Microbial dissimilatory iron(III) reduction: Studies on the mechanism and on processes of environmental relevance

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Microbial dissimilatory iron(III) reduction: Studies on the mechanism and on processes of environmental relevance

Abstract: Many microbes are able to respire aerobically oxygen or anaerobically other electron acceptors for example sulphate, nitrate, manganese(IV) or Fe(III). As iron minerals are widespread in nature, dissimilatory iron(III) reduction by different microorganisms is a very important process of anaerobic respiration.

The general goal of this work was to improve the knowledge of processes, in which iron-reducing microbes are said to play an important role. For this purpose, in one part the focus was made on anaerobic contaminant degradation and in the other part on studies on the mechanism of microbial iron(III) reduction. Both parts were investigated in growth and cell suspension experiments with different microorganisms.

At former industrial sites, monoaromatic hydrocarbons, such as benzene, toluene, ethylbenzene, and xylene (BTEX), are widespread contaminants, which cause different problems due to their high solubility in water and strong toxicity. At such sites, where usually anoxic conditions prevail, the anaerobic degradation of these compounds is a very important process. In this study, the anaerobic degradation of BTEX compounds by dissimilatory iron-reducing microorganisms was examined. In order to isolate new bacterial strains, enrichment cultures with the different BTEX substrates added as sole carbon and energy source were prepared. Successful enrichment cultures were obtained for all BTEX substrates both in the presence and absence of AQDS (9,10-anthraquinone-2,6-disulfonic acid). The electron balances showed a complete anaerobic oxidation of the aromatic compounds to CO₂. This is the first report on the anaerobic degradation of o-xylene and ethylbenzene in sediment free iron-reducing enrichment cultures. Also, the successful isolation of a pure strain, which is able to degrade toluene, and another pure strain, which is able to degrade toluene or o-xylene, was reported in this work. In a case study, the oxidation capacity for different iron-reducing BTEX-degrading cultures with water and soil samples from a contaminated site was determined.

Cyanide or cyanide-metal complexes are frequent contaminants of soil or aquifers at industrial sites. Toxic cyanides can be released from such sites by outgasing or transport with the groundwater. Cyanides form very stable complexes with iron, such as ferrocyanide [Fe(CN)₆]₄⁻ and ferricyanide [Fe(CN)₆]₃⁻. Together with ferri iron, ferrocyanide forms an insoluble blue pigment, the so called Prussian Blue (Fe₄[Fe(CN)₆]₃), which may occur in the subsurface of contaminated sites as a blue coating on mineral surfaces. It was shown in this study that the insoluble and colloidal iron(III)-cyanide-complex Prussian Blue is readily reduced and utilized as electron acceptor by the dissimilatory iron-reducing organisms Geobacter metallireducens and Shewanella alga strain BrY. The microbial reduction of the dark blue pigment Prussian Blue lead to the formation of a completely colourless solid mineral, presumably Prussian White (Fe₄[Fe(CN)₆]₃), which could be reoxidized through exposure to air, regaining the dark blue colour. In addition, the microorganisms were able to grow with Prussian Blue, using it as the sole electron acceptor. G. metallireducens could also reduce Prussian Blue coatings on sand, which was sampled from a cyanide-contaminated site.

As iron(III) minerals are only poorly soluble at neutral pH, it is still under discussion how dissimilatory iron(III)-reducing microorganisms are able to use the different iron(III) species. In the last years, several mechanisms of electron transfer from microbial cells to iron mineral surfaces have been discussed. In this work, an additional completely new mechanism is proposed and investigated: The transfer of electrons from bacterial cells to iron minerals is performed via colloidal iron(III) particles, which are naturally abundant in many aquifers. It could be shown that colloidal iron(III) was
an effective electron acceptor in cell suspension experiments with washed *Geobacter metallireducens* cells. The reduction of the colloidal ferrihydrite particles was much faster than the reduction of bulk ferrihydrite mineral. When *Geobacter* cells were grown on amorphous ferrihydrite, the addition of colloidal Prussian Blue mediated an increase of the electron transfer rate to the solid minerals. The presented data strongly indicate that colloids might play a significant role in microbial iron reduction processes.

The data presented for the degradation of the different contaminants might help to improve or develop further strategies for natural attenuation and bioremediation of contaminated sites. Also the finding of the mediated iron reduction by iron colloids might lead to a completely different look at iron reduction processes and thus might also be used in newly developed remediation techniques. However, still further experiments are needed under conditions, which are closer to the *in situ* situation of different environments to elucidate the real contribution of iron(III) reduction to the different processes mentioned compared to other forms of anaerobic respiration.
Mikrobielle dissimilatorische Eisen(III)-Reduktion: Untersuchungen des Mechanismus und verschiedener umweltrelevanter Prozesse


Aufgrund der Tatsache, dass Eisen(III)-Minerale bei neutralem pH-Wert nur sehr schlecht löslich sind, ist es immer noch Gegenstand lebhafter Diskussionen, wie Eisen(III)-reduzierende Mikroorganismen

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Chapter 1

1 General Introduction

1.1 General properties of iron

Iron is the most abundant element on our planet. In the earth crust it is the second abundant metal after aluminium and the fourth abundant element after oxygen, silicon and aluminium. Iron belongs to the transition metals, which have d-orbitals that are not completely filled with electrons. Therefore iron occurs in different oxidation numbers (0, +2, +3, sometimes +4 or +6). In nature, it is found in many minerals mainly in the oxidation states +2 or +3 (Römpp, 1990). The fact that iron(III) minerals are very colourful, mostly reddish, is mainly due to charge-transfer-absorption of light energy, during which electrons are transferred from Fe$^{2+}$ to Fe$^{3+}$ or from O$^{2-}$ to Fe$^{3+}$. In minerals, that contain transition metals, the energetic levels of the five d-orbitals are splitted into two groups: Three orbitals with a lower (t$_{2g}$) and two with a higher energetic level (e$_{g}$). Generally, complexes with a total electron spin that is as high as possible are preferred (Hundt’s Rule). In this so-called “high-spin” (outer-orbital) complexes, e. g. [FeF$_6$]$^{3-}$, iron has the d$^5$-configuration and all d-orbitals are occupied with one single electron with parallel spin. In this case, only spin forbidden transitions from t$_{2g}$ to e$_{g}$ are possible. In contrast, in the so-called “low-spin” (outer-orbital) complexes, e. g. [Fe(CN)$_6$]$^{3-}$, the e$_{g}$-orbitals are not occupied, because the energetic barrier for the electrons is too high to reach the levels of the upper orbitals. The low-spin complexes have four paired electrons and one unpaired electron in the t$_{2g}$-orbitals. In this case, if enough energy is added to the system, spin allowed transitions to the e$_{g}$-orbitals might be possible (Römpp, 1990).

Iron is also said to have played an important role in earth’s early chemistry of life in an oxygen free atmosphere. It is even proposed that the most ancient form of all living cells might be descendent of iron-sulphur metalloclusters, which could have formed closed compartments that were necessary for the development of different metabolic processes. Thus, iron might have played a major role in the origin of life in cellular structures (Russell and Hall, 1997; Crichton and Pierre, 2001; Martin and Russell, 2003; Rees and Howard, 2003).

1.2 Iron cycling and geochemical impact

The cycling of iron in the environment is to a large extent controlled by dissimilatory iron(III)-reducing microorganisms (Burdige, 1993; Thamdrup et al., 1994; Krebs et al., 1997). These organisms are able to gain energy for growth and other metabolic processes from the oxidation of different substrates and the reduction of ferric iron to ferrous iron. Such microorganisms have a great geological impact in terms of biomineralization, as they reduce and solubilize iron(III) present in many minerals in nature, e. g., ferrirhydrite (Fe$_5$HOH$_8$ · 4 H$_2$O), goethite (α-FeOOH), hematite (α-Fe$_2$O$_3$) or clay minerals (Roden and Zachara,
1 General Introduction

1996; Zachara et al., 1998; Kostka et al., 1999; Zachara et al., 2002; Dong et al., 2003). Another important aspect of iron reduction in marine sediments is the contribution to the cycling of iron, carbon, sulphur, phosphorus, and trace elements. Thus, this process has an important influence on marine ecosystems, regarding for example the growth of phytoplankton, which strongly influences global food chains (Burdige, 1993; Thamdrup et al., 1994; Morel and Price, 2003).

Other minerals are precipitated from dissolved ferrous iron, which results from microbial iron reduction. The most prominent are vivianite (Fe₃(PO₄)₂ · 8 H₂O), siderite (FeCO₃) and also mixed-valent Fe(II)/Fe(III) minerals like green rusts (Fe(II)/Fe(III) (hydr)oxides) or magnetite (Fe₃O₄), which is the most important ferromagnetic mineral found in nature (Fredrickson et al., 1998; Parmar et al., 2001; Ona-Nguema et al., 2002; Hansel et al., 2003).

The role of iron-reducing microbes in the formation of banded-iron formations, which are very important iron ores, was also discussed (Croal et al., 2004). Also the adsorption of ferrous iron onto surfaces of ferric minerals might also lead to transformations into other minerals, as was shown by (Jeon et al., 2003).

1.3 Role of ferrous iron

The production of ferrous iron from the reduction of ferric iron may have different effects. For one part, high concentrations of dissolved iron in pore waters up to millimolar levels may cause different problems. For example, ferrous iron is inhibitory for microbial growth in high concentrations. Another important economical problem is due to the reoxidation of ferrous iron, when anoxic groundwater gets into contact with air, which may cause clogging of tubes through precipitation of ferric iron (Thamdrup et al., 1994). Waters, which contain high concentrations of dissolved iron, are also not appropriate for drinking water purposes and have to be pre-treated before use.

For the other part, ferrous iron is a very reactive species, especially when it is adsorbed to mineral surfaces. It plays a major role in different chemical processes of environmental relevance, for example dehalogenation or nitroaryl reduction, and different contaminants are transformed after contact with iron(II). This transformation could lead to contaminant degradation or in some cases species might be formed, which are much more hazardous and poisonous for the environment, e. g. vinyl chloride or trichloromethane (Pecher et al., 2002; Hofstetter et al., 2003; Elsner et al., 2004b; Elsner et al., 2004a; Klupinski et al., 2004).

Apart from the reduction of ferric iron, the opposite process, which is the anaerobic oxidation of ferrous iron to ferric iron by phototrophic iron-oxidizing bacteria (Widdel et al., 1993; Straub et al., 1996; Ratering and Schnell, 2001), is also a very important process regarding the cycling of iron in nature (Thamdrup et al., 1994; Gadd, 2000; Benner et al., 2002; Weiss et al., 2003). Also the formation of different minerals might be due to microbial oxidation of iron(II). For example, the appearance of large banded-iron formations, which have been formed already in the Proterozoic but mainly in the Precambrian, at times, when the atmosphere was still almost free of oxygen, are probably of biogenic origin and have been formed after oxidation of ferrous iron (Brown et al., 1995; Croal et al., 2004). Others found that if the oxidation of ferrous iron was coupled to denitrification, biogenic magnetite was formed (Chaudhuri et al., 2001).
1.4 Dissimilatory iron(III) reduction

Microbial iron reduction is known since the 1920s, but only after the isolation of the first Geobacter strain, Geobacter metallireducens in 1987, this metabolic group of organisms was studied in more detail (Lovley et al., 1987; Lovley and Phillips, 1988). Dissimilatory iron-reducing microorganisms where found throughout the phylogenetically distinct kingdoms Archaea and Bacteria (Nealson and Little, 1997; Lovley et al., 2004) and they are widespread in many different marine or freshwater environments (Coates et al., 1996; Venkateswaran et al., 1999). Most strains, that are able to gain energy from the reduction of ferric iron minerals, are heterotrophs and so far only hyperthermophilic strains were isolated, that are able to grow autotrophically with hydrogen as electron donor without addition of an organic carbon source (Lovley et al., 2000; Kashefi et al., 2002). After the discovery of autotrophic thermophilic iron-reducing archaees, which use CO$_2$ to build up biomass, the process of autotrophic iron reduction was discussed as one of the potentially most ancient forms of microbial metabolism (Vargas et al., 1998).

Up to the present, no mesophilic autotrophic iron reducer could be isolated, which grows with hydrogen gas as electron donor and carbon dioxide as sole carbon and energy source.

1.5 Iron reduction and contaminant degradation

At many contaminated sites, anoxic conditions prevail and the anaerobic degradation of pollutants is the most important process for the removal of contaminants from groundwater aquifers or soils (Christensen et al., 2000; Cozzarelli et al., 2001). Many pollutants are fuel-derived hydrocarbons, such as alkanes or aromatic hydrocarbons, or halogenated aliphatic hydrocarbons, such as trichlorethylene (TCE) or pentachlorethylene (PCE) (Eganhouse et al., 1996; Coates et al., 1997; Bjerg et al., 1999; Zheng et al., 2001). Especially the monoaromatic hydrocarbons, like benzene, toluene, ethylbenzene, xylene (BTEX), which are major contaminants at former industrial sites, were thought to be non-degradable under anoxic conditions several years ago (Dua et al., 2002; Chakraborty and Coates, 2004). Nowadays, several nitrate- or sulfate-reducing microorganisms are known and available as pure cultures that are able to degrade the different BTEX compounds (Kazumi et al., 1997; Harwood et al., 1998; Spormann and Widdel, 2000; Widdel and Rabus, 2001; Gibson and Harwood, 2002; Kniemeyer et al., 2003). However, for dissimilatory iron-reducing microorganisms there are still only two pure Geobacter strains available, Geobacter metallireducens and Geobacter grbiciae, which are able to degrade toluene (Lovley et al., 1993; Coates et al., 2001) and no pure strains have been isolated for benzene, ethylbenzene, or xylene degradation. There are only reports on microbial enrichment cultures, which where able to degrade benzene under iron-reducing conditions (Lovley et al., 1989; Kazumi et al., 1997; Lovley, 2000). This is a surprising fact, because in the process of anaerobic BTEX degradation, iron reduction is thought to play a major role (Lovley et al., 1989; Lovley, 1993; Anderson et al., 1998; Anderson and Lovley, 1999; Kao and Wang, 2000; Lovley, 2000; Zheng et al., 2001).

