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Extracellular Electron Transfer in *in situ* Petroleum Hydrocarbon Bioremediation

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Additional information is available at the end of the chapter

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1. Introduction

1.1. Environmental pollution with petroleum hydrocarbons

Anthropogenic contamination of soil, subsurface and surface waters and atmosphere with toxic organic chemicals is an environmental issue of world wide concern. While energy and goods production from fossil hydrocarbon sources is one of the driving factors of global economy, the often adverse environmental effects of exploration, production, transport and processing of crude and shale oils, tar sands, coal and natural gas seldom come into the focus of attention.

Chemically, the term *hydrocarbons* encompasses a large variety of compounds. Saturated and unsaturated structures composed of hydrogen and carbon make up the largest fraction of organic compounds derived from fossil sources, including crude oil, its derivatives and distillates as well tar oils originating from coal gasification. These are often referred to as petroleum hydrocarbons (Cermak, 2010), also encompassing mono- and polycyclic aromatic structures of environmental and toxicological concern (Loibner et al., 2004).

In the US alone, between 5,000 and 7,000 spills of hazardous material occur every year (Scholz et al., 1998). Although the US-EPA's *Spill Prevention Control and Countermeasure Program* for inland oil spills has reduced the amount of undesired hydrocarbon release to less than 1% of the total volume of oil handled each year (US-EPA, 2012), considerable surface areas and contaminated ground water bodies remain to be decontaminated. Microbial, physical and chemical remediation strategies are available.

1.2. *In situ* bioremediation of petroleum hydrocarbons

For soil and water bodies contaminated with organic pollutants, *bioremediation* refers to the engineered exploitation of an ecosystem's intrinsic capacity for the attenuation of adverse

biogeochemical influences. In *in situ* remediation soil, water and contamination remain in place¹. These processes are driven by a large variety of microorganisms, including bacteria, fungi and archaea (*microbial bioremediation*) and may also involve plants (*phytoremediation*). The targeted addition of enriched or modified microorganisms (*bioaugmentation*) is justified in case the resident microbial population is incapable or incapacitated to perform substantial contaminant transformation or, more importantly, mineralisation. In contrast, the naturally present microbial population at a long-term contaminated site is expected to have the potential metabolic capacity for the degradation of aromatic and aliphatic petroleum hydrocarbons under both aerobic and anaerobic conditions (Widdel and Rabus, 2001). In *biostimulation* efforts, growth factors are administered to the contaminated area, including electron acceptors or donors for biochemical contaminant oxidation or reduction, respectively, and / or of micro- and macronutrients, vitamins and trace elements and others (Bartha, 1986; Cerniglia, 1992). A variety of strategies is in application or under development.

Implementing *in situ* bioremediation is an intricate task for designing and executive engineers, while decreasing the burden to economy and environment in lessening the need for soil excavation. Some strategies of physical, chemical and also microbial remediation involve harsh and cost-intensive measures effecting substantial changes to the biogeochemical conditions in the treated environments. Thus, the optimization of existing strategies and the development of novel approaches to deal with environmental pollution in sustainable ways is required both from economic and environmental viewpoints.

1.3. Objective

This review is dedicated to elucidating mechanisms for extracellular electron transfer to geogenic electron acceptors, which can potentially be coupled to oxidative hydrocarbon detoxification. An increase in the accessibility of naturally present electron accepting compounds to hydrocarbon-degrading bacteria could substantially improve bioremediation efficacy of anaerobic petroleum hydrocarbon contaminated aquifers.

2. Petroleum hydrocarbon degradation under different terminal electron accepting conditions

Petroleum hydrocarbons such as normal, branched and cycloalkanes and aromatic hydrocarbons are degradable *in situ* via biochemical oxidation both under aerobic and anaerobic conditions (DeLaune et al., 1980), provided the degradative activity is not inhibited by a lack of nutrients (nitrogen, phosphorus, potassium), electron acceptors, trace elements and moisture, or if pH, temperature, salinity and contaminant concentrations are outside certain boundaries (Bartha, 1986; DeLaune et al., 1980). In contrast, hydrocarbons carrying strongly electron-withdrawing substituents are preferentially degraded via

¹ In contrast, *ex situ* bioremediation refers to manipulation strategies of excavated or delocalized contaminated matrices (e.g. in biopiles, biofilters and others) intended to decrease the contaminant load via microbial degradation.

contaminant reduction, such as sequential *reductive dechlorination* for chlorinated solvents (Maymo-Gatell et al., 1999).

A large range of microorganisms have the metabolic capability of oxidative hydrocarbon degradation using a variety of terminal electron acceptors (TEA) in the vadose and saturated zones of the subsurface (Heider et al., 1998).

2.1. Molecular oxygen as TEA

In the range of naturally occurring TEAs, molecular oxygen yields the largest amount of energy in terms of ATP production and is associated with high degradation rates with aerobic microbial communities. Molecular oxygen, however, has a low aqueous solubility (up to 10 mg/L). Fast oxygen depletion in microbial contaminant oxidation processes is opposed to slow diffusive replenishing in the groundwater. Thus, petroleum hydrocarbon contaminated aquifers are often depleted of molecular oxygen, while other, energetically less favourable TEA are reduced more slowly. Oxygen limitation is thus manifested in low natural contaminant attenuation rates, and anaerobic conditions are often encountered at biologically active contaminated sites.

2.2. Soluble alternative TEA

This has prompted the development of strategies for the stimulation of anaerobic hydrocarbon degradation. One approach is the addition of naturally occurring, well-soluble alternative terminal electron acceptors such as nitrate and sulphate (Hasinger & Scherr et al., 2012; Bregnard et al., 1998). Hydrocarbon oxidation coupled to nitrate reduction, however, yields approximately one tenth of the biochemical energy of aerobic degradation per mole hydrocarbon, and sulphate reduction about 1%. Both terminal electron accepting processes are associated with significantly lower degradation rates compared to aerobic degradation (Widdel and Rabus, 2001). The use of well soluble and less chemically reactive alternative electron acceptors, however, is connected to an increase in influence *radii* when introduced into the aquifer (Hasinger & Scherr et al., 2012).

Considerable amounts of alternative TEA, however, are required for aquifer remediation. On a theoretical mass basis, around 5 g of nitrate and sulphate are needed for the anaerobic mineralisation of 1 g of *n*-hexadecane, a mid-weight petroleum hydrocarbon, although lower demands were observed in practice (Hasinger & Scherr et al., 2012), are required. In addition, possible by-products of alternative TEA consumption, including products from incomplete nitrate reduction and sulphide precipitation, can render the implementation of these strategies under field conditions an intricate task.

2.3. Poorly soluble alternative TEA

Despite their poor aqueous solubility², and thus originally somewhat surprising, naturally abundant solid-phase minerals are known to play a quantitatively important role in

² *Poor aqueous solubility* and *insoluble in water* are often used as synonyms in this context.

subsurface microbial reduction and oxidation³ processes (Weber et al., 2006). *Poorly soluble alternative TEA* refers mainly to iron (Fe) and manganese (Mn) minerals in their tri- and tetravalent oxidation states (Lovley et al., 2004). Their oxides, hydroxides and oxyhydroxides are amongst the most abundant redox-active elements on the earth's surface.

This comes surprising since solubility in water is deemed a prerequisite for chemicals to participate in microbial transformation processes. The participation of poorly soluble minerals in environmental redox-processes, however, is made possible by a variety of bacterial strategies to access and transfer electrons to (and from) poorly soluble mineral surfaces. These strategies include the secretion of chelators, production of electrically conductive protein structures and the participation of redox active natural organic moieties, or *electron shuttles*.

These processes are summarized as mechanisms for *extracellular electron transfer*, and are the subject of this review.

2.4. Potential use of extracellular electron transfer in bioremediation

Poorly soluble, naturally abundant alternative electron acceptors have the potential to be incorporated into efficient bioremediation strategies. The abundance of reducible iron and manganese minerals in the subsurface, may, provided these minerals can be involved into microbial hydrocarbon oxidation, alleviate the need to artificially introduce electron acceptors, such as molecular oxygen, nitrate and sulphate.

