Evaluation of Direct and Indirect Mechanisms of Microbial Influence on Natural Organohalogen Formation

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To my grandparents

whose destiny always reminded me

that the possibility to work on a PhD thesis is a luxury and can

only be considered tough on a relative scale.

“There are known knowns; there are things we know we know.

We also know there are known unknowns; that is to say we know there are some things we do not know.

But there are also unknown unknowns – the ones we don't know we don't know.”

Donald Rumsfeld

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Summary

Volatile halogenated organic compounds (VOX) are known as potent pollutants, ozone-destroying agents and carcinogens of anthropogenic origin - neglecting that many VOX also have natural sources. While VOX are produced naturally through combustion of biomass (e.g. forest fires) and volcanic eruptions, soils also contribute significantly to the emission of several VOX on a global scale. Previous laboratory studies constrained the biogeochemical conditions under which soils can emit VOX, such as acidic pH combined with the presence of chloride, catalytic amounts of iron (Fe) and organic radicals or the activity of halophilic microorganisms in salt lake sediments, which are naturally rich in halides. However, detailed mechanisms of VOX formation in soil remain to be elucidated. Another question that is still open is if, how, and to which extent microorganisms can influence natural VOX formation in terrestrial environments. Therefore, the goals of this thesis were I) to determine whether Fe(III)-reducing microorganisms can indirectly stimulate natural VOX formation by producing reactive Fe phases and organic radicals that initiate abiotic reactions involved in VOX formation, II) to elucidate the role of microorganisms that express halogenating enzymes in salt lake sediments in direct production of VOX, and III) to investigate whether VOX-degrading, dehalorespiring microorganisms, whom naturally produced organohalogens could serve as substrates, are present in salt lake sediments.

One major finding of this thesis is that the potential of microorganisms to reduce Fe(III) at acidic pH (unlike under neutral pH) is not necessarily coupled to their ability to reduce humic substances and to initiate the formation of organic radicals. Further results of this thesis call into question whether, according to our hypothesis, microorganisms play any indirect role in natural VOX formation while it remains hard to judge their direct influence on this process: the addition of neutrophilic Fe(III)-reducing bacteria to a methyl chloride-emitting salt lake sediment sample did not lead to an increase in production of the VOX methyl chloride. However, Fe(III)-reducing as well as Fe(II)-oxidizing and humic substance-reducing microorganisms were

enriched from different salt lake sediments and shown to be active in medium with up to 5 M NaCl, suggesting that independent of their contribution to VOX emissions, these groups of microorganisms probably play an ecological role in salt lake sediments.

Furthermore, it was found that the diversity of microbial genes encoding halogenating enzymes that can produce VOX as determined by DNA sequence variation is too high to study them by PCR-based screening with only one set of degenerate primers. To address this problem, multiple sets of degenerate primers were designed and successfully used to screen selected subgroups of halogenation genes. Halogenation genes were not found in any of the 18 salt lake sediments studied.

One conclusion from this result is that even though the products of these genes likely contribute to global VOX emissions, it is difficult to elucidate the identity of the most significant enzymes, the organisms that harbor them and the conditions under which they are expressed. Furthermore, no signs of presence or activity of chlorinated ethene-respiring bacteria could be detected by PCR and microcosm experiments, suggesting VOX-degrading microorganisms are not important in salt lake sediments.

Although we are still beginning to understand the influence of microorganisms on natural organohalogen formation (and degradation), this work suggests that in salt lake sediments, microorganisms seem to have little role in either production or degradation of VOX.

Zusammenfassung

Flüchtige halogenierte organische Verbindungen (VOX) sind als schädliche, ozonzerstörende Substanzen und Karzinogene anthropogener Herkunft bekannt - wodurch vernachlässigt wird, dass viele VOX auch natürliche Quellen haben. Während VOX bei der Verbrennung von Biomasse (z.B. bei Waldbränden) und bei Vulkanausbrüchen auf natürliche Weise produziert werden, so tragen auch Böden in einem Maßstab zur Emission verschiedener VOX bei, der von globaler Bedeutung ist. Frühere Laborstudien haben die biogeochemischen Bedingungen, unter welchen Böden VOX emittieren, genauer definiert. Hierzu gehören z.B. ein saurer pH in Kombination mit Anwesenheit von Chlorid, katalytischen Mengen an Eisen (Fe) und organischen Radikalen oder die Aktivität von salzliebenden Mikroorganismen in Salzseesedimenten, die natürlicherweise reich an Halogeniden sind. Die genauen Mechanismen, die zur VOX-Bildung im Boden führen, bleiben jedoch noch zu erforschen. Des Weiteren ist die Frage offen, ob, wie und in welchem Ausmaß Mikroorganismen die natürliche Bildung von VOX in terrestrischer Umgebung beeinflussen können. Aus diesem Grund bestanden die Ziele dieser Arbeit darin I) herauszufinden, ob Fe(III)-reduzierende Mikroorganismen die natürliche Bildung von VOX auf indirekte Weise stimulieren können, indem sie reaktive Eisenphasen und organische Radikale produzieren, die wiederum abiotische Reaktionen in Gang setzen, welche letztendlich zur VOX-Bildung führen, II) die Rolle von Mikroorganismen zu klären, welche in Salzseesedimenten halogenierende Enzyme produzieren, die auf direktem Wege VOX produzieren und III) zu untersuchen, ob VOX-abbauende, dehalorespirierende Mikroorganismen, denen auf natürliche Weise produzierte Organohalogene als Substrate dienen könnten, in Salzseesedimenten vorhanden sind.

Ein wichtiges Ergebnis dieser Arbeit besteht darin, dass das Potential von Mikroorganismen, unter sauren pH-Bedingungen Fe(III) zu reduzieren im Gegensatz zu demselben Vorgang bei neutralem pH nicht notwendigerweise mit der Fähigkeit einhergeht, Huminstoffe zu reduzieren

und hierdurch die Bildung organischer Radikale zu initiieren. Weitere Ergebnisse dieser Arbeit stellen infrage, ob Mikroorganismen entsprechend unserer Hypothese eine indirekte Rolle bei der natürlichen VOX-Bildung spielen, während es schwer bleibt, ihren direkten Einfluss auf diesen Prozess zu beurteilen: so führte die Zugabe neutrophiler Fe(III)-reduzierender Bakterien zu einem Methylchlorid emittierenden Salzseesediment nicht zu einem Anstieg der Bildung des Organohalogens Methylchlorid. Allerdings konnten sowohl Fe(III)-reduzierende als auch Fe(II)-oxidierende und Huminstoff-reduzierende Mikroorganismen aus Salzseesedimenten angereichert und deren Aktivität in Medium mit einem Salzgehalt von bis zu 5 M NaCl nachgewiesen werden. Unabhängig davon, ob diese Organismen zu VOX-Emissionen beitragen, legt dieses Ergebnis nahe, dass diese mikrobiellen Gruppen in Salzseesedimenten wahrscheinlich von ökologischer Bedeutung sind.

Darüber hinaus hat sich herausgestellt, dass die Diversität von Genen, welche für halogenierende Enzyme codieren, die VOX produzieren können, auf der Basis von DNA-Sequenzvariationen zu groß ist, um diese mit einem PCR-Screen mit nur einem Set von degenerierten Primern zu untersuchen. Um dieses Problem anzugehen, habe ich mehrere Sets degenerierter Primer designt und erfolgreich dazu verwendet, Untergruppen von Halogenierungsgenen zu screenen. In keinem der 18 untersuchten Salzseesedimente wurden irgendwelche Halogenierungsgene gefunden.

Eine Schlussfolgerung aus diesem Ergebnis ist, dass obwohl die Produkte dieser Gene sehr wahrscheinlich zu globalen VOX-Emissionen beitragen, es sehr schwierig ist, die entsprechenden Gene sowie die Organismen, aus denen sie stammen und die Bedingungen, unter denen sie exprimiert werden, zu identifizieren.

Des Weiteren konnten weder durch PCR noch durch Mikrokosmosexperimente irgendwelche Anzeichen der Anwesenheit oder der Aktivität von Mikroorganismen detektiert werden, welche chlorierte Ethene dehalogenieren, was darauf hindeutet, dass in Salzseesedimenten VOX-abbauende Mikroorganismen ökologisch nicht von Bedeutung sind.

5 Zusammenfassung

Obwohl wir immer noch am Anfang stehen, den Einfluss von Mikroorganismen auf die natürliche Bildung und den Abbau von Organohalogenen zu verstehen, liefert diese Arbeit erste Anzeichen dafür, dass Mikroorganismen in Salzseesedimenten sowohl bei der Bildung als auch beim Abbau von VOX nur eine untergeordnete Rolle zu spielen scheinen.

Introduction

3.1 Biogeochemical cycling of chlorine

In nature, the 17th element of the periodic table occurs in two fundamentally different forms: inorganic chloride (Cl_{in}) and organically bound chlorine (Cl_{org}). The various kinds of transformation processess that can convert Cl from one form into the other are indicated in figure 1. Together with the sizes of and the fluxes between the different Cl pools, these processes shall be the focus of this section.

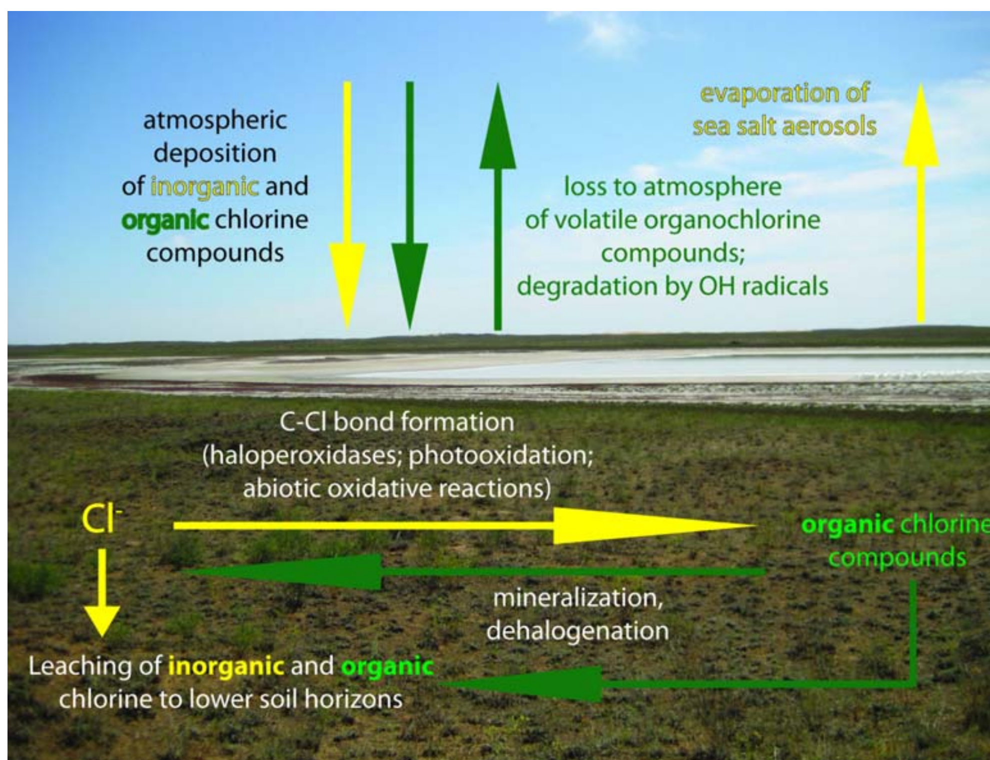

Figure 1: *The natural chlorine cycle with transformation processes of inorganic Cl^- depicted in yellow and organically bound chlorine in green. Modified from (Winterton, 2000)*

Mainly originating from sea spray, between 1 and 100 kg ha^{-1} of Cl_{in} are deposited on land and water per year (Oberg, 2002). According to general understanding, the mobility of Cl_{in} in soil is

controlled by water movements (Oberg, 2003) and only interfered by temporary uptake by plants (Lovett et al., 2005). Origins of organically bound chlorine in soil comprise rain, throughfall, litterfall and intrinsic formation in soil (Oberg and Gron, 1998). In more recent studies, it has been found that both haloperoxidase-like biotic activity (Asplund et al., 1993; Bastviken et al., 2009; Rohlenova et al., 2009) and abiotic reactions (Huber et al., 2009; Keppler et al., 2006; Keppler et al., 2002; Keppler et al., 2000) can mediate the transformation of Cl_{in} to Cl_{org} in the terrestrial environment. The formation of volatile chlorine-containing organic compounds such as chloromethane and chloroform by these reactions represents one major loss process of Cl_{in} from soil. Further loss processes are leaching of Cl_{in} to deeper soil horizons and biomass burning. On the other side, the mineralization of Cl_{org} terminates in the production of Cl_{in} (Oberg, 2002). In terms of budgets, the total amount of Cl_{org} stored in soils ranges between a few micrograms and five milligrams compared to 25 to 100 micrograms of Cl_{in} per g of dry soil (Graedel and Keene, 1996; Oberg, 2003). While these numbers represent averages from several sampling sites around the globe, much less information is available about fluxes between the two pools of chlorine in soil. In a recent study, rates of Cl_{in} transformation into Cl_{org} in a Swedish catchment covered by coniferous forest were estimated by following the fate of $^{36}Cl_{in}$ in different soil fractions over time (Bastviken et al., 2007). Based on the results of this study, a transformation rate of Cl_{in} to Cl_{org} of 1.6 µg per g of dry soil and year (or of 25 % of the total wet deposition of Cl_{in}) was calculated for this catchment. In a different study, it was shown that the residence time of Cl in soils of the temperate climate zone is about fivefold higher if both Cl_{org} and Cl_{in} are taken into account than if Cl_{in} is considered alone (Thiry et al., 2011). This suggests a much lower turnover for Cl_{org} compared to Cl_{in} in the terrestrial compartment, which has been confirmed by another study of Cl fluxes in a Swedish catchment (Svensson et al., 2007). Moving on to the atmosphere, terrestrial and marine emissions of volatile organochlorines represent major sources of atmospheric Cl_{org}. Degradation by •OH radicals and photolysis act as major sinks for these compounds (Montzka and Reimann, 2010). Despite these degradation reactions, the stratospheric lifetime of organochlorines can extend to several decades (Montzka

and Reimann, 2010) due to the chemical recalcitrance of the C-Cl bond. Compared to 2.4 x 10^{10} t of Cl stored in the pedo- and 5.7 x 10^{6} t of Cl stored in the atmosphere, the total amount of 2.6 x 10^{16} t of Cl that can be found in the global ocean (Winterton, 2000) seems impressive. However, most of the oceanic Cl occurs as Cl_{in}, for which a mean residence time of 10^{8} years has been estimated (Pilson, 1998), indicating that oceanic Cl is not very reactive. The discovery of different kinds of halogenating enzymes in marine organisms (Edwards et al., 2004; Eustaquio et al., 2008) as well as the identification of various chlorinated organic compounds released from the sea (Gribble, 2003; Moore, 2003) show that some transformation of Cl_{in} to Cl_{org} does take place in the marine environment, too.

The use of chlorinated hydrocarbons as terminal electron acceptor by certain microorganisms, a process called reductive dechlorination, represents a biotically mediated way for transformation of Cl_{org} to Cl_{in} in aquatic systems (Smidt and de Vos, 2004). Up to date, this process has mainly been studied in contaminated aquifers (Hendrickson et al., 2002; Moe et al., 2009; Scheutz et al., 2008). However, the discovery of *Dehalococcoides*-like Chloroflexi, who are known to perform this kind of reaction, in marine sediment (Inagaki et al., 2009) as well as in other pristine environments (Kittelmann and Friedrich, 2008; Loffler et al., 2000; Swan et al., 2010), indicates that reductive dechlorination could play a larger role than previously thought. In principle, the same kind of transformation reactions that has been described for conversions between organically bound chlorine and inorganic chloride in the different compartments take place for other halogens such as F, Br and I as well (Blasiak and Drennan, 2009; Montzka and Reimann, 2010; Schöler, 2003). However, due to much lower availability of these elements compared to Cl in nature (Harper et al., 2003; Tallmadge et al., 1964), biogeochemical cycling of these other other halogens has been studied to a much lesser extent.

3.2 Volatile organohalogens and their natural formation

Volatile organohalogens (VOX) are mono- or poly-fluorinated, -chlorinated, -brominated or -iodinated C1 and C2 hydrocarbons with relatively low boiling temperatures. Consequently, they occur to a large extent in the gaseous state already at room temperature (http://toxics.usgs.gov/definitions/vocs.html).

VOX play an important role in atmospheric chemical processes including stratospheric ozone destruction (Molina, 1974) and global warming (Montzka and Reimann, 2010; WMO, 2007). Some VOX such as vinyl chloride (C_2H_3Cl) and tetrachloroethene (C_2Cl_4) have also been shown to induce cancer (Maltoni et al., 1974; Stevens and Eisenmann, 1997). Due to these unwanted harmful properties, the industrial production of many compounds of this class has become prohibited or heavily restricted with the 1987 Montréal Protocol. Nevertheless, some VOX, e.g. tri- (C_2HCl_3) and tetrachloroethene (C_2Cl_4), are still industrially produced or form as byproducts of water chlorination (Ballschmiter, 2003). In contrast, e.g. methyl halides have mainly natural sources (Butler, 2000; Khalil and Rasmussen, 1999). With mixing ratios of up to 600 parts per trillion volume (ppt) and an estimated annual emission of five million tons, methyl chloride (CH_3Cl) is by far the most abundant organohalogen in the atmosphere (Harper and Hamilton, 2003).

Known sources for methyl chloride and other VOX comprise both abiotic processes such as biomass burning (Crutzen and Andreae, 1990) and volcanic emissions (Jordan et al., 2000), as well as biotically mediated reactions. Methyl transferase- and haloperoxidase-expressing algae (Class and Ballschmiter, 1988; Lovelock, 1975; Moore, 1977) and fungi (Frank et al., 1989; Harper, 1985; Turner, 1971) have been known for decades to emit volatile organohalogens in the marine and terrestrial environments, respectively. More recently, plants have been added to the plethora of VOX-releasing organisms (Hamilton et al., 2003; Teh et al., 2008; Wishkerman et al., 2008). Plants seem to enrich the global budget of these compounds both directly by producing halogenating enzymes (Saito et al., 2008) as well as indirectly by pectin that becomes subject to abiotic chlorination reactions (Hamilton et al., 2003). By analysis of $^{13}C/^{12}C$ stable

isotope ratios, which are particularly small for CH_3Cl released from tropical plants, it was estimated that this source could account for 30-50 % of all global emissions of CH_3Cl (Keppler et al., 2005; Saito et al., 2008).

Early diagenetic processes in soils and sediments represent an additional terrestrial source of (volatile) organohalogens (Schöler, 2003). The importance of this source for global budgets became clear about ten years ago, when Keppler et al. showed for the first time that soils and sediments can work as natural sources for organohalogen formation in absence of sunlight and microbial activity (Keppler et al., 2000). In the following years, several series of batch experiments revealed conditions under which abiotic halogenation reactions can take place. The carbon substrates used in these experiments included catechol, guaiacol, resorcin, hydroquinone, commercially available humic acids and freeze-dried and milled soil (Huber et al., 2009; Keppler et al., 2002; Keppler et al., 2000). The presence of Cl^- or Br^-, catalytic amounts of Fe^{3+} and a pH below 3.5 were found to be prerequisites for these reactions to occur (Huber et al., 2009; Keppler et al., 2002). In different batch experiments, the formation of CH_3Cl, C_2H_3Cl, C_2H_5Cl, C_3H_7Cl, C_4H_9Cl, $C_2H_3Cl_3$, C_2HCl_3, C_2Cl_4, C_2HCl and CHX_3 (X = Cl or Br) has been observed (Huber et al., 2009; Keppler et al., 2006; Keppler et al., 2002; Keppler et al., 2000). The addition of hydrogen peroxide in the mM range was found to lead to a substantial increase in the VOX yield in several experiments (Huber et al., 2009; Keppler et al., 2006; Keppler et al., 2002). For this reason, it has been speculated that hydroxyl radicals formed by the Fenton reaction play a crucial role in this process (Schöler, 2003). Carbon suboxide (O=C=C=C=O) was identified as an intermediate during oxidative catechol degradation under the conditions applied in the VOX-forming batch experiments (Huber et al., 2007). On the basis of this finding, multiple hydroxylation followed by symmetrical cleavage of the C-C bonds between the hydroxyl groups has been suggested as a possible key reaction leading to the formation of reactive C3 compounds from phenolic C6 rings (Huber et al., 2007). However, mechanistic details of this reaction remain to be elucidated.

In addition to this abiotic way of VOX formation in soils and sediments, similar compounds could also arise from haloperoxidase-mediated halogenation of organic material in soil (Asplund et al., 1993) followed by partial degradation of larger halogenated organic molecules. In summary, some scattered pieces of information about possible ways of natural VOX formation in soils are available. However, it is still unclear which role soils play in the global cycling of VOX and in particular it is not known if, how and to which extent microorganisms influence VOX formation in soils.

3.3 Microbial reduction of Fe(III) and humic substances

Dissimilatory Fe(III)-reducing prokaryotes are phylogenetically widespread and include representatives of both bacterial and archaeal phyla (Straub, 2011). They have been found in all subdivisions of the Proteobacteria, the Firmicutes and separate lineages such as *Geothrix fermentans* or *Geovibrio ferrireducens* as well as within the Eury- and the Crenarchaeota (Straub, 2011). In addition to dissimilatory Fe(III) reduction, where Fe(III) serves as an electron acceptor for anaerobic respiration, some bacteria can also reduce Fe(III) during fermentative growth (Straub, 2011).

Dissimilatory Fe(III) reducing microorganisms have been isolated or detected by molecular techniques in many different pristine and contaminated environments including freshwater and marine sediments, wetlands, soils, aquifers, the deep subsurface, hydrothermal vents, hot springs (Lovley et al., 2004) and acid mine drainage systems (Johnson, 1998). At neutral pH, the redox potential of the Fe(III) in poorly crystalline Fe(III) oxides ranges between +100 and -100 mV (Canfield et al., 2005). This renders the use of Fe(III) as a terminal electron acceptor for anaerobic respiration in environmental systems of circumneutral pH thermodynamically less favorable compared to O_2, NO_3^- and Mn(IV), but more favorable than SO_4^{2-} and CO_2 (Canfield and Thamdrup, 2009).

Neutrophilic Fe(III)-reducers have to cope with a poorly soluble electron acceptor, i.e. Fe(III) minerals. To overcome this problem, three strategies for electron transfer are known to exist

within neutrophilic Fe(III)-reducers. These include direct contact between outer membrane Fe(III)-reductases and the Fe(III) mineral (DiChristina et al., 2002), the excretion of organic ligands that solubilize Fe(III) which can then be taken up by the bacteria and reduced by a Fe(III) reductase located in the periplasm or in the inner membrane (Fennessey et al., 2010; Pitts et al., 2003) as well as the use of microbially produced or external electron shuttles (e.g. dissolved or solid-phase humic substances (Jiang and Kappler, 2008; Roden et al., 2010)). Humic substances (HS) are polymeric, heterogeneous redox-active organic compounds formed during the degradation and transformation of biopolymers such as lignin, proteins and carbohydrates (Stevenson, 1994). Due to the multitude of their chemical properties and the ubiquity of their distribution, HS play a crucial role for the fate of contaminants in the environment in many respects. In particular, their influence on redox transformations of chlorinated hydrocarbons (Kappler and Haderlein, 2003) and metal(loid)s such as arsenic (Jiang et al., 2009) have been subject to detailed studies.

Microbial reduction of Fe(III) and humic substances are linked both by mechanistic and ecological aspects. Mechanistically, reduction of Fe(III) and humic substances by outer membrane reductases that depend on electron flow over several quinone- and *c*-type cytochrome-containing proteins in the inner membrane and the periplasm have recently been shown to be based on the same molecular machinery (Lies et al., 2005; Voordeckers et al., 2010). For this reason, it is not surprising that all neutrophilic Fe(III)-reducers tested so far were also found able to reduce humic substances or the humic model quinone AQDS (Coates et al., 1998; Finneran et al., 2002; Francis et al., 2000; He and Sanford, 2003; Lovley et al., 1996; Nevin and Lovley, 2000). In addition to Fe(III)-reducers, the group of microorganisms that can reduce dissolved humic substances at circumneutral pH includes fermenting bacteria (Benz et al., 1998), toluene degraders, sulfate-reducers and methanogens (Cervantes et al., 2002). It has been shown recently that even solid-phase humic substances can serve as electron acceptors for bacteria (Roden et al., 2010). The potential of humic substances to mediate the electron transfer between

bacteria and Fe(III) minerals has been discovered about 15 years ago (Lovley et al., 1996). Further studies revealed quinone moieties as the most likely electron acceptors within the humic substance molecules (Scott et al., 1998). It was also shown that electron transfer reactions via quinones involve the formation of semiquinone radicals (Scott et al., 1998), whose stability increases with increasing pH (Jiang et al., 2009). Figure 2 illustrates the process of electron shuttling via quinones including the different redox states of the quinone moieties that are relevant in this process.

The plethora of interesting findings made in the field of microbial Fe(III) and humic substance reduction still left some questions unanswered equally as it opens up new ones. Besides the heavily debated question whether Fe(III)-reducing microorganisms produce conductive cell appendices, so-called nanowires, which could be involved in the electron transfer between cells and Fe(III) minerals (Gorby et al., 2006), environmental boundary (geochemical) conditions such as maximum levels of salinity up to which microbial Fe(III) reduction can still take place remain to be elucidated. Concerning humic substances, it would be thrilling to find out if they could play a role in the development of electric currents that have been measured in sediments following changes between oxic and anoxic incubation conditions (Nielsen et al., 2010). This would involve a more detailed investigation of the mechanism of electron shuttling, about which it would also be interesting to know whether it takes place at acidic pH as well where semiquinones are not very stable (Jiang et al., 2009) and Fe(III) can occur as dissolved Fe^{3+} in solution (Kappler and Straub, 2005).