Also the in situ degradation of BTEX contaminants could be shown through isotope fractionation of the compounds during degradation (Richnow et al., 2003a; Richnow et al., 2003b; Griebler et al., 2004; Heidrich et al., 2004). Furthermore, the presence of iron-reducing communities at hydrocarbon-
contaminated sites was found several times (Rooney-Varga et al., 1999; Lovley and Anderson, 2000; Snoeyenbos-West et al., 2000). Due to the lack of BTEX-degrading strains, it is necessary to isolate new contaminant-oxidizing cultures to be able to study the mechanism of anaerobic contaminant degradation processes in more detail. Also for natural attenuation of contaminated sites, this new strains might be helpful tools to improve and speed up the remediation of former industrial sites.

Dissimilatory iron-reducing microorganisms are also able to reduce other metals or metalloids and thus alter the solubility of the compounds in water, which may lead either to mobilization or to removal of contaminants from the groundwater by precipitation. Examples for these processes are microbial reduction of U(VI) to U(IV), Tc(VII) to Tc(V) or Tc(IV), As(V) to As(III), Cr(VI) to Cr(III), and Se(VI) or Se(IV) to Se(0) (Lovley, 1993; Lloyd and Macaskie, 1996; White et al., 1997; Stolz and Oremland, 1999; Kashefi and Lovley, 2000; Wielinga et al., 2001; Oremland and Stolz, 2005).

Other forms of iron present at many industrial sites are iron-cyanide complexes (Young and Theis, 1991; Shifrin et al., 1996). Iron forms very stable complexes with cyanides, which may either be soluble, such as ferrocyanide [Fe(CN)₆]⁴⁻ and ferricyanide [Fe(CN)₆]³⁻ (Mansfeldt and Dohrmann, 2001), or present as solids. Ferrocyanide together with iron(III) forms an insoluble blue pigment, the so called Prussian Blue (Fe₄[Fe(CN)₆]₃)(s), which can be found at contaminated sites in high concentrations as blue coatings on mineral surfaces (Ghosh et al., 1999; Mansfeldt and Dohrmann, 2001; Ghosh et al., 2004). Although free cyanide, ferricyanide or ferrocyanide are readily degraded, the reduction of the insoluble pigment Prussian Blue by dissimilatory iron-reducing organisms was not reported so far (Boucabeille et al., 1994; Ebbs, 2004). The equilibrium constant for the reductive dissolution of Prussian Blue is very low and was determined as $K^0 = -85.4$ (Meeussen et al., 1992). The solubility of Prussian Blue is strongly pH and pE-dependant and increases with increasing pH (Meeussen et al., 1995). Thus, microbial reduction of Prussian Blue to Prussian White (Fe₂[Fe(CN)₆]) might also affect the mobility of cyanide contaminants and in that way the toxicity, because on one hand, Prussian White could also precipitate and form white coatings on other minerals under anoxic reducing conditions (see equation 1.1). On the other hand, during the microbial reduction of Prussian Blue to Prussian White, the soluble cyanide complex ferrocyanide ([Fe(CN)₆]⁴⁻ might be formed, which would lead to a further mobilization of cyanide from the weakly soluble Prussian Blue mineral (see equation 1.1).

**Equation 1.1**

$Fe_4[Fe(CN)_6]_3 + 4e^- \rightarrow 2Fe_2[Fe(CN)_6] + [Fe(CN)]^{4-}$

Different iron cyanide complexes adsorb pH-dependently onto mineral surfaces, for example onto goethite or ferrihydrite (Rennert and Mansfeldt, 2001a, b). Changes in the pH value of the soil therefore influence the mobility of such compounds and thus the bioavailability and toxicity of cyanide contaminants in the environment. The reduction of insoluble Prussian Blue coatings in the subsurface by iron-reducing microbes, which so far has not been described, might have a great influence on the
degradation and transport of cyanide contaminants in the subsurface, as on one hand the reduction leads to the production of the soluble compound ferrocyanide, which can be transported over long distances and is easily used by microorganisms, and on the other hand to Prussian White, which is an insoluble mineral that might precipitate and form white coatings on other minerals.

1.6 Mechanisms of dissimilatory iron(III) reduction

Ferric iron, in contrast to other soluble electron acceptors such as nitrate, sulfate, and oxygen, is virtually insoluble in pure water at neutral pH-values predominating at most natural environments (see figure 1.1) (Thamdrup, 2000; Pierre et al., 2002).

![Figure 1.1: Effect of the pH value on the solubility of amorphous Fe(OOH)(s) (from (Stumm and Morgan, 1996)). The different hydrolysis products of amorphous Fe(OOH) greatly enhance the solubility. However, the overall Fe(III) concentration Fe(III)_{tot} is still only about 10^{-10} M at neutral pH.](image)

Thus, iron transport into the microbial cells via diffusion would be inefficient and the electrons rather have to be transferred to an electron acceptor outside the cell. The mechanism of extracellular electron transfer from the cell to the insoluble iron mineral is still under discussion and several suggestions have been made (see (Hernandez and Newman, 2001; Nevin and Lovley, 2002a; Lovley et al., 2004) for reviews).

The most accepted ones are the following (see figure 1.2): The transfer of electrons from the cell to the iron minerals is performed through direct contact between cell and mineral surface (Nevin and Lovley, 2000) (figure 1.2 A). This mechanism is favoured for the different Geobacter strains (Straub and Schink, 2003; Lovley et al., 2004).

However, this mechanism has different disadvantages, because the cells have to be in close proximity to the mineral surface and if the iron minerals are reduced, the bacteria have to move to so far unreduced surfaces. A special strategy to overcome this problem could be the expression of certain genes, which induce the synthesis of structures like flagella or pili in order to improve the motility of the cells. This strategy could be shown for Geobacter metallireducens cells, which expressed flagella and accessed insoluble iron minerals via chemotaxis (Childers et al., 2002). So far, this is the only example for such behaviour and it is also very energy consuming for cells, which usually live in oligotrophic environments, to synthesize such structures.

A different mechanism favours the dissolution of bulk iron minerals with soluble iron-chelating substances like NTA (nitritetriacetic acid) or EDTA (ethylendiaminetetraacetic acid) (Lovley et al., 1994; Lovley and Woodward, 1996) (figure 1.2. B). Other chelators, which had been investigated, had different efficiencies for the enhancement of
iron(III) reduction (Dobbin et al., 1995; Dobbin et al., 1996). Through the influence of chelators, the bacteria are now for one part able to access the dissolved iron(III) more easily and for the other part the chelated iron is transported to the cells by diffusion. Another mechanism is the transfer of electrons from the cell to the iron mineral through redox-active electron-shuttling substances, like quinones, humic acids or natural organic matter (Lovley et al., 1996; Lovley et al., 1998; Royer et al., 2002b) (figure 1.2 C). The electron shuttles are reduced at the bacterial surface where they take over electrons. After diffusion to the mineral surface, they chemically reduce the iron(III) phase through the release of electrons. The reduced shuttles diffuse back to the cell surface and another redox cycle might start. The addition of humic acids or quinone-like substances like AQDS (9,10-anthraquinone-2,6-disulfonic acid) strongly enhanced iron reduction by different microbes (Lovley et al., 1996; Scott et al., 1998; Zachara et al., 1998; Royer et al., 2002a; Kappler et al., 2004). Humic substances are ubiquitous in nature and therefore could be an efficient tool for the microbes to transfer the electrons to bulk ferric minerals. Also redox-active antibiotics were shown to promote the bacterial reduction of crystalline iron(III) oxides (Hernandez et al., 2004). Other authors reported the stimulation of the reductive dissolution of iron(III) oxides after addition of cysteine (Doong and Schink, 2002). The electron-shuttling mechanism is favoured for different Shewanella strains (Lovley et al., 2004). Another mechanism is the production of electron-shuttling substances by the microorganisms themselves. Such substances would be synthesized inside the cells and excreted into the surrounding medium (figure 1.2. D). Indications for such substances were found in cultures of Geothrix fermentans cells (Nevin and Lovley, 2002b). Other authors found indications in agar shakes, which were set up with ferrihydrite and inoculated with different iron-reducing enrichment cultures. During growth of the bacteria in the shakes, clear sphere-like zones appeared in the reddish ferrihydrite agar, which could be due to the excretion of shuttling substances from the microbial cells (Straub and Schink, 2003). In different batch experiments could be shown that increasing concentrations of dissolved iron(II) in the medium have an inhibitory effect on microbial growth. The removal of the Fe(II) produced by exchange of the medium or by complexion of the Fe(II), which is released during iron(III) reduction, could be another mechanism to promote microbial growth on different iron(III) minerals (Roden and Urrutia, 1999, 2002; Royer et al., 2002a). A common disadvantage of all the systems including shuttling or chelating substances is the potential risk of loosing the mediators through diffusion, especially in groundwater aquifers. The loss of those substances would be a great waste of energy for the cells in a very nutrient poor environment. As all mechanisms presented are more or less hypothetical and, as was discussed in more detail, all mechanisms seem to have certain advantages and disadvantages, the iron-reducing microbes could also use completely different mechanisms to access the insoluble iron minerals. To find out different completely new mechanisms, which might be less energy intensive than the ones presented, might be a valuable field of investigation in order to learn more about microbial energetic and also evolutionary strategies, which organisms might develop to overcome certain problems and restrictions they are facing in the different environments.
Figure 1.2: Different proposed mechanisms of microbial dissimilatory iron(III) reduction. A). The reduction of iron(III) minerals via direct contact between microbial cell and mineral surface. B). Iron(III) reduction after solubilization of ferric iron by iron-chelating substances. C). Reduction of iron(III) via shuttling of electrons from the microbial cell surface to ferric minerals with soluble humic substances. D). Iron(III) reduction via a microbially produced soluble electron shuttle, which is excreted from the cells.

1.7 Objectives of this work and methodology

The purpose of this work was to investigate the following questions. First, how important is the metabolic microbial group of dissimilatory iron-reducing microorganisms for processes of environmental relevance, e.g. contaminant degradation at former industrial sites. Second, how is the process of dissimilatory iron reduction done by microorganisms, which have to face different difficulties using insoluble iron(III) minerals as sole electron acceptor.

Therefore, in my thesis I focused on three parts, which dealt with different aspects of iron(III) reduction. The purpose of the first part was to isolate new dissimilatory iron-reducing cultures that are able to degrade aromatic hydrocarbons and thus might be significant in terms of natural attenuation and bioremediation processes. The focus is made on monoaromatic hydrocarbons, like benzene, toluene, ethylbenzene, and xylene (BTEX), because these are major pollutants at many contaminated sites. Enrichment cultures with sludge from
contaminated sites together with an easily bioavailable iron(III) mineral have to be performed for different contaminants and hopefully new cultures might be enriched from this setup. Following the enrichment procedure, the goal is to isolate new BTEX-degrading cultures and to describe the new strains in detail.

The second part deals with another important group of contaminants, the iron-cyanide complexes. Those are also widespread at many former gasworks sites and toxic cyanides, such as HCN, might be volatilised from such sites by outgasing. The aim of the thesis is to investigate, if the dissimilatory iron-reducing microbes are able to use one of the most abundant of these cyanide complexes, Prussian Blue, which is an insoluble blue mineral. Therefore, iron reduction and growth experiments with known and well-investigated iron-reducing bacterial strains have to be made, using Prussian Blue as the sole electron acceptor. As the iron reducers are highly abundant in nature, the use of cyanide minerals by such organisms might have great influence on the distribution and degradation of cyanide pollutants in the environment.