There is evidence that iron and manganese respiration can be coupled to anaerobic contaminant oxidation of monoaromatic compounds (Kunapuli et al., 2008; Lovley et al., 1989). Under field conditions, however, pronounced iron reduction coupled to the oxidation of higher molecular weight hydrocarbons is seldom reported (Aichberger et al., 2007). These data, however, indicate that hydrocarbon oxidation coupled to solid-state mineral respiration is possible, however at limited rates. The rates of Mn and Fe respiration, on the other hand, can be significantly increased via the enhancement of extracellular transport mechanisms. *Geobacter sulfurreducens*, a model dissimilatory metal reducing bacterium (DMRB) transfers electrons 27 times faster to a transient electron storage, an electron shuttle or mediator, than to iron hydroxide (Jiang and Kappler, 2008). The introduction of such electron transfer facilitators is one of the key issues where extracellular electron transport mechanisms could be incorporated into petroleum hydrocarbon bioremediation. Extracellular electron shuttles are reversibly reduced and oxidized in many subsequent cycles. Thus, only substoichiometric amounts would theoretically be required to achieve substantially increased degradation rates.

Provided a comprehensive understanding of the underlying mechanisms, efficient bioremediation strategies may be devised to enhance extracellular electron transfer from polluting organic substances to poorly soluble mineral surfaces, adjoined by oxidative pollutant transformation and detoxification. The mechanisms of electron shuttling and other

³ Reduction / oxidation processes are also abbreviated as *redox*-processes.

extracellular electron transfer strategies devised by DRMBs, thermodynamic considerations and microorganisms involved are reviewed in Chapters 3 through 8.

In summary, possible strategies to enhance anaerobic petroleum hydrocarbon degradation using naturally occurring, poorly soluble TEA include the addition of electron shuttling compounds (ES) of natural or anthropogenic origin, as was demonstrated for the anaerobic oxidation of dichloroethene (Scherr et al., 2011) before, to contaminated aquifers. The addition or targeted stimulation of microorganisms equipped with the metabolic capability to transfer electrons to mineral surfaces, via secretion of chelators and microbial ES or expression of conductive protein appendages, such as *Geobacter* or *Shewanella*, represents another approach.

A tentative collection of bioremediation strategies incorporating extracellular electron transfer for petroleum hydrocarbon contaminated aquifer remediation is provided in Chapter 9.

3. Interactions in extracellular electron transfer

3.1. Possible participants and pathways

Different reactions can be mediated between participants of subsurface redox-processes via extracellular electron transfer, involving microbes as well as soluble and insoluble, organic and inorganic electron acceptors and donors. Extracellular electron transport is required in case the electron donor, electron acceptor or both can not be taken up by the bacterial cell, be it due to poor solubility, or if a direct contact between the microbe and electron sources or sinks is not possible, e.g. due to occlusion in pore spaces too small for microorganisms to enter. Possible extracellular pathways include, besides direct contact between cell and mineral surfaces:

- Secretion of chelators for, e.g. Fe(III) and Mn(IV) minerals
- Electron shuttling, i.e. reversible electron uptake and release by redox-active organic compounds excreted by microbes or in the natural background
- Expression of electrically conductive, pilus-like bacterial assemblages, also termed *nano-wires*

These will be discussed in more detail in Chapter 5.

3.2. Routes for extracellular electron transfer in the saturated zone

Electrons may be transported extracellularly between any of the parties participating in redox-processes: (i) microbes and poorly soluble bulk (ii) sinks and (iii) sources of electrons. Where either electron donor or acceptor are well soluble, usually no extracellular transport pathway is required. Figure 1 shows four possible flow paths or *routes* between the parties. Where extracellular transport is required, is indicated by solid lines. Dashed lines represent pathways for well-soluble participants.

Electron route A: electron donor to microbe

Via route **A** electrons are transported from an electron source to a microbe, a typical half-reaction pathway for the oxidation of zero-valent iron (ZVI). In case soluble polluting and

non-polluting organic compounds (petroleum hydrocarbons, acetate and others) yield electrons, no extracellular pathway is required (dashed lines).

Route B: microbe to electron acceptor

In the oxidation of petroleum hydrocarbons coupled to iron or manganese reduction, the microbe gains energy from donating the electron obtained directly from the pollutant donor (dashed line to green microbe) to the energetically favourable, poorly soluble terminal electron acceptor (route **B**).

In case the terminal, inorganic electron acceptor is insoluble, as in the case for Fe(III) and Mn(IV) minerals, and/or no direct contact between cellular and electron accepting surface can be established, electron transfer rates can significantly be increased via extracellular electron transport vehicles (Jiang and Kappler, 2008). This can be aided by the production of electrically conductive pilus-like assemblages, also termed microbial *nanowires* (Reguera et al., 2005), electron shuttles or metal chelating agents chelators, as depicted in Figure 1.

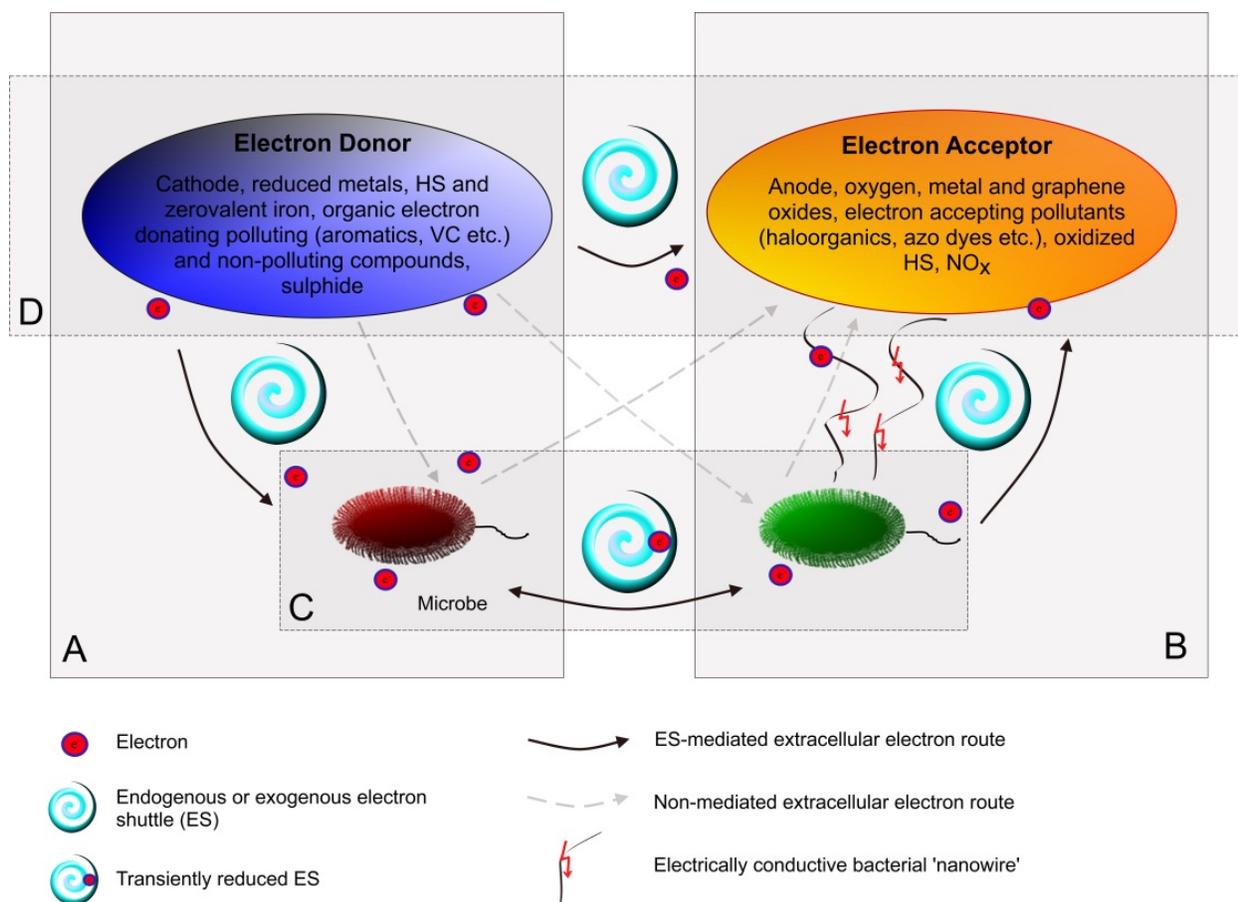


Figure 1. Electron routes in biogeochemical redox-processes: extracellular electron transport (A) from an electron source (donor) to microbe, (B) from microbe to electron sink (acceptor), (C) between microbes and (D) abiotic source/sink exchange. Solid lines indicate extracellular electron transport, dashed lines represent direct transport, usually via uptake of donor or acceptor. HS = humic substances, VC = vinyl chloride.