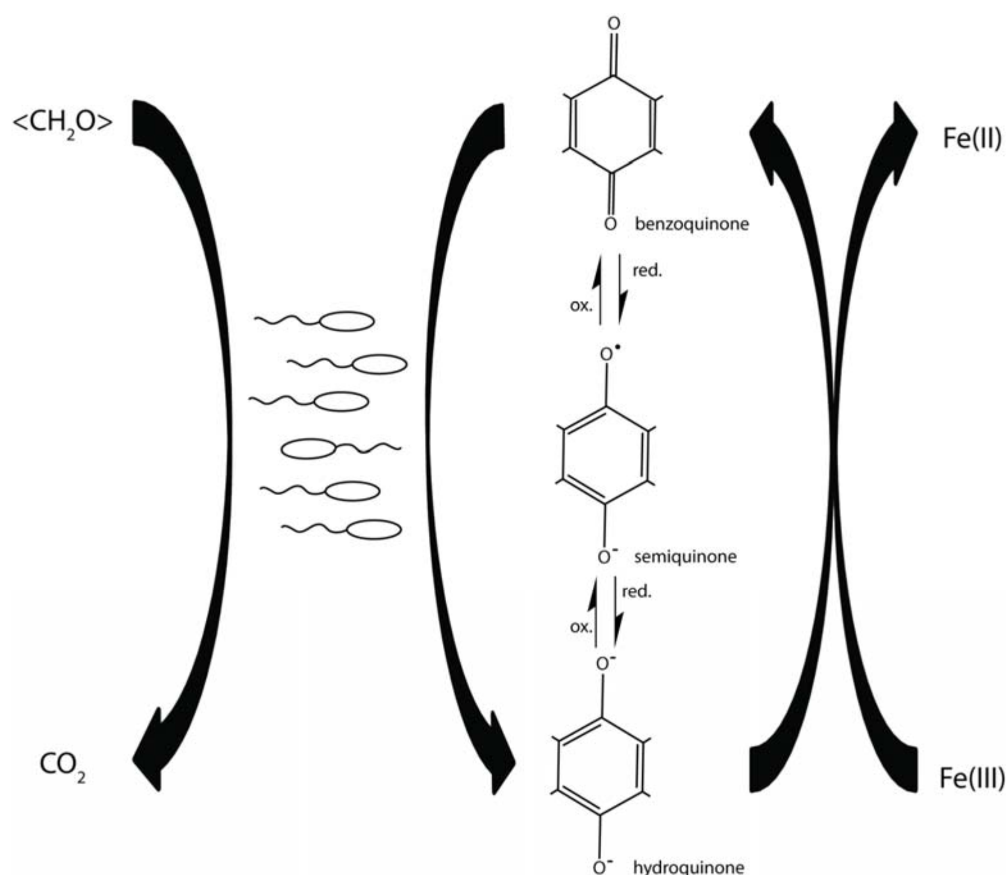

Figure 2: *Scheme illustrating electron shuttling via quinone moieties as they occur in humic substances.*

3.4 Salt lakes

Salt lakes are temporary or permanent epicontinental water bodies characterized by their salinity which can range from below that of seawater (35 g of dissolved salt L^{-1}) to salt saturation (e.g. 474 g of dissolved salt L^{-1} in Antarctic Lake Don Juan (Yamagata, 1963-65; Oren, 2001). On a global scale, nearly as much water is stored in salt lakes as in freshwater lakes (Hammer, 1986). Dependent on the predominant anions, salt lakes can be classified as chloride-sulfate lakes or carbonate-chloride lakes (Hutchinson, 1937). The pH range of salt lakes extends from pH < 2 (e.g. some lakes in parts of Western Australia and Victoria (Bowen and Benison, 2009)) to 12 (Grant, 1992). Hypersaline alkaline lakes, which are typically dominated by sodium

(bi)carbonate, are known as soda lakes (Sorokin et al., 2011). Saline waters can either be of marine origin (= thalassohaline habitats) or develop at the termini of inland fresh water drainage basins where high evaporation rates lead to an increase in salt concentrations (= athalassohaline lakes). For these reasons, most salt lakes can be found in arid and semi-arid regions (Williams, 2002). Figure 3 shows in which regions on the globe salt lakes can be found.

Figure 3: *Shaded areas indicate the distribution of salt lakes around the world. Adopted from (Williams, 2002)*

Due to climate change, the area covered globally by salt lakes is expected to increase in the near future. Many freshwater lakes will turn into salt lakes and existing salt lakes will increase in salinity due to increased levels of evaporation (Williams, 2002). Despite representing extreme habitats for microbial life (Litchfield and Gillevet, 2002; Rothschild and Mancinelli, 2001), high primary productivity rates were often measured in hypersaline systems showing that salt-adapted species do thrive regardless of the environmental conditions they are exposed to (Schidlowski, 1988; Sorokin et al., 2007). Representatives from all three domains of life, Bacteria, Archaea and Eukaryotes, have been found in salt lakes (Oren, 2002). They can be divided into halotolerant species that can tolerate moderate salt levels even though they thrive better at lower salt

concentrations and halophilic organisms who depend on a minimum of salt to survive (Margesin and Schinner, 2001). Even though organisms need special mechanisms in order to cope with high extracellular salt concentrations, the diversity of both phylogenetic and metabolic groups of microorganisms that have been found active in moderately saline environments is still pretty high (Oren, 2001; Oren, 2010): both oxygenic and anoxygenic photosynthesis, aerobic respiration, denitrification, reduction of sulfate, manganese, arsenate and selenate, fermentation, acetogenesis, methanogenesis, oxidation of reduced sulfur compounds, autotrophic arsenite oxidation and aerobic methane oxidation have been observed in laboratory studies with pure cultures in medium with more than 100 g of salt L^{-1} (Oren, 2010). However, at salt concentrations over 300 g L^{-1}, the majority of these processes do not take place any more, presumably since they do not deliver enough free energy to make up for the costs the microorganisms that catalyze the respective reactions need for haloadaptation (Oren, 2010). From a phylogenetic perspective, halotolerant and halophilic microorganisms can be found in the different branches of the Proteobacteria, cyanobacteria, the *Flavobacterium-Cyophaga* branch, the spirochaetes, the actinomycetes and the Firmicutes (Oren, 2002; Ventosa et al., 1998). The maybe most extreme group of halophiles, however, belongs to the Archaea and consists of members of the order Halobacteriales (Oren, 2002). Even though there are studies of halophilic environments that are dominated by Bacteria (Hollister et al., 2010) while others are dominated by Archaea (Jiang et al., 2007; Swan et al., 2010), a general trend of increasing dominance of Archaea over Bacteria with increasing salinity has been observed (Jiang et al., 2007; Ventosa et al., 1998).

In the past, high concentrations of halogen oxides have been detected over salt lakes. Mixing ratios in the range of 20-80 ppt of bromine oxide (BrO) have been measured over the Dead Sea (Hebestreit et al., 1999; Matveev et al., 2001) and Salar de Uyuni, Bolivia (Honninger et al., 2004). In the lower troposphere over the Great Salt Lake in Utah, USA, elevated concentrations of BrO and chlorine oxide (ClO) have been detected (Stutz et al., 2002). The formation of

bromine oxide, whose atmospheric concentrations have been found to be negatively correlated with ozone concentrations, has been attributed to the interaction of atmospheric oxidants with bromide at the surface of the large salt pans (Matveev et al., 2001). More recently, a microcosm study demonstrated the release of highly chlorinated C1 and C2 hydrocarbons from salt lake sediments originating from the kalmykian steppe in Russia (Weissflog et al., 2005). Interestingly, the formation of some of these compounds seemed to critically depend on the activity of halophilic microorganisms since it neither occurred in sterilized setups nor in setups that were incubated with water instead of 4 M NaCl (Weissflog et al., 2005). However, so far nothing is known about the mechanism how halophilic microorganisms can lead to the release of volatile organochlorines from salt lake sediments.

3.5 Potential mechanisms of microbial influence on natural organohalogen formation

Considering what has been described in the previous paragraphs, it seems very likely that prokaryotic microorganisms do have an influence on the release of volatile organohalogens in soils and sediments. However, nobody has ever studied possible ways how bacteria and archaea could stimulate natural VOX formation.

Based on the results of previous studies that have been summarized in sections I.2 and I.4, two fundamentally different ways would be feasible how microorganisms could contribute to the formation of halogenated hydrocarbons including volatile ones. These two possibilities are illustrated in figure 4. Even though the oxidative breakdown of quinone compounds followed by their halogenation that occurs at low pH has only been studied in abiotic batch systems so far, microorganisms might still influence this process in nature. Production of VOX via this route is assumed to be stimulated by hydroxyl radicals generated from a Fenton reaction between Fe^{2+} and H_2O_2 (Schöler, 2003). This renders it tempting to speculate whether semiquinone radicals that are formed during the reduction of humic substances e.g. by Fe(III)-reducing bacteria (section I.3) could take over the role of the hydroxyl radicals in this reaction and enhance VOX formation

in humic- and Fe-rich, acidic environments. A similar indirect stimulation of natural VOX formation by Fe(III)- and HS-reducing microorganisms would be feasible to occur in VOX-emitting salt lake sediments, a process that has already been shown to depend on microbial activity (Weissflog et al., 2005). In addition, microorganisms could also produce volatile and larger halogenated hydrocarbons directly via halogenating enzymes, the activity of which has already been proven in soil (Asplund et al., 1993).

Unspecifically halogenating haloperoxidases and methyl halide-producing methyltransferases could directly lead to the formation of halogenated C1 and C2 compounds. These compounds could theoretically also form if larger organic molecules are halogenated by haloperoxidases and later on degraded into smaller molecules. Consequently, the groups of microorganisms that are particularly interesting with respect to their potential influence on natural VOX formation comprise acidophilic and halophilic Fe(III)- and HS-reducing microorganisms as well as microorganisms that express halogenating enzymes.

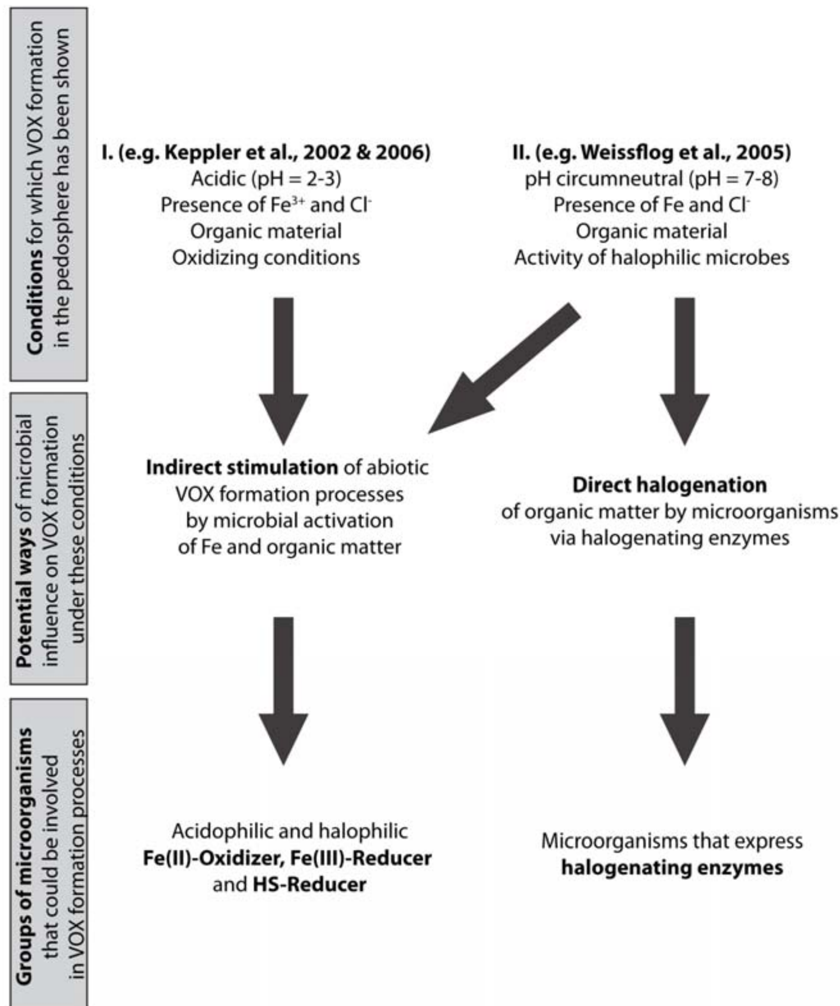

Figure 4: *Possible ways how different groups of microorganisms could influence the two mechanisms of VOX formation in the terrestrial environment that are presented in the first paragraphs of this chapter.*

3.6 Research questions addressed in this study

Since there is an obvious knowledge gap concerning the influence of microorganisms on natural VOX formation, it was the goal of this study to narrow this gap by evaluating the different ways how microorganisms could potentially contribute to the overall budget of VOX that are released

to the atmosphere that were presented in section I.5. More specifically, research questions addressed in this study included:

- Under which conditions can microorganisms indirectly stimulate natural VOX formation?
- Do Fe(III)-reducing microorganisms also reduce humic substances at acidic pH and does this lead to the formation of semiquinone radicals as it is the case at neutral pH?
- Does anaerobic microbial Fe(II) oxidation and Fe(III) reduction take place in salt lake sediments? If yes, up to which salinity do the respective reactions occur and which microorganisms catalyze them?
- What is the diversity of genes for halogenating enzymes in salt lake sediments?
- Is there any evidence of activity of reductive dechlorinators in salt lake sediments that might degrade chlorinated hydrocarbons of natural origin in this environment?

These questions were addressed in different sets of batch and microcosm experiments. For identification and quantification of VOX, gas chromatography-mass spectrometry (GC-MS) systems were used. Semiquinone radicals were quantified using electron spin resonance (ESR) spectroscopy. For the identification of Fe-metabolizing microorganisms in enrichments from salt lake sediments as well as for the detection of genes for halogenating enzymes, molecular techniques such as polymerase chain reaction (PCR) were applied.

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4

Identification and quantification of volatile organohalogens released from batch setups with Fe(III)-reducing microorganisms and field samples from salt lake sediments

Maren Emmerich & Andreas Kappler

Abstract

While it has been established by now that natural sources of volatile organohalogens (VOX) exist, knowledge concerning the quantitative contribution of the different sources and detailed mechanisms of natural VOX formation is still very limited. This holds particularly true for soils and sediments, where it is e.g. unclear if and to which extent microorganisms contribute to VOX emissions. One possible mechanism of microbial stimulation of natural VOX formation would be an activation of Fe phases and humic substances enabling the subsequent halogenation and fractionation of the latter. In the present study, we tested the hypothesis whether Fe(III)-reducing microorganisms can enhance VOX formation via this indirect pathway using batch setups as well as microcosm experiments and GC-MS measurements. While we were not able to reproducibly quantify the halogenated hydrocarbons formed in the batch setups, addition of Fe(III)-reducing bacteria to VOX-emitting sediments did not lead to an increase in the release of these compounds. Therefore, our results do not support the hypothesis of a stimulation of natural VOX formation by Fe(III)-reducing microorganisms.

Introduction

Halogenated organic compounds, most famous due to their contribution to stratospheric ozone depletion (Molina, 1974), were thought for a long time to be exclusively of anthropogenic origin. Since the middle of the 1970s (Lovelock, 1975), more and more natural sources for fluorinated, chlorinated, brominated and iodinated hydrocarbons started to be explored. Today, more than 4,700 halogenated hydrocarbons of natural origin have been described (Gribble, 2010) including both short-chain volatile organohalogens (VOX) and larger polar compounds (POX).

While representatives of both groups are known to be carcinogenic (IARC, 1997; Maltoni et al., 1974), the volatile organohalogens pose additional concern due to their high global-warming potentials (e.g. 1400 for CCl_4 in comparison to CO_2; (WMO, 2007)) as well as ozone-destruction activities. The realization of the latter lead to considerable reductions in industrially emitted VOX since many of these compounds became forbidden or restricted in their production with the 1987 Montréal Protocol. However, considerable amounts of VOX continue to be measured in the atmosphere with values in the ppt (parts per trillion volume) range in the case of the monohalomethanes CH_3Cl, CH_3Br and CH_3I (Harper, 2003). The relatively long retention times of these compounds in the atmosphere can only partly explain this phenomenon. This has made scientists realize the importance of natural sources of volatile organohalogens for the global budget of these compounds, which can be differentiated into biotic and abiotic ones.

Purely abiotic sources mainly comprise biomass burning and volcanic eruptions with biomass burning accounting for 0.9 x 10^9 g of annual CH_3Cl emissions (Butler, 2000; Crutzen and Andreae, 1990). In addition, di- and trichloromethane, chloroform and methyl chloroform are generated by this source (Lobert et al., 1999). A quantitative study of fumaroles and lava gas emitted from four different volcanoes in Italy and Japan revealed the presence of 5 fluorinated, 100 chlorinated, 25 brominated and 4 iodinated compounds with highest concentrations of chlorinated methanes, chloroethene and chlorobenzene of up to 100 ppb in the fumes. However, on a global scale the amounts of halogenated hydrocarbons from volcanic sources are too small to contribute significantly to the atmospheric burden (Jordan et al., 2000).

A recent study demonstrated that methyl chloride is also released abiotically upon photoirradiation of seawater rich in colored dissolved organic matter (Moore, 2008). Biotic sources for halogenated hydrocarbons that have been defined so far comprise marine algae (Wuosmaa and Hager, 1990) and terrestrial plants (Teh et al., 2008) as well as fungi (Cowan et al., 1973). In these organisms, the halogenation reactions are carried out by enzymes such as methyl transferases (Harper, 1985) and haloperoxidases (Urhahn and Ballschmiter, 1998). Another indirect biotic way of VOX formation which might account for a great part of the yet unattributed sources of methyl chloride is the abiotic chlorination of plant pectin (Hamilton et al., 2003). Apart from the sources of halogenated hydrocarbons mentioned so far which can be clearly distinguished into biotic and abiotic ones, soils have also been found to be VOX-emitters (Hoekstra et al., 1998; Rhew et al. 2000). Rhew et al. (2000) measured high emissions of monohalomethanes over coastal salt marshes in California, and quantification of methyl chloride concentrations in the atmosphere at three different locations strongly suggests a land-based CH_3Cl source in the tropics (Yokouchi et al., 2000). Following the outcome of studies where VOX formation in soil was analyzed in detail, two sets of conditions under which soils can emit VOX can be defined: on the one hand, at low pH (2-3), in the presence of catalytic amounts of Fe^{3+} and a halogenide source, organic material can be oxidatively degraded and chlorinated in a purely abiotic reaction (Huber et al., 2009; Keppler et al., 2002; Keppler et al., 2006). The exact mechanism of this reaction, which has been shown to release mono- as well as di- and trihalogenated C1 and C2 compounds as well, remains to be elucidated. Interestingly, VOX yields increase tremendously with the addition of H_2O_2, suggesting a Fenton-like reaction and a crucial role of hydroxyl- or semiquinone radicals. The fact that radicals can be involved in the formation of organohalogens due to their characteristics as powerful oxidants had already been suggested by Fahimi et al. (2003).

Apart from this low-pH-dependent pathway of VOX-release from soil, highly chlorinated C1 and C2 compounds have been found to be released from a chemically completely different

terrestrial environment, namely salt lake sediments (Weissflog et al., 2005). The authors had performed microcosm experiments with pH-neutral salt lake sediments from southern Russia and noted a dependency of VOX release on the activity of halophilic microorganisms. Interestingly, a catalytic role of dissolved Fe is proposed in this publication as well, since the sediments of the lakes in which Fe concentrations exceeded the detection limit of 16 µM emitted the highest amounts of VOX (Weissflog et al., 2005).

In summary, the crucial role of Fe is recognized in both the radical-dependent and the microbe-dependent scenario of VOX formation in soils or sediments. In addition it is known that Fe(III)-reducers, besides other groups of microorganisms, can transfer electrons to humic substances as well (Lovley et al., 1996) and produce semiquinone radicals during this process (Scott et al., 1998). Therefore, we hypothesize that Fe(III)-reducing microorganisms can indirectly stimulate natural organohalogen formation by activating Fe minerals and humic substances which leading to abiotic chlorination of organic material. In detail, the goal of this study was to identify and quantify VOX released from abiotic batch setups containing Fe(III), commercially available humic substances or functional analogues thereof and KCl at low and neutral pH in presence and absence of mM concentrations of H_2O_2 as a radical generator. The second goal of this study was to quantify to which extent the addition of known Fe(III)-reducing microorganisms can increase organohalogen production in H_2O_2-free setups. Finally, we intended to determine the effect of adding known Fe(III)-reducing microorganisms to VOX-emitting salt lake sediments which had been sterilized by freeze-drying and grinding.

Materials and Methods

Bacteria

Shewanella oneidensis MR-1 was cultured in lysogeny-broth (LB) medium (10 g L^{-1} tryptone, 5 g L^{-1} yeast extract, 5 g L^{-1} NaCl, pH = 7) at 28°C. Prior to an experiment, a single colony was

picked from an LB agar plate containing 1.2 % agar and transferred into 50 mL liquid LB where it was incubated overnight. In the beginning of an experiment, cells were quantified by optical density (OD) measured at 600 nm according to a calibration curve plotting OD_{600} against microscopic cell counts with a Neubauer chamber (cell number per ml = OD_{600} * 10^9). 1.2 times as many cells as needed (to account for the loss during centrifugation/washing) were centrifuged for 10 minutes at 9 000 g, washed with SBM medium (1.3 mM K_2HPO_4, 1.65 mM KH_2PO_4, 7.87 mM NaCl, 1.7 mM $(NH_4)_2SO_4$, 30 mM $NaC_3H_5O_3$, 0.47 mM $MgSO_4$ x 7 H_2O, 10 mM HEPES, NaOH added until pH = 7.2), centrifuged again and resuspended in an appropriate amount of SBM medium (in a typical setup, a total of 10^8 cells resuspended in 100 µL SBM were added to a total setup volume of 10 mL giving rise to a final cell density of 10^7 cells per ml).

Soil sample

Two experiments were performed with sediment from lakes Kasin (N47°36.165' E047°27.129') and Elton (N49°09.057' E046°48.001'), Russia, sampled in August 2009. Following collection, the sample from lake Kasin was sieved (2 mm), while the sample from lake Elton was freeze-dried, milled and sieved (<315 µm). A total of 3.5 g of sieved lake Kasin sediment (field wet weight) or 1.0 g of the milled sediment from lake Elton (dry weight) was used per experimental setup, respectively.

Solutions, media & mineral suspensions

Pahokee Peat Humic Acids (PPHA) were purchased from the International Humic Substances Society (IHSS). Aldrich Humic Acids were purchased from Aldrich. 10 or 100 mg mL^{-1} anoxic stock solutions were prepared in SBM medium or in Millipore-H_2O. After adjustment of pH to pH = 7 with 1 M NaOH or HCl, stock solutions were transferred into 20 ml glass vials, closed with butyl rubber stoppers and metal crimp caps and made anoxic by 3 times alternating 3 min degassing and 1min flushing with N_2. Solutions were then sterilized by autoclaving and stored for up to 2 months in the dark at 4°C.

Apart from pH adjustment and autoclaving, 50 mM stock solutions of catechol and 500 mM solutions of $Fe_2(SO_4)_3$ were prepared and stored in a similar way in Millipore-H_2O. Anoxic solutions were sterilized by filtration (cellulose ester, ⌀ 0.22 µm) into evacuated and autoclaved glass vials which had been sealed with butyl rubber stoppers and metal crimp caps. KCl was prepared anoxically as well as an aqueous 500 mM stock solution and autoclaved for sterilization. 2-line ferrihydrite was prepared as described before (Cornell and Schwertmann, 2003) and suspended in Millipore-H_2O to a final concentration of 500 mM. The suspension was deoxygenated as described above and if ferrihydrite was used in experiments that were incubated for more than 24 h, it was additionally autoclaved. When H_2O_2 was added to any setups, 500 mM stock solutions were freshly prepared with Millipore-H_2O directly before pipetting the reaction mixture together. 50 mM HEPES was prepared as an oxic solution in Millipore-H_2O. pH was adjusted to a pH of 7 with 1 M NaOH followed by autoclaving. Anoxic media (SBM and salt lake medium) were prepared sterilely and anoxically using a Widdel flask and by cooling the autoclaved medium to room temperature under N_2 atmosphere. Salt lake medium contained 5 M NaCl, 100 µM $MgSO_4$ x 7 H_2O, 3 mM KCl, 100 µM KBr, 5 mM NH_4Cl and 1.9 mM $MgCl_2$. After autoclaving, 15 mL L^{-1} of 1 M $NaHCO_3$ that had been autoclaved separately was added as a buffer as well as 1 ml each from the following 1000 x sterile stock solutions: vitamin solution (Widdel and Pfennig, 1981), trace element solution (Tschesch, 1984), selenate-tungstate solution (Widdel, 1980), 1 M $CaCl_2$, 1 M $NaHPO_4$, 214 µM NH_4VO_3 and 500 mM $Na_2S_2O_3$. If necessary, pH was adjusted to a value between pH=7.2 and pH=7.4 with sterile and anoxic 1 M NaOH or 1 M HCl after adding all other ingredients.

General setup of experiments

All experiments were set up in glass vials or serum bottles of which half the volume was filled with liquid (i.e. 10 mL total reaction volume in 20 mL glass vials, 50 mL reaction volume in 100 mL serum bottles or 100 mL reaction volume in 200 mL serum bottles). All glass vials and serum bottles were sealed with PTFE-layered butyl rubber septa and metal crimp caps. Anoxic setups

were set up in an anoxic chamber (glovebox) under N_2-atmosphere using anoxic solutions. Oxic setups were set up with the same anoxic solutions which were taken out anoxically with syringes and needles on the lab bench. For both oxic and anoxic setups, the order of addition of ingredients was as follows: medium/buffer/water, KCl, ferrihydrite/$Fe_2(SO_4)_3$, HA/catechol, H_2O_2, cells. If experiments included incubation steps, these were carried out at 28°C without shaking. Whenever indicated samples were shaken before measurements, this occurred on a rotary shaker at 500 rpm and 35°C or 40°C as indicated.

Setup of individual experiments

Experiment 1: stimulation of abiotic VOX-formation by MR-1 (batch-setup)

10^7 cells per ml were added anoxically to setups in SBM medium amended with 10 mM ferrihydrite and 1mg/mL PPHA (final concentrations). The total volume was 10 mL in 20 mL glass vials, and setups lacking one or two of the three ingredients (cells, ferrihydrite, PPHA) were prepared as negative controls. Setups were incubated for 7 and 14 days at 28°C, after which volatile compounds formed were quantified by purge-and-trap-assisted GC/MS.

