (2008) showed for a number of different HS samples that the reducing capacities obtained after chemical reduction with H_2/Pd and after microbial reduction by a soil extract and by *Geobacter sulfurreducens*, respectively, were very similar. From this they conclude that chemical and microbial reduction both transfer electrons to essentially the same redox-active moieties in HS. These findings indicate that chemically reduced HS or HS-analogs can be used as a proxy for microbially reduced HS or HS-analogs, as it is commonly done in laboratory studies (e.g. Benz et al. 1998; Lovley et al. 1998, 1999). However, it should be noted that chemical reduction, especially in the presence of a palladium catalyst, can alter physicochemical properties and potentially even leave chemical traces (e.g. Pd-ions) in the HS which might affect the redox properties of the treated HS. A further possibility of abiotically reducing HS is by the use of an electrochemical cell, where the HS are reduced at an electrode surface (Kappler and Haderlein 2003). This method has the advantage that it allows precise control of the electrochemical endpoint of the oxidation and reduction. Further methodological developments by Aeschbacher et al. (2010) include the use of a glassy carbon working electrode, which prevents the reduction of H^+ leading to H_2 formation and, thus, enables the exact quantification of the number of electrons transferred to the HS. They also introduced the use of chemical mediators to facilitate electron transfer between organic matter and the electrode.

The first microorganisms with a demonstrated capacity for reducing HS were the dissimilatory Fe(III) reducers *G. metallireducens* and *Shewanella alga* (Lovley et al. 1996). In a subsequent study, Lovley et al. (1998) tested a number of different Fe(III)-reducing bacteria for their ability to reduce anthraquinone-2,6-disulfonate (AQDS), a model compound for quinone moieties in HS, and found that all Fe(III)-reducers studied were able to use AQDS as an electron acceptor. To our knowledge, this statement still holds true and there is still no report of any neutrophilic Fe(III)-reducer that is unable to reduce HS. However, Emmerich and Kappler (2012) recently demonstrated that the acidophilic Fe(III)-reducer *Acidiphilium* SJH was neither able to reduce HS nor AQDS. In addition to Fe(III)-reducers, fermenting (Benz et al. 1998), halorespiring, sulfate-reducing, and methanogenic microorganisms (Cervantes et al. 2002) have been shown to be able to reduce HS. Thus, the indirect reduction of Fe(III) via electron shuttling by HS is not restricted to microorganisms that are also able to directly reduce Fe(III).

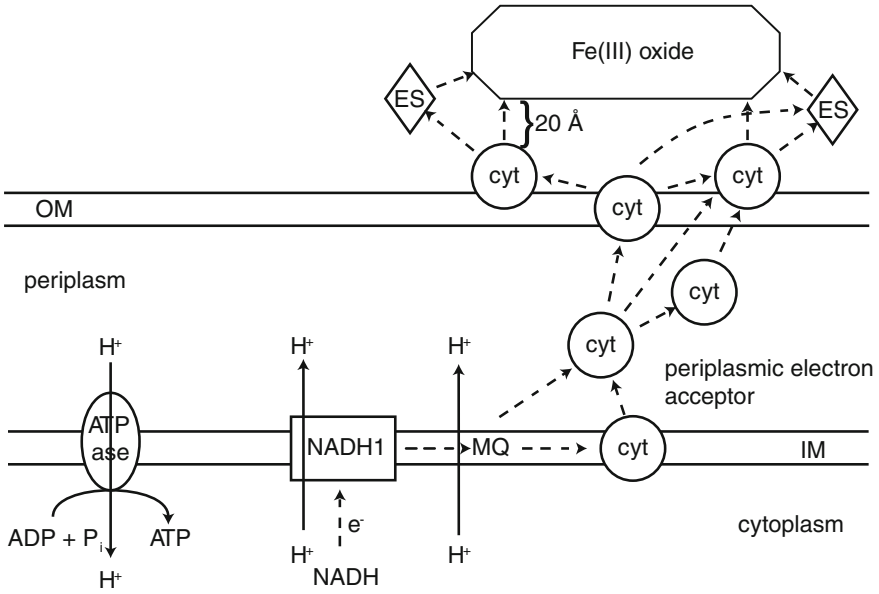

Fig. 4 Schematic illustration of the pathway of electron flow to poorly soluble Fe(III) oxides and dissolved electron shuttles in Fe(III)-reducing bacteria. Reduction of both electron acceptors [Fe(III) oxides and electron shuttles] proceeds largely via the same electron transfer system. *OM* outer membrane, *IM* inner membrane, *NADH1* NADH dehydrogenase, *MQ* menaquinone pool, *cyt* cytochrome of the electron transfer system, *ES* dissolved electron shuttle. *Dashed arrows* indicate electron flow