Route C: electron transport between microbes

Shuttling of electrons between microbes is represented by route C. Electron shuttles have been hypothesized to syntrophically link diverse organisms in nature (Lovley et al., 2004). This may occur, for example, between outer and inner layers of a biofilm (Lies et al., 2005). Intraspecies electron transfer has been observed with a naphthoquinone moiety as shuttle in the gastrointestinal tract (Yamazaki et al., 1999); its functionality in the subsurface has not been investigated yet.

Route D: electron transfer between abiotic parties

Moreover, electron shuttles may participate in purely abiotic electron transfer reactions between electron donors and acceptors (route D in Figure 1).

Route D symbolises the abiotic reduction of metals by microbially reduced humic substances (HS), in case the HS-oxidation process is literally *terminal*. In case of microbial 'regeneration', i.e. re-oxidation of HS, it serves as an electron shuttle in route B. Route D also describes abiotic chlorophenol degradation via ZVI, mediated by the presence of humic and fulvic acids (Kang and Choi, 2009), or shuttle-mediated abiotic reductive dechlorination mechanisms (Lee and Batchelor, 2002). This route also refers to the ability of natural organic matter to shuttle between oxidized and reduced forms of mercury that has only recently been recognized (Gu et al., 2011; Zheng et al., 2012).

4. Breathing solids: microbial respiration of poorly soluble iron and manganese minerals in uncontaminated environments

4.1. Not attached: respiration from a distance

Electrons need to be associated to a vector to participate in reactions beyond the immediate bacterial surface, i.e. for travelling distances exceeding 0.01 μm (Gorby et al., 2006; Gray and Winkler, 1996). Soluble electron acceptors commonly used for petroleum hydrocarbon degradation include oxygen, nitrate and sulphate. These compounds can easily diffuse into the cell where they are reduced. Unlike these, the prevalent forms of iron and manganese in the environment are insoluble and remain extracellular, rendering the direct contact of cell and mineral surfaces an apparent necessity for respiration. This is contrasted by the ability of a number of microorganisms for dissimilatory reduction of undissolved mineral oxy(hydr)oxides, entailing electron transfer over distances of 50 μm and up. As an example the reduction of physically trapped or occluded iron(III) crystals was observed (Lies et al., 2005; Nevin and Lovley, 2002b). Different strategies enabling for long-distance electron transfer have been identified; they will be discussed in Chapter 5.

Microbes capable of dissimilatory metal reduction (DRMB) are phylogenetically dispersed throughout the *Bacteria* and *Archaea*; the most prominent members belong to *Geobacter*, *Shewanella* and *Geothrix* (see Chapter 7).

4.2. Biogeochemical role of poorly soluble iron minerals

Iron, comprising approximately 3.5% per mass of the earth's crust (Martin and Meybeck, 1979; Reimann and de Caritat, 2012) is the most abundant redox-active metal in the modern environment. While the main iron fraction is located in silica structures, approximately one third of iron atoms are present as amorphous or crystalline oxides in surface rocks (Canfield, 1997). At a circumneutral pH, iron is present primarily as quasi-insoluble minerals in di-valent ferrous (Fe(II), e.g. wüstite) trivalent ferric (Fe(III), e.g. goethite) or mixed-valence (Fe(II,III), e.g. magnetite) oxides, hydroxides and oxihydroxides (Scheffer and Schachtschabel, 2002).

Quasi-insoluble refers here to log K values of around -40 for goethite, ferric hydroxide and hematite (Morel and Hering, 1993). In the face of the poor solubility of iron oxides, it may come surprising that iron redox cycling in soil and sedimentary environments is now assumed to be dominated by microbial action (Arrieta and Grez, 1971; Chaudhuri et al., 2001; Weber et al., 2006). In fact, Fe(III) represents one of the most important terminal electron acceptors for microbes under anaerobic conditions (Lovley et al., 2004; Weber et al., 2006).

Iron, in its various oxidized or reduced states, has a high versatility in energy-creating processes in oxygen-rich and suboxic environments, as an electron donor for iron-oxidizing microbes under oxic and anoxic conditions as well as a terminal electron acceptor under anoxic conditions, and also influences other biogeochemical cycles. Microbial iron redox-cycling, also termed the *iron redox wheel*, is mechanistically connected to carbon, nitrogen, phosphorous and sulphur redox cycling (Li et al., 2012).

While poorly crystalline oxides appear to be readily metabolized (Phillips et al., 1993), the microbial utilization of highly crystalline oxides as electron acceptors in a (real) soil or sediment environment appears to be thermodynamically unfavourable (Thamdrup, 2000).

4.3. Dissimilatory manganese reduction

Mineral-bound manganese is significantly less abundant than iron in our environment, contributing approximately 0.07% by mass to surface rocks (Martin and Meybeck, 1979; Reimann and de Caritat, 2012).

The main fraction of manganese in the earth's surface, roughly 75%, is present as oxides (Canfield, 1997) with the remainder located in silicates. Minerals contain varying amounts of both Mn(III) and Mn(IV), with poorly crystalline manganite, vernadite and birnessite minerals as most abundant minerals (Friedl et al., 1997; Zhu et al., 2012). Similarly to iron cycling, biotic manganese oxidation and reduction are commonly encountered processes in soils and sediments. It appears that the mechanisms for manganese respiration and oxidation are similar to those for iron, as most organisms that can respire Fe can also respire Mn and vice versa (Lovley et al., 2004; Shi et al., 2007).

4.4. Behaviour of reduced manganese and iron

Following reduction, both Fe(II) and Mn(II) are re-oxidized biologically or chemically rather quickly. The thermodynamics of Fe(II) regeneration appear more favourable than those for

Mn(II) oxidation. Adding to this, Mn(II) has higher aqueous solubility than Fe(II), rendering microbe-driven post-depositional redistribution by reductive dissolution and transport from anoxic to oxic interfaces, where precipitation takes place, a main mechanism governing manganese localization (Thamdrup, 2000). Every atom may participate in subsequent reduction / oxidation cycles as many as 100 times (Thamdrup, 2000).

4.5. Side note: Bacterial reduction and oxidation of heavy metals

Besides iron and manganese, also polluting metals can be involved into bacterially mediated oxidation and reduction processes. These approaches can be used for bioremediation and the facilitated recovery of metals from waste water streams due to precipitation (Lovley and Phillips, 1992).

Uranium reduction

Some microbes can also reduce uranium (Cologgi et al., 2011; Lovley and Phillips, 1992; Lovley et al., 1991) at rates comparable to those of Fe(III) reduction (Lovley and Phillips, 1992). In contrast to manganese and iron, uranium precipitates during reduction from its hexavalent to its tetravalent form. The simultaneous precipitation of uranium with the reduction of Fe(III) has been observed for *Geobacter sulfurreducens* (Cologgi et al., 2011), leading to the assumption that similar mechanisms are governing both processes.

Reduction of other elements by DRMB

Similarly, *Shewanella* has been observed to precipitate reduced chrome (Cr(III)), likely involving the same enzymes as for Fe(III) reduction (Belchik et al., 2011). It can be hypothesised that the expression of extracellular electron transport pathways for soluble, oxidized electron acceptors precipitating during reduction, such as chrome and uranium was intended to keep the precipitates from forming inside the cells.