The last part deals with the mechanism of dissimilatory iron reduction. This process of anaerobic respiration is quite different compared to others, like sulphate or nitrate reduction, because iron(III) minerals are virtually insoluble at neutral pH values predominating at most environments. A completely new mechanism for microbial iron reduction was hypothesized, which is investigated in this work: Iron-reducing bacteria, in this case Geobacter metallireducens, might use colloidal iron(III) particles, which work as a mediator between bacterial cell and mineral surface, in order to solve the problems that result from the low solubility of the electron acceptor iron(III). A mediator, or electron shuttle, takes over electrons from the bacteria, gets reduced, and diffuses to the mineral where the electrons are released again. After that, the shuttle is ready for another reduction-oxidation cycle. Examples for a similar shuttle mechanism have been reported for the use of humic acids by different iron-reducing bacteria. As iron(III) colloids are particulate, the new hypothesis favours a completely new way of electron-shuttling via colloidal particles. In order to deal with the new hypothesis, first the colloidal iron particles need to be prepared. Second, conditions have to be found, under which the iron(III) colloids remain in stable suspension during the cultivation of the bacteria. This is the most critical point, because colloids have very special properties, especially at cultivation conditions of high ionic strength or elevated temperatures common in the cultivation of bacteria. In following cell suspension experiments with washed Geobacter metallireducens, a well described iron reducer, the potential electron-shuttling capacity of iron(III) colloids has to be elucidated. Therefore the reduction of iron in the presence and absence of colloids has to be compared, which theoretically should be better in the presence of colloids due to the transfer of electrons by these electron-shuttling particles. The improved reduction after addition of iron(III) colloids would give strong indication that the postulated hypothesis might in fact work out.
1.8 References


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2.1 Anaerobic degradation of benzene, toluene, ethylbenzene, and o-xylene in sediment-free iron-reducing enrichment cultures

2.1.1 Introduction

The degradation of aromatic hydrocarbons in contaminated aquifers is of general interest because some of the compounds are toxic or even carcinogenic. Due to anoxic conditions typically found in organic contaminant plumes (Christensen et al., 2001), anaerobic degradation of aromatic compounds plays a major role for bioremediation processes and increased knowledge on the anaerobic oxidation of aromatic compounds has been acquired recently (Harwood et al., 1998; Spormann and Widdel, 2000; Widdel and Rabus, 2001; Zheng et al., 2001; Gibson and Harwood, 2002). Beside the reduction of soluble electron acceptors such as oxygen, nitrate, and sulfate, iron(III) reduction is thought to play a major role for the reduction of aromatic hydrocarbons at contaminated sites (Lovley, 1993; Kao and Wang, 2000). Biodegradation of different BTEX compounds, i.e. benzene, toluene, ethylbenzene, and xylene, in the field under iron-reducing conditions in situ could be shown (Johnson et al., 2003; Richnow et al., 2003; Heidrich et al., 2004). However, since the first description of a pure culture of a dissimilatory iron-reducing microorganism growing with toluene as the sole carbon and energy source (Lovley and Lonergan, 1990) little progress was made on the isolation of other iron-reducing bacteria that are able to degrade aromatic hydrocarbons (Coates et al., 2001). Iron-dependent degradation of some BTEX compounds could only be shown in contaminated sediments (Kazumi et al., 1997; Anderson and Lovley, 1999; Rooney-Varga et al., 1999) and in laboratory batch experiments containing aquifer matrix (Lovley et al., 1996a). A sediment free benzene-oxidizing enrichment culture was reported, which showed the evolution of CO$_2$ from radiolabelled benzene as substrate (Lovley et al., 1994; Rooney-Varga et al., 1999). No enrichments have been reported so far, which are able to grow with ethylbenzene or xylene as the sole carbon and electron source and ferric iron as the electron acceptor.

In this work, the enrichment of different iron-reducing cultures, which are able to oxidize all BTEX compounds respectively, is reported. Also electron balance experiments are presented for the oxidation of four different aromatic hydrocarbons, i.e. benzene, toluene, ethylbenzene, and o-xylene in highly enriched sediment free cultures proving a total oxidation of the compounds to CO$_2$. This is the first report on the degradation of ethylbenzene or o-xylene by sediment-free iron-reducing enrichment cultures.

Following the enrichment procedure, two of the aromatic hydrocarbon-degrading cultures were successfully isolated. One of the new isolates is able to grow with toluene as sole
2.1 Anaerobic degradation of benzene, toluene, ethylbenzene, and o-xylene in sediment-free iron-reducing enrichment cultures

carbon and energy source, the other isolate is able to degrade either toluene or o-xylene to CO$_2$. The second isolate is the first pure culture of an o-xylene-degrading dissimilatory iron-reducing microorganism reported so far.
2.1 Anaerobic degradation of benzene, toluene, ethylbenzene, and o-xylene in sediment-free iron-reducing enrichment cultures

2.1.2 Experimental Procedures

2.1.2.1 Setup of enrichment cultures

The inoculum for the enrichment of iron-reducing cultures was taken from a tar-oil contaminated former gasworks site in Stuttgart, Germany. Sludge was sampled from the bottom of highly contaminated groundwater monitoring wells, covered with anoxic groundwater, and kept in the dark at 4°C until the enrichment cultures were prepared.

The enrichment cultures were grown in a carbonate buffered mineral medium consisting of 1.0 g/L NaCl, 0.4 g/L MgCl₂ * 6H₂O, 0.2 g/L KH₂PO₄, 0.25 g/L NH₄Cl, 0.5 g/L KCl, 0.15 g/L CaCl₂ * 2H₂O. (Widdel et al., 1983) with a pH between 7.2 and 7.4. The medium was supplemented with 1 ml/L of trace element solution SL10 (Widdel et al., 1983), 1 ml/L of a selenite-wolframate solution (Widdel, 1980), and 0.5 ml/L of a 7-vitamine-solution (Widdel and Pfennig, 1981). Sulphate (10 μM) was added as sulphur source and FeCl₂ (1 mM) was added as a reductant in order to eliminate traces of oxygen in the medium. The medium was anoxically transferred to 100 ml serum bottles in portions of 50 ml, the bottles were purged with an 80/20 (v/v) mixture of N₂-CO₂ gas and sealed with butyl rubber stoppers. Amorphous Fe(III) hydroxide was prepared as described in (Lovley and Phillips, 1986a) and was added as the bulk electron acceptor to each bottle at a final concentration of 50 mM.

Each bottle contained the solid adsorber resin Amberlite-XAD7 (0.3 g) (Fluka, Buchs, Switzerland) as a substrate reservoir, to keep hydrocarbon concentrations at a moderately low level during bacterial growth (Morasch et al., 2001). The XAD7 was added to the bottles and autoclaved prior to the addition of the medium. One of the different BTEX-compounds, i.e. benzene, toluene, ethylbenzene, or o-xylene was injected through the stopper with a gastight syringe as the sole carbon and energy source at a nominal initial total concentration of about 1 mM, calculated for the volume of the aqueous phase without consideration of the adsorption to the XAD7 resin. The bottles were equilibrated for one week before incubation. All enrichments were inoculated with 5 ml of sludge, which had been taken from the bottom of a highly polluted groundwater monitoring well at a tar-oil contaminated former gasworks site in Stuttgart, Germany. Some of the enrichment cultures contained AQDS (2 mM) as an additional agent to enhance microbial iron reduction (Lovley et al., 1996b; Coates et al., 1998; Zachara et al., 1998; Royer et al., 2002). All enrichments were cultivated independently in parallel, either with or without AQDS, at 30 °C in the dark. Active enrichments were repeatedly transferred into fresh medium and ferrous iron production was monitored.

2.1.2.2 Electron balance experiments

After four subsequent transfers the enrichments were sediment free and were taken for electron balance experiments (figure 2.2, A – D). To this end, the enrichments (5 ml) were transferred to fresh medium, which contained about 150 μM of one of the BTEX compounds and no XAD7 adsorber resin. The bottles were sealed with Viton rubber stoppers (Maag Technik, Dübendorf, Switzerland) and iron(II) production and BTEX degradation were measured over time. Each electron balance was determined in three parallel
2.1 Anaerobic degradation of benzene, toluene, ethylbenzene, and o-xylene in sediment-free iron-reducing enrichment cultures

2.1.2.3 Iron analysis

Iron(III) reduction was monitored through development of Fe(II) in the culture medium. Samples of 100 µl were taken from the culture medium, added to 900 µl of 1 M HCl, and shaken overnight at room temperature. Fe(II) concentrations in the samples were measured with ferrozine (Stookey, 1970). The total iron content was measured with the same procedure after complete reduction of Fe(III) with hydroxylamine hydrochloride solution in 1 M HCl (10% w/w). Samples of 100 µl, which had been incubated in 1 M HCl as described above, were added to the hydroxylamine solution, incubated for 15 min. at room temperature, and measured with ferrozine.

2.1.2.4 BTEX analysis

The BTEX concentrations in the bottles were measured with high-performance-liquid-chromatography (HPLC) and UV/vis detection at 210 nm. A Hyperchrome-HPLC-column NC-03-200 (200 * 3.0 mm) with a filling of PRONTOSIL 120-3-C18-H 3.0 µm (Bischoff Chromatography, Leonberg, Germany) was used as the stationary phase and a mixture of 70/30 (v/v) acetonitrile/water was used as the mobile phase at a constant flow rate of 0.6 ml/min. The concentrations of the aromatic hydrocarbons were calculated using external calibration with the pure substances.

2.1.2.5 Isolation of new strains

Following the enrichment of the new cultures, also new strains of iron-reducing microorganisms were successfully isolated. In order to isolate the new strains, dilution series with the enrichment cultures were done. The standard cultivation medium, as described above, and also ferrihydrite (40 mM) as electron acceptor were used. One of the different BTEX compounds was added as sole carbon and electron source, except the bottles for the isolation of an autotrophic iron-reducing strain. These bottles contained hydrogen gas as electron donor and only CO2 as carbon source. All bottles contained the standard medium, the ferric mineral, the BTEX pollutant or CO2 + H2 gas, and AQDS (1 mM), except the dilution series with toluene as substrate. The dilution series were incubated in the following way (see figure 2.1): From a well grown enrichment culture, which, as was assumed, contains about 10^7 cells per ml, an aliquot of 0.1 ml was taken out with a syringe and transferred into a fresh bottle, which now contained 10^6 cells in total. Out of this bottle, an aliquot of 0.5 ml was taken out and transferred to a new bottle. The third dilution step was done in three parallel bottles and 5 ml of the previous bottle were added as inoculum to each bottle. The next steps were also diluted tenfold and cultivated as three parallels. All bottles were grown at 30 °C in the dark, until the reduction of the iron mineral was clearly measurable. After about three months, the first dilution series was transferred into a second series and incubated another three months. The cultures of the second dilution series were assumed to be pure and tested for purity in an extra master thesis (see master thesis of Monika Cieślińska: “Partial characterization of two iron(III)-reducing microorganisms: the autotrophic strain AUTO-1 and the o-xylene-degrading strain MJOX-5” (2005)). The isolation of the strains resulted in one pure...
2.1 Anaerobic degradation of benzene, toluene, ethylbenzene, and o-xylene in sediment-free iron-reducing enrichment cultures
toluene-degrading strain, one pure strain, toluene, and one mesophilic autotrophic iron-
which is able to degrade either o-xylene or reducing culture.

Figure 2.1: Scheme of a dilution series for the isolation of new strains from the iron-reducing enrichment cultures. The squares symbolize the single culture bottles, the numbers describe the theoretical total number of cells in each bottle. The bottles from $10^0$ cells on are presumably pure cultures.
2.1.3. Results and Discussions

After successful enrichment of several iron-reducing cultures, electron balance experiments for the different pollutants, i.e., benzene, toluene, ethylbenzene, or o-xylene, were performed, one set in the presence of AQDS and the other in the absence of AQDS. In order to correct the BTEX concentrations measured in the aqueous phase for gas exchange with the headspace of the culturing bottles the following values for dimensionless Henry’s law constants at 30 °C were used (calculated from data given in reference (Shiu and Ma, 2000)): 0.268 for benzene, 0.307 for toluene, 0.414 for ethylbenzene, and 0.253 for o-xylene.

Of special interest were the experiments with benzene-degrading enrichment cultures, because benzene is known to be the most stable BTEX compound with respect to anaerobic degradation. The degradation of benzene to CO$_2$ by two different enrichment cultures could, however, be shown (figure 2.2 A). Enrichment culture benz AQDS was cultivated in the presence of AQDS through all enrichment steps. It showed a lag phase of only 16 days and a complete degradation of benzene within 77 days of incubation. Culture benz K11 that was enriched without addition of AQDS showed a lag phase of 61 days before degradation of benzene started. After 115 days the degradation slowed down and stopped after 162 days before complete exhaustion of benzene. The calculated electron recoveries are listed in table 2.1. In contrast to former studies where a mineralization of benzene to CO$_2$ of 25 % by a highly purified benzene-oxidizing enrichment culture was shown (Lovley and Anderson, 2000), the benzene-degrading enrichment cultures presented here showed a recovery of electrons from benzene oxidized in ferric iron reduced of 82 % and 74 %, respectively.

The degradation of toluene started immediately in both cultures without any significant lag phase as is indicated by the increase of the iron(II) concentration and the corresponding decrease in toluene concentration (figure 2.2 B). In the culture tol AQDS, which was enriched in the presence of AQDS, the degradation appeared to be slightly faster than in culture tol K21 with amorphous ferric iron only. In both cultures, the toluene concentration was depleted already after 39 days of incubation and no further change in the Fe(II) concentration could be measured. The sterile control experiment showed no decrease in toluene concentration and no increase in Fe(II) over the whole period of the experiment. Ethylbenzene was degraded by two different iron-reducing enrichment cultures (figure 2.2 C). After relatively long lag phases of 61 days in case of the enrichment in the absence of AQDS (culture ebenez K21) and of 93 days with AQDS (culture ebenez AQDS), the substrate was used by both bacterial enrichments. After the lag phase, ethylbenzene was completely degraded to CO$_2$ within 69 days (ebenz AQDS). Enrichment culture ebenez K21 did not show a complete depletion of the substrate within 162 days. In contrast to culture ebenez AQDS the degradation of ethylbenzene and also the increase in Fe(II) concentration was very slow.
2.1 Anaerobic degradation of benzene, toluene, ethylbenzene, and \( o \)-xylene in sediment-free iron-reducing enrichment cultures

**Figure 2.2:** Iron(III) reduction by enrichment cultures growing with four different aromatic hydrocarbons as sole carbon and energy source and amorphous iron(III) hydroxide as the electron acceptor. The presented data are the means of triplicate experiments. Hydrocarbon concentrations (solid symbols) and ferrous iron concentrations (open symbols) are shown. As substrates were used A) benzene, B) toluene, C) ethylbenzene, and D) \( o \)-xylene. The control experiments were not inoculated (diamonds). Some enrichment cultures contained AQDS (triangles): A) benz AQDS, B) tol AQDS, C) ebenz AQDS, D) ox AQDS. Parallel enrichments were performed without AQDS (circles): A) benz K11, B) tol K21, C) ebenz K21, D) ox K11. The error bars represent the standard deviation of the three parallel experiments.