Experiment 2: stimulation of methane formation by MR-1 (batch-setup)

10^7 cells per ml were added anoxically to SBM medium amended with 10 mM ferrihydrite and 1 mg/mL PPHA (final concentrations). The total volume was a) 10 mL in 20 mL glass vials and b) 100 mL in 200 mL glass vials. Setups lacking one or two of the three different ingredients (cells, ferrihydrite, PPHA) were prepared as negative controls. Setups were incubated for 7 days at 28°C, after which methane was quantified by GC.

Experiment 3: abiotic VOX formation

10 mM KCl and $Fe_2(SO_4)_3$, 2 mM catechol, 50 mM H_2O_2 and 1 mg/mL HA were mixed in a total volume of 10 mL under oxic conditions and either directly analyzed by GC-MS after closing the vial or shaken on a rotary shaker at 40°C and 500 rpm for 30 minutes. One setup was incubated for 24 h at 28°C before the measurement to check for VOX formation over time. In

order to increase the sensitivity of the analysis, another setup was purged with He for 30 instead of 7.5 minutes (see section “Analytical Methods” for a detailed description of the GC-MS protocol). Setups lacking one or more of the ingredients were prepared as negative controls.

Experiment 4: microcosm experiment

3 g of 2 mm sieved sediment from lake Kasin, Russia were incubated with 8.5 mL of medium which had the same salinity as the salt lake water (salt water medium, see above) and 0.1 ml of a stock solution containing 500 mM of both lactate and acetate. Six parallel setups for this experiment were prepared under oxic conditions since it was unclear whether O_2 was necessary for VOX formation. Setups were then closed air-tight as described above and incubated for four weeks. Three parallel setups each were then analyzed for VOX formed by GC-MS and GC-ECD, respectively.

Experiment 5: stimulation of abiotic VOX-formation by *Shewanella oneidensis* MR-1 (cell suspension-setup)

5 x 10^8 MR-1 cells/mL were added to 1 g of freeze-dried and milled Elton sediment in presence and absence of additional Fe(III) (added as $Fe(OH)_3$, 5 mM final concentration), 10 mg/mL final concentration of Aldrich HA and/or 5 mM final concentration of H_2O_2. All setups had a total volume of 10 mL and were prepared in 50 mM HEPES buffer (pH 7) under oxic conditions in 20 mL glass vials. Before quantification of methyl chloride by GC-MS, setups were incubated for 2 h on a rotary shaker at 500 rpm at 40°C or 35°C to spare the bacteria.

Analytical methods

GC/MS analyses were performed on a Varian STAR 3400 Cx gas chromatographic system connected to a Saturn 2000 ion trap mass spectrometer. Sampling, preconcentration and injection of the volatile analytes were controlled by a custom-made purge and trap system. The GC was equipped with a BP624 (25 m; 0.53 mm i.d.; 3.0 µm film thickness) and a BPX5 (60 m; 32 mm; i.d.; 1.0 µm film thickness) capillary column, both connected in series. Stainless steel needles

served both as inlet and as outlet through the septum. If not indicated otherwise, volatile compounds were purged for 7.5 min with a helium stream at 7 mL/min which was regulated with a flowcontroller and retarded on a cooled glass lined tubing trap at -190°C. Moisture was removed on a magnesium perchlorate water trap from the samples stream. The loaded preconcentration GLT_trap was then purged by the GC carrier gas (He, 2 mL/min) and heated to 220°C in 45 seconds. The GC oven temperature program started automatically: 35°C hold 8 min, 35°C to 150°C at 5.5°C/min, hold 5 min, 150°C to 210°C at 30°C/min, hold 10 min. Detection limits of this instrumentation were 530 pg for methyl chloride and 510 pg for vinyl chloride (S. Huber, personal communication). GC/ECD analyses were carried out on a gas chromatograph (GC 8000 series Fisons Instruments) equipped with an ECD-detector (Carlo Erba, HT25). The chromatographic separation was performed using a BP624 (25 m; 0.53 mm i.d.; 3.0 µm film thickness) capillary column. The carrier gas was N_2 (2 mL/min), and the ECD make up gas Ar/CH_4 (95/5 %, 20 mL/min). The injector had a temperature of 150°C and the GC oven temperature program proceeded as follows: 50°C, hold 10 min, 50°C to 200°C at 25°C/min, hold 14 min. When sampling, aliquots of 2 mL gas phase were injected in splitless mode (1 min) into the GC.

Methane measurements of 250 µL headspace samples were performed with a Varian 3800 gas chromatograph equipped with an Alltech 13939 column (length: 30 m, inner diameter: 0.53 mm, AT™-Q) and an FID detector. The injector had a temperature of 200°C and the temperature program was as follows: 60°C, hold 3 min, 60°C to 200°C at 75°C/min, hold 3 min. The flow rate was 5 mL/min at a flow pressure of 3.2 psi. The detection limit of this instrument was 10 µg/L.

Results

In order to test the hypothesis that Fe(III)-reducing microorganisms can indirectly stimulate natural organohalogen formation by activating Fe minerals and humic substances which then stimulate abiotic reactions leading to chlorination of organic material, we started with batch setups using a neutrophilic Fe(III)-reducing strain that is known to be able to transfer electrons to humic substances, i.e. *Shewanella oneidensis* MR-1 (Jiang and Kappler, 2008) (experiment 1). According to results from experiments where Fe(III) reduction had been followed, 5 % of the initial 10 mM Fe(III) had been reduced after 28 days in the microbially active setups without PPHA, while in the PPHA-amended setups 60 % of Fe(III) had been reduced after 7 days of incubation (figure A1). After 14 days, 10 and 60-70 % of the Fe(III) were reduced in the setups without and with PPHA, respectively. In the sterile setups, no Fe(III) had been reduced.

Table 1: *Identification and quantification of volatile compounds formed in batch setups containing the Fe(III) mineral ferrihydrite ($Fe(OH)_3$) and Pahokee Peat humic acid (PPHA) in presence or absence of Shewanella MR-1 cells in SBM medium. GC-MS-measurements were performed after 14 days of incubation. "Blank" values refer to measurements of an air-filled vial. Concentrations are given in kilocounts corresponding roughly to ng per L concentrations; two values indicate duplicate setups.*

Composition of setups	Cells $Fe(OH)_3$ PPHA	$Fe(OH)_3$ PPHA	Cells PPHA	Cells $Fe(OH)_3$	Cells	$Fe(OH)_3$	PPHA	Medium	Blank
Methyl bromide	25; 7	27; 0	13	19	22	--	17	13; 18	72
Isoprene	20; 13	0; 24	9	10	4	--	--	--	--
Carbon disulfide	240; 125	210; 170	170	430	70	--	200	36; 26	11
Furan	10; 0	5; 7	7	--		--	7	--	--
Methyl furan	240; 40	110; 15	90	65	175	--	40	28; 44	165
Chloroform	11; 10	8; 12	--	--	--	--	--	--	--

In addition to the Fe redox state, we identified and quantified the volatile compounds formed within 14 days in these setups (Table 1). The first column, which depicts the results of the actual

experiment containing ferrihydrite, humic acids and microbial cells, shows that detectable amounts of the volatile organohalogens methyl bromide and chloroform could be identified in the setups and in addition, some further volatile compounds such as isoprene, carbon disulfide and methyl furan were present. However, the same substances were found to be present in the abiotic controls with concentrations of the same order of magnitude. With the exception of chloroform that was present only in the cells/ferrihydrite/HA and in the ferrihydrite/HA setups and with the exception of the ferrihydrite-only setup that did not show any volatile organic compounds at all, all identified volatile substances measured appeared in some of the controls at similar concentrations as in the cells/ferrihydrite/HA setup. Even in the blank and in the SBM-medium only-setup, some methyl bromide, carbon disulfide and methyl furan could be detected. Measurements after 7 days of incubation gave similar results (data not shown). In summary, no significant difference in identity or quantity of VOX and other volatile compounds formed could be found between abiotic and microbially stimulated setups with ferrihydrite and PPHA after 7 or 14 days of incubation under anoxic conditions at neutral pH.

Since methane functions as a substrate for methyltransferases and could serve as a substrate for both biotic and abiotic halogenation reactions, we determined if this potential precursor for VOX is formed in the same setups as described in experiment 1. However, in headspace samples of 10 mL setups (experiment 2a), no methane formation could be measured that exceeded background values after 7 days of incubation with a detection limit of around 10 µg/L. To exclude that any formed methane remained undetected due to the detection limit of the instrument, 100 mL setups were prepared in 200 mL serum bottles with all other parameters remaining the same as before, and methane measurements were performed again after 7 days of incubation (experiment 2b). However, even in the setups with higher volume, no methane could be detected in any experimental setup or control.

Since neither microbial stimulation of VOX nor methane formation could be found in these first experiments, we tested conditions for which abiotic VOX formation had been shown to occur

before, namely pH 2-3 and presence of KCl, dissolved Fe(III), humic acids or catechol and H_2O_2 under oxic conditions (Keppler et al., 2002; Keppler et al., 2006) (experiment 3). Figure 1 shows the results of the GC-MS-based quantification of volatile compounds detected in the individual setups.

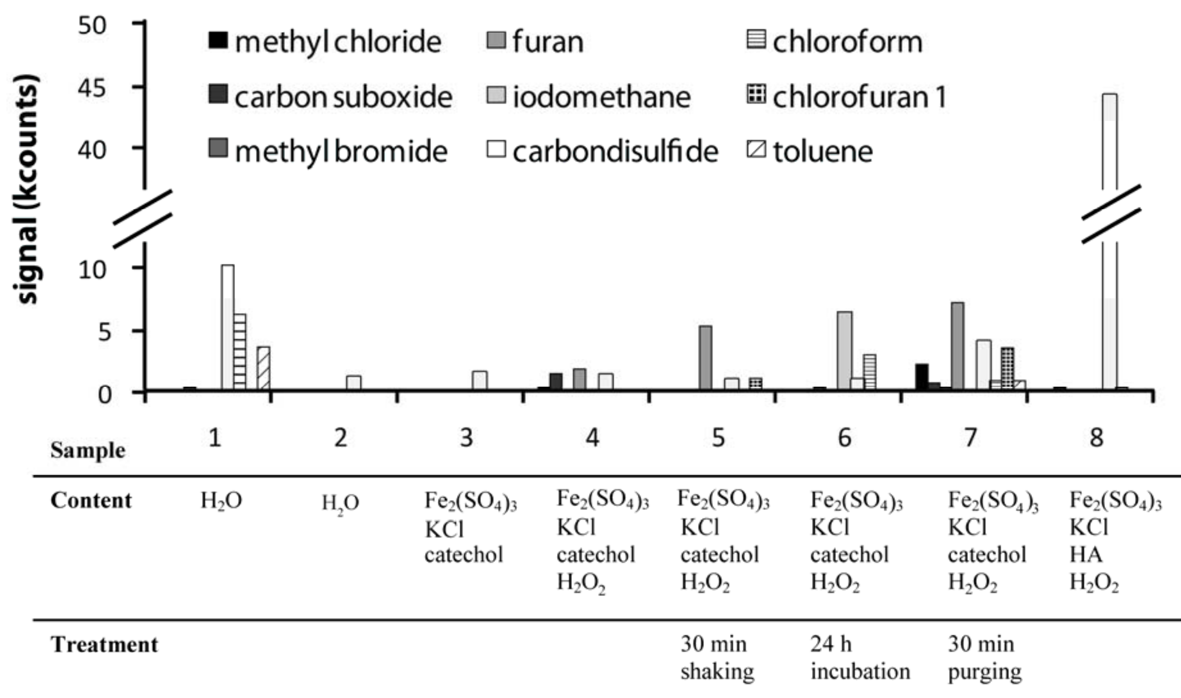


Figure 1: *Quantification of abiotically formed volatile organic substances by GC-MS in setups consisting of dissolved Fe(III), KCl, catechol or humic acids (HA) in presence or absence of H_2O_2. Setups were prepared under oxic conditions and directly measured after closure of the vials if not indicated otherwise under "treatment". "Blank" values refer to measurements of an air-filled vial.*

As in experiment 1, most striking are again the signals from carbon disulfide which occur in every measurement including blanks. Aditionally, methyl chloride, methyl bromide, iodomethane and chloroform could be detected in some setups. However, since methyl bromide and chloroform were also measured in one of the water samples at up to twofold higher concentrations, it cannot be concluded that these compounds formed in any of our setups. The highest concentration of iodomethane was measured in the setup which had been incubated for 24 h, while the other

setups had not been incubated at all. Methyl chloride was mainly measured in the setup that was purged for 30 minutes instead of 7.5 minutes. Unfortunately, 30 minutes of purging time could not be applied to a higher number of samples due to the enormous consumption of He. Therefore, we tried to improve the detection limit for VOX in our samples by increasing the volume of the setups to 50 mL in 100 mL serum bottles and leaving final concentrations of the ingredients unchanged. Three parallel setups of this kind were measured as before without any additional pretreatment. Unfortunately, neither iodomethane nor methyl chloride could be detected in these samples (data not shown). The only VOX signals in these samples came from methylene chloride and were in the 10 kcount order of magnitude. However, a 3.5 kcount signal for methylene chloride appeared in a water sample as well, which is why we can again not claim that these compounds formed in our setups. Most notably, we did not detect any vinyl chloride or chloroethyne as it was detected by Keppler et al., (2002) and Keppler et al., (2006) in comparable setups. In summary, with this series of experiments, we were unable to verify abiotic VOX formation as observed by (Keppler et al., 2002).

Since salt lake sediments had been shown to emit highly chlorinated C1 and C2 hydrocarbons before in dependence of microbial activity (Weissflog et al., 2005), we wanted to analyze this phenomenon in more detail. To this end, we incubated sediment from lake Kasin, Russia with medium which had the same salinity as the salt lake water and added lactate and acetate as electron donors for 4 weeks (experiment 4). Chemical properties of lake Kasin sediment are shown in table 3. We expected this sediment to be likely to emit VOX due to its high Fe and Cl^- content. Setups for this experiment were prepared under oxic conditions because we did not know whether O_2 was necessary for the VOX formation to occur. However, no VOX could be detected in any setup neither by GC-ECD nor by GC-MS analysis (data not shown). In addition to this experiment which had been performed with field-wet sediment, in total five sediments sampled in Russia were analyzed for abiotic VOX formation by shaking 1 g of freeze-dried and milled sediment with 10 mL of H_2O followed by GC-MS-measurement. One of the

sediments originating from lake Elton (characteristics of which can be found in table 2) showed considerable release of methyl chloride with peak heights corresponding to 1-3 ng of methyl chloride released per g dried sediment. Focusing on our initial hypothesis that Fe(III)-reducing microorganisms might indirectly stimulate natural VOX formation by providing reactive Fe(II)/Fe(III) phases and organic radicals, we wanted to test this hypothesis with the methyl chloride-emitting sediment to which we added Fe(III)-reducing MR-1 cells in high numbers (experiment 5).

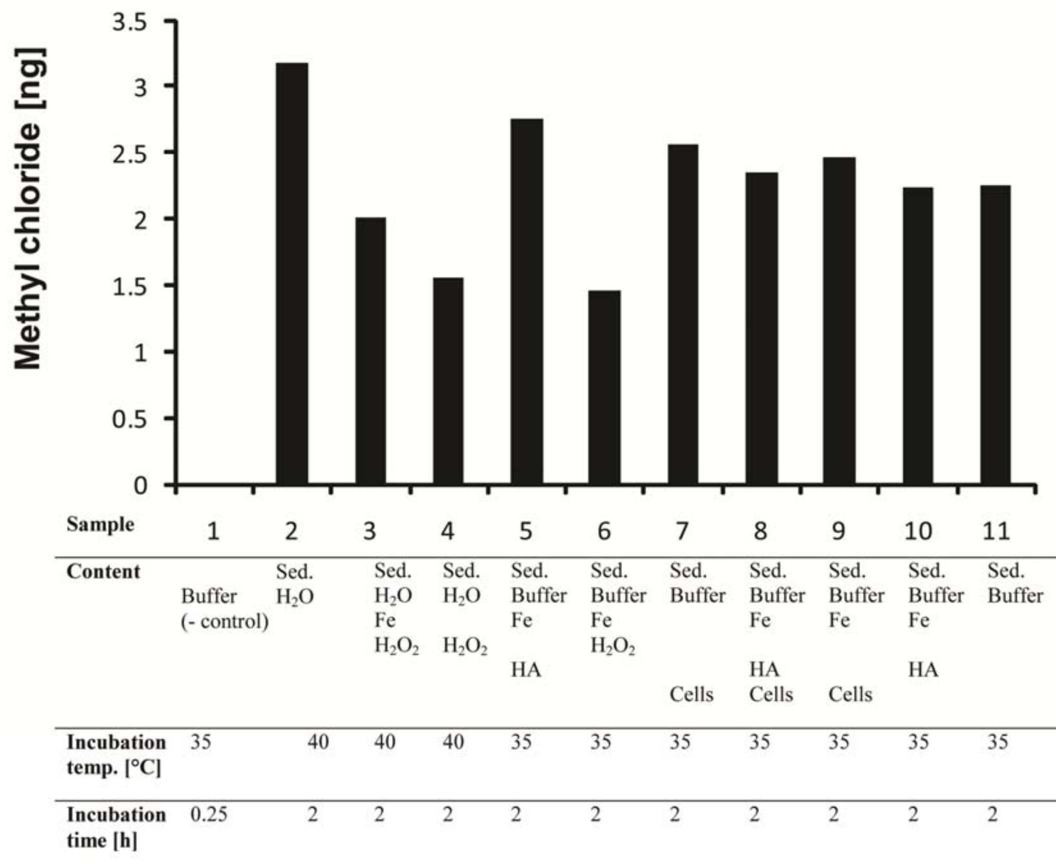
Table 2: *Location and geochemical properties of sediment samples from lakes Kasin and Elton.*

	Kasin	**Elton**
geographic position	N47°36.165’ E047°27.129’	N49°09.057’ E046°48.001’
pH[a]	7.86	7.43
Fe-content[b]	1.13 %	0.86 %
Cl^{-}[b]	1.51 %	2.5 %
Br^{-}[c]	0.01 %	0.05 %
C_{org}[d]	0.11 %	0.2 %
C_{inorg}[e]	1.85 %	6.73 %
AOX[f]/C_{org}[d]	39.25 mg/g	15 mg/g

[a]determined with 0.01 M $CaCl_2$ [b] [weight %] quantified by XRF
[c]quantified by IC from modified porewater after (Jiang et al., 2006]
[d]determination via weight loss at titration with HCl
[e]quantification with C/N-analyzer of HCl-titrated sample
[f]adsorbable organically bound halogens; determined according to DIN EN ISO 9562 at the hygiene institute, Bonn

If our hypothesis was true, we would expect both the addition of bacteria alone and in particular in combination with the further additions to increase the amount of methyl chloride measured in the setups. The results of the methyl chloride measurements are illustrated in Figure 2. The main result we can derive from this figure is that in all sediment-containing setups, there are

no considerable differences in the amount of methyl chloride released from the individual setups, independent of the addition of bacteria, Fe(III), HA, H_2O_2 or any combination of these.



Sample	1	2	3	4	5	6	7	8	9	10	11
Content	Buffer (- control)	Sed. H_2O	Sed. H_2O Fe H_2O_2	Sed. H_2O H_2O_2	Sed. Buffer Fe HA	Sed. Buffer Fe H_2O_2	Sed. Buffer Cells	Sed. Buffer Fe HA Cells	Sed. Buffer Fe Cells	Sed. Buffer Fe HA	Sed. Buffer
Incubation temp. [°C]	35	40	40	40	35	35	35	35	35	35	35
Incubation time [h]	0.25	2	2	2	2	2	2	2	2	2	2

Figure 2: *GC-MS-based quantification of methyl chloride emitted from 1 g of freeze-dried sediment from lake Elton, Russia. Setups were shaken in 10 ml HEPES-buffered aqueous solution for 2 h at 35 or 40°C in presence or absence of additional Fe(III), HA, H_2O_2 and S. oneidensis MR-1 cells before measurement (experiment 5).*

Discussion

Even though we were able to detect some halogenated hydrocarbons in individual batch setups, this was in no case reproducible and we sometimes even got signals for VOX in negative controls. An exact repetition of the experiments performed by Keppler et al., (2002) and Keppler et al., (2006) did not lead to the detection of any vinyl chloride or chloroethyne, as it would have been expected from the results of these previous studies. This could either be explained by a lack of formation of these compounds in our setups due to small differences in the kinds of chemicals used etc. or by an lower sensitivity of the analytical system used in our study. Keppler et al., (2002) had also used a purge and trap-assisted GC-MS device with a preconcentration trap and reported detection limits of 2-5 pg total amount for chloromethane, vinyl chloride, trichloroethylene and 1,1,1-trichlorethane. The highest amounts of vinyl chloride these authors measured in 10 mL setups amended with catechol, Fe(III), KCl and H_2O_2 were in the range of 40 pg. As far as methyl chloride is concerned, they detected 600 pg in the same setups, both values clearly exceeding their detection limits. The detection limits of the GC-MS we used were 530 pg total amount for methyl chloride and 510 pg for vinyl chloride (S. Huber, personal communication). Thus, if the same amounts of these compounds had formed in our setups as in the setups of Keppler et al., (2002), we should just have been able to detect methyl chloride, but not vinyl chloride. This expectation was verified by the results from setup 7 in experiment 1 illustrated in Figure 1. This setup, which had the same initial chemical composition as setups 4-6 had been purged with He for 30 minutes before the measurement. Vinyl chloride has an air-water partitioning coefficient of 0.912 (Schwarzenbach et al., 2003), meaning that more than half of it remained in the liquid phase in our setups which had atmospheric pressure and a liquid-to-headspace ratio of 1:1. The aim of the purging process is to change this ratio leading to a transition of possibly all VC and other VOX into the headspace to render it available for detection by GC-MS. Since we could measure a higher number of VOX when we increased the purging time to 30 minutes, the 7.5 minute purging time we applied to parallel setups were obviously not sufficient to transfer all VOX into the headspace. Unfortunately, we could not

extend our purging times to 30 minutes for every setup due to the high costs of the He gas. Since Keppler et al., (2002) used purging times of up to 60 minutes at a helium stream of 20 mL/min while our stream was only 7 mL/min, this might have helped these authors to transfer an even higher fraction of VOX into the headspace and from there to the preconcentration trap and the GC-MS than we managed for the setup that we purged for 30 minutes. However, this still does not explain the more than 100-fold higher sensitivity of the instrumentation Keppler et al. (2002) used as compared to the analytical system used in our study.

In the end, we were unable to reproduce formerly made experiments with our GC-MS, which shows that the sensitivity of this instrument is not sufficient to use it in order to test our hypothesis concerning indirect stimulation of natural VOX formation with batch setups. However, if more sensitive instruments become available more easily, it would be of high interest to repeat our experiments and test our hypothesis of microbial stimulation of natural VOX formation anew.

A further point of concern in our experiments is the fact that CH_3Br, carbon disulfide and methyl furan were detected in most setups including controls that consisted of water only (Table 1; Figure 1). This means that there is either some background contamination of these compounds in the instrument itself or the water or septa we used did indeed release some CH_3Br, carbon disulfide and methyl furan. Even if this implies a very high sensitivity of the GC-MS for detecting traces of these compounds, in combination with the fact that only very small amounts of VOX are expected to be formed in our batch setups (i.e. in the pg range per 10 mL setup) we have to admit that it will be a challenge to differentiate between signals stemming from background contamination and signals from volatiles produced in the setups. Concerning experiment 3, it is interesting to note that in some of the setups (setups 4, 7 and 8; Figure 1), the unstable compound carbon suboxide (O=C=C=C=O; C_3O_2) was detected. By far the highest amount of C_3O_2 was measured in setup 6 which had been purged for 30 minutes, indicating again that the increased purging time improved the sensitivity of the measurement.

Carbon suboxide, which had been measured before in similar setups, has been proposed to be formed as an intermediate during oxidative degradation of aromatic compounds in soil (Huber et al., 2007). Thus, the results from our study strengthen these findings. The only experiment where we could reliably measure any VOX in the form of methyl chloride concerned the setups where freeze-dried and milled sediment from lake Elton was shaken in buffer for 2 h at 40 or 35°C. However, neither addition of Fe(III)-reducing bacteria, Fe(III), HA, H_2O_2 or any combination of these increased the amount of methyl chloride that was measured. Consequently, so far we did not get any support for our hypothesis of microbial stimulation of natural VOX formation by forming reactive Fe(III)/Fe(II) phases and organic radicals. This is true at least for the tested neutral-pH-scenario. Considering the facts that I) hardly any microbial cells from the sediment itself had survived the freeze-drying and milling procedure (microscopic analysis of freeze-dried and milled sediment with Dead/Live staining) and that II) the milled sediment had been stored for months before the experiment was performed, we expect it to be unlikely that the methyl chloride we detected in the setups was formed anew during the 2 h incubation period. Instead, we rather assume that the incubation at 35 or 40°C in combination with the shaking enhanced the release of methyl chloride from inclusions within the sediment. However, the repetitive measurement of methyl chloride in setups with lake Elton sediments clearly shows that some natural chlorination processes must occur or have occurred at this field site which is far from any possible industrial source of VOX. The up to 100-fold higher ratio of adsorbable organically bound halogens (AOX) to organic carbon in this and also in lake Kasin sediment as compared to average soils (Asplund and Grimvall, 1991) strongly points into the same direction. Results from our study rather argue against a formation of these halogenated hydrocarbons by a pathway that depends on reactive Fe(III)/Fe(II) phases and organic radicals originating from microbial activity at least in these pH-neutral environments. Alternatively, microorganisms could contribute to halogenations processes in the salt lake sediments by the activity of halogenating enzymes as they have been detected in soils (Asplund et al., 1993), or some purely abiotic halogenation reactions might take place. However, due to analytical

limitations including the inability to reproduce results from previous experiments we cannot completely rule out our initial hypothesis either. Therefore, further work with more sensitive analytics is necessary to elucidate whether and how microorganisms can influence natural VOX formation.