Dissimilatory iron-reducing bacteria were also found to participate in the reduction of technetium (Istok et al., 2004), cobalt, gold (Kashefi et al., 2001) and other metals, metalloids and radionuclides (Lloyd, 2003) as well as graphene oxide reduction (Jiao et al., 2011). These abilities may be used for the recovery or removal of metal resources from waste water streams or *in situ* remediation.

The ability to grow on these metals as sole electron acceptors has not been adequately demonstrated. Reducible iron and manganese minerals, however, will quantitatively dominate in most contaminated environments over heavy metals (Lovley et al., 2004) as terminal electron acceptors.

5. Microbial strategies to enhance extracellular electron transfer

In this chapter, mechanisms devised by DMRB for accessing poorly soluble electron acceptors are reviewed. Most of our understanding of these processes is derived from investigations of dissimilatory iron and manganese reduction under anaerobic conditions. *Geobacter* and *Shewanella* are considered as model organisms in this respect (cf. Chapter 7).

In the course of microbial energy generation, electrons are transferred from an electron source to a sink, usually inside a cell. DMRB, in contrast, are faced with the problem of how to effectively access an electron acceptor that can not diffuse to the cell. In case the electron donor or acceptor do not permeate through the membrane, as in the case of insoluble metals, or where the presence of redox-products inside the cell is not desired, such as for uranium precipitates, cells can rely on active processes to access poorly soluble TEAs or utilize extracellular electron shuttles. These are naturally present or anthropogenically administered compounds that have the potential to facilitate extracellular electron transfer.

Several mechanisms to overcome the physiological challenges for the dissimilatory reduction of poorly soluble electron acceptors have been identified.

5.1. Direct transport and electrochemical wiring

Direct electron transport

One route for extracellular electron transfer is the direct transport of electrons between the cellular envelope and electron accepting mineral surfaces, however requiring direct contact between cell and mineral surfaces. In these reactions, electrons have been found to travel over distances of 10 to 15 Å (Kerisit et al., 2007).

This direct transfer is mediated by outer-membrane cytochromes directly to the mineral's surface, and are typical of Gram-negative bacteria such as *Geobacter* and *Shewanella* (Hernandez and Newman, 2001). *Geobacter* and also *Shewanella* are representatives of a family of ferric iron reducers that were found in a variety of anaerobic environments (more about them in Chapter 7). The involved multiheme c-type cytochromes are essential electron-transferring proteins, rendering the journey of respiratory proteins from through the cell to the outer membrane possible (Myers and Myers, 1992; Shi et al., 2007) and forming a respiratory chain extended beyond the cell's physiological periphery.

Directly nano-wired

Both *Shewanella* and *Geobacter* were observed to form pilus-like assemblages extending to the mineral surface when grown on insoluble electron acceptors, representing a special case of direct transport.

Geobacter was reported to produce monolateral pili to access iron and manganese oxides physically, i.e. via chemotaxis (Childers et al., 2002). Mineral reductases are located on the outer cell membrane but also on electrically conductive pilus-like assemblages prominently termed *nano-wires* (see Figure 1). These conductive protein nanostructures are 'wiring' the cell and the mineral phase, enabling for electron transport via a physical extension of the cell, thus outsourcing direct contact between cell and mineral surface to thin conductive appendages. Evidence for the production of *nano-wires* has been reported for both *Shewanella* (Gorby et al., 2006) and *Geobacter* (Reguera et al., 2005), however with distinct molecular mechanisms for the electron transport.

Different strategies are required for iron reducing microorganisms lacking conductive surface cytochromes, requiring for alternative strategies for extracellular electron transfer.

5.2. Electron shuttles and metal chelating agents

Electron shuttles are small molecules capable of undergoing repeated reduction and oxidation processes. An electron shuttle can be an organic or inorganic compound that is reversibly redox active and has the right redox potential, i.e. poised between the E_0' of the reductant and the oxidant. It serves as an electron acceptor and, once reduced, can itself transfer electrons to other organic or inorganic electron acceptors, whereupon it becomes re-oxidized. Such a shuttle provides a mechanism for an indirect reduction or oxidation process. In principle, a single shuttle molecule could cycle thousands of times and, thus, have a significant effect on the turnover of the terminal acceptor in a given environment.

Extracellular electron shuttles may be produced by microorganisms, originate from the humification of plant and animal matter or be anthropogenically added. While most electron shuttles are organic, heterocyclic aromatic structures, theoretically also inorganic, nonenzymatic compounds such as sulphide and reduced uranium, and organic compounds equipped with sulfhydryl groups (Nevin and Lovley, 2000) may serve as electron shuttles themselves.

In Figure 2 the redox-active groups of frequently encountered microbial, natural and anthropogenic electron shuttles are displayed. Heterocyclic aromatic compounds with redox-active functional groups, with di-ketone moieties in quinones, and nitrogen for phenazines and viologen, are common structures. Isoalloxazine represents the reactive structure in flavins.

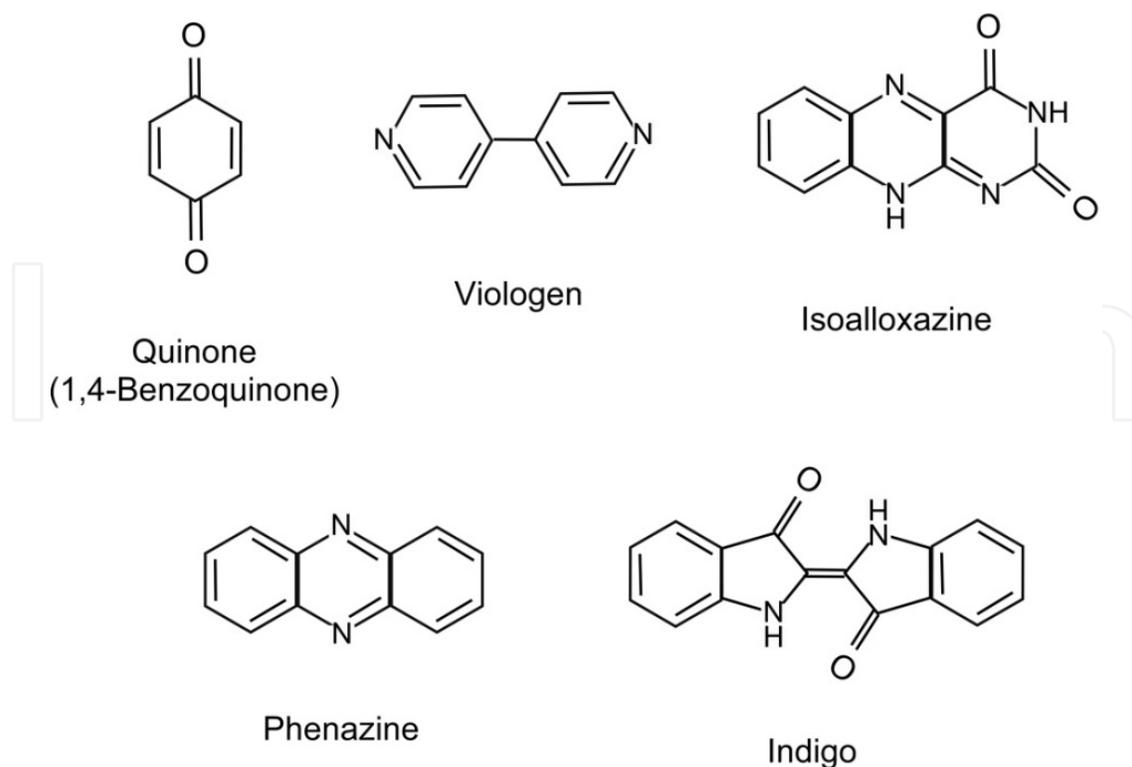


Figure 2. Main redox-active structures found in extracellular electron shuttles

5.2.1. Microbially produced extracellular electron transporters

In case the electron acceptor is physically detached from the cell, e.g. oxides occluded in pores, direct contact is not possible for a large fraction of bacteria, e.g. in biofilms, or the microbe lacks the enzymatic toolkit to respire electron acceptors, electrons are encouraged to take alternative routes through extracellular space by using transport facilities provided by the microorganism itself, such as electron shuttles and / or chelating agents.