Electron shuttling thus increases the number of microorganisms that are able to indirectly reduce Fe(III) and, therefore also potentially increases the importance of Fe(III) reduction in the environment.

To date, the molecular mechanism of electron transfer from microbes to HS and other electron shuttles is not completely understood. There are several studies indicating that electrons are transferred to dissolved electron shuttles largely via the same protein complexes that are used for direct metal reduction (Gescher et al. 2008; Lies et al. 2005; Voordeckers et al. 2010). This electron transfer proceeds via a number of quinones and *c*-type cytochromes that transport the electrons from the oxidoreductase in the cytoplasm through the cytoplasmic membrane and the periplasm to the surface of the outer membrane (Fig. 4; chapter "The Biochemistry of Dissimilatory Ferric Iron and Manganese Reduction in *Shewanella oneidensis*"). These outer membrane cytochromes are particularly important in the reduction of poorly soluble electron acceptors such as iron and manganese oxides and oxyhydroxides, whereas some dissolved electron acceptors are expected to penetrate the outer membrane and take up electrons directly from the proteins located in the periplasm (Gescher et al. 2008). While there is some evidence for uptake of HS and AQDS into cells (Kulikova et al. 2010; Shyu et al. 2002), the main pathway of electron transfer to HS and AQDS seems to involve electron transfer at the surface of the outer membrane (Fig. 4).

Electron transfer through outer membrane proteins was demonstrated by the diminished ability of omc-mutants (defective in outer membrane proteins) to reduce AQDS or to indirectly reduce iron minerals by the use of electron shuttles (Gescher et al. 2008; Lies et al. 2005; Voordeckers et al. 2010). However, deletion of only one outer membrane cytochrome in *G. sulfurreducens* was not sufficient to completely inhibit HS and AQDS reduction, indicating that different cytochromes contribute to the reduction of electron shuttles (Voordeckers et al. 2010). The pool of proteins and reactive sites within proteins that are able to reduce dissolved electron shuttles also seems to include some sites which are either located in the periplasm or otherwise protected from access, making them inaccessible for poorly soluble minerals (Lies et al. 2005; Voordeckers et al. 2010). In the environment, electron shuttling to poorly soluble electron acceptors such as Fe(III) minerals via reduction of soluble electron shuttles such as HS might even take place simultaneously with the direct reduction of the Fe(III) minerals (Clarke et al. 2011; MacDonald et al. 2011).

The second step of the electron shuttling process is the transfer of electrons from reduced shuttles to the terminal electron acceptor. This means that after the HS are reduced microbially, they can be reoxidized by transferring their electrons for example to Fe(III) minerals (Fig. 1), leading to the indirect microbial reduction of Fe(III) minerals. While there are many studies on microbial HS reduction (see above), systematic studies of the kinetics and thermodynamics of abiotic electron transfer from reduced HS to different Fe(III) minerals are sparse. It is known from microbial iron mineral reduction experiments in the absence and presence of electron shuttles (HS, AQDS) that reduced shuttles are able to transfer electrons to a variety of different Fe(III) minerals and Fe(III) phases, including some phases such as goethite, hematite, and structural iron in clay minerals that are scarcely reducible by direct microbial reduction (Lovley et al. 1998).