Conclusions & Outlook

Overall, we could only partially answer the research questions stated at the end of the introduction. The identification of VOX released from abiotic batch setups containing Fe(III), commercially available humic substances or functional analogues thereof, KCl at low and neutral pH in presence and absence of mM concentrations of H_2O_2 as a radical generator was rendered difficult since the measurements were not reproducible and some VOX measured in the experimental setups were detected in the negative controls as well. Our second goal, which was to quantify to which extent the addition of known Fe(III)-reducing microorganisms can increase organohalogen production in H_2O_2-free setups, could not be achieved since we could not even reproduce results from VOX-formation experiments in cell-free setups with 50 mM H_2O_2 as performed by Keppler et al., (2002). Thirdly, we wanted to determine the effect of adding known Fe(III)-reducing microorganisms to VOX-emitting salt lake sediments that had been sterilized by freeze-drying and grinding. However, no effect could be detected. Since these unsatisfactory results are most probably due to the fact that the sensitivity of our analytics was not high enough to detect the trace amounts of VOX formed in our setups, one recommendation for further studies would be to repeat these experiments with an analytical system that is sensitive enough to allow at least reproduction of the results obtained from the experiments performed by Keppler et al., (2002). Another idea would be to restrict oneself to experiments with field samples that can reproducibly be shown to emit VOX such as lake Elton sediment. These samples could then be used to test the influence of the addition of different kinds of microorganisms (including leaving the indigenous microbial communities intact by

circumventing the freeze-drying procedure), varying incubation conditions etc. on the identity and quantity of released VOX.

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4

Identification and quantification of volatile organohalogens released from batch setups with Fe(III)-reducing microorganisms and field samples from salt lake sediments

Maren Emmerich & Andreas Kappler

APPENDIX

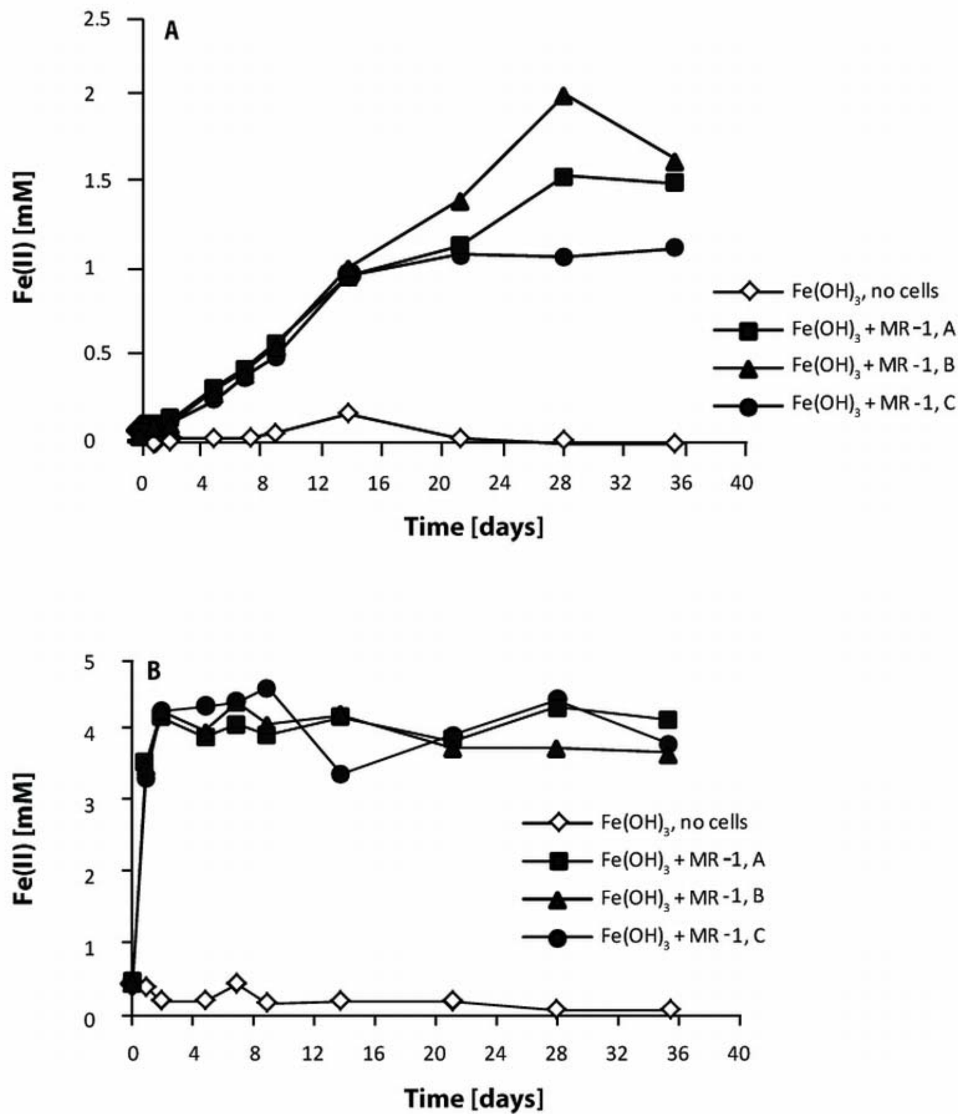

Figure A1: *Reduction of initially 10 mM $Fe(OH)_3$ in SBM medium with 30 mM lactate as an electron donor by an inoculums of 10^7 cells/mL Shewanella oneidensis MR-1 under anoxic conditions A) in absence and B) in presence of 1 mg/mL PPHA. Filled symbols illustrate the increase of Fe(II) (in mM, y-axis) over time (in days, x-axis) for three parallel setups while open symbols represent data from an abiotic control. Note different values on the y-axes.*

5

Absence of humic substance reduction by the acidophilic Fe(III)-reducing strain *Acidiphilium* SJH: implications for stimulation of natural organohalogen formation and for the mechanism of Fe(III) reduction at acidic pH

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humic and fulvic acid reduction; natural organohalogen formation

Abstract

A vast amount of volatile organohalogens (VOX) has natural origins. Both soils and sediments have been shown to produce VOX via redox reactions between Fe(III) and quinones in the presence of halide anions, particularly at acidic pH. We tested whether acidophilic Fe(III)-reducers might indirectly stimulate natural VOX formation at acidic pH by providing reactive Fe and quinone species. However, it is unknown whether acidophilic Fe(III)-reducers can reduce humic acids (HA) or fulvic acids (FA). We therefore tested the ability of the acidophilic Fe(III)-reducer *Acidiphilium* SJH to reduce macromolecular, suspended HA and dissolved FA at pH 3.1-3.3. We found that i) SJH can neither reduce HA/FA nor the humic model quinone anthraquinone-2,6-disulfonic-acid (AQDS) nor stimulate the formation of FA radicals, ii) at acidic pH, significantly more electrons are transferred abiotically both from native and reduced FA to dissolved Fe(III) than from native or reduced HA, and iii) the presence of strain SJH does not stimulate VOX formation. Our results imply that the acidophilic Fe(III)-reducer SJH either uses an enzyme for Fe(III) reduction that can neither be used for HA/FA nor for AQDS reduction or that the location of Fe(III) reduction is inaccessible for these compounds. We further conclude that microorganisms such as strain SJH probably do not indirectly stimulate natural VOX formation at acidic pH via the formation of reactive quinone species.

Introduction

Volatile halogenated organic compounds (VOX) play an important role in atmospheric chemical processes including stratospheric ozone destruction (Molina, 1974) and global warming (WMO, 2010). Some of these compounds have mainly natural sources, e.g. methyl chloride (Butler, 2000). Several recent studies suggest that a considerable amount of CH_3Cl might originate from decaying plant material in tropical soils (Keppler *et al.*, 2005, Saito et al., 2008, WMO, 2010). However, details of reaction pathways, the exact role soils play in the global cycling of VOX as

well as the parameters controlling emissions from this source remain to be elucidated. In batch experiments and soil incubations performed at pH 2-3, VOX yields increased tremendously with the addition of H_2O_2 (Keppler et al., 2002, Keppler et al., 2006, Huber et al., 2009), suggesting a Fenton-like reaction and an important role of hydroxyl- or semiquinone radicals. Since the presence of an active microbial community has been shown to be crucial for VOX emission from sediments (Weissflog *et al.*, 2005), we hypothesized that microbial radical formation via humic substance reduction could stimulate VOX formation and therefore intended to evaluate the influence of microorganisms on VOX emissions from soil and sediment.

Humic substances (HS) are polymeric, heterogeneous redox-active organic compounds formed during the degradation and transformation of biopolymers such as lignin, proteins and carbohydrates. HS consist of three operationally defined fractions: humins, which are non-soluble at both acidic and alkaline pH; humic acids (HA), which are alkaline-soluble and acid-insoluble (with a small portion being soluble at neutral pH); and fulvic acids (FA), which are soluble at both acidic and alkaline pH (Stevenson, 1994). The group of microorganisms that can reduce dissolved humic substances at circumneutral pH includes Fe(III)-reducers (Lovley et al., 1996), fermenting bacteria (Benz et al., 1998), toluene degraders, sulfate-reducers and methanogens (Cervantes *et al.*, 2002). It has been shown recently that even solid-phase humic substances can serve as electron acceptors for bacteria (Roden et al., 2010).

All neutrophilic Fe(III)-reducing bacteria so far tested have been able to transfer electrons to humic substances or to the humic model quinone AQDS (anthraquinone-2,6-disulfonate) (Lovley et al., 1996, Coates et al., 1998, Francis et al., 2000, Nevin and Lovley, 2000, Finneran et al., 2002, He and Sanford, 2003). During this process, organic radicals are formed (Scott et al., 1998). In order to analyze if microorganisms could indirectly stimulate natural organohalogen formation in low-pH soil environments by providing organic radicals, we tested whether one specific acidophilic Fe(III)-reducer, *Acidiphilium* strain SJH, can reduce HS and produce semiquinone radicals similar to neutrophilic Fe(III)-reducers.

For neutrophilic Fe(III)-reducers it has been demonstrated in a recent study that under pH neutral conditions, the same *c*-type cytochromes distributed over both cell membranes and the periplasm are involved in electron transfer to both humic substances and Fe(III) oxides (Voordeckers et al., 2010). However, the reduction of chelated, and therefore dissolved Fe(III), which can enter the periplasm e.g. via ligand-gated outer membrane-receptors (Andrews et al., 2003), does not necessarily depend on the cytochrome electron transport chain (Leang et al., 2005, Mehta et al., 2005). This strongly suggests that an additional Fe(III)-reduction mechanism must exist within neutrophiles that mediates electron transfer to dissolved Fe(III) which might be located in the periplasm and has been suggested to be used for AQDS reduction as well (Lies et al., 2005). At this point, the question arises whether acidophilic Fe(III)-reducers can also reduce macromolecular HA and FA, or at least the quinone model compound AQDS, as it is small enough to enter the periplasm via porins.

Recent research has shown that the potential for dissimilatory ferric iron reduction is widespread among acidophilic heterotrophic bacteria (Coupland and Johnson, 2008). This makes sense considering the fact that Fe(III) in the acidic solutions represents a favorable electron acceptor for energy generation by microorganisms at low pH. An explanation for this can be found in the standard redox potential of the ferrous/ferric iron couple being +770 mV at pH 2 in comparison to -112 mV at neutral pH (assuming 1 mM dissolved Fe(II)) (Stumm and Morgan, 1996). In our study we used an acidophilic bacterium with particularly high rates of Fe(III) reduction, *Acidiphilium* strain SJH. SJH is a heterotrophic, facultative anaerobic α-proteobacterium which has been isolated from acid mine fluids (Johnson and McGinness, 1991). This strain can reductively dissolve a variety of Fe(III) minerals such as goethite and akageneite (Bridge and Johnson, 2000) and couple Fe(III) reduction to growth under both oxic and anoxic conditions (Johnson and Bridge, 2002). The mechanism of Fe(III) reduction in SJH is only poorly understood. This bacterium was suggested to excrete a heat-stable compound (e.g. a small organic ligand but not an enzyme) that enhances the dissolution of Fe(III)-containing minerals,

but does not catalyze Fe(III) reduction itself (Bridge and Johnson, 2000). The localization of the Fe(III)-reducing enzyme(s) in this acidophile remains subject of further research.

In order to elucidate whether microbial radical formation via humic substance reduction at acidic pH (by acidophilic Fe(III)-reducers) could stimulate VOX formation, in the present study we intended to determine i) whether non-dissolved HA can serve as electron acceptors for the acidophilic Fe(III)-reducer *Acidiphilium* SJH. Further we wanted to test, ii) whether this strain can reduce fulvic acids or the model quinone AQDS, both of which are dissolved under acidic conditions, and iii) whether VOX are formed in cultures of SJH in presence of Fe(III), HA/FA and chloride. These results will then allow to draw conclusions regarding the potential indirect microbial influence of acidophilic Fe(III)-reducing microorganisms such as strain SJH on natural VOX formation.

Material and Methods

If not stated otherwise, sterile and anoxic conditions were maintained for preparation of all solutions as well as during sampling. All experiments were set up in an anoxic chamber (glovebox) under N_2 atmosphere and incubated at 28°C in the dark. Details concerning composition and incubation conditions of the experimental setups are given in table 1.

Bacterial cultures and media

Acidiphilium strain SJH was kindly provided by DB Johnson (Bangor University) and cultivated in liquid medium containing 15 mM $(NH_4)_2SO_4$, 1.3 mM KCl, 3 mM K_2HPO_4 and 2 mM $MgSO_4$ x 7 H_2O (medium 269 of the German Collection of Microorganisms and Cell Cultures, DSMZ). The pH was adjusted to pH 3.0 with 1 M H_2SO_4 and if the medium was used for cultivation, glucose and yeast extract were added after autoclaving to final concentrations of 0.1 % and 0.03 % (w/v), respectively. Cultures were kept at 28°C under oxic conditions and shaken at 200 rpm. For reduction experiments, the same mineral medium was prepared in a Widdel flask

and cooled to room temperature under an N_2 stream. If necessary, pH was readjusted to pH 3.0 with 1 M H_2SO_4, before the medium was dispensed into 50 ml glass bottles without addition of glucose or yeast extract. 10 mM glycerol was added from a 500 mM stock solution. Experiments were set up with 5-day old cultivation cultures that were harvested at the end of their logarithmic growth phase. In the beginning of an experiment, cells were quantified by optical density (OD) measured at 600 nm according to a calibration curve plotting OD_{600} against microscopic cell counts with a Neubauer chamber (cell number per ml = $6 * 10^{8} * OD_{600} + 10^{7}$). 1.2 times as many cells as needed (to account for the loss during centrifugation/washing) were centrifuged for 10 minutes at 9,000 g and washed with mineral medium twice. After the third centrifugation step, cell pellets were resuspended in mineral medium to a concentration that required 100 or 250 µl of the resulting cell suspension to be added per setup in order to give rise to final cell densities of 10^{7} or 5×10^{8} cells per ml, respectively. Final cell densities were verified by microscopic counts of DAPI-stained cells for selective setups at the beginning and end of batch experiments.

Preparation of native and chemically reduced humic and fulvic acid solutions

Hohloh Lake fulvic acids were kindly provided by Christian Zwiener (University of Tübingen). A detailed characterization of these fulvic acids is published in (Gul et al., 2003). Pahokee Peat humic acids (PPHA) were purchased from the International Humic Substance Society (IHSS). HA suspensions and FA solutions were prepared in mineral medium 269.

Final concentrations of 5 or 10 mg/ml of the respective HA and FA were prepared in medium under constant stirring. During this process, all FA dissolved while the majority of HA remained in suspension. The pH was adjusted to pH 3.0 with NaOH until it remained stable for at least 10 min. FA solutions and HA suspensions were transferred into 100 ml glass bottles in batches of 60 ml and closed with butyl rubber stoppers and metal crimp caps. Native (non-reduced) HA suspensions and FA solutions were deoxygenated by applying 3 cycles of 3 min vacuum followed by 1 min flushing with N_2. For chemical reduction of FA solutions and HA suspensions, 5

palladium-coated aluminum pellets (0.5 % Pd, Merck) were added per bottle prior to degassing by application of vacuum for 30 min. FA solutions and HA suspensions were then flushed with H_2 for 2 min and put on a rotary shaker for 16 h. Prior to the use of FA solutions and HA suspensions in further experiments, headspaces were exchanged with N_2 as described above in order to avoid Fe(III) reduction by residual H_2 in the Fe(III)-NTA assay (Roden et al., 2010). Both native and chemically reduced FA solutions were additionally filtered through 0.22 µm cellulose ester filters into evacuated and sterilized bottles. HA suspensions were not filtered in order to leave the precipitated HA particles in the reaction mixture. For determination of the redox state of chemically reduced HA (see below), HA samples were taken directly from the suspensions after brief shaking by hand followed by rapid sedimentation of the Pd-Al-pellets to the bottom of the vial thus leaving the palladium-coated alumina pellets behind.

Setup of microbial HA and FA reduction experiments

The experiments to determine if SJH can reduce HA and FA (HA and FA reduction experiments) were set up in 20 ml glass vials with 10 ml total volume. Chemically reduced HA suspensions and FA solutions (for preparation see previous section) served as positive controls for the microbial reduction experiments. First, glycerol was added to the HA- or FA-containing medium followed by addition of the cells. Both incubation as well as sampling at t=0 and t=5 h and the Fe(III)-NTA assay took place within the anoxic chamber. The content of dissolved organic carbon (DOC) was quantified, after filtration and appropriate dilution of samples, by a total organic carbon (TOC) analyzer (Elementar, Hanau, Germany).

Table 1: *Overview about composition and incubation conditions of five separate experimental setups and the respective control experiments (two different control experiments per experimental setup).*

Experiment	Composition and number of setups	Composition and number of controls	Incubation conditions
HA reduction experiment	Medium 269 5 mg/ml native PPHA 10 mM glycerol 5 x 10^8 cells per ml (3 setups)	Medium 269, 5 mg/ml chemically reduced PPHA 10 mM glycerol (3 setups) -- Medium 269, 5 mg/ml native PPHA 10 mM glycerol (3 setups)	anoxic, 5 h
FA reduction experiment	Medium 269 5 mg/ml native FA 10 mM glycerol 5 x 10^8 cells per ml (3 setups)	Medium 269, 5 mg/ml chemically reduced PPHA 10 mM glycerol (3 setups) -- Medium 269, 5 mg/ml native PPHA 10 mM glycerol (3 setups)	anoxic, 5 h
AQDS reduction experiment	Medium 269 2 mM AQDS 10 mM glycerol 10^7 cells per ml (6 setups)	50 mM phosphate buffer pH = 4.7 2 mM chemically reduced AQDS 10 mM glycerol (1 setup) -- Medium 269, 2 mM AQDS 10 mM glycerol (2 setups)	anoxic, 4 weeks oxic, 4 weeks
VOX formation experiment	Medium 269 5 mg/ml native PPHA 10 mM glycerol 35 mM dissolved Fe(III) 5 x 10^8 cells per ml (3 setups)	Medium 269, 5 mg/ml chemically reduced PPHA 10 mM glycerol 35 mM dissolved Fe(III) (3 setups) -- Medium 269, 5 mg/ml native PPHA 10 mM glycerol 35 mM dissolved Fe(III) (3 setups)	anoxic, 3 weeks
ESR experiment	Medium 269 10 mg/ml native FA 10 mM glycerol 5 x 10^8 cells per ml (3 setups)	Medium 269, 10 mg/ml chemically reduced PPHA 10 mM glycerol (3 setups) -- Medium 269, 10 mg/ml native PPHA 10 mM glycerol (3 setups)	anoxic, 5 h

Determination of redox state of humic and fulvic acids by the Fe(III)-NTA assay

The redox state of HA suspensions and FA solutions before and after 5 h incubation with SJH as well as before and after chemical reduction was determined with Fe(III)-NTA as described by (Roden et al., 2010) with the only modification that 0.5 ml of sample was incubated with 1 ml of 5 mM Fe(III)-NTA.

Quantification of VOX formation in Fe-HA experiments

Experiments to quantify volatile organohalogens (VOX) formed in microbially stimulated versus abiotic setups were prepared in the same way as the HA reduction experiments. However, here 35 mM dissolved Fe(III) was added from a 500 mM $Fe(III)_2(SO_4)_3$ stock solution to every setup and the glass vials were closed with PTFE-layered butyl rubber septa to enable GC-MS-measurements of volatile organohalogens. Organohalogens were then quantified by GC-MS as described by (Huber et al., 2010). Details about instrumentation, temperature program and detection limits are given in online resource 5.

AQDS reduction experiments

The experiments to determine if SJH can reduce AQDS were set up in a similar way to the FA and HA reduction experiments, i.e. in 20 ml glass vials with 10 ml total volume. 3 parallel setups were prepared anoxically and closed using butyl rubber stoppers. 3 parallel setups were covered by loose aluminum caps enabling exchange with atmospheric O_2 during incubation. AQDS reduction to the hydroquinone form was followed visually by a color change from translucent (AQDS) to yellow (AH_2QDS). Chemically reduced AH_2QDS in the same medium was prepared as a positive control by reduction via the Pd/H_2-treatment described above. Since the pH increased from pH 3 to pH 7 due to the addition of Pd pellets (even without/before H_2 addition), chemical reduction of AQDS with Pd/H_2 was also performed in 50 mM phosphate buffer that

was adjusted initially to pH 3. However, even in the 50 mM phosphate buffer, the pH increased after addition of Pd pellets from pH 3 to pH = 4.7.

Electron spin resonance (ESR) spectroscopy

For quantification of organic radicals by ESR spectroscopy, 3 parallel setups containing 10 mg/ml FA were adjusted inside an anoxic chamber to pH 12.0 with NaOH to stabilize the organic radicals that had formed before and after 5 h incubation with strain SJH. Samples from chemically pre-reduced and native FA solutions in absence of SJH were prepared for comparison. Samples were added to glass capillaries (Blaubrand Intramark Mikropipettes, Brand GmbH, Germany) and sealed with a vinyl sealing kit (Haematocrit Sealing Compound, Brand GmbH, Germany). They were then placed into quartz tubes with an inner diameter of 4 mm and closed with plastic caps (Magnettech GmbH, Berlin, Germany). To limit penetration of O_2 into the samples throughout transport and measurement, the tubes were additionally sealed with parafilm. Quartz tubes were then analyzed at the Federal Institute for Materials Research and Testing (BAM) in Berlin where ESR spectra were recorded at 25°C using an ESR spectrometer (MiniScope MS 300, Magnettech GmbH, Berlin, Germany) at a modulation amplitude of 2000 mG, a damping of 20 dB and an amplification of 900 with 3 runs of 30 s per spectrum.

Results and Discussion

In order to evaluate whether acidophilic microorganisms might stimulate natural organohalogen formation by producing organic radicals, we tested the ability of *Acidiphilium* strain SJH to reduce HA, FA and AQDS in comparison to its ability to reduce Fe(III).

Fe(III) reduction by strain SJH under both oxic and anoxic conditions

In order to first evaluate the Fe(III) reduction capacity of SJH, we monitored Fe(III) reduction over time both under anoxic and microoxic conditions. Since we were interested in maximum rates of Fe(III) reduction, we used Fe(III) sulfate as electron acceptor, which is almost completely soluble at pH 2-3 leading to high concentrations of dissolved Fe(III) at the pH at which our experiments were conducted. It has been shown before that SJH cannot use sulfate as an electron acceptor (Johnson and McGinness, 1991). Figure S1A shows that an initial inoculum of 10^7 cells/ml reduced 4 mM Fe(III) to Fe(II), i.e. approx. 80 % of the initially present 5 mM Fe(III), with 10 mM glycerol as an electron donor within 35 days.

In order to compare the extent of Fe(III) reduction by SJH under microoxic and anoxic conditions, we repeated the same experiment under microoxic conditions. Figure S1C shows that all Fe(III) got reduced under microoxic conditions within 27 days, while under anoxic conditions, only around 10 % got reduced within the same time. This can be explained by the around 14-fold increase in cells numbers we observed in the microoxic, but not in the anoxic setups (figure S2).

To find out whether cell suspension experiments can be applied to test reduction by strain SJH, we first determined whether strain SJH can reduce Fe(III) in cell suspensions, in addition to the observed Fe(III) reduction in growing batch cultures. In these cell suspension experiments, we found that with 5 x 10^8 cells/ml, approximately 1 mM of dissolved Fe(III) was reduced within 5 h under anoxic conditions (figure S1B).

Absence of reduction of fulvic and humic acids and AQDS by strain SJH

As strain SJH reduced Fe(III) efficiently under anoxic conditions, we determined whether strain SJH could also reduce fulvic and/or humic acids under the same conditions. To this end, SJH cells were incubated with Pahokee Peat HA or Lake Hohloh FA as electron acceptor and glycerol as electron donor. Even though SJH had been shown to reduce Fe(III) at much higher rates under oxic than under anoxic conditions, anoxic conditions were chosen for these kinds of

experiments in order to avoid reoxidation of FA, HA and AQDS by atmospheric oxygen. As positive controls, HA and FA were reduced chemically by Pd/H_2 and were incubated with glycerol under the same conditions as in the experimental setups, but without addition of cells. Since it had been previously demonstrated that the reducing capacities of HA after chemical reduction are very similar to the values obtained for microbial reduction (Peretyazhko, 2006), we considered chemically reduced HA and FA as suitable positive controls to assess the capacity of strain SJH to reduce them microbially. At the beginning and at the end of incubation of strain SJH with the HA and FA, the reduction state of the humic or fulvic acids was determined by quantifying the amount of electrons that were transferrable from the HA and FA to Fe(III)-NTA (figure 1).