The degradation of \( o \)-xylene by two different enrichment cultures started immediately after incubation of the bottles (figure 2.2 D). With xylene, the reaction was much faster for the enrichment with AQDS than for the enrichment without AQDS. Already after 39 days, \( o \)-xylene was depleted with AQDS as electron shuttle (ox AQDS) whereas in culture ox K11, which was not supplemented with AQDS, the degradation was much slower and did not lead to a complete depletion even after 162 days of incubation. In the control culture, neither a decrease of \( o \)-xylene nor an increase of the Fe(II) concentration was observed.
2.1 Anaerobic degradation of benzene, toluene, ethylbenzene, and \( o \)-xylene in sediment-free iron-reducing enrichment cultures

Electron balances for the different experimental set ups were calculated for a complete oxidation of the aromatic hydrocarbons to \( \text{CO}_2 \) (table 2.1). In all eight cases, the electron balances were very similar and consistent with the general rule that about 70% to 90% of a hydrocarbon substrate is usually used to produce energy. Up to 30% of the hydrocarbon added to the cultures was probably used to produce biomass, as other studies of anaerobic degradation of hydrocarbons have shown (Lovley and Lonergan, 1990; Galushko et al., 1999; Kniemeyer et al., 2003). A significant accumulation of intermediates, i.e., an incomplete oxidation of the substrate, can be ruled out, because this would result in less ferrous iron than was measured in the cultures. For example, if acetate was produced instead of \( \text{CO}_2 \) only 20-25% of the actual Fe(II) production would have been expected for the amount of substrate oxidized.

In contrast to most earlier studies on the degradation of aromatic hydrocarbons by iron-reducing microbes (Kazumi et al., 1997; Weiner and Lovley, 1998; Anderson and Lovley, 1999; Zheng et al., 2001), we used sediment-free enrichment cultures and not microcosm studies. For that reason, we were able to exclude the influence of substances from the sediment samples, e.g. dissolved organic carbon. In the cultures supplied with AQDS the concentration of ferrous iron did not increase any further after the complete depletion of the aromatic hydrocarbons. This indicates that the AQDS was not used as carbon or energy source by the bacterial enrichment cultures. This first report on the complete degradation of ethylbenzene and \( o \)-xylene under iron-reducing conditions supports the hypothesis that dissimilatory iron reduction may play an important role in the degradation of aromatic hydrocarbons \textit{in situ}. The fact that we were able to enrich dissimilatory iron reducers degrading BTEX compounds other than toluene shows that the enrichment strategy presented here was very successful, probably due to the combination of two effects.

<table>
<thead>
<tr>
<th>Enrichment culture</th>
<th>Electron equivalents calculated from measured concentration of aromatic hydrocarbons (( \mu \text{M} ))</th>
<th>Electron equivalents recovered in ferrous iron measured (( \mu \text{M} ))</th>
<th>Calculated electron recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>benz ( K11 )</td>
<td>3339</td>
<td>2732</td>
<td>82 ± 20</td>
</tr>
<tr>
<td>benz AQDS</td>
<td>5081</td>
<td>3771</td>
<td>74 ± 9</td>
</tr>
<tr>
<td>tol ( K21 )</td>
<td>5564</td>
<td>4774</td>
<td>86 ± 12</td>
</tr>
<tr>
<td>tol AQDS</td>
<td>5184</td>
<td>5282</td>
<td>102 ± 4</td>
</tr>
<tr>
<td>ebenz ( K21 )</td>
<td>4216</td>
<td>2670</td>
<td>79 ± 3</td>
</tr>
<tr>
<td>ebenz AQDS</td>
<td>5191</td>
<td>4183</td>
<td>80 ± 8</td>
</tr>
<tr>
<td>ox ( K11 )</td>
<td>2463</td>
<td>2032</td>
<td>83 ± 22</td>
</tr>
<tr>
<td>ox AQDS</td>
<td>4616</td>
<td>3710</td>
<td>79 ± 8</td>
</tr>
</tbody>
</table>

Table 2.1: Calculated electron balances for the different enrichment cultures. The table displays the percentage of electron equivalents recovered in ferrous iron produced assuming complete oxidation of BTEX compounds to \( \text{CO}_2 \). The errors for the calculated electron balance represent the standard deviation from the average of the triplicate cultures for the different electron balance experiments.
2.1 Anaerobic degradation of benzene, toluene, ethylbenzene, and o-xylene in sediment-free iron-reducing enrichment cultures

First, the concentration of the BTEX compounds was kept at a moderate level of about 60 µM due to the addition of the adsorber resin XAD7 (Morasch et al., 2001), which reduces the cytotoxicity of the harmful BTEX substances. With time, a substrate pool adsorbed to the resin, equivalent to about 1 mM of total substrate if all the hydrocarbon would have been dissolved in the aqueous phase, was steadily released to the organisms leading to high cell densities in the bottles. This setup resembles more closely in situ conditions occurring at contaminated sites including those present at the specific site where the sludge was taken from, where usually low substrate concentrations are predominant (Christensen et al., 2001; Griebler et al., 2004). In other studies, cultivation with low substrate concentrations resulted in the isolation of different bacterial strains as compared to the cultivation in nutrient-rich media, probably because many microorganisms are adapted to nutrient-poor environments (Busmann et al., 2001; Connon and Giovannoni, 2002).

Second, the addition of the electron-shuttling substance AQDS, which has already been described several times by different authors to accelerate iron reduction and substrate oxidation by iron-reducing bacteria (Lovley et al., 1996b; Coates et al., 1998; Zachara et al., 1998; Royer et al., 2002), proved useful for the enrichment of new iron-reducing microorganisms. Iron reduction and substrate utilization was also much faster in our set of experiments compared to the cultures without addition of AQDS. Recently, iron reduction by reduced sulphur species resulting from an internal sulphur cycle was reported (Straub and Schink, 2004). Although we have no indication that such processes occur in our assays we cannot rule out such mechanism, as we added 10 µM SO$_4^{2-}$ as sulphur source to our experiments.

The successful purification of one toluene-degrading and one toluene or o-xylene-degrading strain offers diverse possibilities in terms of investigating different contaminant degradation pathways. It could also be a challenge to compare those to contaminant degradation pathways of other groups of anaerobic respiration, like sulphate reduction or the nitrate reduction. Especially the investigation of the strain, which is able to use two different aromatic hydrocarbons, seems worth, in order to get possible insight into near-in situ conditions, because at most sites many pollutants are present at the same time and the regulation of the different degradation pathways might be helpful for future remediation efforts. Similar experiments have been done by other authors with contaminant-degrading sulphate-reducing strains (Meckenstock et al., 2004; Morasch et al., 2004).

In summary, our results demonstrate that BTEX-oxidizing iron-reducing microorganisms are naturally occurring at contaminated sites and can be enriched with the appropriate methods. However, it remains unclear what their ecological significance is compared to other respiratory classes of organisms because the presented data are derived from batch experiments and the results cannot be transferred to natural systems assuming similar microbial growth. We used single aromatic compounds as the sole carbon and electron source which does not reflect the in situ situation at most sites contaminated with petroleum derived hydrocarbons. At present, there are only limited data available about microbial degradation of mixtures of aromatic hydrocarbons that represent conditions that are closer to in situ conditions in contaminant aquifers (Heidrich et al., 2004; Meckenstock et al., 2004), and future research is needed to address the effects of multiple substrates on enrichment techniques and contaminant degradation.
2.1 Anaerobic degradation of benzene, toluene, ethylbenzene, and o-xylene in sediment-free iron-reducing enrichment cultures

In addition to the two BTEX-degrading pure cultures, an autotrophic, mesophilic iron-reducing strain was isolated. This is the first report on the purification of such an organism and so far only thermophilic autotrophic strains have been reported in literature (Zhou et al., 2001; Kashefi et al., 2002). Also only hydrogen-utilizing mesophilic strains have been described, which need an additional organic carbon source for growth (Caccavo et al., 1992; Coates et al., 2001). The isolation of a mesophilic autotrophic strain also supports the hypothesis that iron reduction might be an ancient metabolic process, which might have developed already on early earth. It might also be fruitful to compare the metabolism of autotrophic iron reduction to other classes of autotrophs, for example the methanogens or the homoacetogens.
2.2 Case study: Determination of the oxidation capacity of iron-reducing BTEX-degrading microorganisms

2.2.1 Introduction

Amorphous iron minerals, like ferrihydrite, are usually easier reducible by microorganisms than crystalline minerals, like goethite, hematite, or lepidocrocite (Zachara et al., 1998; Zachara et al., 2002). Thus, the amorphous minerals are said to be the bioavailable iron(III) phase in soils and sediments (Lovley and Phillips, 1986b, 1987). Therefore, the concentration of the chemically extractable amorphous iron(III) should directly correspond to the microbially reducible iron phase in a sediment.

In order to investigate, how much of the chemically extractable amorphous iron(III) is in fact reduced by BTEX-degrading iron-reducing cultures, reduction experiments with sediment samples and groundwater from a kerosene-contaminated site at Brand (Brandenburg, Germany) were performed. The experiments were done under conditions, which were as close to the in situ situation at the site as possible, using water and soil samples from the field to prepare the cultivation medium.

2.2.2 Experimental Procedures

2.2.2.1 Site description

The soil samples and the natural water for the preparation of a groundwater medium were taken from a former airfield of the soviet army at Brand (Brandenburg, Germany). At this site, a vast amount of kerosene was spilled and diffused into the ground, forming a big pool on top of the aquifer of up to two metres thickness. Part of the kerosene was already pumped out of the ground and was reused, but still big contaminant plumes are present in the soil and in the aquifer. In the saturated zone of the aquifer, anoxic conditions prevail and high concentrations of dissolved ferrous iron are measured, giving strong indication that iron reduction processes are in progress.

2.2.2.2 Preparation of the medium

The groundwater medium for the cultivation of microorganisms was prepared, using the original water from the contaminated site...
without autoclaving, because a preceding experiment had shown that after autoclaving the water, a lot of black substances precipitated in the flask, probably polyaromatic hydrocarbons (PAHs), and the medium became very smelly (see appendix for the analysis of the water samples).

One litre of the medium consisted of 1 L aquifer water, 1 ml of trace element solution SL10 (Widdel et al., 1983), 1 ml of a selenite-wolframate solution (Widdel, 1980), and 1 ml of a 7-vitamine-solution (Widdel and Pfennig, 1981). A nitrogen source was added (10 µM NH₄Cl) and also a sulphur source (10 µM Na₂SO₄). The medium was carbonate buffered with 30 mM NaHCO₃ at a pH between 7.2 and 7.4. The medium was distributed in portions of 50 ml into culture bottles, which were purged with N₂/CO₂ gas and closed with butyl rubber stoppers. Each bottle contained the solid adsorber resin Amberlite-XAD7 (0.3 g) (Fluka, Buchs, Switzerland) as a substrate reservoir, to keep hydrocarbon concentrations at a moderately low level during bacterial growth (Morasch et al., 2001). The XAD7 was added to the bottles and autoclaved prior to the addition of the medium. One of the different BTEX-compounds, i.e. benzene, toluene, ethylbenzene, o-xylene, or m-xylene, was injected through the stopper with a gastight syringe as the sole carbon and energy source at a nominal initial total concentration of about 1 mM, calculated for the volume of the aqueous phase without consideration of the adsorption to the XAD7 resin. The bottles were equilibrated for one week before incubation. After preparation of the medium, 6 ml of a contaminated soil sample were added into the bottles as the sole electron acceptor. Three parallel bottles were incubated with different BTEX-degrading cultures, mostly cultures, which had been successfully enriched beforehand. 5 ml of each preculture were added to the culture bottles and incubated at 30 °C in the dark. For toluene degradation, Geobacter metallireducens was used as inoculum, which is one out of two pure cultures available that are able to degrade toluene (Lovley et al., 1993). The m-xylene-degrading preculture was provided by Michael Safinowski and the precultures for benzene, ethylbenzene, and o-xylene degradation were taken from the enrichments described in part 2.1. The iron concentration was measured at the beginning of the experiment and after some months, until no further increase in iron(II) concentration was observed. For all BTEX compounds, a sterile culture was also prepared, which only contained the medium, XAD7, one of the BTEX contaminants and the soil sample without addition of precultures. The controls were autoclaved at three subsequent days, to sterilize them and to prevent growth of spore forming organisms.