However, the number of electrons that are transferred from reduced HS to different Fe(III) minerals and complexed Fe(III) depends on the redox potential of the respective Fe(III) phase (Bauer and Kappler 2009). Furthermore, Liu et al. showed that the electron transfer from reduced AQDS to hematite was limited, or at high concentrations even prevented, by sorption of Fe(II) and phosphate to the hematite surface (Liu et al. 2007). This indicates that the electron transfer between reduced shuttles and Fe(III) minerals strongly depends on geochemical conditions such as concentrations of different ions that might sorb to the Fe(III) minerals and the identity of the Fe(III) minerals themselves, such as biogenic versus abiogenic minerals. Therefore, it is unclear how fast and to what extent Fe(III) minerals in the environment are reduced by HS.

4 Advantages and Disadvantages of Electron Shuttling Versus Direct Contact Fe(III) Mineral Reduction

As discussed previously (chapter "Minerals and Aqueous Species of Iron and Manganese As Reactants and Products of Microbial Metal Respiration"), at near neutral pH values, Fe(III) minerals have very low solubilities and Fe(III) is

therefore present in the environment mostly in the form of solid (oxyhydr)oxides. This imposes a limitation on the rate and extent of direct microbial Fe(III) reduction, since microbes have to come within a distance of approximately 20 Å from the iron mineral surface in order to directly transfer electrons (Gray and Winkler 2005). Therefore, the addition of electron shuttling compounds such as HS is expected to stimulate microbial Fe(III) reduction by those cells that are not close enough for direct electron transfer to minerals. However, even when cells are attached to the mineral, the distance between the electron donating cytochromes and the electron accepting mineral surface might in some cases still be larger than 20 Å (Fig. 4). HS or other electron shuttles may facilitate electron transfer in this situation as well. Indeed, several studies have found increased rates of microbial Fe(III) reduction in the presence of HS and AQDS (e.g. Lovley et al. 1996; MacDonald et al. 2011; Wolf et al. 2009).

Jiang and Kappler (2008) compared the rates of direct ferrihydrite reduction by *Geobacter sulfurreducens* to the rate of HS reduction by *G. sulfurreducens* and to the rate of abiotic electron transfer from reduced HS to ferrihydrite. They found that HS are reduced 27 times faster than ferrihydrite and that the electron transfer from reduced HS to ferrihydrite proceeds at least seven times faster than the electron transfer from *G. sulfurreducens* to ferrihydrite. Thus, they showed that the overall electron shuttling process from *G. sulfurreducens* via HS to ferrihydrite is limited by the second, abiotic electron transfer step, but that it still proceeds at least seven times faster than the direct microbial ferrihydrite reduction. However, HS do not in all cases increase the Fe(III) reduction rate (see also Sect. 7). Studies with low concentrations of HS showed that at these concentrations HS can even lead to decreased microbial Fe(III) reduction rates compared to setups without HS (Amstaetter et al. 2012; Piepenbrock et al. 2011). These observations were attributed to the sorption of the HS to the mineral surface, thus reducing the bioavailable mineral surface area either by directly blocking surface sites or by increasing aggregation of the ferrihydrite particles.

Wolf et al. (2009) studied the effects of different model quinones on microbial Fe(III) reduction and found that the kinetics were mainly controlled by the redox potential of the shuttling compound. They hypothesized that there is an ideal redox potential for the electron shuttle as the most efficient shuttles all had a redox potential between –137 and –225 mV. This is high enough to provide sufficient redox potential difference to the electron donor (lactate or acetate in case of *Shewanella* and *Geobacter* sp., respectively) to allow the necessary amount of ATP synthesis for microbes but at the same time the redox potential of the shuttle is low enough to make the rate-limiting second electron transfer step to the terminal electron acceptor favorable. (Wolf et al. 2009).

When electron shuttling occurs, Fe(III) can be reduced at a higher rate than in the absence of electron shuttles. However, the energy that microbes can gain from a redox reaction depends on the redox potential difference between the electron donor and the microbial electron acceptor. As shown by Wolf et al. (2009), the redox potential of the electron shuttle must be between the standard redox potential of the electron donor and the electron acceptor in order to efficiently