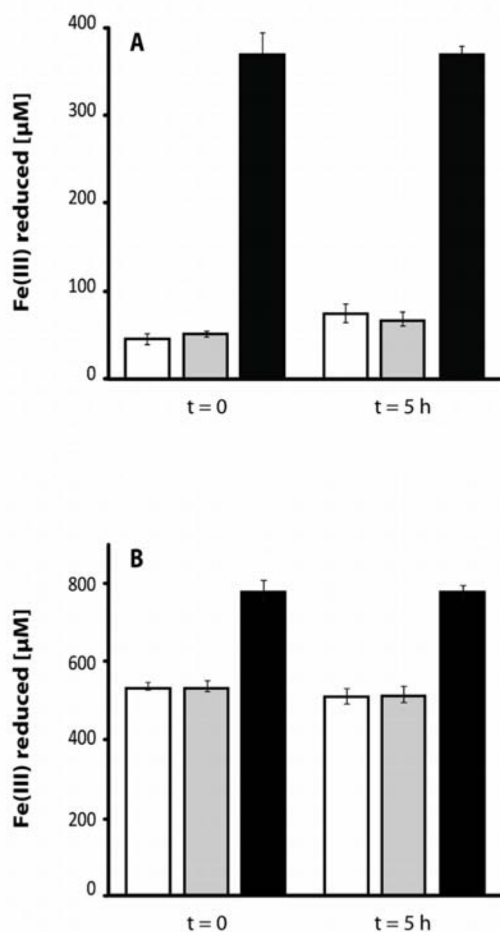

Figure 1. *Amount of Fe(II) formed by incubation of humic acids (A) and fulvic acids (B) with Fe(III)-NTA before (0 h) and after incubation (5 h) with (grey bars) and without (white bars) the acidophilic Fe(III)-reducing strain SJH and glycerol as electron donor. Black bars represent humic or fulvic acid solutions which had been first chemically reduced by* H_2*/Pd for 18 h and then incubated for 0 or 5 h with glycerol (no microbial cells present). Experiments were performed with A) Pahokee Peat humic acid and B) Lake Hohloh fulvic acid. Please note the different maximum values at the y-axes. Standard deviation/error bars were calculated from results of triplicate setups.*

We found that native (non-reduced) HA reduced only low amounts of Fe(III) in the Fe(III)-NTA assay before and after incubation with glycerol, demonstrating that the glycerol did not chemically reduce the HA (figure 1). In contrast, chemically reduced HA reduced approximately 8 times more Fe(III) than the native HA. Incubation of the reduced HA with glycerol in the absence of cells did not lead to an increased reducing capacity again indicating that the glycerol did not reduce the HA chemically. We then tested whether addition of SJH cells changed the reducing capacity of the HA. However, incubation of native HA in the presence of SJH and glycerol showed no evidence for electron transfer from the cells to the HA. We therefore concluded that strain SJH is the first Fe(III)-reducer tested that is unable to reduce humic acids, although it can reduce Fe(III). Because the cells were present during the short 1 min Fe(III)-NTA assay, we performed control experiments with cells alone (no HA and no FA) to determine whether they influenced the Fe(III)-NTA assay and could show that no detectable microbial Fe(III) reduction by cells from biotic setups takes place within the 1 minute reaction time (data not shown).

Potential reasons for the absence of humic substance reduction by strain SJH

In order to understand the absence of HA reduction by strain SJH the solubility and molecular size of the HA have to be considered. Even though the pH of our medium and HA, as well as FA stock solutions, had been adjusted to 3.0, the different compositions and reactions within the individual setups caused slight pH changes over the incubation periods. Therefore, we measured a final pH of 1.8 in the Fe(III) reduction setups, a pH of 3.1 in the FA and AQDS reduction setups and a pH of 3.3 in the HA reduction setups. The pH values of the positive controls where the FA and HA solutions had been reduced by Pd-H_2 were slightly (0.2 to 0.4 pH units) higher than the pH values of the experimental setups after incubation. However, these slightly higher pH values were also measured in control setups with HA and FA solutions which had been incubated with Pd under N_2 atmosphere (in absence of H_2) for 16 h. Samples from these control

setups did not reduce any additional Fe(III) in the Fe(III)-NTA assay compared to samples from setups prepared with native FA/HA solutions that had never encountered any Pd and had a slightly lower pH (figure S4). Therefore, we can rule out any effect of small pH deviations or residual traces of Pd in some setups on the Fe(III)-NTA assay including Fe quantification.

Within the pH range of our experiments, FA molecules are soluble, in contrast to HA for which only a very small part is dissolved. DOC measurements of 0.22 µm-filtered setups without glycerol showed that approximately 4.5 mg/ml FA but only 0.5-0.7 mg/ml HA were in solution in the individual setups and that most of the HA were present in non-dissolved state. Since also under neutral pH conditions most humic substances are non-dissolved but can still be used by Fe(III)-reducing microorganisms as electron acceptor (Kappler et al., 2004; Roden et al., 2010), our data suggest that not all Fe(III)-reducing microorganisms can reduce dissolved and non-dissolved humics. This implies that different Fe(III) reduction pathways are present in known neutrophilic Fe(III)-reducers than in the acidophilic Fe(III)-reducing strain analyzed in this study.

Since it had been suggested that the poorly described second Fe(III) reduction pathway for dissolved, and thus easily accessible, Fe(III) that occurs within neutrophilic Fe(III)-reducers might be involved in reduction of soluble quinones (Voordeckers et al., 2010), we hypothesized that strain SJH can reduce AQDS and FA. While the FA are dissolved but probably too large to enter the periplasm to a large extent, AQDS is both dissolved and small enough to enter the periplasm (Shyu et al., 2002). However, our experiments showed that strain SJH can neither reduce FA (figure 1B) nor the model quinone compound AQDS (no color change to yellow could be observed neither upon incubation under oxic or anoxic conditions while chemically reduced AH_2QDS turned clearly yellow even at acidic pH (figure S4). This suggests that even if the enzyme that reduces Fe(III) in acidophiles is located in the periplasm, it is not only unable to reduce quinones due to a lack of accessibility (in the case that they are too large to enter the periplasm) but also due to a lack of specificity for quinone moieties.

Interestingly, in the experiments with FA we observed a much higher background Fe(III) reduction in the Fe(III)-NTA assay by the native FA when compared to the HA experiments. More than 10 times more Fe(II) was formed by native FA (values of 531 μM) than by native HA (values in the range of 46 μM), independent of whether cells were present or whether the samples had been taken before or after incubation with cells (figure 1).

Chemically reduced FA also produced twice as much Fe(II) as chemically reduced HA. This data first suggests a general higher reducing capacity of the FA compared to the HA used in our experiments, and second, points towards a relatively reduced redox state of the native FA.

This is in agreement with previous experiments showing that native FA can reduce several times more Fe(III) at acidic pH than native HA (Szilágyi, 1971, Skogerboe and Wilson, 1981). These authors explain this finding by citing the higher redox potential of HA suspensions than of FA solutions at pH 2. The reduction potential of a HA suspension at pH 2 versus the normal hydrogen electrode has been determined to be +700 mV (Szilágyi, 1973), while for FA a value of +500 mV has been reported (Skogerboe and Wilson, 1981). These values are slightly below the redox potential of the Fe^{3+}/Fe^{2+}-couple of +770 mV at pH 2 (Stumm and Morgan, 1996) but definitely positive enough to render their reduction with glycerol as an electron donor thermodynamically favorable.

Abiotic processes between Fe(III)-NTA, glycerol and HA/FA

In order to determine to which extent abiotic reactions, in particular glycerol-HA and glycerol-FA interactions might have influenced the Fe(III)-NTA assay, we set up batch experiments where dissolved Fe(III) was incubated with either FA or HA in the presence and absence of glycerol. For comparison, dissolved Fe(III) was incubated with glycerol alone. We found that glycerol alone did not reduce any Fe(III) (figure 2).

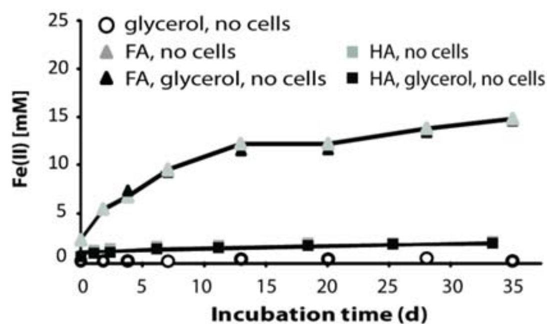

Figure 2. *Fe(II) formation over time during abiotic incubation of 40 mM Fe(III) with 10 mM glycerol (white circles), 5 mg/ml FA (grey triangles), 5 mg/ml HA (grey squares), 10 mM glycerol plus FA (black triangles), or 10 mM glycerol plus HA (black squares). Setups were prepared sterilely without addition of cells and incubated for 35 days under anoxic conditions.*

However, within a couple of days, we quantified 6 mM Fe(II) in the HA-containing setups and even 15 mM Fe(II) in the FA-containing Fe(III)-NTA setups both in the presence and absence of glycerol a) confirming the reduction of Fe(III) by native HA and FA as described above and b) suggesting that the glycerol did not further increase or influence this reaction, i.e. HA and FA are not reduced by the glycerol.

Absence of radical formation during incubation of humic substances with strain SJH

In addition to the quantification of electron transfer from humic substances to Fe(III), we used radical measurements by ESR spectroscopy to investigate microbial reduction of FA under acidic pH conditions. In contrast to microbial reduction experiments with FA and HA at neutral pH where high radical concentrations were found (Jiang et al., 2009), in our setups where solutions of 10 mg/ml FA were incubated with strain SJH at acidic pH, no radical formation could be detected by ESR measurements (data not shown). Moreover, incubation of FA with H_2/Pd at pH 2.3 also did not lead to increasing concentrations of radicals, although increasing reducing capacities were observed (figure 1). This suggests that radical measurements are not suited to follow reduction of HA and FA under acidic conditions since under these conditions reduction obviously leads – in contrast to reduction at neutral pH - exclusively to the hydroquinone state

without significant accumulation of semiquinone radicals. This is in line with results from a modeling study by (Rosso et al., 2004) who calculated that at acidic pH, a two-electron transfer to AQDS to the hydroquinone AH_2QDS is thermodynamically much more favorable than a one-electron transfer to form semiquinone. Additionally, (Ratasuk and Nanny, 2007) showed that below pH 6.5, most likely non-quinone functional groups serve as electron acceptors in humic substances, which additionally explains why we did not observe any semiquinone radical formation at pH 2.3.

Microbial influence on natural organohalogen formation

In order to test our initial hypothesis, namely whether VOX are formed in cultures of Fe(III)-reducers in presence of Fe(III), HA and chloride, we performed GC-MS measurements of setups with native PPHA in presence and absence of SJH and with chemically reduced PPHA without bacteria. With regard to our target compounds, namely volatile organohalogens that have been shown to be produced naturally in previous studies, chloromethane and bromomethane were detected in some setups in the low ng range (data not shown). However, calculated in pptv, the CH_3Cl concentrations we measured were about twofold lower than environmental background concentration of about 600 pptv as given by WMO (2010). For CH_3Br, the concentrations we measured exceeded the environmental background concentration of 7.5 pptv (WMO, 2010) about 200-fold. However, similar amounts of these compounds were detected in all setups including both abiotic control setups and microbially active setups. This means that bromomethane might have formed in our setups but its formation was not enhanced by the presence of the acidophilic Fe(III)-reducing strain *Acidiphilium* SJH. Since SJH can neither reduce HA/FA or AQDS at acidic pH nor cause semiquinone formation of these compounds at pH 3, this result could be expected. Consequently, we propose that this acidophilic Fe(III)-reducer cannot enhance natural organohalogen formation in acidic environments by providing organic radicals. However, (Huber et al., 2009) showed that the Fe(III)- and radical-dependent

oxidative way of VOX formation strongly decreases with increasing pH, such as at pH >3.6, and hardly any trihalomethane formation could be observed any more. Therefore, based on our results, we hypothesize that microorganisms do not stimulate the radical-dependent mechanism of organohalogen formation via formation of reactive Fe and humics species. Instead we propose that in the pH-neutral salt lake sediments, where a clear dependency of VOX formation on the presence of an active microbial community has been shown (Weissflog et al., 2005), microbes rather mediate the organohalogen formation in a direct way, e.g. via haloperoxidase-like enzymes, which have already been shown to be present in soil (Asplund et al., 1993).

Implications for mechanisms of electron transfer to Fe(III) in neutrophilic and acidophilic Fe(III)-reducing microorganisms

The present study also revealed details regarding the potential electron transport pathway(s) to Fe(III) and the selectivity of the responsible Fe(III)-reducing enzymes in the acidophilic Fe(III)-reducing microorganism *Acidiphilum* strain SJH in comparison to neutrophilic Fe(III)-reducers (figure 3). Neutrophilic Fe(III)-reducers have to cope with a poorly soluble electron acceptor, i.e. Fe(III) minerals. To overcome this problem, three strategies for electron transfer are known to exist within neutrophilic Fe(III)-reducers. These include direct contact between outer membrane Fe(III)-reductases and the Fe(III) mineral and electron transfer mediated by outer membrane cytochromes (DiChristina et al., 2002; Clarke et al., 2011), the use of microbially produced or external electron shuttles (e.g. dissolved or solid-phase humic substances (Jiang and Kappler, 2008; Roden et al., 2010) as well as the excretion of organic ligands that solubilize Fe(III) which can then be taken up by the bacteria and reduced by a Fe(III) reductase located in the periplasm or in the inner membrane (Pitts et al., 2003, Fennessey et al., 2010).

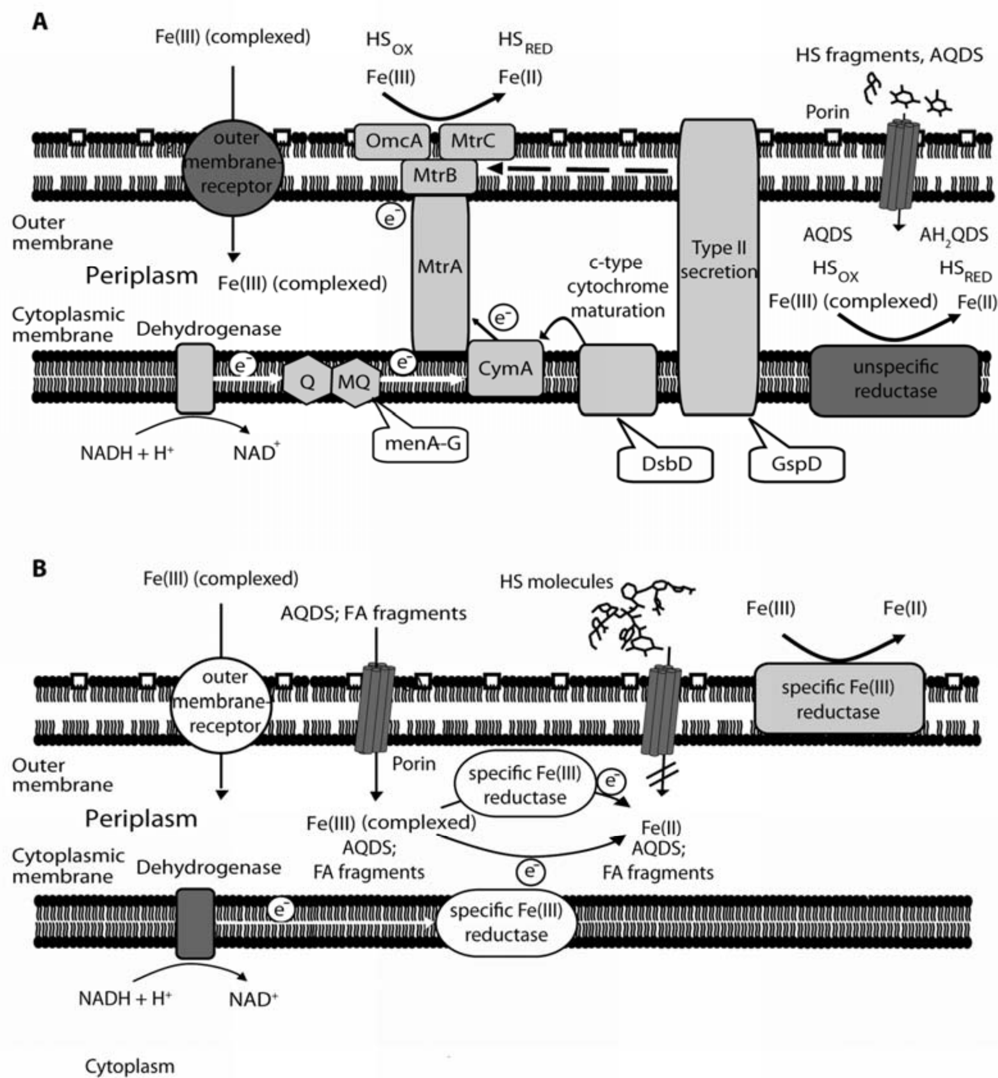

Figure 3. *Mechanisms of microbial Fe(III) and humic substance reduction A) by neutrophilic Fe(III)-reducers and B) by the acidophilic Fe(III)-reducer Acidiphilium strain SJH as proposed in this study. Previous studies indicate that at least 2 ways for Fe(III) and humic substance reduction exist in neutrophiles including 1) cytochrome-dependent electron transfer leading to a terminal reductase at the outer membrane (involved proteins are depicted in light grey) and 2) reduction of membrane-permeable quinones and dissolved (complexed) Fe(III) in the periplasmic space or at the inner membrane (involved proteins are depicted in dark grey). Reduction of complexed Fe(III) by a periplasmic- or inner-membrane reductase is also a possible mechanism in acidophiles (hypothetically mediated by proteins depicted in white in panel B). Alternatively, this enzyme could also be located at the outer membrane (depicted in light grey). Based on our results, the Fe(III) reducing enzyme of SJH does not accept any quinones as electron acceptor, as it does in the case of neutrophiles.*

Reduction of Fe(III) and humic substances by outer membrane reductases that depend on electron flow over several quinone- and *c*-type cytochrome-containing proteins in the inner membrane and the periplasm have recently been shown to be based on the same molecular machinery (Lies et al., 2005, Voordeckers et al., 2010).

Electron transfer to dissolved Fe(III) by periplasmic electron transfer components has been suggested to represent an additional pathway of Fe(III) reduction for neutrophiles and to mediate reduction of dissolved and outer-membrane-permeable quinones such as AQDS (Lies *et al.*, 2005). It is very well feasible that at neutral pH even FA and possibly also HA fragments can be reduced by this mechanism. This means that no Fe(III) reductase is known to exist within neutrophiles that could not transfer electrons either to HA/FA or to dissolved quinones.

The lacking ability to reduce HA/FA and AQDS by the Fe(III) reductase of strain SJH could point either to a difference in localization or specificity of this Fe(III) reductase in comparison to Fe(III) reductases of neutrophiles. The main difference between Fe(III) reduction at acidic versus neutral pH is the up to 10^{18} fold higher availability of dissolved iron (Fe^{3+}) in acidic as compared to neutral environments which goes along with an astoundingly large number and diversity of Fe(III)-uptake systems of acidophilic microorganisms (Osorio et al., 2008). This suggests that Fe(III) reduction in acidophiles could occur in the periplasm or even in the cytoplasm. However, since acidophiles maintain a circumneutral pH in the cytoplasm as neutrophiles do (Hsung and Haug, 1977, Oshima et al., 1977, Cox et al., 1979), an uptake of the Fe(III) into the cytoplasm would lead to a loss of the thermodynamic advantage to reduce dissolved (free) Fe^{3+} at low pH (since the Eh of redox couples of Fe^{2+}/complexed Fe(III) are less positive than the Eh of Fe^{2+}/Fe^{3+}). This renders a cytoplasmic location of the Fe(III) reductase in acidophilic Fe(III)-reducing microorganisms highly unlikely, even though we cannot completely exclude this possibility. The fact that filtered heat-killed cells and SJH spent medium could mediate dissolution, but not reduction of Fe(III)-containing minerals (Bridge and Johnson, 2000) suggests that the Fe(III) reductase of this strain is not a secreted enzyme either. This means that

if the lacking ability of SJH to reduce HA/FA and AQDS is attributable to a localization of the Fe(III) reductase that renders it accessible for dissolved and/or complexed Fe(III), but not for HA/FA and AQDS, it would make sense to expect the enzyme to be located in the periplasm (or at the periplasmic side of the inner membrane). The most common way for small molecules to enter the cytoplasm is via porins which allow the passage of particles with a size up to 600 Da into the periplasm (Nikaido, 1992). This means that AQDS, which is below this size, should be able to enter the periplasm. The size of fulvic acids varies between 500 and 2000 Dalton (Stevenson, 1994), implying that at least a fraction of them should be able to pass the porins. Humic acids are generally believed to consist of larger molecules than fulvic acids with a size of up to 250 kDa (Stevenson, 1994) and therefore it would not be expected that they can enter the periplasm to a significant extent. It has to be mentioned, however, that recent studies have suggested that HA are rather large aggregates of relatively low molecule size molecules than large polymers (Sutton and Sposito, 2005). This means that small fragments could be released from these aggregates and could potentially be taken up into the periplasm. Based on the conclusion that AQDS, a part of the FA and potentially even some HA molecules probably enter the periplasm but still did not become reduced, we infer that if the Fe(III)-reducing enzyme of acidophiles such as SJH is located at the outer membrane or in the periplasm, it obviously differs from Fe(III) reductases of neutrophiles in terms of a narrower specificity for complexed Fe(III) excluding quinone compounds.

In summary, this suggests that the Fe(III) reduction pathways of neutrophilic Fe(III)-reducers can also transfer electrons to quinones such as AQDS and HA/FA, but this is not the case for the Fe(III) reduction mechanism of the acidophilic strain used in this study. Further studies are needed to elucidate whether this finding can be generalized, and if the mechanism of Fe(III) reduction in acidophiles is fundamentally different from the mechanisms of Fe(III) reduction that have been described for neutrophiles.

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5

Absence of humic substance reduction by the acidophilic Fe(III)-reducing strain *Acidiphilium* SJH: implications for stimulation of natural organohalogen formation and for the mechanism of Fe(III) reduction at acidic pH

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ONLINE RESOURCES (=APPENDIX)

Online Resource 1

Setup of Fe(III) reduction experiments

For Fe(III) reduction experiments, $Fe(III)_2 (SO_4)_3$ was added from a 500 mM stock solution to final concentrations of 5, 35 or 40 mM Fe(III), respectively. Experiments were conducted in 20 ml glass vials with initial total volumes of 10 ml (or 12.5 ml in the case of setups from which 1 ml samples were taken in the beginning for initial cell counts). Medium was added first, followed by glycerol and $Fe(III)_2 (SO_4)_3$. Cells were added last, and glass vials were closed with butyl stoppers. The vials were removed from the anoxic chamber after all components had been added and t=0 samples were taken immediately. In order to create microoxic conditions in some setups, butyl rubber stoppers were removed for 5 seconds twice per week under sterile conditions to let the headspace refill with air. For sampling, 200 µl were taken from each setup with N_2-flushed syringes and needles and added directly to 200 µl of 1 M HCl to stabilize Fe(II). After centrifugation for 5' at 14,000 g, Fe(total) and Fe(II) in the supernatant were quantified spectrophotometrically by the ferrozine assay (Stookey, 1970).

Online Resource 2

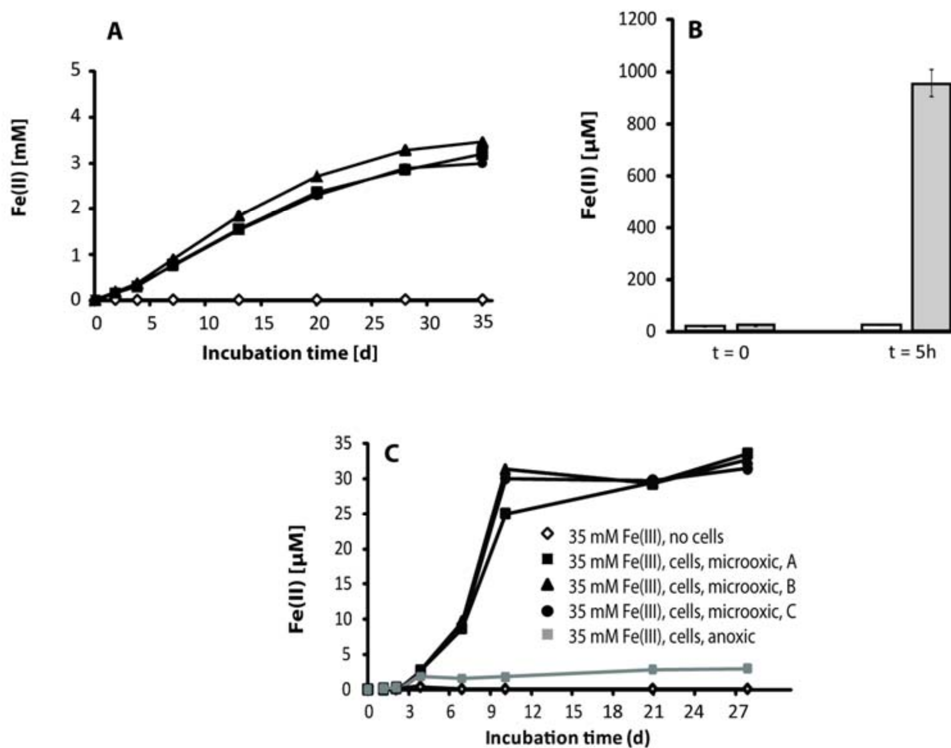

Figure S1. *Fe(III) reduction by Acidiphilium strain SJH monitored over time. A) Kinetics of Fe(III) reduction in three parallel batch experiments performed under anoxic conditions with 10^7 cells/ml each (filled symbols) compared to an abiotic control (open symbols). B) Cell suspension Fe(III) reduction experiment with 5 x 10^8 cells/ml. The amount of Fe(II) formed in biotic setups before and after 5 h of incubation is shown by the grey bars. Abiotic controls are shown in white. Standard deviation/error bars were calculated from results of triplicate setups. All setups contained 2.5 mM $Fe(III)_2(SO)_3$ and 10 mM glycerol as electron donor. C) Reduction kinetics of 35 mM dissolved Fe(III) with 10 mM glycerol in presence of an initial cell number of strain SJH of 10^7 cells/ml (filled black symbols; data from triplicate setups (labeled A, B and C) incubated under microoxic conditions; filled grey symbols: data from single setup incubated under anoxic conditions). Open symbols represent data from an abiotic control containing 35 mM dissolved Fe(III) and 10 mM glycerol.*