The production of electron transport mediators is known for many microbes. Beside melanin, produced by *Shewanella alga* (Turick et al., 2002), phenazine derivatives (Hernandez et al., 2004), flavins (von Canstein et al., 2008), quinone-type structures (Nevin and Lovley, 2002a) and possibly also siderophores (Fennessey et al., 2010; Kouzuma et al., 2012) were shown to enhance anaerobic ferric iron respiration.

Phenazine and its derivatives

Phenazines are small redox-active molecules secreted by bacteria and functionally similar to those of anthraquinones. They are, due to their antibiotic effect, believed to be part of the organisms' biological warfare arsenal (Chin-A-Woeng et al., 2003; Hernandez and Newman, 2001). They are able to reductively dissolve iron and manganese (Hernandez et al., 2004; Wang and Newman, 2008), and are applied in microbial fuel cells (Pham et al., 2008). Phenazines are, however, of toxicological concern, such as pyocyanin, a blue pigment excreted by *Pseudomonas aeruginosa*, rendering the application of phenazines in environmental biotechnology an intricate task.

Flavins

Flavins have a midpoint potential of -0.2 to -0.25 V and can reduce most iron oxides and soluble forms of ferric iron, contributing to metal acquisition in plants and yeasts (Marsili et al., 2008). They have been proven to mediate the reduction of iron hydroxides inaccessible to microbes, via penetration through pores too small to allow for microbial passage (Nevin and Lovley, 2002b). Their isoalloxazine group acts as metal chelator.

The production of iron-solubilizing ligands has first been observed for *Shewanella putrefaciens* (Taillefert et al., 2007), which were shortly afterwards simultaneously identified as flavins by von Canstein and Marsili (Marsili et al., 2008; von Canstein et al., 2008). Also other γ -proteobacteria and *Marinobacter* are able to produce flavins, which may be used or *pirated* by other organisms directly (Hirano et al., 2003; von Canstein et al., 2008) and from flavin-coated surfaces (Marsili et al., 2008). The role of flavins in electron shuttling has recently been reviewed (Brutinel and Gralnick, 2012).

Side note: flavin or not flavin

Flavins were identified to have a major role in iron respiration of *Shewanella oneidensis*. In this context, both riboflavin (RF, Vitamin B₂) and flavin mononucleotide (FMN) were detected in iron-respiring *Shewanella* cultures. Flavins are now well-studied (Brutinel and Gralnick, 2012; Marsili et al., 2008; von Canstein et al., 2008) examples of endogenously synthesized electron transporting compounds deployed to donate respiratory electrons to a

distant mineral phase. Here, electrons are conveyed to extracellular flavins via cytochromes that are re-oxidized upon direct contact to Fe(III), resulting in increased electron transfer rates than at direct contact to the mineral surface (Brutinel and Gralnick, 2012).

Experimental evidence for the functionality of flavin-mediated enhanced iron respiration was manifested in the presence of flavins in the supernatant of *Shewanella* reducing poorly soluble Fe(III). A stoichiometric ratio of 1 (FMN oxidized) to 2 (Fe(II) produced) was noted (von Canstein et al., 2008). The nature of the force bringing these compounds into the outer cell space remains disputed in that FMN and RF may either be actively secreted (Marsili et al., 2008; von Canstein et al., 2008) or merely be released during cell lysis as part of the common intracellular enzymatic mix. In this context, the former hypothesis is corroborated by different concentrations and species of intra- and extracellular flavins detected in the supernatant and the proportionality of extracellular flavin concentrations to live rather than dead cell density (von Canstein et al., 2008). These arguments were, in turn, challenged recently (Richter et al., 2012).

Siderophores

Siderophores are low molecular weight compounds produced by bacteria, fungi and plants when exposed to low-iron stress conditions, and are very strong Fe(III) chelating agents. Most of them contain catechol or hydroxamate groups as iron-chelating groups (Neilands, 1995). In forming Fe(III)-siderophore complexes, they are scavenging Fe(III) from the environment, rendering it more accessible for bacterial uptake. Its role in increasing metal respiration is, to our knowledge, uncertain.

Although siderophores were found to catalyze the mineralisation of carbon tetrachloride, it is not known whether microorganisms can gain energy from the process (Lee et al., 1999). Recent research suggests indirect effects of siderophores on respiration in increasing iron uptake, which is in turn required for the synthesis of cytochromes for manganese respiration (Kouzuma et al., 2012), while others indicate no effect of siderophore-related iron solubilisation on its respiration (Fennessey et al., 2010). It appears likely that iron-containing shuttles participate in iron solubilizing mechanisms, where the cation remains with the redox-active centre, thus corresponding to permanent rather than transient iron complexation.

5.3. Exogenous electron shuttles and chelators

5.3.1. Types of electron shuttles

For a large variety of naturally occurring redox-active compounds, at least some functionality as extracellular electron shuttles was described (Guo et al., 2012; O'Loughlin, 2008; Van der Zee and Cervantes, 2009; Watanabe et al., 2009; Wolf et al., 2009). Main functional structures are depicted in Figure 2. Frequently encountered shuttles encompass the following:

- Quinones, including
 - anthraquinones, their sulfonates, carboxylic acids and chlorinated moieties

- juglone,
- lawsone,
- menadione and other naphthoquinones
- ubiquinone, coenzyme Q₁₀
- Humic substances
- Phenazines cores, including
 - neutral red
 - methylene blue
- Vitamine cores, including
 - corrin
 - isoalloxazine
- Indigo sulfonates and other derivatives
- Viologens, including
 - methyl viologen
 - benzyl viologen
- Cysteine and other thiol-containing molecules

Amongst these, the role of quinones and humic substances are discussed in more detail below.

5.3.2. Quinones

Structure

Chemically, quinones are fully conjugated cyclic di-ketone structures derived from the oxidation of aromatic compounds, where an even number of $-\text{CH}=\text{}$ groups is converted into $-\text{C}(=\text{O})$ groups. This is adjoined by a re-arrangement of double bonds and connected to the loss of aromaticity. Quinones may also be considered as products from polyphenol reduction (Figure 3). Quinones are stable throughout a number of repeated oxidation and reduction processes. In their redox-cycling, one- and two-electron reduction reactions lead to the formation of free semiquinone radicals and hydroquinones, as displayed in Figure 3 (Michaelis and Schubert, 1938).

Occurrence

Quinones are abundant structures in nature, participating in a wide variety of reactions in pro- and eukaryotic organisms. Membrane-bound ubiquinones and menaquinones shuttle electrons between respiratory protein complexes in bacteria. Phylloquinone participates in the electron transfer chain of in photosynthesis. Other quinones may have antimicrobial (Lown, 1983; Zhou et al., 2012) or even toxic (Shang et al., 2012) effects, such as juglone. Isoalloxazine, the reactive structure in flavins, is also a quinone (Muller et al., 1981).

Role of quinones in electron shuttling

The hydroquinone moiety chemically reduces Fe(III) to Fe(II) while it is simultaneously oxidized to a quinone (Scott et al., 1998). In turn, it is reduced back to hydroquinone by microbes deriving electrons from hydrocarbon oxidation and other processes. This is a type

B reaction (Figure 1), with the electron donor being a hydrocarbon and the acceptor an insoluble mineral, such as a Fe(III) or Mn(IV) mineral. Although the energy gain from quinone reduction is lower than from direct reduction of Fe(III) minerals, the use of extracellular shuttles enables for significantly increased reduction rates, i.e. a higher bacterial energy gain per unit time (Jiang and Kappler, 2008).