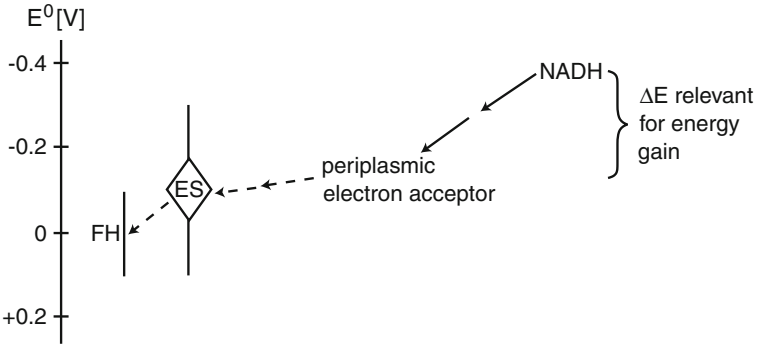

Fig. 5 Redox potential of the most important components involved in electron flow from NADH to ferrihydrite in the model for Fe(III) reducers presented in Fig. 4. *Solid arrows*, electron flow associated with ATP production; *dashed arrows*, electron flow without ATP production; *ES*, dissolved electron shuttle; *FH*, ferrihydrite

stimulate Fe(III) reduction. Hence, if bacteria reduce the shuttle instead of directly reducing the Fe(III) mineral, the redox potential difference is smaller and they can be expected to gain less energy from the reaction. However, this only holds true if the electron is transported through a long electron transport chain in the membrane, in the course of which protons are translocated out of the cytoplasm and a proton motive force is built up for ATP synthesis.

In the case of the most commonly studied Fe(III)-reducing bacteria, *Geobacter* and *Shewanella*, however, the electron transport chain involved in Fe(III) reduction seems to be rather short and proton translocation takes place only until the electron reaches the periplasm (Fig. 4) (chapter "The Biochemistry of Dissimilatory Ferric Iron and Manganese Reduction in *Shewanella oneidensis*"). Thus, the redox potential difference relevant for the energy gain is not the one between the electron donor and the terminal electron acceptor, but the one between the electron donor and the periplasmic electron acceptor (Fig. 5) (Bird et al. 2011). As the electron transport chain seems to be the same for the reduction of HS and Fe(III) (see Sect. 3, Fig. 4), the redox potential difference between electron donor and periplasmic electron acceptor and hence the overall energy gain should be the same for the direct reduction of Fe(III) minerals and the reduction of Fe(III) minerals via electron shuttles. Further evidence for this comes from studies with electrochemical cells which show that *G. sulfurreducens* yielded the same energy gain when grown at higher versus lower electrode potentials (Marsili et al. 2010).

In summary, the presence of electron shuttles (above a certain minimum concentration, see below) can increase the rate of microbial Fe(III) reduction. If the rate of microbial metabolism thus increases, the growth rate (increase in cell number per time) of bacteria that perform the electron shuttling can be expected to increase likewise. Hence, the use of electron shuttles provides an ecological advantage for bacteria as it enables them to outgrow other species. This is especially the case if there is indeed no loss in energy gain for the bacteria when

reducing the shuttle instead of reducing the mineral directly as discussed above. Thus, they can increase the rate of electron turnover and still generate the same amount of energy per electron transferred.

5 Reduction of Solid-Phase Humics

Until now, most research on HS electron shuttling has focused on dissolved HS. However, the highest fraction of HS in natural soils and sediments is in the solid form (Stevenson 1994). Kappler et al. (2004) presented the first evidence that solid-phase HS are also redox-active. They determined the reducing and electron accepting capacities of HS extracted from sediments with 0.1 M NaOH, and thus, they also extracted a fraction of HS that is particulate at circumneutral pH. They found that the HS were in a more reduced state in the deeper layers of the sediment. Although these authors did not determine to what extent the solid HS fraction contributed to the measured reducing capacities, this study showed that at least a fraction of the solid-phase redox-active humics was reduced by microorganisms.

Roden et al. (2010) were the first to systematically study the microbial reduction of solid-phase HS. They found that the two Fe(III)-reducers *G. sulfurreducens* and *S. oneidensis* were able to transfer electrons to Fe-stripped wetland sediments containing solid-phase HS. With a series of control experiments, they ruled out the possibility that the electron accepting capacity stemmed from inorganic constituents in the sediment. Although the electron accepting capacities determined per mg sediment were a lot lower than those for dissolved HS, the addition of the Fe-stripped sediments that contained solid-phase humic substances to microbial Fe(III) reduction experiments significantly increased the microbial Fe(III) reduction rates (Roden et al. 2010). Based on this evidence the authors suggested that solid-phase HS can also function as electron shuttles between microorganisms and poorly soluble terminal electron acceptors such as Fe(III) minerals. However, the relevance of microbial solid-phase HS reduction and electron shuttling in environmental systems is unknown and must be determined through future studies.