Online Resource 3

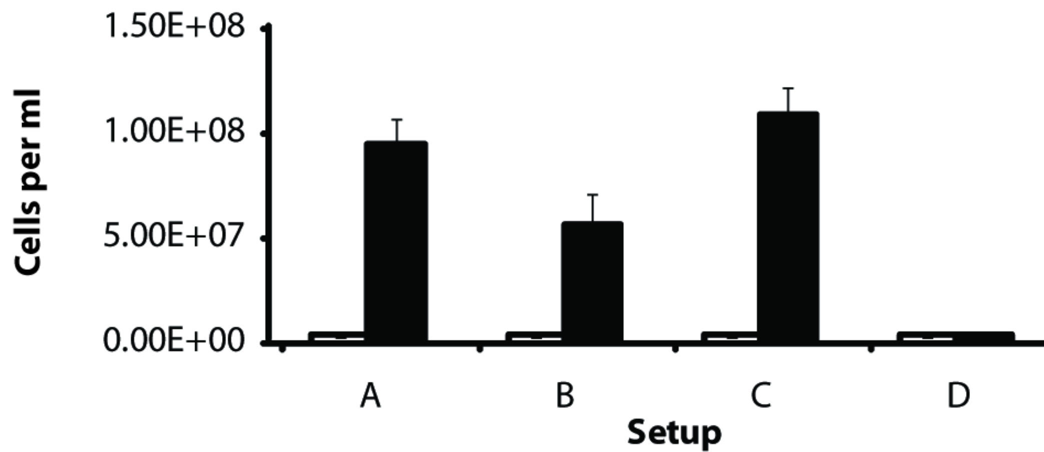

Figure S2: *Change in cell numbers during microaerophilic versus anaerobic Fe(III) reduction by SJH as determined by cell counts at t=0 and t=4 weeks. An initial number of 10^7 cells/ml had been incubated with 35 mM dissolved Fe(III) and 10 mM glycerol under conditions of limited oxygen supply (three parallel microaerophilic setups A-C) or without oxygen (setup D). Grey bars represent cell counts at t=0; black bars represent cell counts at t=4 weeks. After 4 weeks, ≈ 100% of the Fe(III) had been reduced in the microoxic setups while in the anoxic setup, only 10% of the Fe(III) had been reduced (Fig S1). Standard deviation/error bars were calculated from cell numbers calculated according to counts in 10 fields of view per DAPI-stained filter.*

Online Resource 4

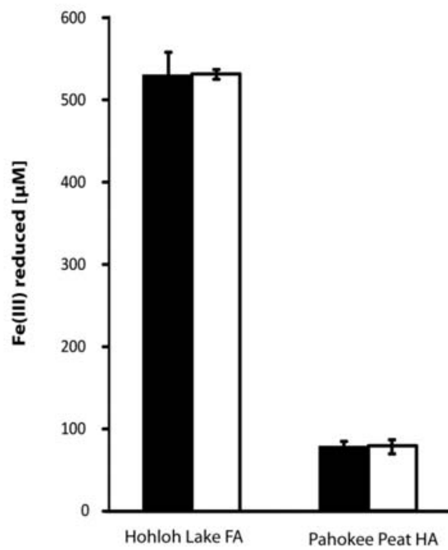

Figure S3: *Concentration of Fe(II) formed by incubation of fulvic and humic acids that had either been exposed to Pd or not (in the absence of H_2) with Fe(III)-NTA. Headspaces of all fulvic and humic acid solutions were exchanged by N_2 in both setups to which Pd had been added and in Pd-free setups. Pd-containing setups were then incubated under shaking for 16 h, after which headspaces were again exchanged by N_2. Neither glycerol nor cells were added before anoxic incubation with Fe(III)-NTA. Data from Pd-containing setups are indicated by black bars, while white bars depict data from setups without Pd. Standard deviation/error bars were calculated from results of triplicate setups.*

Online Resource 5

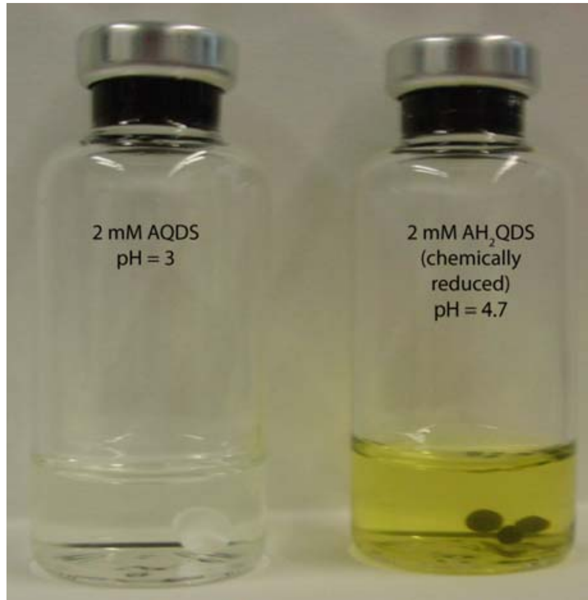

Figure S4: *2 mM AQDS in 50 mM phosphate buffer at pH = 3 in oxidized (left vial) and chemically reduced (right vial) state (the pH increased from 3 to 4.7 during the reduction of the AQDS to the AH_2QDS). Setups containing 2 mM oxidized AQDS in medium 269 to which 10^7 cells/ml and 10 mM glycerol had been added remained colorless for 4 weeks.*

Online Resource 6

Quantification of volatile organohalogens: instrumentation

As described by (Huber et al., 2010), GC/MS analyses were performed on a Varian STAR 3400 Cx gas chromatographic system connected to a Saturn 2000 ion trap mass spectrometer. In contrast to (Huber et al., 2010), a DB-5 capillary column (60m; 0.32 mm i.d.; 1.0 µm film thickness) was used instead of a BP624+BP X5 column.

Quantification of volatile organohalogens: experimental procedure

GC/MS measurements were carried out as described by (Huber et al., 2010) with the following changes: the flow rate of the helium stream during purging was increased to 10 ml/min. The temperature of the cooling trap was decreased to -196°C. The temperature program of the GC

oven was as follows: 30°C hold 15.5 min, 30 to 114°C at 5°C/min, 114 to 210°C at 30°C/min, hold 1.5min.

Quantification of volatile organohalogens: detection limits

Detetion limits of all compounds measured by GC/MS were defined as $\sigma+3\delta$ while limits of quantification were set as 2 x ($\sigma+3\delta$). For volatile organohalogens whose natural production had been shown before and that were detected in our setups, detection limits corresponded to the following concentrations (given in ng purged from a sample of 10 ml liquid volume and 10 ml headspace):

CH_3Cl 5.07 ng

CH_3Br 2.16 ng

$CHCl_3$ 2.01 ng

6

Abundance, distribution, and activity of Fe(II)-oxidizing and Fe(III)-reducing microorganisms in hypersaline sediments of Lake Kasin, Southern Russia

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Abstract

The extreme osmotic conditions prevailing in hypersaline environments result in decreasing metabolic diversity with increasing salinity. Various microbial metabolisms have been shown to occur even at high salinity, including photosynthesis, sulfate and nitrate reduction. However, information about anaerobic microbial iron metabolism in hypersaline environments is scarce. We studied the phylogenetic diversity, distribution, and metabolic activity of iron(II)-oxidizing and iron(III)-reducing bacteria and archaea in pH-neutral, iron-rich salt lake sediments (Lake Kasin, Southern Russia; salinity 348.6 g L^{-1}) using a combination of culture-dependent and -independent techniques. 16S rRNA gene clone libraries for Bacteria and Archaea revealed a microbial community composition typical for hypersaline sediments. Most probable number counts confirmed the presence of 4.26×10^2 to 8.32×10^3 iron(II)-oxidizing bacteria and 4.16×10^2 to 2.13×10^3 iron(III)-reducing microorganisms per gram dry sediment. Microbial iron(III) reduction was detected in the presence of 5 M NaCl extending the natural habitat boundaries for this important microbial process. Quantitative real-time PCR showed that 16S rRNA gene copy numbers of total Bacteria, total Archaea, and species dominating the iron(III)-reducing enrichment cultures (relatives of *Halobaculum gomorrense*, *Desulfosporosinus lacus,* and members of the *Bacilli*) were highest in an iron oxide-rich sediment layer. Combined with the presented geochemical and mineralogical data, our findings suggest the presence of an active microbial iron cycle at salt concentrations close to the solubility limit of NaCl.

Introduction

Hypersaline aquatic environments are abundant worldwide and include inland salt lakes and marine coastal areas such as marshes and solar salterns. On a global scale, nearly as much water is stored in salt lakes as in freshwater lakes (Hammer, 1986). Due to climate change, the area

covered by salt lakes is expected to increase in the near future. Many freshwater lakes will turn into salt lakes and existing salt lakes will increase in salinity due to increased evaporation (Williams, 2002). With salinities ranging from below seawater salinity (i.e. 35 g/L^{-1} dissolved salt) to NaCl saturation (i.e. 304 g/L^{-1} or 5.2 M) (Oren, 2001), salt lakes represent extreme habitats for microbial life (Rothschild and Mancinelli, 2001). However, high rates of primary production show that salt-adapted microorganisms are active regardless of the extreme environmental osmotic conditions (Sorokin et al., 2007).

A major challenge for microorganisms living in hypersaline environments is to prevent desiccation caused by osmotic stress. The various modes of adaptation to extreme osmotic conditions can pose a high energy burden on microorganisms in hypersaline environments (Oren, 2001 and 2002; Paul et al., 2008). The high energy requirements to counteract the osmotic pressure have been used to explain the often observed decrease in metabolic diversity with increasing salinity and led to the hypothesis of an upper limit of salinity for every metabolic process as determined by thermodynamic constraints (Oren, 1999; 2001 and 2011). In other words, metabolic processes from which sufficient energy can be gained, such as nitrate reduction (2716 kJ are generated per 4.8 mol of nitrate reduced with 1 mol of glucose under standard conditions) are expected to occur up to higher environmental salt concentrations than processes from which less metabolic energy can be derived such as methanogenesis from H_2 and HCO_3^- (-34 kJ/mol of HCO_3^-, (Oren, 2001)). So far, the majority of observations made in hypersaline environments strengthen the aforementioned hypothesis (Oren, 2011). Denitrification, for example, has been observed in laboratory cultures in medium with more than 300 g NaCl L^{-1}, while sulfate reduction has been shown to occur up to 240 g NaCl L^{-1} and methanogenesis from H_2 and HCO_3^- could not be detected at salinities beyond 120 g NaCl L^{-1} (Oren, 2011). While metabolic processes of microorganisms involved in the biogeochemical cycling of sulfur, including dissimilatory sulfate reduction in salt lake water and sediments (Kulp et al., 2006; Sorokin et al., 2010), have been studied extensively, knowledge on the use of oxidized metal ions, such as iron

(Fe) in the form of Fe(III), as electron acceptors in anaerobic hypersaline environments is scarce. An upper salinity limit for microbial Fe(III) reduction has not been defined yet (Oren, 2011). The use of Fe(III) in the form of $Fe(OH)_3$ as electron acceptor is more thermodynamically favorable than the use of sulfate (ΔG = -48 kJ per mole of electrons transferred from acetate to Fe(III) at pH 7 in comparison to -7 kJ per mole of electrons transferred from acetate to sulfate under the same conditions (Madigan and Martinko, 2006)). Consequently, from a thermodynamic point of view, as long as bioavailable Fe(III) is not limiting, microbial Fe(III) reduction should be more energetically favorable than sulfate reduction in hypersaline environments. With typical concentrations of 1-5% of sediment dry matter, Fe(III) (oxyhyd)roxides have indeed been shown to represent important electron acceptors in freshwater (Schink and Benz, 1999) and also in marine sediments, where Fe can constitute up to 20% of the sediment by weight (Ussher et al., 2004). In hypersaline sediments similar amounts of Fe(III) hydroxides as in freshwater lake sediments have been found (De Koff et al., 2008). In a previous study, these iron oxide minerals were identified as jarosite, goethite and hydrous iron-oxides (Long, et al., 1992). However, these studies only consider abiotic aspects of Fe geochemistry and neglect the possible role of microorganisms on Fe mineral transformations. To date, a few isolates from hypersaline environments have been reported to be capable of Fe(III) reduction. Often the isolates were shown to reduce dissolved Fe(III) but were not tested for the reduction of any solid Fe(III) phases (Switzer Blum et al., 1998 and 2009, Pollock et al., 2007), which represent the dominant form of Fe(III) at neutral or even alkaline pH values of most salt lake environments (Stumm and Morgan, 1996). In other cases, isolates were found to be halotolerant rather than halophilic. Bacteria such as *Geoalkalibacter ferrihydriticus* can only grow and reduce Fe(III) in up to 50 g/L^{-1} NaCl (Zavarzina et al., 2006). The same applies to several *Bacillus* strains (Switzer Blum et al., 2009; Kanso et al., 2002) as well as to an Fe(III)-reducing isolate from the sediment of the hypersaline Lake Chaka (China), because the strain originated from

880 cm depth where salinity was only slightly elevated compared to freshwater (Jiang et al., 2007).

While knowledge about microbial Fe(III) reduction in hypersaline environments is scarce, even less is known about Fe(II)-oxidizers in these habitats. Previous reports indicated an inhibitory effect of Cl^- at seawater concentration on microaerophilic (Cameron et al., 1984) and phototrophic (Newman and Poulain, 2009) Fe(II)-oxidizers. McBeth et al. [2011] recently presented the first study of a microaerophilic Fe(II)-oxidizing strain associated with the *Zetaproteobacteria* isolated from a microbial mat in the Great Salt Bay where salinity ranges between 0% and 2.5% (McBeth et al., 2011). However, our current knowledge on microbial Fe(II) oxidation, Fe(III) reduction and the role of both processes in iron cycling in hypersaline sediments is very limited (Coby et al., 2009, Sobolev and Roden, 2002, Straub et al., 2004). Therefore, the goals of the present study were 1) to determine the diversity of Fe-metabolizing microorganisms in pH-neutral, NaCl-saturated Lake Kasin sediments, 2) to analyze the abundance and distribution of Fe-metabolizing microorganisms within a geochemically heterogeneous sediment profile and 3) to determine the activity of microbial Fe(II)-oxidizers and Fe(III)-reducers in order to evaluate their ecological role in the cycling of iron at a salt lake in Southern Russia, Lake Kasin.

Materials and Methods

Field measurements and sampling

Lake Kasin is a shallow hypersaline lake located approximately 250 km southeast of Volgograd (Russia) within the district of Astrachan. The region forms a part of the North- or Pre-Caspian Depression, a low-elevation flatland north of the Caspian Sea (GPS: N47°36.165' E047°27.129'). A detailed description of the study site is given in the supplementary material.

The sampling site was located about 50 m east of the water-covered area of Lake Kasin and can be described as exposed lakebed (figure S1). In addition to samples from Lake Kasin, reference samples were taken from two other salt lakes in the same area (Lake Elton and Lake Baskunchak).
From all visually distinguishable horizons of the individual sampling sites (figure S1), sediment was mixed in a 1:1 ratio with deionized water for determination of pH with color indicator strips (pH range 5.0-10, Merck) and electrical conductivity with a portable electrode (SenTix ORP as part of a MultiLine P4 universal meter). Groundwater samples were tested for NO_3^- and NO_2^- with color indicator strips (Merckoquant®) and amended with 1 M HCl and ferrozine in a ratio of sample:HCl:ferrozine of 1:1:2 in order to do a qualitative test for the presence of Fe(II) (Stookey, 1970).

Zero to ten cm composite samples of the salt pan and sediment sites were taken with a spatula and transported in UV-sterilized plastic bags. Lake water was sampled with a UV-sterilized plastic bottle. The water-covered sediment was found to release some gas upon tramping, which was sampled in 20 mL glass vials that were closed under water with PTFE-layered butyl rubber septa and metal crimp caps. Sediment composite samples as well as water and gas samples were immediately placed into an insulated transport box, cooled on site and kept at about 4°C during transportation to Tübingen, Germany. In Tübingen, composite samples were sieved (2 mm diameter) and stored in plastic bags at 4°C in the dark.

At site Kasin, two 15 cm sediment cores (named "A" and "B") were drilled with UV-sterilized plastic tubes of 3 cm diameter and cut into 0.5 cm (up to 5 cm depth) or 1 cm (from 5 to 15 cm depth) thick segments. Samples were transferred into sterile 15 mL plastic tubes on site and immediately transferred to a battery-driven portable refrigerator box set to -20°C. The same was done with a 0-10 cm composite sample for the clone library construction. Samples were

transported to Tübingen, Germany within a few days after sampling and remained frozen until arrival. In Tübingen, Germany, samples were stored at -20°C until further analysis.

Laboratory chemical analysis of sediment samples

All geochemical analyses of sediment samples were performed in duplicates. Determination of the water content, pH, total organic carbon (TOC) and total inorganic carbon (TIC) as well as X-ray fluorescence analysis (XRF) of the sieved composite samples were performed as previously described by Porsch and Kappler (2011).

1 g of each sieved, freeze-dried and milled sample from the different horizons of Lake Kasin sediment was amended with 10 mL of double distilled water (ddH_2O) and shaken horizontally for 24 h. After centrifugation and 1:20 to 1:40 (v/v) dilution with ddH_2O, the eluates were used to quantify HCO_3^-, Cl^-, Br^-, IO_3^-, NO_3^-, PO_4^{3-} and SO_4^{2-} by ion chromatography (Dionex DX 120 equipped with an AS9HC column and a AG9HC precolumn). From this analysis the concentrations of water-leachable ions in the sediment samples were calculated. Lake water was filtered through a cellulose ester filter with a pore size of 0.45 μm and diluted 1:5,000 with Millipore®-water before determining concentrations of dissolved ions by ion chromatography with a Dionex DX 120 device equipped with an AS14 column, ASRS 300 suppressor and a conductivity detector (Dionex, Germering, Germany). From the sieved, freeze-dried and milled samples of the different horizons, pH was measured according to DIN ISO 10390 (German Institute for Standardization, 2005) and electrical conductivity was determined following DIN ISO 11265 (German Institute for Standardization, 1997) in order to confirm the values measured on site.

In order to quantify bioavailable (0.5 M HCl extractable) versus crystalline (6 M HCl extractable) iron in the sediment profile, Fe extractions were performed using the sequential extraction protocol previously described by Porsch and Kappler (2011). The sequential extractions were

performed in an anoxic (N_2) glove box in order to prevent samples from oxidation. Fe concentrations were quantified with the ferrozine assay (Stookey, 1970).

Since the sediment samples were too dry to obtain pore water by established methods, "artificial pore water" was created by leaching 1 g of the 0-10 cm composite samples from Lake Kasin three times with 30 mL of autoclaved Millipore®-water for 4 h. Analysis was performed in triplicates as described by Jiang et al. (2006). Ionic composition of this "artificial pore water" was determined by ion chromatography (see above for analytical details) and organic acids (e.g. acetate, lactate, formate) were quantified by HPLC (instrument type "Class vp" from Shimadzu, Duisburg, Germany equipped with a Microguard cation H cartridge pre-column and a Aminex HPX-87H Ion exclusion column 300 mm x 7.8 mm from Bio-Rad, Munich, Germany). The total amount of ions and organic acids leached from 1 g of sediment was back-calculated to the water content of the sample.

Analysis of gas samples from the water-covered part of the lake sediment for methane was performed with a Varian 3800 gas chromatograph equipped with an Alltech 13939 column (length: 30 m, inner diameter: 0.53 mm, AT™-Q) and an FID detector. The injector had a temperature of 200°C and the temperature program was as follows: 60°C, hold 3 min, 60°C to 200°C at 75°C/min, hold 3 min. The flow rate was 5 mL/min at a flow pressure of 3.2 psi.

Mössbauer spectroscopy

A Fe-oxide rich layer between 1 and 2.5 cm in depth was observed in the sediment profile of Lake Kasin. Because the sample contained less than 3% (w/w) of total Fe, unlikely to be detected by XRD, Fe speciation was analyzed by ^{57}Fe Mössbauer spectroscopy. For this purpose, about 1 g of sample was transferred into an anoxic glovebox and preserved between two layers of O_2-impermeable Kapton® tape (Polyfluor Pastics BV, Oosterhout, Netherlands). The measurement was performed with a ^{57}Co source at room temperature with linear acceleration in transmission mode as described previously (Larese-Casanova et al., 2010). The spectrometer was constructed

by WissEL (Wissenschaftliche Elektronik GmbH, Starnberg, Germany). A Janis closed-cycle cryostat with a helium atmosphere was used to vary the temperature of the sample. Spectra were calibrated against spectra of α-Fe(0) foil. Recoil® software (University of Ottawa, Canada) and Voigt-based models were used for spectra interpretation.

Most probable number counts and enrichments of Fe(II)-oxidizing and Fe(III)-reducing microorganisms

Anoxic salt water media (SWM) containing 5 M and 0.5 M NaCl (pH 7.2 - 7.4) was used to enumerate anaerobic nitrate-reducing Fe(II)-oxidizers (anFeOx) as well as anaerobic Fe(III)-reducers (FeRed) by MPN counts. Dilution series of sediment suspensions of the 0-10 cm composite sample were set up in 96 well plates. The anoxic salt water media contained: 100 µM $MgSO_4 \times 7 H_2O$, 3 mM KCl, 100 µM KBr, 5 mM NH_4Cl and 1.9 mM $MgCl_2$, 15 mM $NaHCO_3$, 1 mM $CaCl_2$, 1 mM $NaHPO_4$, 0.2 µM NH_4VO_3 and 0.5 mM $Na_2S_2O_3$ as well as 1 x vitamin solution (Widdel and Pfennig, 1991), trace element solution (Tschesch and Pfennig, 1984) and selenate-tungstate solution (Widdel, 1980).

The following electron donors and acceptors were added to the media from stock solutions: For anFeOx the final media contained 4 mM $NaNO_3$ and 10 mM $FeCl_2$ as well as 0.5 mM Na-acetate to allow growth of mixotrophic Fe(II)-oxidizers. For FeRed the media contained 5 mM of 2-line ferrihydrite prepared according to Straub et al. (2005) and a mixture of 5 mM Na-acetate and 5 mM Na-lactate. Setup and analysis of the MPN counts are described in detail in the supplements.

Gradient tubes for enumeration and enrichment of microaerophilic Fe(II)-oxidizers were prepared as described by Emerson and Floyd (2005) with some modifications which are also described in the supplements.

DNA extractions

For DGGE and qPCR analyses, DNA from sediment samples was extracted as follows: In order to remove salts 0.3 g of sediment were washed three times with 1.5 mL of TE buffer (10 mM Tris-EDTA, pH=7.0). Following centrifugation for 10 min at 7,200 g the supernatant was collected and filtered through a 0.22 µm polyethersulfone (PES) membrane filter. Washed sediments and membrane filters were separately extracted using the PowerSoil DNA isolation kit® (MoBio Laboratories, Carlsbad, CA) following the protocol of the manufacturer. At the end of the protocol DNA extracted from sediment and filters were sequentially eluted in 2 × 25 µL elution buffer (buffer C6).

For 16S rRNA gene clone library construction DNA was also extracted following the protocol described by Zhou et al. (1996). Each extraction was performed in duplicates. The two DNA extracts obtained using the PowerSoil kit and Zhou et al. protocol were pooled in order to minimize a methods-immanent extraction bias. Pooled DNA extracts were further purified using the QUIAEX2 kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. DNA from cell pellets of the liquid enrichment cultures was extracted using the UltraClean DNA isolation kit® (MoBio).

DGGE

Partial 16S rRNA genes from the DNA extracts of the liquid enrichment cultures were amplified using the primers 341GCF (51) and 907R (De Long, 2006) for amplification of general bacterial 16S rRNA gene fragments. The primers 20F (Massana et al., 1997) and 1392R (Lane, 1991) were used for amplification of general archaeal 16S rRNA gene fragments. Amplified archaeal 16S rRNA gene fragments were used as template for a nested PCR with primers 344GCF (Stahl and Amann, 1991) and 519R (Lane, 1991). Details about PCR and DGGE conditions can be found in the supplementary information. Prominent DGGE bands were cut from the gel, re-amplified, cloned, and sequenced.

Clone library construction and phylogenetic analysis

DNA from the 0-10 cm composite sample from Lake Kasin sediment was PCR-amplified with general primers GM3F (Muyzer et al., 1995) and 1392R (Lane, 1991) for bacterial 16S rRNA gene fragments and 20F (Massana et al., 1997) and 958R (De Long, 1992) for archaeal 16S rRNA gene fragments. Reaction mixtures contained 1× PCR buffer with 1.5 mM $MgCl_2$ final concentration (Promega), 200 µM dNTP mix (New England Biolabs), 200 nM of each primer, 1.25 U Taq DNA-Polymerase (Promega) and 10 ng of DNA in a total volume of 25 µL. The following thermocycler program was used for amplification of 16S rRNA gene sequences: hot start at 70°C; initial denaturation at 95°C for 5 min; 25 cycles of denaturing (95°C for 1 min bacteria/ 2 min archaea), annealing (44°C bacteria/ 58°C archaea for 1 min) and elongation (72°C for 3 min bacteria/ 1.5 min archaea) and a final elongation at 72°C for 10 min. PCR products were purified with the Wizard® PCR Clean-Up System (Promega laboratories). All PCRs were performed in duplicates and pooled after purification. The purified PCR products cloned using the TOPO TA cloning kit and TOP10 competent cells (Invitrogen). *E. coli* were sent for sequencing of the 16S rRNA gene insert to GATC Biotech (Konstanz). Forward and reverse reads were assembled and trimmed using the program "DNA Baser" (http://www.dnabaser.com/). The total length of the sequences after trimming was ~900 bp for Archaea and ~1300 bp for Bacteria. All sequences were checked for chimeras by using Bellerophon (Huber et al., 2004) and Slayer (Haas et al., 2011). Chimeras were removed from the data sets. Sequences were aligned and analyzed using the SINA aligner of the SILVA rRNA database project (Pruesse et al., 2007) and the ARB software package (version 5.2) (Ludwig et al., 2004) with the corresponding SILVA SSURef 106 database (Pruesse et al., 2007) following the standard operating procedure published by Peplies et al. (2008). Tree construction was carried out with up to 200 sequences using the neighbor joining and maximum likelihood (RAxML, AxML and fastDNAml) methods in ARB. Tree topology was further tested by the application of positional variability filters for Archaea and Bacteria, respectively, as well as with 50 % positional

conservatory filters that were created for Archaea and *Firmicutes*, respectively (Peplies et al., 2008). The archaeal tree was calculated with 152 sequences based on 7123 valid columns (50 % conservation filtering) with fastDNAml. The bacterial tree was calculated with 200 sequences based on 6370 valid columns (50 % conservation filtering) with RAxML (model: GTRCAT). Partial sequences were added to both trees using the ARB parsimony tool. A multifurcation was introduced manually into the bacterial tree in one case where the tree topology could not be unambiguously resolved. For clarity, only selected subsets of the sequences used for treeing are shown in the figures. Rarefaction curves were constructed using the software MOTHUR (Schloss et al., 2009).

qPCR

Copy numbers of 16S rRNA genes in the environmental DNA extracts were quantified on an iQ5 real-time PCR cycler (BioRad Laboratories GmbH, Munich, Germany) using the SsoFast Eva Green detection kit (BioRad). Table S1 of the supplements lists the primers used in the different qPCR assays. For quantification of general bacterial 16S rRNA gene copies, 75 nM of primer 341F and 225 nM of primer 797R were used per reaction. All other primers were added to final concentrations of 250 nM. In addition, reaction mixes contained 10 µL of SsoFast Eva Green master mix, 2 µL of template, standard or DNAse-free water in a final volume of 20 µL. All thermal cycler programs started with 2 min at 98°C followed by 40 cycles of the respective programs listed in table S2 of the supplements. Table S2 also contains information on the standards used in the different qPCR assays. Data analysis was performed with the iQ5 optical system Software, Version 2.0 (Bio-Rad, 2006) as described by Behrens et al. (2008).