### 2.2.2.3 Iron analysis

Iron(III) reduction was monitored through development of Fe(II) in the culture medium. Samples of 100 µl were taken from the culture medium, added to 900 µl of 1 M HCl, and shaken over night at room temperature, in order to extract and dissolve the amorphous iron(III) minerals from the sediment and the iron(II) phases formed during the experiments (Heron et al., 1994). Fe(II) concentrations in the samples were measured with ferrozine (Stookey, 1970). The total iron content was measured with the same procedure after complete reduction of Fe(III) with hydroxylamine hydrochloride solution in 1 M HCl (10% w/w). Samples of 100 µl, which had been incubated in 1 M HCl as described above, were added to the hydroxylamine solution, incubated for 15 min at room temperature, and measured with ferrozine.
2.2.2.4 Balance of the iron(III) reduction

The determination of the total oxidation capacity of the different BTEX-degrading cultures for the reduction of natural iron(III) minerals was done through balancing the concentration of total iron in the bottles at the beginning of the experiments and the concentration of reduced iron(III) at the end of the experiments. Thus, the percentage of reduced amorphous iron(III) is calculated and the reduction efficiency of the different BTEX-degrading cultures is compared. The balancing is made with the average values out of three parallel experiments, the error represents the standard deviation (see table 2.2).
2.2.3 Results and Discussion

The reduction of contaminated soil samples with iron-reducing enrichment cultures, grown in a groundwater medium of low ionic strength, showed that only the amorphous iron(III) minerals in the soil samples were reduced by the different BTEX-degrading cultures and thus were bioavailable. The more crystalline iron(III) minerals were probably reduced very slowly, if they were reduced at all. Already after six months of incubation, almost no further increase in Fe(II) concentration was measurable and still after eleven months, the iron(II) concentration did not rise any further.

The balancing of reduction experiments showed, that all the different enrichments or the pure culture reduced the amorphous iron(III) completely (see table 2.). This result further supports the assumption that the amorphous iron minerals, that are extractable with 0.5 M HCl, represent the bioavailable iron(III) phases in the environment, if no electron-shuttling or iron-chelating substances are present (Lovley and Phillips, 1986b). If electron-shuttling substances, for example humic acids, are available for the microorganisms, the reduction of crystalline iron(III) minerals, like goethite or hematite, might be greatly enhanced, as was shown in batch experiments with different iron minerals (Lovley et al., 1994; Zachara et al., 1998). Our results show that it might be possible to determine the concentration of bioavailable iron(III) in different soils simply by using chemical extraction techniques. In the following, the concentration of chemically extracted iron(III) might be used to estimate the total oxidation capacity for iron(III) reduction at a given contaminated site very easily. Thus, also the concentration of BTEX contaminants, which might be degraded by the iron-reducing microorganisms, might be roughly estimated, simply based on the chemical extraction of amorphous iron(III) minerals in contaminated soils and aquifer systems.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Total iron at the beginning of the experiment (µM)</th>
<th>Fe(II) at the beginning of the experiment (µM)</th>
<th>Fe(II) at the end of the experiment (µM)</th>
<th>Percentage of bioavailable iron(III), which is reduced (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>benzene</td>
<td>7153</td>
<td>5207</td>
<td>8013</td>
<td>112 ± 3</td>
</tr>
<tr>
<td>toluene</td>
<td>9627</td>
<td>6807</td>
<td>10047</td>
<td>104 ± 2</td>
</tr>
<tr>
<td>ethylbenzene</td>
<td>10780</td>
<td>6053</td>
<td>10467</td>
<td>97 ± 8</td>
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<tr>
<td>o-xylene</td>
<td>9800</td>
<td>6787</td>
<td>9853</td>
<td>101 ± 4</td>
</tr>
<tr>
<td>m-xylene</td>
<td>7720</td>
<td>5420</td>
<td>8133</td>
<td>105 ± 1</td>
</tr>
</tbody>
</table>

Table 2.2: Oxidation capacity of the different iron-reducing enrichment cultures for the degradation of different BTEX compounds, using natural soil as sole electron acceptor.
2.2 Case study: Determination of the oxidation capacity of iron-reducing BTEX-degrading microorganisms

Also the fact that all the different iron-reducing enrichment cultures, which used very different contaminants as a substrate, for example benzene or \( m \)-xylene, showed the complete reduction of the amorphous iron present in the soil sample could be a strong indication that also at contaminated sites under \textit{in situ} conditions only amorphous iron minerals are readily degraded and mainly contribute to the total oxidation capacity at such sites. However, the influence of electron-shuttling or iron(III)-chelating substances on the reduction of crystalline iron minerals under \textit{in situ} conditions might also be important.
2.4. References


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3 Reduction of Prussian Blue by the two iron-reducing microorganisms *Geobacter metallireducens* and *Shewanella alga*

### 3.1 Introduction

Contaminations with cyanides are widespread at many former gasworks sites and coke ovens (Shifrin et al., 1996). They also result from processes of coal carbonisation and from removing HCN from product gas streams through treatment with iron hydroxide particles. These cyanide-containing wastes used to be deposited on landfills and therefore tend to leach into the environment (Theis et al., 1994). Metal cyanides are found in large quantities of several weight percent of total cyanides in the soil at such contaminated sites (Young and Theis, 1991). However, Fe-cyanide complexes are usually not found in natural systems and therefore are solely of anthropogenic origin (Fuller, 1985). Cyanides are present in different speciations and at most sites iron(II) or iron(III) complexes predominate (Mansfeldt and Dohrmann, 2001). These complexes are very stable and are only dissociable in very strong acids. At contaminated sites mainly complexes of ferrocyanide ([Fe(CN)₆]⁴⁻), ferricyanide ([Fe(CN)₆]³⁻) or a recently identified third complex ([Fe(CN)₅NHCH₃]⁴⁻) are found (Theis et al., 1994; Ghosh et al., 2004). Free cyanide, ferrocyanide, and ferricyanide are readily degradable by different microorganisms (Boucabeille et al., 1994; Ebbs, 2004), but the degradation or the reduction and growth of dissimilatory iron-reducing microbes on insoluble iron(III)-ferrocyanide-complexes, such as Prussian Blue, has not been reported so far. The equilibrium constant for the reductive dissolution of Prussian Blue (see equation 3.1) is very low and was determined as log $K^0 = -85.4$ (Meeussen et al., 1992). From this constant and from the half reaction in equation 3.2 (Beck, 1987), we calculated the solubility product of Prussian Blue as log $K_{sol} = -66.5$, which is very low.

### Equation 3.1

$$\text{Fe₄}[\text{Fe(CN)}₆]_{3(5)} \rightleftharpoons 4\text{Fe}^{3+} + 3[\text{Fe(CN)}₆]^{3-} + 3e^-$$

$\log K^0 = -84.5$

### Equation 3.2

$$[\text{Fe(CN)}₆]^{3-} + e^- \rightleftharpoons [\text{Fe(CN)}₆]^{4-}$$

$\log K^0 = -6.0$
The solubility of Prussian Blue is strongly pH- and pE-dependant and increases with increasing pH (Meeussen et al., 1992; Meeussen et al., 1995). However, in natural water samples with neutral pH and with pE values between −5 and +5, i.e., at anoxic conditions, the concentration of total dissolved cyanide was very low and the undissociated form of Prussian Blue was found to be highest abundant (Ghosh et al., 1999; Ghosh et al., 2004). This matches perfectly with the blue mineral, which is found in the soil at many cyanide-contaminated sites. At many anoxic contaminated sites where reducing conditions prevail (Christensen et al., 2000), a reduced species, Prussian White, also might precipitate. Different iron-cyanide complexes sorb pH-dependently onto iron(III) mineral surfaces, such as goethite or ferrihydrite (Rennert and Mansfeldt, 2001a, b). The pH therefore controls adsorption and desorption of iron cyanides, which strongly influences the bioavailability and thus the toxicity of the different cyanide species.

Prussian Blue has several important applications. For example it is used as a chemical sensor and biosensor in electrochemical, optical or catalytic processes (Koncki, 2002). Prussian White is also used as a catalyst for the electrochemical reduction of hydrogen peroxide and for monitoring of this substance in medicine, environmental control or industry (Fiorito et al., 2005). In histology and cellular biology Prussian Blue staining is a standard technique for the staining of single cells or tissues in light microscopy.

Here, we report on the reduction of Prussian Blue by two different and phylogenetically distinct dissimilatory iron-reducing bacteria, *Geobacter metallireducens* and *Shewanella alga* strain BrY. Both strains were also able to grow on Prussian Blue as the sole electron acceptor. The reduction rates were two orders of magnitude larger as compared to ferrihydrite, which is said to be the insoluble iron mineral accessed best by bacterial cells.
3.2. Experimental Procedures

3.2.1 Growth experiments

A basal mineral medium was prepared according to (Widdel et al., 1983) consisting of 1.0 g/l NaCl, 0.4 g/l MgCl₂ · 6H₂O, 0.2 g/l KH₂PO₄, 0.25 g/l NH₄Cl, 0.5 g/l KCl, 0.15 g/l CaCl₂ · 2H₂O. The medium was buffered with 30 mM sodium bicarbonate and sulphate (10 µM) was added as sulphur source. FeCl₂ was added as a reductant (1 mM) in order to remove traces of oxygen in the medium. After adjusting the pH between 7.2 and 7.4, the medium was anoxically distributed to 100 ml serum bottles in portions of 50 ml each. The bottles were purged with an 80/20 (v/v) mixture of N₂-CO₂ gas and sealed with butyl rubber stoppers. Bottles for growth experiments with *S. alga* also contained 20 g/l NaCl. Sodium acetate was added (10 mM) as carbon and electron source in all experiments with *G. metallireducens* and sodium L-lactate (10 mM) was added to all experiments with *S. alga*. The bottles each contained 6 mM Prussian Blue (Aldrich, Taufkirchen, Germany). After reduction of the Prussian Blue the blue sediment turned white and the cultures were transferred into new bottles with fresh medium.

3.2.2 Iron analysis

Iron(III) reduction was monitored through Fe(II) production in the culture medium using a ferrozine assay (Stookey, 1970). We could show that ferrozine reacts with dissolved Fe(II) and with Prussian White and forms a purple complex, but it does not react with Prussian Blue or with ferrocyanide (data not shown).

For iron analysis, aliquots of 100 µl were taken from the culture medium, added to 900 µl of 1 M HCl, immediately mixed with a Vortex mixer, and incubated for ten minutes. After incubation, 100 µl of the supernatant were added to 900 µl of ferrozine reagent, carefully mixed, and incubated for another ten minutes. The samples were centrifuged at 14000 rpm for 5 minutes and the Fe(II) concentration in the supernatant was measured by UV/vis spectroscopy at 562 nm.

3.2.3 Electron balance experiments

The bacteria were grown in the standard medium described above. The electron balance experiments with *Geobacter* cells contained sodium acetate (0.25 mM) and the experiments with *Shewanella* cells contained sodium L-lactate (1.2 mM) as sole carbon source. In both sets Prussian Blue was added in excess (5 mM) as the sole electron acceptor. The increase in ferrous iron in the culture medium was monitored over time. After completion of Prussian Blue reduction, the electron balance was calculated taking into account the measured Fe(II) production and total depletion of the substrate.

3.2.4 X-ray diffraction analysis of Prussian Blue samples

X-ray diffraction spectra were measured for untreated Prussian Blue (Aldrich, Taufkirchen, Germany) and for reoxidized samples of reduced Prussian Blue from bacterial cultures. A grown culture of *G. metallireducens* or *S.*
3 Reduction of Prussian Blue by the two iron-reducing microorganisms *Geobacter metallireducens* and *Shewanella alga*

*alga* was used respectively, where the blue sediment had turned completely white. The bottles were opened in the glove box and one drop of each culture was dried on a glass carrier. The drops were covered with a layer of wax in order to prevent immediate reoxidation of the Prussian White by oxygen from air during the time of the X-ray diffraction measurement. Spectra were measured for the same sample at different times, during reappearance of the blue colour.

### 3.2.5 Comparison of different iron(III) phases as electron acceptor

In this set of experiments the bacterial growth of *G. metallireducens* with different electron-accepting systems was investigated. After preparation of bottles with basal mineral medium and addition of sodium acetate as substrate for bacterial growth, the different electron acceptors were added: Ferric citrate (5 mM), Prussian Blue (5 mM), amorphous Fe(III) hydroxide (5 mM), and amorphous Fe(III) hydroxide (5 mM) plus AQDS (1 mM).

### 3.2.6 Reduction of environmental Prussian Blue

A sample of cyanide-contaminated sand from a contaminated site was used to monitor the reduction of environmental Prussian Blue. The sand grains were sorted manually and only grains, which were covered with a blue coating, were used for the experiment. The grains were added to a tenfold-diluted mineral medium, and the bottles were shaken over night to wash off the blue coating, in order to be able to visually monitor the reduction of Prussian Blue in the supernatant. Due to the particulate nature of the Prussian Blue it was impossible to measure the absorbance of the pigment directly with a spectrophotometer. The following day the supernatant was slightly blue. Washed cells of *G. metallireducens* were added and the disappearance of the blue colour in the supernatant of the bottles was documented with a digital camera. After removal of the stoppers the bottles were shaken to reoxidize the Prussian White to Prussian Blue.
3 Reduction of Prussian Blue by the two iron-reducing microorganisms *Geobacter metallireducens* and *Shewanella alga*

### 3.3 Results and Discussion

The reduction of one mol Prussian Blue \((\text{Fe}_4[\text{Fe(CN)}_6]_3)\) needs 4 mols of electrons if the ferric form is completely reduced to Prussian White \((\text{Fe}_2[\text{Fe(CN)}_6])\) (see equation 3.3).