Evidence for the existence of long distance electron transfer via redox-active constituents comes from a recent study that showed electrons being transferred from sulfide produced within a marine sediment to oxygen present at the sediment surface over distances of more than a centimeter (Nielsen et al. 2010). The sulfide profile with depth in the sediment measured with microelectrodes showed an immediate response to the presence and absence of O_2 at the sediment surface, suggesting a direct redox coupling of sulfide oxidation to the overlying O_2. The very fast electron transfer rules out diffusion of dissolved redox-active molecules as the underlying mechanism, but requires electron transfer via a conductive network, as could be formed for example by solid-phase HS (Fig. 6) or (as suggested by the authors) by conductive bacterial nanowires or redox-active pyrite particles. However, if and to what extent solid-phase (and also dissolved) HS contribute to this electron transfer over cm-long distances remains currently unknown.

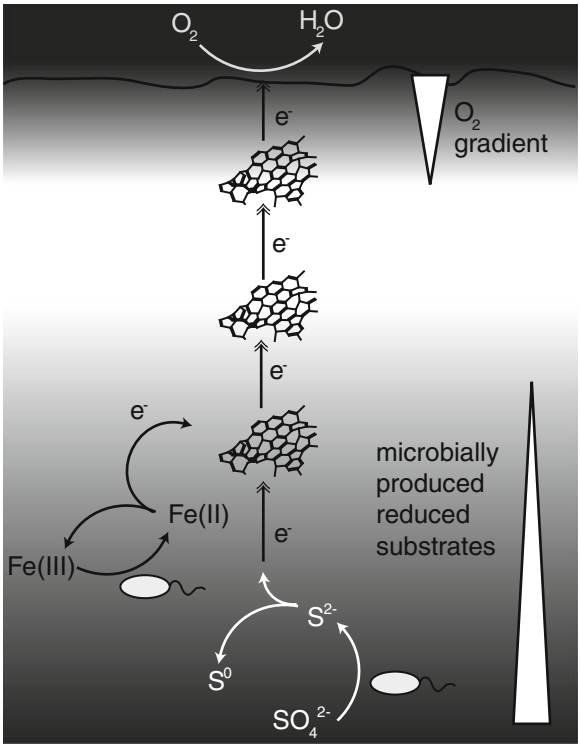

Fig. 6 Electron transfer from microbially produced reduced metabolites (Fe(II), S^{2-}, etc.) to electron acceptors with a more positive redox potential, e.g., O_2 over mm- or even cm-long distances via a conductive network including redox-active dissolved and solid-phase humic substances in sediments or soils

6 Other Extracellular Electron Shuttles and Humic Model Compounds

Besides HS, other organic and inorganic redox-active compounds such as sulfur species have been suggested to function as electron shuttles and to stimulate microbial Fe(III) reduction. In a comparative study, Nevin and Lovley (2000) analyzed the potential of U(IV) and several different sulfur species to function as electron shuttles for microbial Fe(III) reduction. They showed that the addition of U(IV) could stimulate Fe(III) reduction in cell suspension experiments with *G. metallireducens* and synthetic Fe(III) hydroxides as the electron acceptor. However, unlike HS or AQDS, uranium did not stimulate the reduction of Fe(III) present in aquifer sediments under environmentally relevant conditions. The same was true for S^0 species. These authors also observed that sulfur-containing amino acids at environmentally relevant concentrations did not stimulate Fe(III) reduction in cell suspension experiments (Nevin and Lovley 2000). In contrast, Straub and Schink (2004) proposed a model of electron shuttling by an unidentified sulfur species. They found that at low thiosulfate concentrations (50 μM) microbial reduction of the thiosulfate by *S. deleyianum* lead to the reoxidation of the produced sulfides by ferrihydrite leading to the formation of ferrous iron and oxidized

sulfur species, possibly polysulfides (Straub and Schink 2004). The identification of the oxidized sulfur species has not been accomplished and is an open question to be answered in future studies.