Quantification of archaeal 16S rRNA gene copies was performed once in triplicates. All other qPCRs were performed in triplicates twice. Cell numbers per g dry sediment were calculated from the qPCR 16S rRNA gene copy numbers considering the average ribosomal rRNA operon numbers of the respective taxa (Bacteria, Archaea, *Bacillaceae, Peptococcaceae, Halobacteriaceae*) as

listed in the Ribosomal RNA Operon Copy Number Database (http://rrndb.mmg.msu.edu/index.php).

Nucleotide sequence accession numbers

The partial 16S rRNA gene sequences determined in this study have been deposited in GenBank under accession numbers HE604643 to HE604939 (Bacteria) and HE604411 to HE604642 (Archaea). 16S rRNA gene sequences of the Fe(III)-reducing enrichments have the accession numbers HE604940 to HE604952.

Results

Geochemistry of Lake Kasin, Southern Russia

The exact geographic location of the sampling site at Lake Kasin is given in Table 1 and Figure S1 of the supplements. The ionic strength of the wet sediment was 6.09 M mainly comprised of sodium and chloride ions (about 5 M). The normalized electrical conductivity at 25°C of the 0-10 cm composite sediment sample was 438.5 mS cm^{-1}. Based on the measured conductivity the salinity of the sediment was 348.6 g L^{-1} (Williams and Sherwood, 1994) (Table 1). A ferrozine test performed on site revealed the presence of at 100 µM Fe(II) in the porewater at about 20 cm depth. Neither NO_2^- nor NO_3^- could be detected in the groundwater with color indicator strips, which had a detection limit of 160 µM for NO_3^- and 45 µM for NO_2^-. However, as indicated in Table 1, 130 µM of water-leachable NO_3^- was measured using ion chromatography. The leacheate also contained 37.2 +/- 4.6 mM acetate and 10.6 +/- 1.6 mM formate, but no lactate.

Table 1: *Location and geochemical properties of Lake Kasin sediment (0-10 cm depth composite sample)*

geographic position	N47°36.165’ E047°27.129’
pH[a]	7.86
Fe-content[b]	1.13 %
Ionic strength[c]	6.09 M
Conductivity[d]	438.5 mS cm^{-1}
Salinity[e]	348.6 g L^{-1}
H_2O content	14.65 %
Cl[b]	1.51 %
SO_4^{2-}	968 mM[c]
NO_3^- [c]	130 µM
C_{inorg} [f]	1.85 %
C_{org} [g]	0.11 %

[a]determined with 0.01 M $CaCl_2$
[b][weight % of dry sediment] quantified by XRF
[c]quantified by IC from modified pore water after (30)
[d]average value horizon H1 to H4 (0-10 cm depth) at 25°C
[e]calculated from conductivity after Williams et al. (1994) (100)
[f]weight % determined by weight loss during titration with HCl
[g]weight % as quantified by a C/N-analyzer using a HCl-titrated sample

During sampling, a strong scent of H_2S was noticeable at Lake Kasin (odor detection threshold 4.7 ppb or 15 µM (Powers, 2004)). Together with the high concentration of water-leachable sulfate in the sediment (968 mM) this might be an indication for the presence of an effective redox cycling of sulfur at Lake Kasin. About 1.87 mmol/L of methane were measured in gas samples from the water-covered part of the sediment. This corresponds to approximately 45,000 ppm of methane, which exceeds atmospheric concentrations by three orders of magnitude. These observations suggest that in addition to sulfate reduction, methanogenesis takes place in the sediments as well. In addition, based on thermodynamics Fe(III) reduction is also expected to occur given the fact that bioavailable Fe(III) is present. Fe extractions of the composite sample

gave a total Fe content of 0.85% (w/w) (dry weight) in the top 10 cm of Lake Kasin, of which 19% were Fe(II).

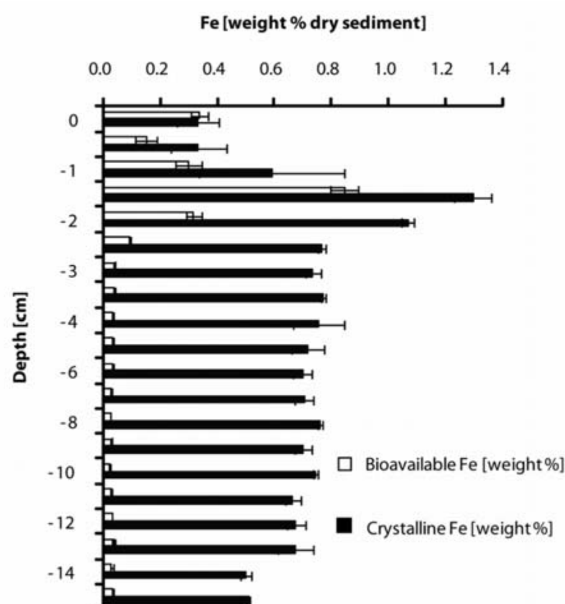


Figure 1: *Concentration of different iron fractions in a sediment profile of Lake Kasin. White bars, 0.5 M HCl-extractable (=" bioavailable") iron. Black bars, "crystalline" iron, extracted during incubation in 6 M HCl at 70°C for 24 h. Iron concentrations in the extracts were determined with the ferrozine assay (82). Error bars represent standard deviations calculated from duplicate samples.*

The total Fe content determined by XRF was slightly higher (1.13% (w/w) dry weight, Table 1). Upon visual inspection of the sediment profile, an Fe oxide-rich layer could be identified between one and three cm depth (figure S1). We quantified both 0.5 M HCl-extractable ("bioavailable") and 6 M HCl extractable ("crystalline") Fe throughout the sediment profile and found clear maxima of both bioavailable and crystalline Fe at 1.5 cm depth where the two iron phases constituted 0.85% (w/w) and 1.30 % (w/w) of the sediment dry weight, respectively (figure 1).

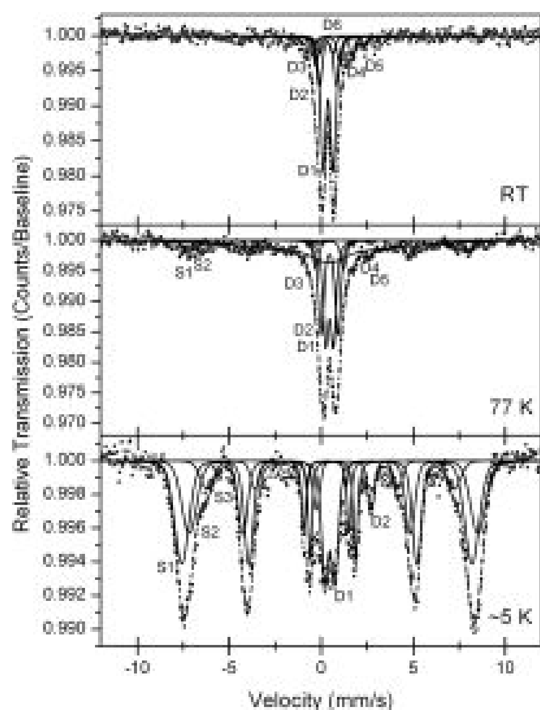


Figure 2: *Mössbauer spectra of the Fe-rich layer of Lake Kasin sediment (1.5 cm depth) recorded at room temperature RT (upper panel), 77 K (middle panel) and 5 K (bottom panel). Sextets (S) and doublets (D) are labeled as listed in Table S3.*

Figure 2 shows Mössbauer spectra of the Fe-rich layer at 1.5 cm depth obtained at room temperature (RT), 77 K and at ~5 K. The spectra show predominantly Fe(III) phases, but also small amounts of Fe(II). We modeled all three spectra of the Fe(III) phase using parameters for akaganéite reported by Murard and Johnston (Murad and Johnston, 1987), Chambaere et al. (1979) and Barrero et al. (2006), and parameters reported by Feder et al. (2005), Génin et al. (1998), Refait et al. (1991), and Rusch et al. (2008) for the green rust Fe(II) phases (Table S3). Akaganéite (β-FeO(OH)), can incorporate up to 7 mol % of Cl^- ions as additional constituents that stabilize tunnels within the crystal structure (Cornell and Schwertmann, 2003). Since the presence of Cl^- or F^- ions is necessary for the formation of akaganéite (Cornell and Schwertmann, 2003), its natural occurrence is generally restricted to environments with high concentrations of Cl^- or F^- ions such as, e.g. hypersaline lakes. However, the Mössbauer parameters reported for lepidocrocite (e.g. Murad and Cashion, 2004) and for Fe(III) in green rust (Feder et al., 2005;

Genin et al., 1998 and Refait et al., 1991) are very similar to those of the akaganéite doublet D1 (Table S3), the akaganéite sextets S3 (lepidocroite), and S1 (green rust). A quantification of these different iron mineral phases based on Mössbauer spectra was therefore not possible. The Mössbauer spectra did not provide any evidence for the presence of iron sulfide minerals.

Abundance and activity of culturable Fe(III)-reducers and Fe(II)-oxidizers

The results of the most probable number (MPN) counts are shown in figure 3.

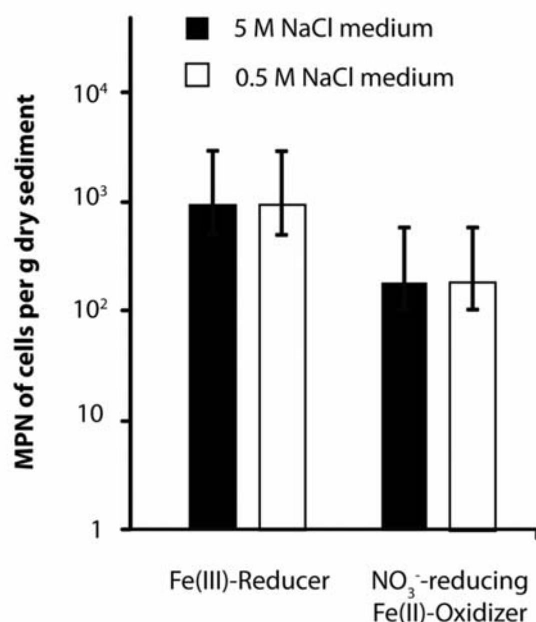


Figure 3: *Most probable number (MPN) counts of Fe(III)-reducing (FeRed) and anaerobic Fe(II)-oxidizing (anFeOx) microorganisms from the top 10 cm of Lake Kasin in mineral medium with 5 M (black bars) or 0.5 M NaCl (white bars), respectively. Medium for FeRed was supplemented with 0.5 M of ferrihydrite as electron acceptor and 0.5 M of lactate and acetate each as electron donors. For anFeOx, 10 mM $FeCl_2$ was added as electron donor and 0.4 M NO_3^- as electron acceptor. The Fe(II)-oxidizer medium further contained 0.05 M acetate as a carbon source. Error bars denote 95% confidence intervals determined from seven replicate samples according to Klee (1993).*

The main finding of this experiment was that similar numbers of anaerobic Fe(II)-oxidizers (anFeOx) and Fe(III)-reducers (FeRed) (210 and 943 cells per g dry sediment, respectively) grew in medium with 0.5 M NaCl and in medium with 5 M NaCl. We also performed MPN counts to quantify microaerophilic Fe(II)-oxidizers from Lake Kasin in gradient tubes, but we did not observe growth in any tube. From selective wells of the FeRed and anFeOx MPN-plates, cultures were transferred into fresh media in order to pursue further enrichment. Enrichment cultures were consecutively transferred into fresh medium as soon as Fe(II)-oxidizing or Fe(III)-reducing

activity was observed, which was on average every 8 to 12 weeks. While the activity of anFeOx could not be maintained over repeated transfers, several FeRed enrichments performed well. After three transfers, the most dominant microorganisms in these enrichment cultures were identified by sequencing prominent DGGE bands.

From DNA extracts of a Fe(III)-reducing enrichment culture in medium with 5 M NaCl, only archaeal 16S rRNA genes could be amplified by PCR. The two most prominent DGGE bands (figure S3 A) were excised from the gel, re-amplified, cloned and sequenced. However, since these 175 bp-sequences were too short to allow accurate classification, full-length 16S rRNA gene amplicons were generated from the original DNA extract of the enrichment culture. Six out of the ten sequenced clones had 97 to 98% 16S rRNA gene sequence identity to *Halobaculum gomorrense* strain JCM 9908. *Halobaculum gomorrense* has first been isolated by Oren et al. in 1995 from the Dead Sea (Oren et al., 1995). The other four sequences grouped within the *Halobacteriaceae* family. The closest cultivated relatives to these sequences were *Halogranum rubrum*, *Halomicrobium katesii* and *Halobacterium noricense*.

Only bacterial but no archaeal 16S rRNA gene amplicons could be amplified from DNA extracts of another Fe(III)-reducing enrichment in medium with 0.5 M NaCl. Only one dominant band was visible on a DGGE gel (figure S3 B). The band was excised, re-amplified, cloned, and sequenced. Even though they originated from one single DGGE band, the three clones that were sequenced were found to be phylogenetically distinct from each other: One of the 16S rRNA gene sequences was 97% identical to a *Dehalobacter restrictus* strain from an anaerobic co-culture that had been enriched from a hexachlorocyclohexane-polluted soil (van Doesburg et al., 2005). The second sequence was 99% identical to the 16S rRNA gene sequence of *Lactobacillus fabifermentans* (De Bruyne et al., 2009). The closest cultivated relative (97% 16S rRNA gene sequence identity) to the third sequence from our enrichment was *Desulfosporosinus lacus*, a

sulfate-reducing bacterium isolated from sediments of Lake Stechlin, Germany (Ramamoorthy et al., 2006).

In the two Fe(III)-reducing enrichment cultures, between 0.46 and 2.49 mM of Fe(II) were formed within 44 days. These values correspond to 0.01 to 0.06 µmoles of Fe(II) produced per mL of culture daily (Table 2).

Archaeal and bacterial diversity as determined by 16S rRNA gene clone libraries
We constructed 16S rRNA gene clone libraries for both Bacteria and Archaea. These libraries revealed a much higher diversity among Bacteria (12 different phyla and five uncultured "candidate divisions") as compared to Archaea (2 phyla).

In the bacterial clone library, *Gammaproteobacteria* represented the most abundant group (169 out of 299 sequences) (figure 4B; Table S4). The majority of the gammaproteobacterial clones (144 sequences) belonged to the genus *Halothiobacillus.* Members of this genus are obligate chemolithoautotrophs. They tolerate high concentrations of solutes and obtain energy from oxidizing reduced sulfur species (Kelly et al., 2000). The second major phylum within the Bacteria were the *Firmicutes* comprising 35 clone sequences, which fell into two different classes, namely *Bacilli* (30 sequences) and *Clostridia* (5 sequences). *Firmicutes* comprise many halophilic, thermophilic, anaerobic, and fermentative bacteria capable of forming spores and toxins (Dworkin et al., 2006).

As for the archaeal clone library (figure 4 A), 16S rRNA gene sequences of both *Euryarchaeota* (227 sequences) and *Crenarchaeota* (4 sequences) were found. Of the *euryarchaeotal* sequences, 209 belonged to the class of *Halobacteria*, extreme halophiles that grow even at saturated salt concentrations (DasSarma, 2007).

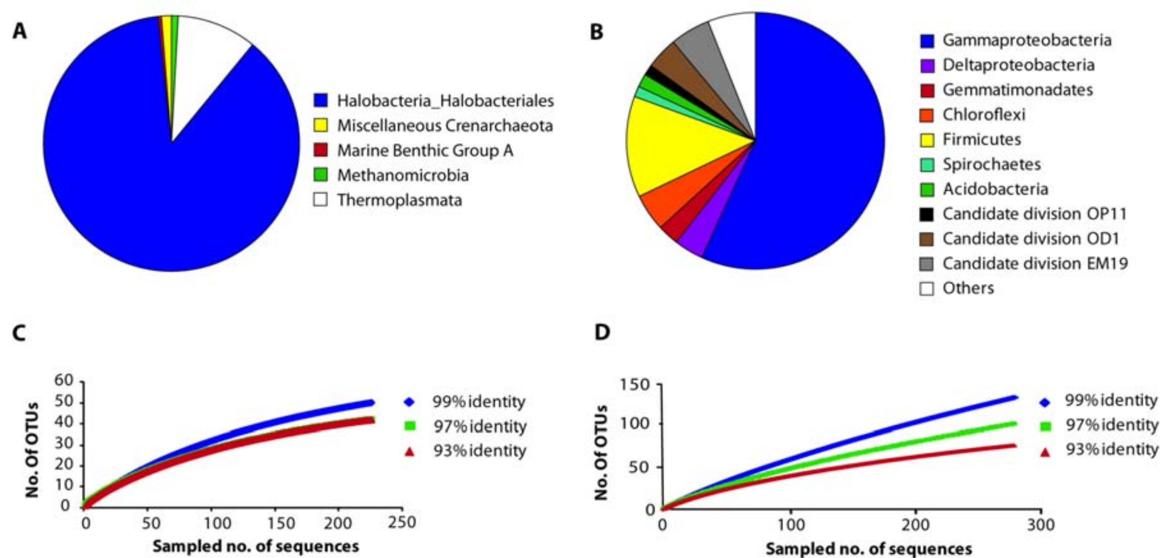


Figure 4: *Classification of 231 archaeal (A) and 299 bacterial (B) full length 16S rRNA gene sequences retrieved from a 0-10 cm composite sample of Lake Kasin. C) and D) show rarefaction curves for the archaeal and bacterial sequences from the respective clone library for three different sequence identity cutoff values (99%, 97%, and 93%). The archaeal rarefaction curve for the 97% cutoff value in (C) is not visible because it exactly resembles the 93%-curve. Rarefaction curves were calculated with the program MOTHUR (Schloss et al., 2009).*

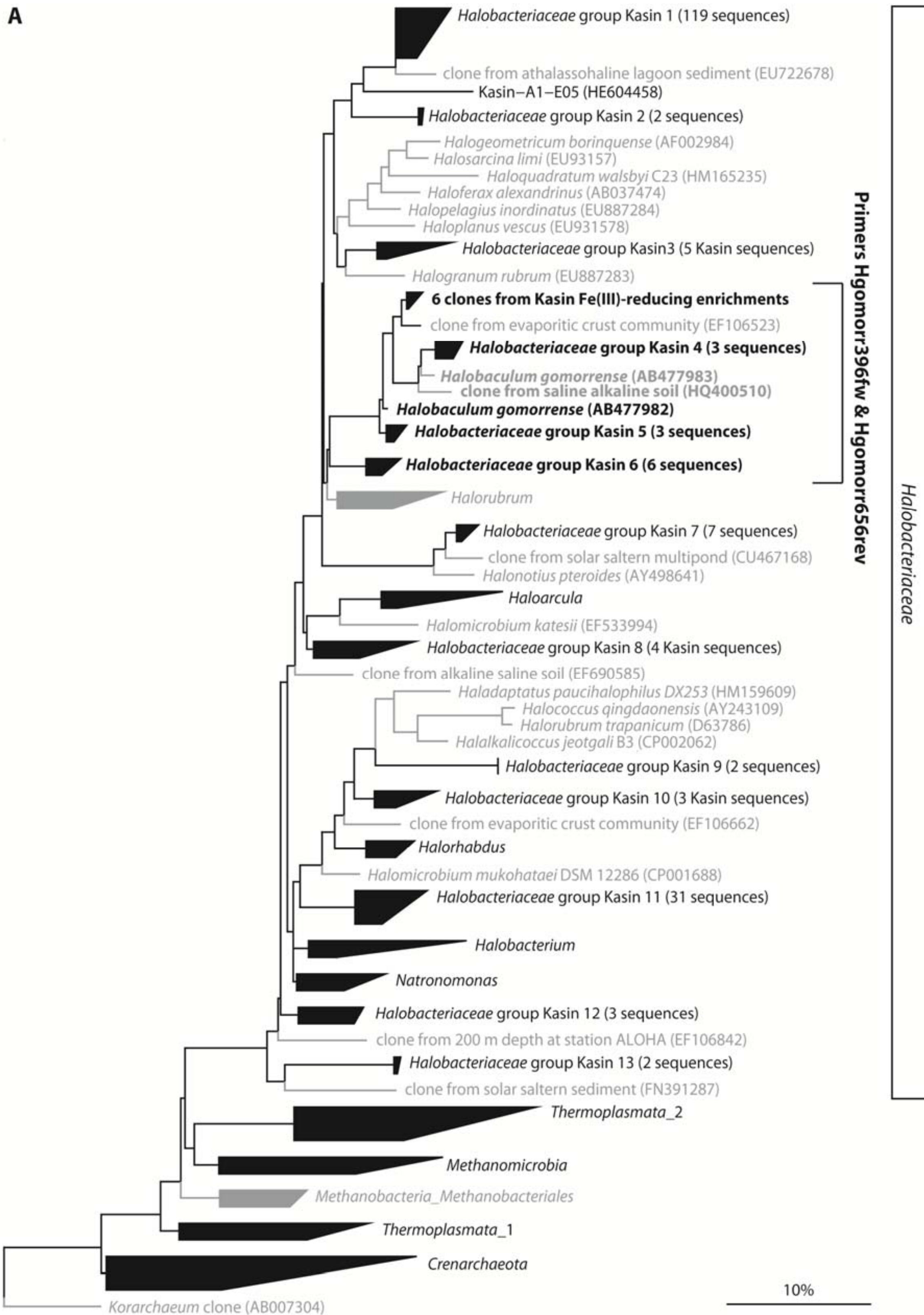
Rarefaction curves (figures 4 C and D) and Chao indices indicated that the diversity of Archaea in Lake Kasin has been covered by the 16S rRNA gene clone library to a larger extent than the bacterial diversity. Interestingly, none of the bacterial sequences recovered were closely related to known and cultured dissimilatory Fe(III) reducers or Fe(II) oxidizers. The sequences obtained from the DGGEs of the bacterial Fe(III)-reducing enrichments were distinct from those in the clone library. However, 10.4% of the sequences in the clone library were affiliated with the class *Bacilli*, a representative of which, *Bacillus infernus*, has been shown to reduce Fe(III) (Boone et al., 1995). Furthermore, Fe(III)-reducing enrichment cultures that were inoculated with sediment from other Russian salt lakes (Lake Elton and Lake Baskunchak) were also dominated by strains which showed 98% 16S rRNA gene sequence identity to *Bacillus alkalidiazotrophicus* and *Anaerobacillus alkalilacustris*, respectively (data not shown). For these reasons and since most

of the cultivated *Bacilli* have not been tested for their ability to reduce Fe(III), it is conceivable that *Bacilli* might be contributing to Fe(III) reduction at Lake Kasin.

With respect to the Archaea, the dominant strain in our Fe(III)-reducing enrichment culture with 5 M NaCl, *Halobaculum gomorrense*, was also the closest cultivated relative of six clones from our archaeal 16S rRNA gene clone library with sequence identities exceeding 97%. Based on the results from our cultivation-dependent and -independent experiments, species affiliated with the *Bacilli* and *Desulfosporosinus* spp. as well as species affiliated with *Halobaculum gomorrense* were considered potential candidates contributing to Fe(III) reduction in Lake Kasin sediment.

Abundance and distribution of Fe(III)-reducing microorganisms along the sediment profile
In order to analyze abundance and vertical distribution of *Desulfosporosinus* spp.-relatives, *Bacilli* and *Halobaculum gomorrense*-relatives in the sediment, we designed 16S rRNA gene primers specific to the obtained sequences, their cultivated relatives and a few sequences from uncultivated strains that belonged to the same sequence clusters. While the "*Bacilli*" quantitative real-time PCR (qPCR) primers targeted most sequences within this class, the number of targeted sequences of primers "Desulfosp." and "Hgomorr" were much smaller and did not include all species of the genera *Desulfosporosinus* and *Halobaculum*, respectively (Table S1). Figure 5 shows maximum likelihood trees of the archaeal (figure 5 A) and bacterial (figure 5 B) 16S rRNA gene sequences from Lake Kasin sediment and the Fe(III)-reducing enrichment cultures. Brackets indicate target sequences of the group-specific qPCR primers.

The total number of cells (16S rRNA gene copy numbers of Bacteria and Archaea corrected for average rRNA operon numbers) ranged from 1.1×10^6 to 6.7×10^7 cells per g dry sediment with the highest numbers found at 1.5 to 2 cm depth. Bacteria outnumbered Archaea by factors two to three throughout the entire sediment profile.