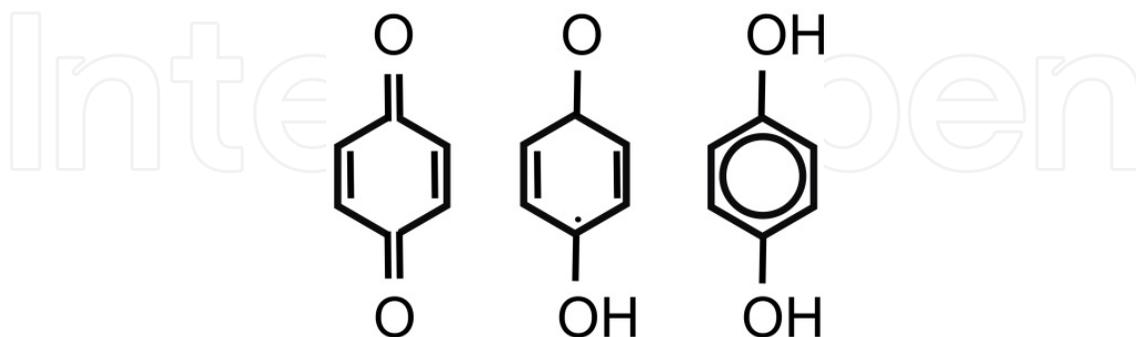


Figure 3. Simple quinoid structures: 1,4- or para-benzoquinone (left), its semiquinone (center) and its hydroquinone (right)

The manifestation of a type **A** route would reflect electron shuttling from an organic electron donor to a halogenated or nitrous contaminant.

In summary, electron shuttles participate in facilitating the electron transfer from the microbe towards the electron acceptor in the type **B** reaction and from electron donor to microbe for sulphide, reduced metals or the cathode for a type **A** reaction; in both contexts, quinones are often encountered.

Iron-reducing microorganisms are able to grow on extracellular quinones as the sole source of energy. Humic substance-derived quinones are possible the most important natural source of extracellular shuttles for Fe(III) reducers (Lovley et al., 1996a; Scott et al., 1998).

In many studies, anthraquinone disulfonate (AQDS) was used as a model humic substance (Aulenta et al., 2010; Bhushan et al., 2006; Collins and Picardal, 1999; Kwon and Finneran, 2006; Kwon and Finneran, 2008; Scherr et al., 2011) to study extracellular electron transfer in the reduction of halogenated and nitrous organic compounds.

5.3.3. Humic substances

Humic substances (HS) are a heterogeneous family of dark-coloured, biologically largely refractory compounds originating from the incomplete biodegradation of plant, animal and microbial debris. They account for up to 80% of soil and sedimentary organic carbon (Schnitzer, 1989). Humic substances consist of structurally diverse macromolecules, containing a variety of functional groups including hydroxy- and carboxygroups as well as aromatic and aliphatic structures that can be resolved using infrared and magnetic resonance spectroscopy, amongst others (Ehlers and Loibner, 2006; Nguyen et al., 1991).

On a more general scale, the classification of humic substances is commonly performed based on isolation procedures rather than on molecular characteristics, usually by their acid

(in)solubility, with brown to black *humic acids* (HA) defined as insoluble at a pH <2, with yellow-coloured *fulvic acids* (FA) completely soluble and *humins* insoluble both under all pH conditions (Scheffer and Schachtschabel, 2002).

Multiple interactions occur between the organic and inorganic soil matter fractions, including encapsulation (Baldock and Skjemstad, 2000), formation of adducts with organic contaminants (Karickhoff et al., 1979), chelation of metals (Arrieta and Grez, 1971; Gu et al., 2011; Lovley et al., 1996b), and as terminal and subterminal electron acceptors for abiotic and biotic reduction processes (Benz et al., 1998; Jiang and Kappler, 2008; McCarthy and Jimenez, 1985), amongst others. The multiple functions of humic substances have been proposed to be incorporated in different remediation technologies for contaminated sites, including bioremediation, reactive barriers and *in situ* immobilization (Perminova and Hatfield, 2005).

In terms of contributing to extracellular electron transfer enhancing contaminant transfer, humic substances play multiple roles.

Humic acids as chelators

Humic acids were found to increase the concentration of soluble Fe(III) by forming chelates (Lovley et al., 1996b). Chelated Fe(III) has a higher redox potential, rendering Fe(III) reduction more thermodynamically favourable (Thamdrup, 2000) and rendering the cation more accessible to microbes for reduction (Dobbin et al., 1995). It is, however, not clear whether extracellular chelated Fe(III) can be used by the cell directly via surface contact or absorption by the periplasm is required prior to reduction (Haas and Dichristina, 2002; Lovley et al., 1996b).

Terminal electron acceptors versus electron shuttles

Although humic substances are rather refractory to microbial breakdown, microorganisms interact with a variety of their functional groups, including the donation of electrons to reactive humic moieties. The ability to reduce humic substances and iron by bacteria and archaea appears to be closely linked (Benz et al., 1998; Lovley et al., 1998). The nature of electron transfer to humic or fulvic acids may be terminal or subterminal. In the latter case, microbially reduced humic matter abiotically transfers electrons to the Fe(III) surface, being re-oxidized themselves in the process (Lovley et al., 1996a; Lovley et al., 1996b) and thus function as natural, biogenic extracellular electron shuttles (see Figure 1). Reduced humic matter can also be „pirated“ by other microorganisms (Lovley et al., 1999), i.e. those that did not create them (route C in Figure 1) and be used as electron donors for microbially mediated reactions (route A).

Originally, the effect of humic substances in promoting biodegradation of organic pollutants was assumed to be dominated by chelation (Lovley et al., 1996b). Humic substances, however, are only weak chelators of iron. The role of humified organic matter as electron shuttle was first raised in 1989 (Tratnyek and Macalady, 1989) and is also referred to as the *Tratnyek-Macalady hypothesis* (Sposito, 2011). In fact, the function of fulvic acids of different origin can be explained as an initial contribution by electron shuttling, further enhanced by iron complexation in latter stages of contact (Royer et al., 2002).

Quality and origin of humic matter

Different humic substance fractions and origins have different effects on the rate and extent of extracellular electron shuttling. While there might be no effect at all by neither humic nor fulvic acids (O'Loughlin, 2008), humic acids, on a mass unit base, were found to stimulate iron reduction more strongly than fulvic acids, also in environmentally relevant concentrations, due to thermodynamic mechanisms (Wolf et al., 2009). On the other hand, aquatic humic substances appear to have a higher electron accepting capacity than those derived from terrestrial environments (Royer et al., 2002; Scott et al., 1998). On a general basis, the shuttling capacity of humic matter is determined mainly by with its aromaticity (Aeschbacher et al., 2010; Chen et al., 2003), where carbohydrate fractions were found to be less redox-active than polyphenolic fractions (Chen et al., 2003)

Soil organic matter aromaticity is also one of the main functional groups in determining sorption of petroleum hydrocarbons (Ehlers et al., 2010; Perminova et al., 1999). Surface-adsorbed and freely dissolved humic matter were found to exhibit similar shuttling activities (Wolf et al., 2009).

The disputed role of quinones in electron shuttling by humic substances

While the role of humic substances in soil and sediment electron shuttling is unchallenged, the nature of functional structures in humic or fulvic acids conveying the electron shuttling capacity to humic substances is not fully elucidated yet.

Profound influence of humic quinone moieties in conveying shuttling properties to humic substances has been attested by numerous studies (Aeschbacher et al., 2010; Jiang and Kappler, 2008; Kang and Choi, 2009; Perminova et al., 2005; Scott et al., 1998). Quinone moieties such as AQDS are often used as practical and standardized surrogates to model the effect of humic substances in extracellular electron transfer, while representing only the humics' redox properties, since AQDS can not chelate metals. The relevance of quinones, however, can be demonstrated by comparing shuttling efficacy of humic acid and pure AQDS. Two gram per litre of humic acids had a similar stimulating effect on the reduction of poorly crystalline ferric iron as approximately 40 mg / L AQDS (Lovley et al., 1996a).

In the search for other redox-active groups in humic substances, one model proposed three distinct sites participating in the redox activity of humic and fulvic acids (Ratasuk and Nanny, 2007), including two quinone structures with different electronegative properties plus one non-quinone structure. Evidence was supplied in that humic substances artificially depleted of their aromatic structures were not entirely depleted of their reducing capacity. In a different study, the humic substances' content of free radicals accounted for only a small fraction of the observed electron equivalents in shuttling between iron and iodine moieties (Struyk and Sposito, 2001).

These studies corroborate the possibility that also non-quinone structures participate in conveying redox-activity to natural organic matter. The nature of these compounds, however, is unknown, while there is general agreement points towards that quinone moieties are the *main*, however not only redox-active groups in humic substances.