Among organic molecules, on the other hand, there are a variety of compounds that have been shown to stimulate microbial Fe(III) reduction, most of them quinones (Wolf et al. 2009). One of the most interesting of these quinones is AQDS (9,10-anthraquinone-2,6-disulfonic acid), since it is one of the most efficient electron shuttles (Wolf et al. 2009) and has often been used as a proxy for quinoid moieties in HS (e.g. Coates et al. 1998; Lovley et al. 1998). However, the replacement of HS with model quinones such as AQDS should be handled with care since AQDS differs from HS in some very important respects: first, while HS show strong sorption to Fe(III) (oxyhydr)oxides, AQDS sorption to Fe(III) minerals is more than one order of magnitude lower (Wolf et al. 2009). This difference in sorption behavior influences electron shuttling by HS and AQDS. While low concentrations of AQDS led to a significant stimulation of Fe(III) reduction, low HS concentrations, as mentioned above, even decreased the Fe(III) reduction rate (Piepenbrock et al. 2011). This was probably due to the fact that the concentration of dissolved shuttles was not high enough in the experiments with low HS concentrations (see below) and that the accessibility of the ferrihydrite surface was lowered by sorbed HS and potentially by consequential aggregation of the ferrihydrite particles (Amstaetter et al. 2012). The second important difference between AQDS and HS is that AQDS at high concentrations can have a toxic effect for some microorganisms (Shyu et al. 2002), which can lead to reduced Fe(III) reduction rates (Nevin and Lovley 2000). Furthermore, the redox potential of AQDS is close to the ideal redox potential for electron shuttling (Wolf et al. 2009), while most of the redox-active moieties in HS have a higher redox potential (Aeschbacher et al. 2011).

Other examples of extracellular organic electron shuttles are phenazines, which are produced by a variety of soil bacteria, e.g., *Pseudomonas* species, and enable these bacteria to reduce poorly soluble Fe(III) oxides (Hernandez et al. 2004). These and other endogenous electron shuttles are discussed in detail in the chapter "On the Role of Endogenous Electron Shuttles in Extracellular Electron Transfer".

7 Environmental Relevance

HS-reducing bacteria have been enriched and isolated from different environments such as aquifer and lake sediments, wetland soils, and marine sediments (Coates et al. 1998; Kappler et al. 2004; Snoeyenbos-West et al. 2000). Detailed protocols for the enrichment and isolation of these microorganisms can be found in (Straub et al. 2005). In these investigated environments, cell numbers of HS reducers were in the range of 10^4–10^6 cells/g or mL sediment (Coates et al. 1998; Kappler et al. 2004) and were as numerous as fermenting microorganisms, indicating that microbial HS reduction has the potential to contribute significantly to electron

fluxes in the environment (Kappler et al. 2004). The high cell numbers of HS reducers are probably due to the fact that HS are reduced not only by Fe(III) reducers but by a wide variety of different physiological groups of bacteria including fermenting microorganisms, sulfate reducers, methanogens and halorespirers (Benz et al. 1998; Cervantes et al. 2002). Thus, Fe(III) reduction is not restricted to microorganisms that directly reduce Fe(III), but also involves microorganisms that use HS as electron shuttles to indirectly reduce Fe(III). Thus, the number of microorganisms that contribute to Fe(III) reduction increases and also potentially the importance of Fe(III) reduction in the environment.