**Equation 3.3**

\[
\begin{align*}
(\text{Fe}_4[\text{Fe(CN)}_6]_3)_{(s)} & + 4e^- & \rightarrow & \\
(\text{Fe}_2[\text{Fe(CN)}_6])_{(s)} & + [\text{Fe(CN)}_6]^{4-}_{(aq)}
\end{align*}
\]

In growth experiments using limited concentrations of sodium acetate as growth substrate, *G. metallireducens* started to reduce the blue pigment Prussian Blue almost immediately after a short lag phase of only four hours. Prussian Blue was completely reduced after 40 hours of incubation with the cells (figure 3.1 A). The calculated electron balance for *G. metallireducens* (i.e. substrate consumption vs. Fe(II) production) showed a recovery of electrons in Prussian Blue reduced of 85 % +/- 9 % (mean out of three parallel experiments), suggesting a complete reduction of the Prussian Blue to Prussian White. This result matches well with the fact that the bacteria usually use between 70 % and 90 % of a substrate to produce energy, whereas about 10 - 30 % are used to produce biomass (Lovley and Lonergan, 1990; Kniemeyer et al., 2003). Prussian Blue was also reduced by *S. alga* (figure 3.1 B), but the reduction was slower than with *Geobacter* cells. The calculated electron balance from three parallel experiments with *S. alga* was 69 % +/- 3 %, suggesting also a complete reduction of the pigment to Prussian White. As *Geobacter* and *Shewanella* species are widely distributed in freshwater or marine environments (Coates et al., 1996; Lonergan et al., 1996; Venkateswaran et al., 1999; Lovley et al., 2004), the use of the insoluble blue pigment as electron acceptor by such phylogenetically distinct microbial groups gives strong indication that this might be a general feature of most dissimilatory iron-reducing organisms. The data also suggest that in anoxic groundwater environments, where different *Geobacter* or *Shewanella* species are common, the Fe-cyanide complexes should mainly be present in the reduced forms including Prussian White because ferric cyanide complexes are much better reduced than other solid iron oxides. This contrasts with findings that mainly Prussian Blue is found at contaminated sites (Meeussen et al., 1994; Shifrin et al., 1996; Mansfeldt and Doehrmann, 2001). However, this controversy might be due to sampling artefacts, because already very short-term exposure to air is sufficient to reoxidize the Prussian White to the blue pigment. We could also observe the rapid reoxidation of the white precipitate during exposure to air in our experiments (data not shown). With X-ray-diffraction measurements we could show that the original Prussian Blue and the reoxidized blue pigment formed by oxidation of the white precipitate by exposure to air gave the same signal patterns (data not shown). This means that the fully reduced Prussian White is immediately converted to Prussian Blue, when exposed to air.
3 Reduction of Prussian Blue by the two iron-reducing microorganisms *Geobacter metallireducens* and *Shewanella alga*

A)

![Graph A](image1)

B)

![Graph B](image2)

**Figure 3.1:** Reduction of Prussian Blue by two different dissimilatory iron(III)-reducing bacteria. *Geobacter metallireducens* was grown with sodium acetate as sole carbon source (figure 3.1 A) and *Shewanella alga* BrY was grown with sodium L-lactate as sole carbon source (figure 3.1 B). In both sets, Prussian Blue was added in excess in order to obtain an electron balance for the growth experiments. Presented are data from triplicate growth experiments (triangles) and from a sterile control experiment without addition of cells (squares).
Apart from the mere reduction, the two bacterial strains were able to grow on Prussian Blue as sole electron acceptor and sodium acetate in case of *Geobacter* and sodium lactate in case of *Shewanella* as electron donor, even after several transfers into fresh medium with Prussian Blue as the sole electron acceptor (data not shown).

In some preceding experiments, we could show that *G. metallireducens* and *S. alga* BrY cells were still able to completely reduce even 10 mM of Prussian Blue to Prussian White, which resulted in the disappearance of the blue colour in the culture medium (figure 3.2 A). For that reason, in the concentration range we used for the electron balance, the growth of the bacterial cells is probably not inhibited by Prussian Blue, which might interfere with the cellular metabolism in higher concentrations. The use of Prussian Blue for staining of cells in microscopic samples is a well-known and established technique, indicating that Prussian Blue might interfere with the bacterial cell wall. The reduction of a natural sample of Prussian Blue coated sand showed a disappearance of the blue colour in the supernatant of the culture bottles within several hours after addition of the *Geobacter* cells (figure 3.2 B). When the bottles were opened and shaken in order to reoxidize the medium, the blue colour reappeared almost instantaneously. Without addition of cells, the supernatant remained light blue (data not shown).
3 Reduction of Prussian Blue by the two iron-reducing microorganisms *Geobacter metallireducens* and *Shewanella alga*

A)  

**Figure 3.2 A):** Growth on Prussian Blue as sole electron acceptor with *Shewanella alga* BrY or *Geobacter metallireducens*. The cells were grown with sodium L-lactate in case of *S. alga* or sodium acetate in case of *G. metallireducens* as sole carbon and energy source. The picture on the upper left shows a culture grown with *G. metallireducens*, where almost half of the Prussian Blue is reduced, which can be seen from the white layer on top of the unreduced pigment. The picture on the upper right was taken from a culture grown with *S. alga*, which shows that in the very beginning of the reduction the Prussian Blue phase is reduced in a different way by those cells: *S. alga* reduces the Prussian Blue in spheres, as can be seen from the disappearance of the blue colour in the form of white circles.

The lower part shows three bottles with different progress of the reduction grown with *S. alga*. The picture on the right hand side was taken at the very beginning of a growth experiment. The picture on the left hand side was taken after reduction of some part of the Prussian Blue, which is already lighter blue in colour. The picture in the middle shows the bottle after complete reduction of Prussian Blue to Prussian White.
3 Reduction of Prussian Blue by the two iron-reducing microorganisms *Geobacter metallireducens* and *Shewanella alga*

B)

**Figure 3.2 B):** Reduction of Prussian Blue in a natural sandy soil sample by *Geobacter metallireducens* cells. The culture contained sodium acetate as sole carbon and electron source and Prussian Blue coated sand as sole electron acceptor. The bottle on the left shows the culture after shaking the sand sample over night in the culture medium before incubation. The blue colour of the colloidal Prussian Blue particles in the supernatant is clearly visible. The bottle on the right shows the same culture after several hours of incubation with the bacterial cells. The blue colour has completely disappeared.

As other studies have shown, *Geobacter* species are widely distributed in different environments (Coates et al., 1996; Snoeyenbos-West et al., 2000) and therefore should be able to reduce Prussian Blue to Prussian White, which then might precipitate and form coatings on different mineral surfaces. The reduction of Prussian Blue in the natural sample shows that this mineral might also be reduced microbially in the environment. However, the removal of the Prussian Blue coating from the sand by abrasion due to stirring might positively effect the bioavailability of the mineral in terms of accessibility for bacteria and thus the reduction to Prussian White might be enhanced by this treatment.

A comparison of the reduction of Prussian Blue and three other forms of iron showed that reduction rates of Prussian Blue were almost as high as for soluble iron(III) citrate (figure 3.3).
3 Reduction of Prussian Blue by the two iron-reducing microorganisms *Geobacter metallireducens* and *Shewanella alga*

![Graph showing Fe(II) concentration over time for different electron acceptors: Ferricydrite, Ferricydrite + AQDS, Ferric citrate, Prussian Blue.](image)

**Figure 3.3:** Comparison of the reduction of Prussian Blue by *Geobacter metallireducens* with different other electron acceptors. The cultures were grown with sodium acetate (10 mM) as sole carbon and energy source and Prussian Blue (5 mM) (diamonds), ferric citrate (5 mM) (circles), ferrihydrite (5 mM) + AQDS (1 mM) (triangles), or ferrihydrite (5 mM) (squares) as electron acceptor.

The reduction of amorphous ferrihydrite alone was not measurable within that time frame. We also included culture bottles, which were amended with the soluble electron-shuttling substance AQDS. This substance greatly enhances the reduction of solid ferrihydrite, as was already reported several times before (Lovley et al., 1996; Royer et al., 2002). We wanted to compare the effectiveness of microbial iron(III) reduction in the presence of AQDS to Prussian Blue as electron acceptor. In our experiments, AQDS also greatly enhanced the reduction of solid ferrihydrite. The maximum increase of Fe(II) in the medium was comparable to ferric citrate and Prussian Blue, whereas the reduction proceeded slower in the beginning and the maximum was reached only after more than 44 hours compared to seven hours in case of ferric citrate and Prussian Blue, respectively.

Our results show, that the Prussian Blue is a very effective electron acceptor for two different types of dissimilatory iron-reducing bacterial strains, which represent members of the two dominating families of this type of anaerobic metabolism. We also found strong indication that Prussian Blue might also be reduced in the environment very easily and thus affect the toxicity and distribution of cyanide species at contaminated sites.
3 Reduction of Prussian Blue by the two iron-reducing microorganisms *Geobacter metallireducens* and *Shewanella alga*

### 3.4 References


Mansfeldt, T., and Dohrmann, R. (2001) Identification of a crystalline cyanide-


4 Extracellular electron transfer via iron(III) colloids

4.1 Introduction

Unlike other electron acceptors such as oxygen, nitrate, or sulphate, iron(III) is virtually insoluble at neutral pH values predominating at most natural habitats (Thamdrup, 2000). This makes iron(III) minerals poorly bioavailable and represents the limiting factor for dissimilatory iron(III) reduction. Microorganisms therefore need to develop special strategies to transfer electrons to the solid electron acceptor (see (Lovley et al., 2004) for review). Several possibilities for electron-shuttling to iron minerals have been reported in the literature so far. Humic acids or humic acid analogues (quinones) are a well-investigated example for the stimulation of dissimilatory iron(III) reduction by redox active mediators (Lovley et al., 1996). It should be noted that in recent studies some physiological types of microorganisms were found (Benz et al., 1998; Cervantes et al., 2002) that were only able to reduce iron(III) via electron-shuttling through humic acids. In the genus *Shewanella* the excretion of quinone derivatives has been shown and a role of these exudates in extracellular electron transfer was assumed (Newman and Kolter, 2000). However, other authors showed that these compounds probably have a function in the respiratory chain rather than in extracellular electron transfer (Myers and Myers, 2004). In general, the excretion of an extracellular electron shuttle is very energy consuming for the organism and the risk of dispersive loss of the shuttle in groundwater is high. Therefore, this strategy might be ineffective in oligotrophic groundwater environments. For *Geobacter* species, the transfer of electrons from the cell to the mineral via direct contact is favoured (Nevin and Lovley, 2000). This implies that the bacteria need to be motile to move across the mineral surface in order to have sufficient access to the electron-accepting mineral.

Here we provide data that suggest a completely different mechanism of microbial iron(III) reduction: The transfer of electrons from the bacterial cell to insoluble iron minerals via colloidal iron(III) particles (see figure 4.1). Such particles are found in many freshwater and marine habitats and a major part of the so-called soluble iron found through filtering of natural water samples is present in colloidal form (Tipping et al., 1981; Degueldre et al., 1996b; Wu et al., 2001). Colloidal iron particles may be stable in solutions of low ionic strength and at ambient temperatures (Stumm, 1977; Tipping and Ohnstad, 1984) and can be transported over long distances even in porous media (Tipping et al., 1993; Degueldre et al., 1996a; Ryan and Elimelech, 1996).

Colloids also show a strong Brownian movement due to their small size. Our proposed model of electron transfer from microorganisms to iron minerals (see figure 4.1) comprises a diffusive transport of colloidal particles to the cell wall, where they take up electrons and get reduced.
Subsequently, partially reduced colloidal particles detach from the cell wall and diffuse to an iron(III) mineral surface, where they release electrons after collision.

After electron transfer the colloid may detach and move back to the bacterial surface, where it can be reloaded with electrons. In this way, another reduction cycle starts. An abiotic transfer of electrons from Fe(II) to Fe(III) phases is a known process and the transformation of ferric hydroxide into spinel oxides, mixed valent iron(III)/iron(II) minerals, by electron transfer from adsorbed iron(II) was shown (Tronc et al., 1984; Tronc et al., 1992). Also it could be shown in recent experiments with labelled iron that electrons are readily transferred from adsorbed iron(II) to the underlying iron(III) mineral surface (Williams and Scherer, 2004). Other authors reported the presence of nano-sized magnetite precipitates on ferrihydrite surfaces, potentially formed by contact of the mineral surface with reduced colloidal particles (Hansel et al., 2003). In a recent study with *Shewanella putrefaciens*, a non-local electron transfer to bulk hematite surfaces was shown, which did not correlate with the attachment sites of the bacteria. In contrast, the mineral was reduced in a sphere-like form around the microorganisms (Rosso et al., 2003), which could be attributed to a random like transfer of electrons to the mineral surface via colloids.