Further understanding can be expected by the computational and procedural determination of quinone redox properties (Aeschbacher et al., 2010; Cape et al., 2006; Guo et al., 2012).

5.4. Concentrations of electron shuttles

Depending on the stability of the electron shuttling compound, it may undergo multiple cycles of electron take up and release. Thus, concentrations of redox-active moieties in the environment theoretically need to be substoichiometric in comparison to the amount of electron donating hydrocarbon and acceptor.

The oxidation state of a mediator itself also, at least theoretically, plays a role in its efficacy. Thus, an electron shuttle is effective at certain ratios of oxidized : reduced between 1:100 and 100:1 (Meckstroth et al., 1981), although in many studies it is attempted to completely reduce shuttles using harsh methods ahead of experimental use.

Practically, concentrations of pure quinones generally between 0.1 to 100 micromoles are commonly used for shuttling experiments. For some compounds, however, higher concentrations are inhibitory due to antimicrobial effects (Lown, 1983; Wolf et al., 2009). For some of the tested substances tested in a recent study (Wolf et al., 2009), a linear correlation between the normalized reaction rates and the logarithm of their concentrations was observed (humic and fulvic acid and AQDS), while others showed no concentration dependence (carminic acid and alizarin).

There has been criticism that concentrations of humic and fulvic acids commonly employed for experimental shuttling studies would far exceed those relevant under natural conditions, which are in the milligram per litre scale (Wolf et al., 2009), amounting to between 5 and 25 mg C/L (Curtis and Reinhard, 1994; Jiang and Kappler, 2008; O'Loughlin, 2008).

For humic and fulvic acids, enhancing effects on Fe(III) reduction attributed to electron shuttling were observed for concentrations of dissolved fulvic acids as low as 0.61 mg/L and of humic acids of 0.025 mg/L, i.e. at concentrations which do occur under natural conditions (Wolf et al., 2009) such as in pore waters of sediments rich in organic matter.

6. Thermodynamic considerations for extracellular electron shuttles

The ability to shuttle electrons between different organic and inorganic participants in the subsurface is largely dependent on the energies driving the extracellular electron transfer processes in simultaneous oxidation and reduction processes.

6.1. Minimum requirements for ATP production

The relation of electrochemical potentials of electron donor, shuttle and electron acceptor determine the amount of energy, in terms of ATP generation or free energy (ΔG^0), a microorganism may harvest from the electron transfer. A minimum energy yield of -20kJ/mol is required for ATP production (Schink, 1997). This determines the lower threshold amount of energy required to be transformed in the process of microbial electron transfer from donor to

shuttle to allow for ATP synthesis in the cells (Wolf et al., 2009). This first step depends on the potential of the electron donor or, more precisely, of its redox couple, and the shuttle.

In the subsequent, rate-limiting step of electron transport from the shuttle to the electron acceptor, the difference voltage between the mediator and the acceptor couple should be as negative as possible (Wolf et al., 2009). From a theoretical viewpoint, a mediator, assuming a concentration of 1M/L, can efficiently mediate between compounds with a E° range of +/- 118 mV of its own midpoint potential (Meckstroth et al., 1981).

These relations should be regarded in the selection of an electron shuttle. Several publications are dedicated to the description of the redox-properties of a wide range of mediators (Bird and Kuhn, 1981; Fultz and Durst, 1982; Meckstroth et al., 1981).

6.2. Effect of shuttle structure on its midpoint potential

The location and type of the mediator's functional groups influences its potential. As an example, quinones equipped with an increasing number electron donating groups (e.g. -CH₃) become less easily reduced. Redox-potentials (all E°) decrease from 280 mV for the unalkylated *para*-benzoquinone to 180 mV for 2,5-*p*-benzoquinone and to 5mV for the fully alkylated congener (2,3,5,6-tetramethyl-*p*-benzoquinone). On the other hand, electron-withdrawing sulphone groups facilitate reduction, as is reflected by an increasing potential along with increasing sulphonation for anthraquinone (AQ). Here, an increase of the compounds' potential from -225 mV for AQ-2-sulphonate by almost 40 mV to -184 mV for AQ-2,6-disulphonate is connected to the addition of one sulphone group (Fultz and Durst, 1982). The location of electron donating and accepting groups in respect to the keto-groups also determines the shuttles' stability in recurring redox-processes (Watanabe et al., 2009).

6.3. Practical relevance

In practice, however, the translation of thermodynamic principles to complex biogeochemical interactions can seldom be expected to be straightforward. In this case, small-scale inductive effects do not manifest themselves in decisively different shuttling efficacies, however a good correlation of shuttle electrochemical potential to Fe(II) production can be observed (O'Loughlin, 2008; Wolf et al., 2009). Mediation of ferrihydrite reduction with formate as electron donor was found to be most effective for a quinone range with potentials (all E°) of between -137 mV (2-hydroxynaphthoquinone) to -225 mV for AQS as mentioned above (Wolf et al., 2009). These potentials are closely related to those of flavins excreted by *Shewanella*, around -215 mV (Marsili et al., 2008; von Canstein et al., 2008). Thermodynamic issues of extracellular electron transfer are discussed elsewhere in more detail (O'Loughlin, 2008; Watanabe et al., 2009; Wolf et al., 2009).

7. *Geobacter* and *Shewanella*: Well-studied iron and manganese breathers

Dissimilatory iron (III) reducing microorganisms are phylogenetically diverse and can be found within the bacteria and archaeal domains. They are able to gain energy from the

reduction of Fe(III) on the cost of the oxidation of organic substances or molecular hydrogen. Behind their rather modest designation as iron-reducers the ability to participate in a great variety of biogeochemical processes is hiding. Mechanisms required for iron reduction also negotiate interactions with other organic and inorganic chemical species in the subsurface. Thus, the influence of these organisms and their metabolic products extends far beyond biogeochemical carbon and iron cycling, but also in dissolution of inorganic polluting and non polluting metals, transition metals, micro- and macronutrients and of organic pollutants. Phylogenetically distinct DMRB have different mechanisms to access insoluble electron acceptors. It appears that the ability to reduce these acceptors has evolved in evolution several times (Lovley et al., 2004).

Amongst the variety of involved microorganisms in the terrestrial and aquatic environment, the selection of microbial study objects is somewhat biased by their cultivability as pure cultures under laboratory conditions (Amann et al., 1995). The large diversity of DMRB shall not be underestimated by the selection of few cultivable organisms, which represent less than one percent of total bacterial cell counts in soil and sediments (Jones, 1977; Torsvik et al., 1990).

Beside numerous other organisms capable of dissimilatory metal and transition-metal reduction, including *Geothrix* (Coates et al., 1999) and reservoir-borne bacteria (Greene et al., 1997; Greene et al., 2009), the most well studied DMRB belong to *Geobacter* and *Shewanella* species.

7.1. Members of the *Geobacter* genus

Geobacter are a genus of δ -proteobacteria. They are gram-negative and chemoautotrophic strict anaerobes and were found to dominate iron reducing populations in hydrocarbon-polluted environments over other known Fe(III) reducers such as *Shewanella*, *Geothrix* and *Variovorax*. The latter appear to be unable to couple Fe(III) reduction to pollutant oxidation (Rooney-Varga et al., 1999; Snoeyenbos-West et al., 2000).

Thus, *Geobacter* species appear to be the primary agents in Fe(III) and Mn(IV) reduction coupled to the oxidation of organic compounds in anoxic terrestrial environments.

The first description of the *Geobacter* species was published by D. Lovley in 1987 (Lovley et al., 1987) after isolating them from a Potomac river sediment. The species *G. metallireducens* and *sulfurreducens* were described in 1993 (Lovley et al., 1993) and 1994 (Caccavo et al., 1994), respectively. *Geobacter sulfurreducens* can not, in contrast to *G. metallireducens*, reduce Mn(IV), and can not use alcohols or aromatic compounds as electron donors.