In anoxic systems where microbial Fe(III) reduction takes place, Fe(III) (oxyhydr)oxides are expected to be the most important oxidants for the re-oxidation of microbially reduced HS. However, reduced HS can also be oxidized by O_2 (Aeschbacher et al. 2010; Bauer and Kappler 2009; Ratasuk and Nanny 2007), for instance at oxic-anoxic interfaces. Bauer and Kappler (2009) quantified the amount of electrons transferred from reduced HS to O_2 and found that fewer electrons were transferred than would be expected based on the redox potential of O_2. This corresponds well to the finding that chemically or microbially reduced HS that are re-oxidized by O_2 do not return to the same redox state as before reduction, but that some redox-active sites, which can transfer electrons to Fe(III), are protected from rapid re-oxidation by O_2 (Bauer and Kappler 2009; Macalady and Ranville 1998). These findings indicate that electron transfer from HS to Fe(III) and, thus, electron shuttling between microorganisms and Fe(III) is not necessarily restricted to anoxic environments but has the potential to even take place under microoxic conditions, e.g., at oxic-anoxic interfaces (Bauer and Kappler 2009). However, if and to what extent HS electron shuttling really takes place in oxic environments is a question that remains to be answered in future studies.

In order to analyze the potential of HS electron shuttling in the environment, HS were added to microcosm experiments with soils or aquifer sediments (Nevin and Lovley 2000; Rakshit et al. 2009) and Fe(III) reduction rates were quantified with and without addition of electron shuttles. In both studies, microbial Fe(III) reduction rates were significantly higher in the presence of added AQDS and HS, indicating that, in the absence of added shuttles, microbial Fe(III) reduction was limited by the availability of electron accepting Fe(III) minerals (not by their abundance) and that HS also have the potential to increase reduction rates in complex environmental systems. However, these findings also show that the HS content originally present in the soil and sediment samples was not sufficient to exert the maximum stimulation possible on the Fe(III) reduction, since addition of electron shuttles further increased the Fe(III) reduction rates. This indicates that if and to what extent electron flow to Fe(III) in environmental systems proceeds via electron shuttling strongly depends on the HS concentration and on the ratio of HS to iron minerals.

Several studies demonstrated a linear correlation between the concentration of dissolved HS and the Fe(III) reduction rate (Amstaetter et al. 2012; Jiang and Kappler 2008). However, this is only true for a range of HS concentrations between a lower limit, below which no stimulation of Fe(III) reduction occurs (and in some cases even lower reduction rates were observed than in the absence of HS

(see Sect. 4), and an upper limit, above which no further increase of reduction rates with increasing HS concentrations takes place. Jiang and Kappler (2008) and Amstaetter et al. (2012) determined a lower limit for electron shuttling of 10–20 mg dissolved HS/L while Wolf et al. (2009) found a stimulation of the Fe(III) reduction even at 1 mg/L total HS (0.0025 mg/L dissolved HS). This indicates that the HS concentration necessary for stimulating microbial Fe(III) reduction strongly depends on the system, i.e., the iron mineral identity, mineral concentration, the number and type of microbial cells present, the type of HS, etc. The same is true for the upper limit of electron shuttling, as reported values vary between 50 and 240 mg HS/L (Amstaetter et al. 2012; Jiang and Kappler 2008). The HS concentrations necessary for electron shuttling also depend on the mechanism of electron transfer between the bacterial cell and the Fe(III) mineral. There are two models as to how electron shuttling over spatial distances could work (Fig 7): (1) the electron is transferred from the cell to a HS molecule that is located at a certain distance from the mineral surface. The reduced electron shuttle then diffuses to the Fe(III) mineral surface, where it transfers the electron to the mineral. The re-oxidized shuttle then returns (diffuses back) to the cell and can be re-reduced, thus functioning as electron shuttle between the cell and the mineral. In this case the electron transfer would be controlled by the diffusion of the shuttle to the mineral surface and back, and therefore by the distance between the cell and the mineral. (2) Alternatively, an electron is transferred from the cell to a first electron shuttle that is located at a certain distance from the mineral surface. But, instead of diffusion of the shuttle to the mineral surface, the electron is passed from the first HS molecule to the next one and the distance between the cell and the Fe(III) mineral is thus bridged by electron hopping. Since the maximum distance for each of these electron transfer steps is approximately 20 Å (Gray and Winkler 2005), a minimum concentration of HS is required to provide the necessary density of electron accepting sites (Fig 7).