**Figure 4.1:** Proposed mechanism of microbial iron(III) reduction by electron-shuttling via ferric colloids. The colloidal iron particles take up electrons from the bacterial surface and the colloids are transported to the mineral surface via Brownian movement. Subsequently, the coarse iron(III) mineral is partially reduced to iron(II) and the iron colloids are ready for another electron transfer cycle.
4.2 Experimental Procedures

4.2.1 Cell suspension experiments

*Geobacter metallireducens* was cultivated in 500 ml of a low salt medium consisting of 0.1 g/L NaCl, 0.04 g/L MgCl$_2$ * 6H$_2$O, 0.02 g/L K$_2$HPO$_4$, 0.025 g/L NH$_4$Cl, 0.05 g/L KCl, 0.015 g/L CaCl$_2$ * 2H$_2$O. The medium was supplemented with 1 ml/L of trace element solution SL10 (Widdel et al., 1983), 1 ml/L of a selenite-wolframate solution (Widdel, 1980), and 0.5 ml/L of a 7-vitamine-solution (Widdel and Pfennig, 1981). Sulphate (10 µM) was added as sulphur source and the medium was buffered with Tris/HCl (10 mM) at a pH of 7.2. Sodium acetate (10 mM) was added as substrate and 50 mM ferric citrate as electron acceptor. The cells were incubated at 30 °C in the dark and after growth to late exponential phase and harvested after centrifugation at 6500 rpm for 15 minutes. The pellet was resuspended in the low salt medium in a glove box and again centrifuged. The cells were finally suspended in 25 ml medium, added to 50 ml serum bottles to a final density of about 5 x 10$^9$ cells per ml, flushed with nitrogen gas, and sealed with butyl rubber stoppers. Sodium acetate was added at 10 mM as growth substrate together with one of the different forms of Fe(III) electron acceptors, i. e. amorphous ferrihydrite (800 µM [Fe]) in the first experiment (see figure 4.2) or 30 mM in the second experiment (see figure 4.3), colloidal ferrihydrite (800 µM [Fe]), colloidal Prussian Blue (0.44 or 0.87 mM) (Aldrich, Taufkirchen, Germany), and soluble ferric citrate (800 µM [Fe]). The amorphous ferrihydrite was prepared as described in (Lovley and Phillips, 1986). Colloidal ferrihydrite was prepared as described in (Leibl et al., 1999) and stored at 4 °C in order to slow down the coagulation of the particles to bigger aggregates. The surface areas of the colloidal ferrihydrite (253 m$^2$/g) and the non-colloidal ferrihydrite (309 m$^2$/g) were determined by N$_2$-adsorption using the BET method. The average diameter of the ferrihydrite colloids was 200 nm and the diameter of the Prussian Blue colloids was between 50 and 100 nm as determined by raster electron microscopy (REM). Soluble Fe(III) citrate was added from an anoxic stock solution.

4.2.2 Iron analysis

Iron reduction was monitored through the increase in ferrous iron concentrations using the ferrozine method according to (Stookey, 1970). The reagent reacts with dissolved ferrous iron and with Prussian White to form a purple complex. It does not react with any iron(III) species, including iron(III) colloids, which we tested in previous control experiments. The samples were taken through the stoppers of the culturing bottles and immediately added into a 1 M solution of HCl. After mixing and incubation for 10 min., the samples were added to the ferrozine reagent and incubated another 10 min.. After that, the samples were centrifuged for 5 min. at 14500 rpm and the iron(II) concentration in the supernatant was determined with a spectrophotometer at 562 nm.
4.3 Results and Discussion

We performed cell suspension experiments with washed *Geobacter metallireducens* cells and investigated the reduction of colloidal ferrihydrite and colloidal Prussian Blue particles (Fe₄[(Fe(CN)₆]₃, a blue pigment consisting of an iron(III) and iron(II) complex with cyanide, which were used as electron acceptors. A special low salt medium was used in order to keep the colloids in stable suspension. In a first set of experiments, the microbial reduction of ferric citrate, colloidal ferrihydrite or non-colloidal ferrihydrite, added as the sole electron acceptors, were compared (figure 4.2).

Colloidal ferrihydrite particles were reduced much faster than non-colloidal ferrihydrite despite a slightly lower BET-specific surface area of the colloidal ferrihydrite (253 m²/g) compared to non-colloidal ferrihydrite (309 m²/g). Considering the microporous nature of the coarse ferrihydrite leading to a large fraction of inaccessible surface area, this finding is in line with other studies that found an increase in Fe(III) reduction rates with increasing surface areas of iron(III) minerals irrespective of the type of mineral (Roden and Zachara, 1996). In our experiments the reduction of colloidal ferrihydrite was only 8.5 times slower than the reduction of an equimolar ferric citrate solution. Our results show that colloidal iron(III) can be an efficient electron acceptor in artificial groundwater.

![Figure 4.2](image-url)

*Figure 4.2:* Reduction of dissolved ferric citrate (■), colloidal ferrihydrite (●, 22 m²/l), and non-colloidal ferrihydrite (▲, 26 m²/l) in cell suspension experiments with washed *Geobacter metallireducens* cells. The electron acceptors were added at a concentration of 800 µM [Fe]. The iron(II) concentration in solution is plotted over time.
medium free of larger particles and, possibly, also in natural anoxic habitats. In a second set of experiments, we investigated whether iron colloids can transfer electrons to non-colloidal ferric minerals and thus act as an electron-mediator rather than as a terminal electron acceptor. Suspensions of washed *Geobacter metallireducens* cells were prepared in the presence of both colloidal ferrihydrite and coarse ferrihydrite mineral. In these systems, the presence of colloidal ferrihydrite had no measurable effect on the reduction of coarse ferrihydrite mineral (data not shown). Depletion of the colloids from solution by adsorption onto the ferrihydrite surface, as indicated by the disappearance of the red colour of the colloids in the supernatant, may explain the lack of enhanced electron transfer under the given conditions. This result also implies that the concentration of total dissolved iron(III) was not markedly increased due to a potentially higher solubility of small-sized colloidal particles. Previous studies did not find a clear correlation between iron oxide particle size between 100 nm and 700 nm and solubility of ferric iron (Hsu and Marion, 1985).

In order to further test our hypothesis of electron transfer mediation by iron colloids, we performed cell suspension experiments with bulk ferrihydrite and the colloidal Fe(III)/Fe(II) complex Prussian Blue. In contrast to ferrihydrite colloids, a significant fraction of the blue colloid did not adsorb onto the ferrihydrite surface but remained in suspension, as could be seen from the blue colour of the supernatant of the medium in the culturing bottles. The reduction of coarse ferrihydrite was much faster in the Prussian Blue amended bottles than with ferrihydrite alone (figure 4.3).

![Reduction of coarse ferrihydrite (30 mM, 990 m^2/l) in the absence (●) or presence (▲ and ▼) of the colloidal pigment Prussian Blue in cell suspension experiments with washed *Geobacter metallireducens* cells. The Prussian Blue was added at a concentration of 0.44 mM (▲) or 0.87 mM [Fe] (▼) and the increase in dissolved Fe(II) concentrations was measured over time. The control experiment was performed in the absence of cells.](image)

**Figure 4.3:** Reduction of coarse ferrihydrite (30 mM, 990 m^2/l) in the absence (●) or presence (▲ and ▼) of the colloidal pigment Prussian Blue in cell suspension experiments with washed *Geobacter metallireducens* cells. The Prussian Blue was added at a concentration of 0.44 mM (▲) or 0.87 mM [Fe] (▼) and the increase in dissolved Fe(II) concentrations was measured over time. The control experiment was performed in the absence of cells. (▲).
Complete reduction of Prussian Blue to Prussian White (Fe$_2$[Fe(CN)$_6$]) requires the transfer of four electrons (see equation 4.1).

**Equation 4.1**

$$\begin{align*}
(Fe_2[Fe(CN)_6])_3^+ + 4e^- & \rightarrow \\
2(Fe_2[Fe(CN)_6])_2 + [Fe(CN)_4]^{3-}
\end{align*}$$

Thus, the net effect of the increased iron(III) reduction rate through Prussian Blue colloids can be assessed after 46 h of incubation, when the total amount of ferrous iron produced was larger than the oxidation capacity of the Prussian Blue in the assay. The initial rate constants for the reduction of the Prussian Blue were $k \approx 5.5 \times 10^{-2}$ (h$^{-1}$) with the addition of 0.44 mM colloidal Prussian Blue and $k \approx 7.1 \times 10^{-2}$ (h$^{-1}$) with the addition of 0.87 mM colloidal Prussian Blue. The rates dropped about two orders of magnitude after complete reduction of the Prussian Blue to $k \approx 6.3 \times 10^{-4}$ (h$^{-1}$) with 0.44 mM Prussian Blue and $k \approx 7.8 \times 10^{-4}$ (h$^{-1}$) with 0.87 mM Prussian Blue after 46 or 69 hours of incubation, respectively. These colloid-enhanced rate constants are more than twice as large as the rate constant for reduction of ferrihydrite alone ($k \approx 3.0 \times 10^{-4}$ (h$^{-1}$)). After 46 to 94 hours the catalytic effect of the added colloids on the reduction of ferrihydrite gave a surplus of 0.7 to 1.0 mM ferrous iron after subtraction of the iron(II) deriving from the reduction of the Prussian Blue alone. These results show that the poorly adsorbing Prussian Blue colloids on the one hand are very effective electron acceptors and on the other hand are able to mediate the electron transfer to coarse ferrihydrite mineral surfaces.

The chemical transfer of electrons to the ferrihydrite surface by the formed ferrocyanide ion ([Fe(CN)$_6$]$^{4-}$) (see equation 4.1) is not feasible, because the redox potential of the system [Fe(CN)$_6$]$^{4-}$/[Fe(CN)$_6$]$^{3-}$ at pH 7 is $E_0' = +430$ mV and thus much higher than the redox potential of the couple ferrihydrite/Fe$^{2+}$, which is in the range of $E_0' = -100$ to $+100$ mV (data from Straub et al., 2001). We also could show by an experiment with ferrihydrite and addition of potassium hexacyanoferrate ($K_4[Fe(CN)_6]$) under anoxic conditions, that there was no increase in Fe(II) concentration over time measurable with ferrozine (data not shown).

We could show in our lab-experiments that colloidal Prussian Blue acts as an electron-shuttle at the given conditions. However, the role of natural occurring iron colloids in the transfer of electrons in groundwater is still to be elucidated. Usage of poorly adsorptive iron colloids in natural habitats could be an alternative strategy of microorganisms to transfer electrons to ferric minerals besides the use of humic substances as electron shuttles.
4.4 References


In this thesis, different aspects of dissimilatory iron(III) reduction were investigated, in order to improve the knowledge about this group of organisms. Since iron reducers are said to be widely spread in diverse environments and are one of the dominant classes of microorganisms in anoxic environments, further progress in understanding the basic mechanisms of dissimilatory iron reduction and on different metabolic pathways seems to be crucial (Coates et al., 1996; Konhauser, 1998; Venkateswaran et al., 1999; Snoeyenbos-West et al., 2000; Lovley et al., 2004).

As at many contaminated aquifers usually anoxic conditions prevail, the anaerobic degradation of pollutants is the most important process for natural attenuation at most former industrial sites (Christensen et al., 2000). At many contaminated sites, aromatic hydrocarbons, such as benzene, toluene, ethylbenzene, and xylene (BTEX) are major pollutants, due to their high solubility in water and their toxicity. Iron reducers are said to have a significant share on the anaerobic degradation processes and at many polluted sites, contaminant degradation under iron-reducing conditions was measured or even iron-reducing bacteria were detected in the ground (Lovley et al., 1994; Kazumi et al., 1997; Anderson et al., 1998; Rooney-Varga et al., 1999; Johnson et al., 2003; Chakraborty and Coates, 2004). Also during the last years, the in situ degradation of anaerobic microbial attenuation processes was shown with techniques like stable isotope analysis or identification of specific metabolites (Richnow et al., 2003; Griebler et al., 2004; Heidrich et al., 2004). However, despite all the proves of anaerobic degradation of contaminants under iron-reducing conditions, still there are only two Geobacter strains available in pure culture, which are able to use toluene as sole carbon and electron source for growth, Geobacter metallireducens, and Geobacter grbiciae (Lovley et al., 1993; Coates et al., 2001). Therefore, for the understanding of degradation pathways and in order to assess the metabolic versatility of iron reducers to degrade aromatic hydrocarbons, the isolation of new contaminant-oxidizing strains is necessary.