Geobacter can use AQDS as extracellular electron shuttle to increase electron transfer to poorly soluble electron acceptors, and can also precipitate Uranium (VI) (Cologgi et al., 2011). *G. metallireducens* is chemotactic towards the soluble reduction products (Fe(II) and Mn(II)) of iron and manganese minerals (Childers et al., 2002)).

Nano-wiring and charging

Geobacter form pilus-like appendages, so-called bacterial nanowires (Reguera et al., 2005), when grown on insoluble Fe(III) and Mn(IV) oxides, to transfer electron to distant and

insoluble electron acceptors and electrodes. These pili are electrically conductive. This is independent from c-type cytochromes, which are, in contrast, responsible for terminal connections with the electron accepting surfaces. They are also responsible for microbial 'capacitation', which allows *Geobacter* to extracellularly store electrical charge in times of scarceness of electron acceptors (Lovley et al., 2011).

7.2. Members of the *Shewanella* genus

Members of *Shewanella*, originally isolated from dairy products and introduced as *Achromobacter putrefaciens* by Derby & Hammer in 1931, are gram-negative and belong to the class of γ -proteobacteria (Venkateswaran et al., 1999).

They are facultative anaerobic bacteria equipped with a high respiratory versatility, amongst others capable of using oxygen, nitrate, volatile fatty acids and Fe(III), Mn(IV), As(V) and U(VI) as electron sinks (Bencheikh-Latmani et al., 2005; Cruz-García et al., 2007; Lim et al., 2008; Lovley et al., 2004; Myers and Nealson, 1988; Venkateswaran et al., 1999; von Canstein et al., 2008), and supplemented by evidence for Cr(VI) respiration (Bencheikh-Latmani et al., 2005). Extracellular respiration by *Shewanella oneidensis* has also been noted to comprise graphene oxide (Jiao et al., 2011).

Flavins, electron shuttles and nano-wires

The versatility in using electroactive surfaces to donate electrons is reflected in the use of anodes (Logan, 2009), and also the ability to produce electrically conductive nanowires, similar to *Geobacter*, themselves (Gorby et al., 2006).

Shewanella are known to excrete quinones (Newman and Kolter, 2000) that carry electrons from the cell surface to the electron acceptor that is located at a distance from the cell. Excreted flavins chelate Fe(III), rendering it more accessible to the cells (von Canstein et al., 2008), thus promoting Fe(III) reduction at a distance.

8. Anaerobic hydrocarbon oxidation coupled to Fe(III) and Mn(IV) reduction

8.1. Naturally mediated extracellular electron transfer

A variety of easily degradable electron donors can be used for the anaerobic reduction of Fe(III) and Mn(IV), including short chain organic acids, alcohols or sugars (Lovley et al., 1996a). Iron- and manganese reducers are also known to dwell in petroleum reservoirs (Greene et al., 1997; Greene et al., 2009). This suggests that the metabolic pathways for the reduction of poorly soluble electron acceptors are resistant to exposure to potential inhibitors of microbial activity; this is of potential concern since low molecular weight hydrocarbons are known to act as disruptors of biological membranes.

In fact, poorly soluble electron acceptors can also be used for the anaerobic oxidation of less readily degradable organic compounds, including petroleum hydrocarbons. Several studies documented the occurrence of microbial hydrocarbon degradation coupled to the reduction

of Fe(III) and Mn(IV) as sole electron acceptors with aquifer and sedimentary materials. Biodegradation of petroleum hydrocarbons under iron- and manganese reducing conditions has been noted primarily for low molecular weight aromatic compounds. This includes benzene (Kunapuli et al., 2008), toluene (Coates et al., 1999; Edwards and Grbic-Galic, 1994; Langenhoff et al., 1997; Lee et al., 2012), ethylbenzene (Siegert et al., 2011) and *o*-xylene (Edwards and Grbic-Galic, 1994) as well as phenol (Lovley et al., 1989), naphthalene and also liquid alkanes (Siegert et al., 2011).

It can be assumed that natural mechanisms, including chemotaxis, expression of electrically conductive pilus-like assemblages, extracellular chelators and molecular electron shuttles contribute to the occurrence of these processes.

8.2. Role of anthropogenic extracellular electron shuttles

The enhancement of naturally occurring iron and manganese reduction coupled to hydrocarbon oxidation is a promising strategy for petroleum hydrocarbon bioremediation (Lovley, 2011). There is, to our knowledge, only a limited amount of studies where such a strategy was followed. Humic substances and quinones were successfully applied to stimulate the degradation of a variety of aromatic compounds by Cervantes and co-authors, including the increase of toluene oxidation coupled to manganese respiration (Cervantes et al., 2001). Degradation of the fuel additive methyl tert-butyl ether (MTBE) under iron-reducing conditions was stimulated using humic substances (Finneran and Lovley, 2001) as well as quinones for anaerobic toluene oxidation (Evans, 2000). Iron chelation with humic substances increased anaerobic benzene oxidation (Lovley et al., 1996b), however a contribution by electron shuttling was not discerned.

In the light of the large variety of potential applications of these processes for petroleum hydrocarbon bioremediation, more research tackling upon a broader variety of priority pollutants will be performed.

9. Outlook: Potential exploitation of enhanced extracellular electron transfer for *in situ* petroleum hydrocarbon bioremediation

The application of electron shuttles as amendments in a variety of biotechnological processes has gained increased attention in the past 20 years. Extracellular electron shuttles have, for example, been shown to enhance the *reductive* degradation of a large variety of organic contaminants, including azo- and nitro- compounds such as dyes and explosives, of chlorinated hydrocarbons, and to induce the precipitation of metals such as uranium and chromium (reviewed by Field et al., 2000; Hernandez and Newman, 2001; Van der Zee and Cervantes, 2009; Watanabe et al., 2009). Recent developments include the application of novel electron shuttles and the continued investigation to predicting the redox-properties the most complex natural redox mediators, humic substances (Aeschbacher et al., 2010; Kumagai et al., 2012). The exploitation of extracellular electron transfer in anaerobic petroleum hydrocarbon bioremediation is, in contrast, explored to a lesser extent.

The limited availability of electron acceptors constraining oxidative hydrocarbon biodegradation *in situ* can be circumvented by a variety of measures, including *biosparging*, the introduction of oxygen releasing compounds or the use of naturally occurring, well soluble alternative electron acceptors such as nitrate or sulphate.

Moreover, the abundance of solid-phase, however microbially accessible electron acceptors in the subsurface offers the potential for a significant decrease in the amount of reagents to be introduced into an aquifer, provided solid-phase electron acceptors can be efficiently involved in oxidative contaminant biodegradation.

The enhancement of microbially mediated mechanisms to enhance the accessibility of Fe(III) and Mn(IV) can be applied in a variety of engineered approaches for the biodegradation of aquifers contaminated with fuel hydrocarbons, tar oils and other contaminants susceptible to oxidative biodegradation.

The list of possible approaches includes the following:

- i. Injection of electron shuttles carefully designed by type and concentration, into contaminated aquifers, to increase the available pool of native or resident electron acceptors such as Fe(III) and Mn(IV) for microbial hydrocarbon oxidation.
- ii. Addition of electron shuttles to increase the electron accepting function of pre-existing engineered subsurface structures, such as permeable reactive barriers, gates and reactive zones.
- iii. Use of natural or engineered materials slowly releasing electron shuttles by leaching, such as humic substances.
- iv. Engineered approaches to increase the density and activity of microbial communities with the intrinsic capability to reduce poorly soluble native sources of electron acceptors, including but not limited to *Geobacter* species.

These considerations shall mediate research and development of an ever broader variety of potential opportunities to enhance naturally occurring oxidative contaminant degradation under suboxic conditions. The range of available tools for dealing with hydrocarbon contaminated environments may be extended by an additional instrument, the utilization of extracellular electron transport mechanisms designed by nature.

Abbreviations

ATP	adenosine-5'-triphosphate
AQDS	anthraquinone disulfonate
Cr	chrome
DMRB	dissimilatory metal reducing bacteria
ES	electron shuttle
FA	fulvic acids
Fe	iron
FMN	flavin mononucleotide

HA	humic acids
HS	humic substances
Mn	manganese
RF	riboflavin
TEA	terminal electron acceptor
ZVI	zero-valent iron

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