Besides the concentration of HS and the ratio of HS to iron minerals, the ratio of microbial cells to Fe(III) minerals is also expected to be important for electron shuttling. If the Fe(III) minerals are present in excess and the mineral surface area is not limiting for microbial electron transfer (i.e., all cells are attached to the mineral surface), the addition of electron shuttles potentially leads only to a minor stimulation of microbial Fe(III) reduction. Such a stimulation by electron shuttles in a scenario where all cells are associated with the mineral surface could occur by dissolved shuttles functioning where the electron accepting Fe(III) mineral surface and outer membrane cytochromes are not close enough (see Fig 4). On the other hand, if the cells are in excess and the mineral surface area is not sufficient for all cells to attach, electron shuttling has the potential to significantly increase the reduction rate by enabling Fe(III) reduction by those cells that cannot directly transfer electrons to the mineral surface. Indeed, it was recently shown in our laboratory that increases in the rate of ferrihydrite reduction by *S. oneidensis* MR-1 in cell suspension experiments with varying cell densities in the presence of HS were present at both high and low cell densities but the increase was more prominent at high cell densities than at lower cell densities (Rohrbach,

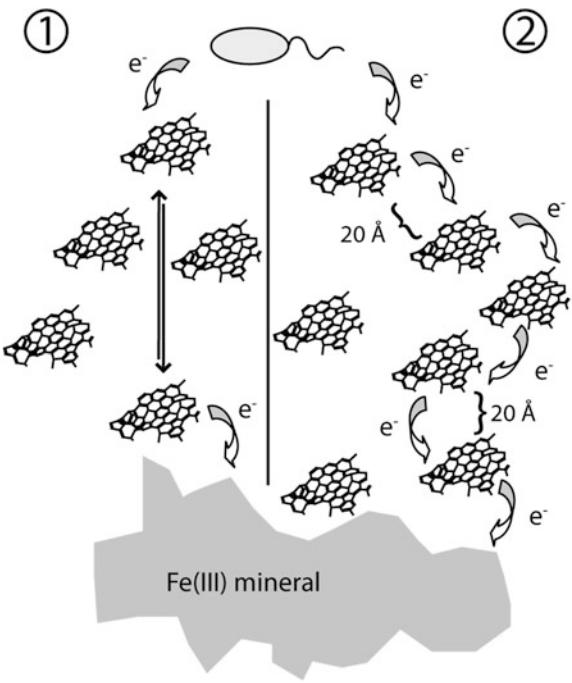

Fig. 7 Models for electron shuttling by humic substances between a microbial cell and a Fe(III) mineral by (1) diffusion of the electron shuttle (*left*) and (2) electron hopping (*right*). Electron hopping requires a maximum distance of approximately 20 Å between the redox-active sites of the involved shuttling molecules which can only be provided at a certain HS concentration

unpublished data). This suggests that at high cell densities, the shuttles enable electron transfer to the mineral surface from cells that are at a distance from the minerals, while at low cell densities the shuttles increase electron transfer to the mineral from cells that are attached to the mineral.

8 Open Questions and Future Research

Humic substances and other extracellular electron shuttles can contribute significantly to the electron fluxes during microbial respiration, in batch systems with pure cultures of microorganisms as well as in complex environmental systems including soil and sediment microcosms. Although a lot of recent research has focused on the role of HS as electron shuttles, there are still several key questions that remain unanswered. Most of them are related to the importance and relevance of electron shuttling in environmental systems, where electron shuttling is often very difficult to assess and quantify. This is particularly due to the absence of a specific enzymatic system that is involved in HS reduction. Therefore, it is not possible to quantify HS reduction in environmental systems via analysis of the expression and activity of functional genes. This is one of the reasons why we cannot easily evaluate which microorganisms are reducing HS in the environment and to what extent the different physiological groups contribute to HS reduction.

Furthermore, it is still unclear whether or not HS concentrations in the environment are really sufficient to function as electron shuttles and what are the contributions of HS reduction to the overall electron flow in the systems. This is especially the case since most of the studies on HS electron shuttling are conducted in batch cultures with single microbial strains and synthetic Fe(III) minerals, while in environmental systems consortia of different microbial strains are present, Fe(III) minerals are also of biogenic origin and the largest fraction of HS present is particulate. It is still unclear to what extent solid-phase HS contribute to electron transfer, especially over long distances (several cm).

These are two of the main topics on which future research should concentrate and which could help us to better understand the importance of HS electron shuttling in environmental systems.

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