In this work, part of this gap of knowledge could be filled by the enrichment and isolation of new dissimilatory iron-reducing and BTEX-degrading cultures. A very efficient method for the enrichment of dissimilatory iron(III)-reducing microorganisms was successfully established. The method combines two strategies: First, the enrichment in the presence of a solid adsorber resin, which works as a substrate pool and keeps the concentration of the toxic contaminants in the medium at a moderately low level (Morasch et al., 2001). Second, the use of the well investigated electron-shuttling compound, AQDS, which mediates the transfer of electrons from the microbial cells to the mineral surface and thus facilitates iron(III) reduction (Lovley et al., 1996; Zachara et al., 1998; Royer et al., 2002). With this setup, new cultures for all different BTEX pollutants, namely benzene, toluene, ethylbenzene, and o-xylene, could be successfully enriched, using sludge from a contaminated site and amorphous ferrihydrite as electron acceptor. For the first time, sediment-free cultures of iron-reducing ethylbenzene and o-xylene degraders could be enriched. This new enrichments further support the hypothesis that iron-reducing microbes might play a significant role in processes of...
anaerobic contaminant degradation and bioremediation in anoxic environments. Also two new pure cultures could be isolated: One toluene-degrading strain and one strain that utilizes either toluene or o-xylene. These isolates might be used in order to study contaminant degradation pathways of the metabolic group of dissimilatory iron(III) reduction in more detail and to compare this to known pathways of other metabolic groups of anaerobic respiration, for example sulphate or nitrate reduction. The new strain that degraded toluene or o-xylene could be a valuable tool in order to study processes of substrate utilization preferences and substrate regulatory effects of the single contaminants, if both pollutants were added at different times or at the same time. This experiments could give very useful insight in metabolic processes at real contaminated sites, which usually are contaminated with a broad mixture of different compounds at the same time and where only few studies have been made so far (Heidrich et al., 2004; Meckenstock et al., 2004; Morasch et al., 2004). In reduction experiments with soil and groundwater samples from a contaminated site and different iron-reducing cultures could be shown that the microbes only used the amorphous iron(III) minerals, which were present in the soil. After reduction of the amorphous phases, which are extractable with weak hydrochloric acid, no further increase in Fe(II) concentration was measurable in all different culture bottles. The part of iron(III) minerals, which is extractable in 0.5 M HCl, in fact seems to correspond directly to the bioavailable portion of iron(III) minerals in the environment, at least as long as no electron-shuttling substances are present, e. g. humic acids. The chemical extraction of this part of iron minerals could thus be used very easily and quickly, to estimate the oxidation capacity of a given site for iron(III) reduction and BTEX degradation.

Apart from aromatic hydrocarbons, another group of contaminants is widely spread, especially at former gasworks sites or coke ovens, the cyanides and metal-cyanide complexes (Shifrin et al., 1996). The most important pollutants are iron-cyanide complexes like soluble ferrocyanide ([Fe(CN)₆]⁴⁻) and ferricyanide ([Fe(CN)₆]³⁻), or insoluble Prussian Blue (Fe₄[Fe(CN)₆]₃) (Theis et al., 1994; Mansfeldt and Dohrmann, 2001; Ghosh et al., 2004). Though the soluble complexes are readily degradable by anaerobic microbes, the use of the insoluble Prussian Blue by dissimilatory iron-reducing microorganisms was not reported so far (Boucabeille et al., 1994; Ebbs, 2004). Also the equilibrium constant for the reductive dissolution of Prussian Blue is very low and was determined as log K⁰ = -85.4 (Meeussen et al., 1992). This facts matches well with the situation that is found in the subsurface at many cyanide contaminated sites, where the Prussian Blue forms blue coatings on mineral surfaces and therefore not seems to be reduced and is probably only weakly dissolved in the aquifer (Ghosh et al., 1999). Thus, the Prussian Blue pigment, should be reduced similar to other insoluble and bulk iron minerals, for example ferricyanide.

In this work, not only the very efficient reduction of Prussian Blue by two phylogenetically distinct iron-reducing strains, i. e. Geobacter metallireducens and Shewanella alga BrY, but also the growth of both strains on this insoluble iron(III) phase, using it as the sole electron acceptor, could be shown. The fact, that both strains, which represent members from the two dominating iron-reducing genera, could utilize Prussian Blue as electron acceptor gives strong indication that this might be a general feature of most other dissimilatory iron-reducing organisms (Venkateswaran et al., 1999; Lovley...
et al., 2004). Moreover, the reduction of the solid Prussian Blue proceeded almost as quickly as the reduction of dissolved ferric citrate and still better than the reduction of ferrihydrite in the presence of the electron-shuttling substance AQDS. Also a natural sample of Prussian Blue coated sand from a contaminated site was reduced by *G. metallireducens* cells. For that reasons, the fact that Prussian Blue is found in the subsurface at most cyanide-contaminated sites might be due to sampling artefacts. In this thesis could be shown that the reduced form of Prussian Blue, the Prussian White (Fe₂[Fe(CN)₆]₃), immediately reacts with oxygen, when exposed to air, and forms Prussian Blue again. So, it is suggested to use different techniques for the sampling of cyanide contaminated soils, for example the use of nitrogen or argon flushed sampling cores or the transport of the soil columns in nitrogen-flushed gastight boxes, in order to prevent the reoxidation of Prussian White. The very effective and complete reduction of Prussian Blue to Prussian White gives strong indication that also in the environment at cyanide contaminated sites, the reduced form in fact might be the dominant species and not the oxidized form, as was reported several times (Ghosh et al., 1999; Ghosh et al., 2004).

One of the main aspects of this thesis dealt with the question, how the dissimilatory iron-reducing microorganisms are able to transfer electrons from the oxidation of different substrates to an insoluble iron mineral surface. The mechanisms of extracellular electron transfer are still subject to intensive discussions by different authors, whereas two hypotheses have been studied in more detail. The first favours a direct contact between cell and mineral surface and the transfer of the electrons with membrane born iron reductases. Several authors suggest this mechanism for the genus *Geobacter* (Nevin and Lovley, 2000, 2002a; Lovley et al., 2004). Another hypothesis includes soluble helper or mediator molecules into the mechanism of extracellular electron transfer, which are redox active molecules that take over electrons at the cellular surface whilst they get reduced. After that, they diffuse to the mineral surface and release the electrons again whilst they are oxidized again and another redox cycle may start. These mediators on one hand could be synthesized by the cells themselves or on the other hand could be naturally abundant or artificially added substances. Examples for the synthesized mediators are chelators or electron-shuttles, examples for the second class of molecules are humic acids or AQDS. Both classes have been shown to greatly enhance microbial iron(III) reduction when added to culture bottles (Lovley et al., 1994; Dobbin et al., 1995; Lovley and Woodward, 1996; Zachara et al., 1998; Newman and Kolter, 2000; Nevin and Lovley, 2002b). By some authors, the electron-shuttling mechanism is the most accepted for the genus *Shewanella* (Lovley et al., 2004). As the mechanisms have certain disadvantages, for example, when direct contact is needed the bacteria always have to take care that they are able to access enough mineral surfaces that have not been reduced yet or are not covered from different coatings. In order to achieve this, the bacteria need to become motile. Indications for such adaptation were found in one single case (Childers et al., 2002). The main problem, when using soluble electron-shuttling substances is the potential risk of loosing the mediators through groundwater flow or diffusion. If the bacteria would loose an electron shuttle, which they synthesized in the cell before, this would mean a great loss of energy in this usually nutrient poor groundwater environment. The use of naturally occurring humic acids would be much better, because the bacteria do not have to use energy for the production of the shuttles. However, some aquifer systems are very poor of humic
substances. Another point to consider is that some humics-reducing bacteria were not able to use iron(III) as sole electron acceptor, and therefore cannot be considered as dissimilatory iron-reducing microorganisms (Benz et al., 1998; Cervantes et al., 2002).

Keeping in mind the different problems the microbes would have to face with the above-mentioned mechanisms, completely different mechanisms seem conceivable. In this work, the hypothesis was investigated, if iron (III) colloids that are highly abundant in aquifers could act as potential electron shuttles from the bacterial cell to the mineral surface (Tipping et al., 1981; Tipping et al., 1993; Degueldre et al., 2000). After successful preparation of the ferrihydrite colloids and adaptation of the culture medium to low ionic strength, in order to stabilize the colloids during the experiments, the iron(III) colloids were shown to be very effective electron acceptors for the dissimilatory iron-reducing bacterium *G. metallireducens*. The colloidal ferrihydrite particles were reduced in much higher rates than bulk ferrihydrite. This implies, that also in nature the highly abundant iron(III) colloids might be reduced. In the following, the mediating capacity of colloidal Prussian Blue particles, which were added to ferrihydrite containing cultures, greatly enhanced iron(III) reduction by *Geobacter* cells. This gives strong indication that also in the environment, the naturally occurring colloidal iron particles could work as electron shuttles between bacterium and mineral surface. The transfer of electrons via a solid redox-active particle would be a completely new way of using an abiotic mediator for a metabolic process. However, the proof that natural occurring colloids also work as mediators between microbial cells and iron(III) mineral surfaces has still to be made.
5.1 References


Appendix
BTEX-measurement of experiment figure 2.2

BTEX-degradation experiment with sediment-free enrichment cultures

BTEX measurements

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BTEX-measurement of experiment figure 2.2, continued

BTEX-degradation experiment with sediment-free enrichment cultures.

BTEX measurements

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Iron measurement of experiment figure 2.2

BTEX degradation experiment with sediment-free enrichment cultures

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Iron measurement of experiment figure 2.2, continued

BTEX degradation experiment with sediment-free enrichment cultures

Fe(II) measurements

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Characterization of contaminated soil material and groundwater from Brand (see chapter 2.2)

Well GWM HT 06
Water level: 10,23m
pH-value: 6,64
Electric conductivity: 537µS/cm
Redox current: -64 (+218) mV = 154mV
Oxygen content: 0,17mg/l

Smell: aromatic
Colour: colourless

Fe(II)-content: 1,725 mM

Soil liners:
Liner 1: 11,35m until 11,95m
Liner 2: 12,65m until 13,20m
Liner 3: 13,9m until 14,4m

Carbon content:

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<th>NPOC (mg/l)</th>
<th>average:</th>
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Ionenchromatography:

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Characterization of contaminated groundwater from Brand (see chapter 2.2)

### BTEX: concentration $[\mu M]$

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<th>MTBE</th>
<th>trans-DCE</th>
<th>cis-DCE</th>
<th>Chloroform</th>
<th>Tetrachlor-kohlenstoff</th>
<th>1,2-Dichlor-ethan</th>
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<th>Isopropyl-benzene</th>
<th>Propyl-benzene</th>
<th>1,3,5-TMB</th>
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### PAH

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Iron measurement of experiment chapter 2.2 (table 2.2)

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Iron measurement of experiment figure 3.1

Reduction of Prussian Blue by *G. metallireducens* or *S. alga* BrY

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### Iron measurement of experiment from figure 3.3

| Incubation time [h] | Iron(II) concentration [µM] | Citrate 1  | Citrate 2  | Prussian Blue 1 | Prussian Blue 2 | Prussian Blue 3 | Ferrihydrite 1 | Ferrihydrite 2 | Ferrihydrite 3 | Ferrihydrite + AQDS 1 | Ferrihydrite + AQDS 2 | Ferrihydrite + AQDS 3 |
|---------------------|-----------------------------|-----------|-----------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|
| 0                   | 1020                        | 1080      | 700       | 770            | 750            | 730            | 700            | 700            | 690            | 680            | 880            | 690            | 680            | 880            |
| 2                   | 2970                        | 3530      | 1830      | 1700           | 1620           | 780            | 720            | 650            | 1160           | 1010           | 1400           | 1710           | 2430           | 1960           |
| 4                   | 3940                        | 4270      | 2600      | 2480           | 2500           | 770            | 760            | 700            | 1710           | 2430           | 1960           | 2450           | 2450           | 2480           |
| 6                   | 3720                        | 4390      | 3690      | 3500           | 3390           | 720            | 700            | 720            | 2450           | 2450           | 2480           | 2450           | 2450           | 2480           |
| 8                   | 3650                        | 3960      | 4420      | 4310           | 3840           | 760            | 930            | 710            | 2830           | 3010           | 3060           | 2830           | 3010           | 3060           |
| 21.5                | 4470                        | 5330      | 3980      | 3810           | 3720           | 770            | 750            | 760            | 3710           | 3440           | 3330           | 3710           | 3700           | 3440           |
| 26                  | 4270                        | 5120      | 4430      | 4280           | 4250           | 740            | 730            | 730            | 3710           | 3700           | 3440           | 3710           | 3700           | 3440           |
| 30                  | 4680                        | 5540      | 5090      | 5030           | 4910           | 760            | 760            | 710            | 3950           | 3460           | 3790           | 3950           | 3460           | 3790           |
| 45.5                | 4690                        | 4770      | 4670      | 4740           | 4110           | 880            | 780            | 800            | 4640           | 4450           | 4270           | 4640           | 4450           | 4270           |
| 54                  |                             |           |           | 890            | 860            | 800            | 4520           | 4320           | 4360           |               |               |                |               |                |                |
| 72                  |                             |           |           | 900            | 910            | 850            | 4770           | 4600           | 4530           |               |               |                |               |                |                |
| 144                 |                             |           |           | 1040           | 710            | 850            | 4280           | 4920           | 4540           |               |               |                |               |                |                |
Iron measurement from experiment figure 4.2

1. Ferric citrate

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2. Colloidal ferrihydrite

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3. Non-colloidal ferrihydrite

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Iron measurement from experiment figure 4.3

Influence of colloidal Prussian Blue on the reduction of bulk ferrihydrite by *Geobacter metallireducens* cells in cell suspension experiments

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<th>Ferrihydrite + Prussian Blue 0.5mM 2</th>
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Curriculum Vitae

Michael Jahn

born 04. 02. 1973 in Fulda


03/2001: Diploma in Biology


07/2003-03/2005: Tübinger Grundwasserforschungsinstitut, Prof. Teutsch (TGF)

10/2001-08/2005: Promotion in Angewandter Geowissenschaft am Lehrstuhl für Umweltmineralogie bei PD Dr. habil. Rainer U. Meckenstock und Prof. Dr. Stefan B. Haderlein. Title of the thesis: “Microbial dissimilatory iron(III) reduction: Studies on the mechanism and on processes of environmental relevance.”

During the Ph.D., I was working on the project “Natural attenuation (NA) and Enhanced Natural Attenuation (ENA) an typischen Mineralölstandorten am Beispiel Brand und Niedergörsdorf.”