A
Halobacteriaceae group Kasin 1 (119 sequences)
clone from athalassohaline lagoon sediment (EU722678)
Kasin–A1–E05 (HE604458)
Halobacteriaceae group Kasin 2 (2 sequences)
Halogeometricum borinquense (AF002984)
Halosarcina limi (EU93157)
Haloquadratum walsbyi C23 (HM165235)
Haloferax alexandrinus (AB037474)
Halopelagius inordinatus (EU887284)
Haloplanus vescus (EU931578)
Halobacteriaceae group Kasin3 (5 Kasin sequences)
Halogranum rubrum (EU887283)
6 clones from Kasin Fe(III)-reducing enrichments
clone from evaporitic crust community (EF106523)
Halobacteriaceae group Kasin 4 (3 sequences)
Halobaculum gomorrense (AB477983)
clone from saline alkaline soil (HQ400510)
Halobaculum gomorrense (AB477982)
Halobacteriaceae group Kasin 5 (3 sequences)
Halobacteriaceae group Kasin 6 (6 sequences)
Halorubrum
Halobacteriaceae group Kasin 7 (7 sequences)
clone from solar saltern multipond (CU467168)
Halonotius pteroides (AY498641)
Haloarcula
Halomicrobium katesii (EF533994)
Halobacteriaceae group Kasin 8 (4 Kasin sequences)
clone from alkaline saline soil (EF690585)
Haladaptatus paucihalophilus DX253 (HM159609)
Halococcus qingdaonensis (AY243109)
Halorubrum trapanicum (D63786)
Halalkalicoccus jeotgali B3 (CP002062)
Halobacteriaceae group Kasin 9 (2 sequences)
Halobacteriaceae group Kasin 10 (3 Kasin sequences)
clone from evaporitic crust community (EF106662)
Halorhabdus
Halomicrobium mukohataei DSM 12286 (CP001688)
Halobacteriaceae group Kasin 11 (31 sequences)
Halobacterium
Natronomonas
Halobacteriaceae group Kasin 12 (3 sequences)
clone from 200 m depth at station ALOHA (EF106842)
Halobacteriaceae group Kasin 13 (2 sequences)
clone from solar saltern sediment (FN391287)
Thermoplasmata_2
Methanomicrobia
Methanobacteria_Methanobacteriales
Thermoplasmata_1
Crenarchaeota
Korarchaeum clone (AB007304)
10%
Primers Hgomorr396fw & Hgomorr656rev
Halobacteriaceae

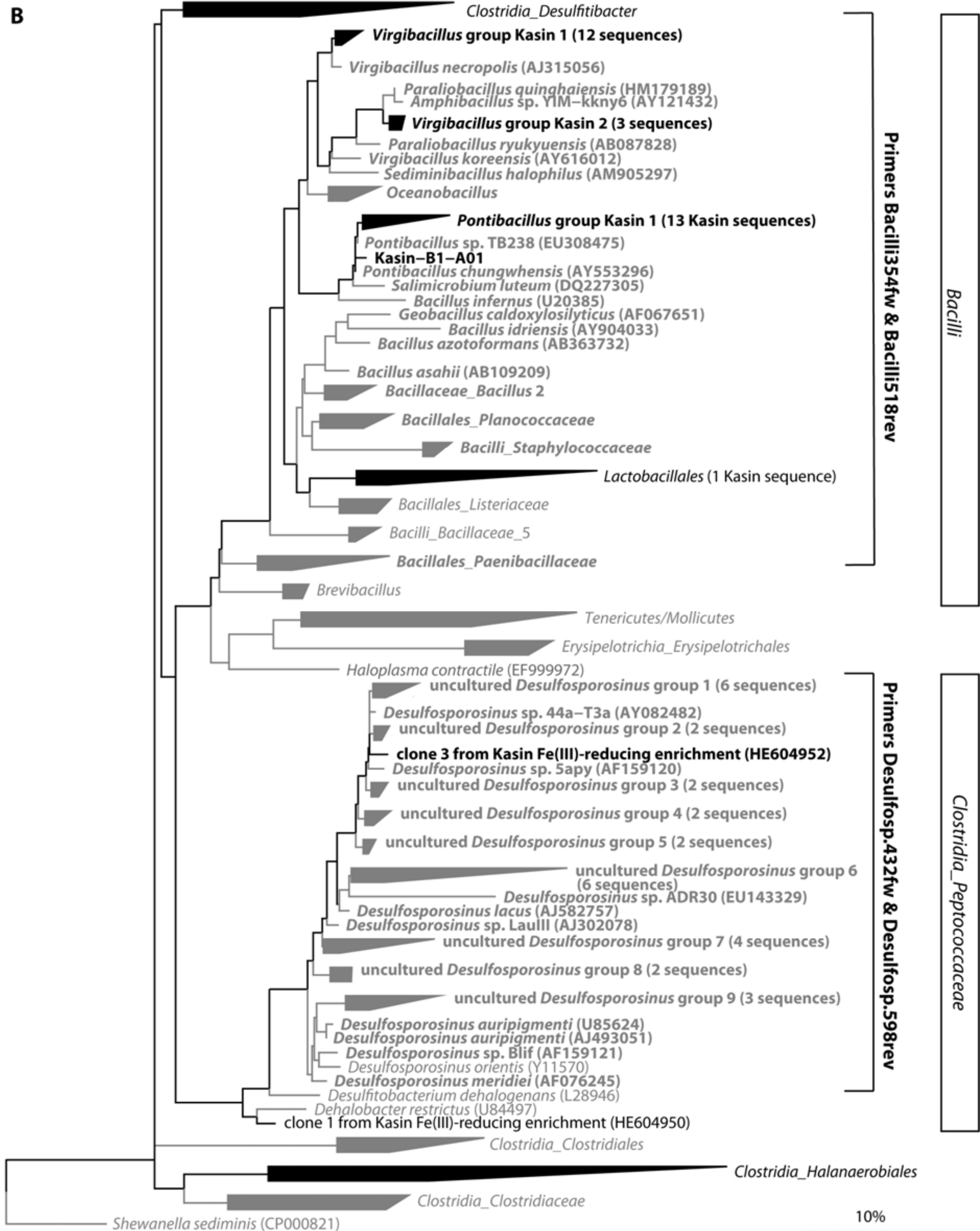

B
Clostridia_Desulfitibacter
Virgibacillus group Kasin 1 (12 sequences)
Virgibacillus necropolis (AJ315056)
Paraliobacillus quinghaiensis (HM179189)
Amphibacillus sp. YIM–kkny6 (AY121432)
Virgibacillus group Kasin 2 (3 sequences)
Paraliobacillus ryukyuensis (AB087828)
Virgibacillus koreensis (AY616012)
Sediminibacillus halophilus (AM905297)
Oceanobacillus
Pontibacillus group Kasin 1 (13 Kasin sequences)
Pontibacillus sp. TB238 (EU308475)
Kasin–B1–A01
Pontibacillus chungwhensis (AY553296)
Salimicrobium luteum (DQ227305)
Bacillus infernus (U20385)
Geobacillus caldoxylosilyticus (AF067651)
Bacillus idriensis (AY904033)
Bacillus azotoformans (AB363732)
Bacillus asahii (AB109209)
Bacillaceae_Bacillus 2
Bacillales_Planococcaceae
Bacilli_Staphylococcaceae
Lactobacillales (1 Kasin sequence)
Bacillales_Listeriaceae
Bacilli_Bacillaceae_5
Bacillales_Paenibacillaceae
Brevibacillus
Tenericutes/Mollicutes
Erysipelotrichia_Erysipelotrichales
Haloplasma contractile (EF999972)
uncultured Desulfosporosinus group 1 (6 sequences)
Desulfosporosinus sp. 44a–T3a (AY082482)
uncultured Desulfosporosinus group 2 (2 sequences)
clone 3 from Kasin Fe(III)-reducing enrichment (HE604952)
Desulfosporosinus sp. 5apy (AF159120)
uncultured Desulfosporosinus group 3 (2 sequences)
uncultured Desulfosporosinus group 4 (2 sequences)
uncultured Desulfosporosinus group 5 (2 sequences)
uncultured Desulfosporosinus group 6 (6 sequences)
Desulfosporosinus sp. ADR30 (EU143329)
Desulfosporosinus lacus (AJ582757)
Desulfosporosinus sp. LauIII (AJ302078)
uncultured Desulfosporosinus group 7 (4 sequences)
uncultured Desulfosporosinus group 8 (2 sequences)
uncultured Desulfosporosinus group 9 (3 sequences)
Desulfosporosinus auripigmenti (U85624)
Desulfosporosinus auripigmenti (AJ493051)
Desulfosporosinus sp. Blif (AF159121)
Desulfosporosinus orientis (Y11570)
Desulfosporosinus meridiei (AF076245)
Desulfitobacterium dehalogenans (L28946)
Dehalobacter restrictus (U84497)
clone 1 from Kasin Fe(III)-reducing enrichment (HE604950)
Clostridia_Clostridiales
Clostridia_Halanaerobiales
Clostridia_Clostridiaceae
Shewanella sediminis (CP000821)
10%
Primers Bacilli354fw & Bacilli518rev
Bacilli
Primers Desulfosp.432fw & Desulfosp.598rev
Clostridia_Peptococcaceae

Figure 5: *Maximum likelihood trees of archaeal (A) and bacterial (B) 16S rRNA gene sequences directly amplified from Lake Kasin sediment or obtained from the Fe(III)-reducing enrichment cultures. (A) ML tree of the Halanaerobiaceae. (B) ML tree of the Bacilli and the Peptococcaceae family of the Clostridia. Groups for which at least one representative sequence was found in Lake Kasin sediment or enrichments are printed in black. Groups with no representatives from Lake Kasin sediment or enrichments are printed in grey. (Groups of) Sequences that match both forward and reverse qPCR primers designed for Halobaculum gomorrense- (A) and Desulfosporosinus spp.- or Bacilli-related sequences (B) are shown in bold face.*

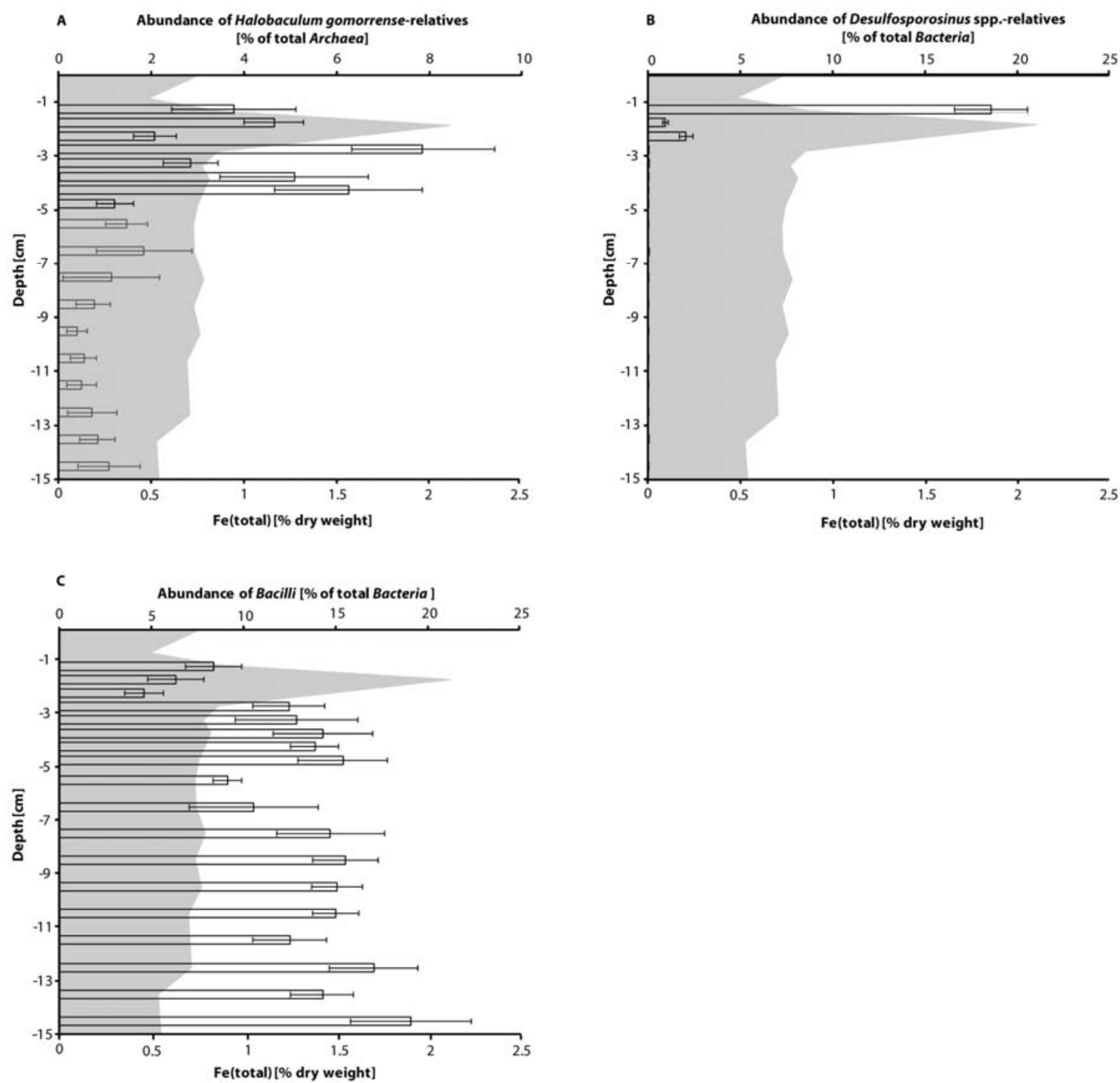


Figure 6: *Changes in relative abundance of potential Fe(III)-reducing taxa in a sediment profile of Lake Kasin. Bars indicate the abundance of Halobaculum gomorrense-relatives (A), Desulfosporosinus spp.-relatives (B) and Bacilli (C) relative to total Archaea (A) or total Bacteria (B + C) cell numbers for each sediment layer. Error bars refer to standard deviations of six individual qPCR measurements recorded in triplicates during two independent runs. The grey-shaded area in the background shows Fe(total) concentrations in % dry weight of the sediment.*

With respect to the distribution of putative Fe(III)-reducers, *Halobaculum gomorrense*-relatives comprised 2-6% of all Archaea in the top 4.5 cm of the sediment (figure 6 A). However, there was no significant correlation between cell numbers of *Halobaculum gomorrense* relatives and the distribution of total Fe (w/w % dry weight) in the upper sediment layers. *Desulfosporosinus* spp.-relatives, on the other hand, were only detected in the Fe-rich top three cm of the sediment. Their abundance was three orders of magnitude higher at 2.5 cm depth than further below (figure 6 B). Between 1 and 1.5 cm depth, up to 20% of all Bacteria were *Desulfosporosinus* spp. relatives. *Bacilli* represented between 5 and 20% of all Bacteria throughout the entire sediment profile without an obvious cell number increase in the upper Fe-rich layers (figure 6 C).

Discussion

Occurrence and speciation of Fe in Lake Kasin sediments

A substantial fraction of the Fe in Lake Kasin occurred in form of the mineral akaganéite (figure 2). Akaganéite has a Néel temperature of 299 K (Murad and Cashion, 2004) and should appear as magnetically split sextets in the spectrum recorded at 77 K. Under these conditions lepidocrocite, which has a Néel temperature of 77 K (Murad and Cashion, 2004), may have contributed to the akaganéite spectrum. Furthermore, green rust minerals, which can contain Cl^-, SO_4^{2-}, and CO_3^{2-} also have similar Mössbauer parameters. The chemistry of Lake Kasin sediment would allow the formation of all three mineral phases. Therefore, a mixture of the three mineral phases seems most likely. The fact that the Mössbauer spectra did not reveal any FeS precipitated

in this system could be due to the precipitation of other Fe(II)-bearing minerals that have a lower solubility product than FeS and are therefore favored to precipitate under *in situ* conditions.

Only minor amounts of akaganéite can be extracted with 1 M hydroxylamine-HCl within 48 h (Poulton and Canfield, 2005). This implies that akaganéite is not expected to contribute to the "bioavailable" Fe fraction of Lake Kasin sediment extracted with 0.5 M HCl. However, the mineral can still be subject to microbial transformations: we observed that the Fe(III)-reducing strain *Shewanella oneidensis* MR1 can reduce akaganéite almost as efficient as poorly crystalline ferrihydrite (unpublished data). This means that akaganéite could also serve as an electron acceptor for Fe(III)-reducing microorganisms in Lake Kasin. Therefore, it is most likely that we underestimated the amount of bioavailable Fe by extraction with 0.5 M HCl.

Apart from Lake Kasin, sediments of other salt lakes have also been found to contain high amounts of Fe minerals: the sediment of Lake Tyrell (Australia) contains hydrous Fe-oxides such as goethite (Long et al., 1992), which can also be subject to microbial reduction (Roden and Zachara, 1996). In addition to goethite, alunite and jarosite were identified in Lake Tyrell sediments (Long et al., 1992). The formation of jarosite in low temperature environments has been related to microbial oxidation of iron and sulfur (Ivarson et al., 1979). In other salt lake sediments, Fe(II) rather than Fe(III) minerals were found to dominate, e.g. in the sediment of Lake Qinghai (China), pyrite was the dominating Fe mineral (Kuno et al., 2002). About 1% dry weight of the sediment of the SO_4^{2-}-rich Salton Sea (USA) was found to consist of reduced Fe phases, which were dominated by greigite (Fe_3S_4; sulfur analogue to magnetite) and pyrite (FeS_2) (De Koff et al., 2008). Fe(II) released during oxidation of pyrite has been shown to also serve as an electron donor for microorganisms (Torrento et al., 2010). The presence of bioavailable Fe(II) and Fe(III) mineral phases in various salt lake sediments could be indicative of both microaerophilic as well as anaerobic microbial Fe(II) oxidation and Fe(III) reduction in these environments.

Fe-metabolizing microorganisms in Lake Kasin

The MPN counts revealed as many Fe(III)-reducers as nitrate-reducing Fe(II)-oxidizers in medium with 5 M NaCl as in medium with 0.5 M NaCl. This was the case for inocula from Lake Kasin sediment (figure 3) as well as for two other reference sediments we investigated (Lake Elton and Lake Baskunchak) (figure S2). Since the microorganisms were exposed to considerable osmotic stress in medium with 5 M NaCl, these results showed that the Fe-metabolizing microorganisms in Lake Kasin sediments were well-adapted to a broad range of NaCl concentrations. A comparison of results from MPN counts to qPCR measurements of total bacterial 16S rRNA gene copies revealed that culturable anaerobic Fe(II)-oxidizers and Fe(III)-reducers represent <0.1% of the total Bacteria present in Lake Kasin sediment.

The conditions in our Fe(III)-reducing enrichment cultures did not allow to distinguish between dissimilatory and fermentative Fe(III) reduction. As consequence, part of the observed Fe(III) reduction in the sediment might be due to fermentation also explaining the relatively high abundance of *Bacilli* and other microbial taxa capable of fermentation.

The absence of growth of microaerophilic Fe(II)-oxidizers in gradient tubes might have been due to limited diffusion of Fe(II) to the upper layers of the gradient tube in the high salinity medium. Since we measured 50 µM of total Fe in the upper part of the agar tubes, it seems more likely that microaerophilic Fe(II)-oxidizers either did not grow under the applied conditions or were not present in Lake Kasin sediments.

Total cell numbers and total Fe content showed a strong linear correlation (R^2=0.91; p=1.77×10^{-8}; according to Spearman's test for non-parametric data). Both parameters were highest at the same sediment depth. The Fe content refers to both bioavailable and crystalline Fe, even though the difference in Fe concentration between the Fe-rich layer and the lower parts of the sediment was more pronounced for the bioavailable than for the crystalline Fe fraction (figure 1). Another strong linear correlation (R^2 value of 0.76) was found between cell numbers and NaCl content. NaCl-dominated salinity continually decreases from 48.7 g Cl^- per kg dry weight in the topmost

horizon (0-2.5 cm) to 10.8 g Cl^- per kg dry weight at 5 cm depth. Correlations between other geochemical parameter (such as temperature, pH, sulfate or carbonate conc.) and cell numbers were not found.

The number of total cells (Bacteria and Archaea) in Lake Kasin sediment (1.1×10^6 to 6.7×10^7 cells per g dry sediment) was rather low in comparison to cell numbers determined in other hypersaline environments, such as sediments of saline Lakes Chaka and Qinghai, (Jiang et al., 2007; Dong et al., 2006), a Californian salt marsh (Cao et al., 2008) and sediments of a hypersaline mud volcano (Lazar et al., 2011). In Lake Qinghai, China, for example, which has a salinity of 12.5 g/L, cell counts revealed 10^7 cells per g wet sediment at 50 cm depth (Dong et al., 2006). Although these numbers refer to wet sediment, the cell numbers at 50 cm depth of Lake Qinghai are still higher than the numbers quantified for the iron-rich layer (1 - 3 cm depth) of Lake Kasin. This might be explained by the low amount of total organic carbon in Lake Kasin sediments (0.11% w/w) compared to e.g. Lake Quinghai (1.8 to 2.4% w/w) sediment (Dong et al., 2006).

In Lake Kasin, Bacteria were more abundant than Archaea throughout the entire sediment profile. This finding is in contrast to results for sediments of Lake Chaka (Jiang et al., 2007) and the Salton Sea (Swan et al., 2010), but similar to La Sal del Rey sediments, where more than 97% of all 16S rRNA gene copies belonged to the Bacteria (Hollister et al., 2010). Only a few comprehensive data sets are available from hypersaline sediments and currently it remains unclear what physicochemical factors determine whether Bacteria or Archaea dominate numerically.

The presence of anaerobic Fe-oxidizers and Fe-reducers in our MPNs and enrichment cultures as well as the strong correlation between Fe content and total cell numbers in Lake Kasin sediments suggest that next to sulfur Fe might also serve as electron donor and acceptor of microbial respiration at high salinities. However, a strong correlation between Fe content and total cell numbers could also reflect sedimentary deposition of iron and carbon over time.

Microbial diversity in Lake Kasin sediments

The 16S rRNA gene clone library revealed a higher bacterial than archaeal diversity in the top 10 cm of Lake Kasin sediment. This is in agreement with clone library data from other saline sediments such as those from the salt Lakes Chaka (Jiang et al., 2007 and 2006) and Qinghai (Dong et al., 2006; Jiang, 2007) as well as sediments from soda lakes of the Wadi An Natrun (Mesbah et al., 2007), the Kenyan-Tanzanian Rift Valley (Rees et al., 2004) and Lonar Lake (Wani et al., 2006). Based on 97% 16S rRNA gene sequence identity higher numbers of bacterial than archaeal OTUs were defined in all clone libraries from the above mentioned saline lakes.

The majority of the sequences in our bacterial clone library were affiliated with *Gammaproteobacteria*. *Gammaproteobacteria*, also comprised up to 10% of all bacterial 16S rRNA gene sequences in clone libraries of other saline sediments (Dong et al., 2006; Hollister et al., 2010; Jiang et al., 2006; Koizumi et al., 2004; Ma et al., 2004; Mesbah et al., 2007; Rees et al., 2004 and Wani et al., 2006). Although high numbers of gammaproteobacterial 16S rRNA gene sequences are also common in clone libraries of other saline sediments, the gammaproteobacterial genus *Halothiobacillus* which comprised 38% of all bacterial 16S rRNA gene sequences in our library (Table S4), did up to date not occur in any other published clone library of saline sediments. The absence of *Halothiobacillus* sequences in all other clone libraries from saline sediments might be due to the fact that we constructed our clone library from exposed lakebed sediments samples while most other published clone libraries (expect for a transect of hypersaline La Sal del Rey, Texas, USA) were constructed from samples of water-logged and anoxic saline sediments. *Halothiobacillus*, is an obligatory aerobic genus which grows by oxidizing reduced sulfur species at up to 4 M NaCl (Sorokin et al., 2006). This means that the presence of H_2S, a salinity close to saturation and the availability of O_2 in the top few cm of the sediment constitute nearly optimal growth conditions for *Halothiobacillus* in Lake Kasin. *Halothiobacillus* species have been described to outcompete other species on the basis of their high growth rate (Sorokin et al.,

2006), which may further explain why so many sequences in our clone library are affiliated with this genus.

Firmicutes accounted for 12% of all bacterial 16S rRNA gene sequences, and were the second-most abundant bacterial phylum in Lake Kasin sediments. *Firmicutes* were also abundant in other saline lake sediments. In sediments from the saline Lakes Chaka (Jiang et al., 2007 and 2006) and Qinghai (Dong et al., 2006 and Jiang, 2007) as well as the soda lakes of the Wadi An Natrun (Mesbah et al., 2007), the Kenyan-Tanzanian Rift Valley (Rees et al., 2004) and Lonar Lake (Wani et al., 2006), *Firmicutes* were the most abundant phylum (constituting 19-42% of all sequences in these libraries).

What is notable in our clone library is the absence of sequences representing the *Cytophaga/Flexibacter/Bacteroidetes* (CFB) group. This group is reported to dominate in many different saline environments including marine water (Kirchmann, 2002), salt lakes (Pagaling, 2009), soda lake sediments (Rees et al., 2004) and hypersaline endovaporitic mats (Sorensen et al., 2005; Sorokin et al., 2006). The main ecological role of the heterotrophic CFB group has been described to be the degradation of organic material due to the capability of many of its members to degrade biopolymers such as cellulose and lignin (Kirchmann, 2002). Since the sediment of Lake Kasin is particularly poor in organic material, this lack of substrate might explain the low abundance of CFB representatives in this environment.

With respect to the Archaea, our clone library is dominated by the order *Halobacteriales* with most representatives belonging to the *Halobacteriaceae* family. 86% of the archaeal 16S rRNA gene sequences from Lake Kasin sediment clustered with this family. *Halobacteriaceae* showed a similar abundance in clone libraries of the Great Salt Plains in Oklahoma (100% of 166 sequences belong to the *Halobacteriaceae*, (Caton et al., 2009)) as well as in sediments of the soda lakes of the Wadi An Natrun (91%) (47) and the Kenyan-Tanzanian Rift Valley (93%) (Rees et al., 2004). In a recent study by Youssef and coworkers (2012) the authors describe that both the alpha- and beta-diversity of *Halobacteriales* populations of five different saline sediments were

much higher than previously thought, suggesting a profound ecological role of this order in saline ecosystems (Youssef et al., 2004).

In other archaeal clone libraries from saline sediments such as the Antrim Shale in Michigan (Waldron et al., 2007), Lake Lonar in India (Wani et al., 2006), Lake Chaka in China (Jiang et al., 2007 and 2006) and the Salton Sea in California (Swan et al., 2010), sequences from methanogenic orders constituted at least 10% of all archaeal 16S rRNA gene sequences. In our library, only one sequence clustered with the order *Methanobacteriales* and two sequences belonged to the *Methanosarcinales*. In the Antrim Shale 67% of all archaeal sequences belonged to methanogenic groups (Waldron et al., 2007). The relatively high concentrations of SO_4^{2-} and Fe(III) in the sediment of Lake Kasin seem to favor microbial sulfate and iron reduction over methanogenesis.

From the archaeal sequences retrieved from Lake Kasin sediment, 10% belonged to the order *Thermoplasmatales*, which has also been shown to be present in other saline sediments (Waldron et al., 2007; Jiang et al, 2007 and 2006; Swan et al., 2010; Dong et al., 2006 and Jiang, 2007). In summary, both the composition of the archaeal and bacterial 16S rRNA clone libraries from Lake Kasin sediment revealed the presence of microbial taxa that have been shown to exist also in other saline habitats. Given the fact that we did not find any sequences of known photoautotrophs together with the scarce vegetation observed at this site, we expect primary production by eukaryotic algae to be the main source of carbon input into the ecosystem.

Interestingly, none of the obtained 16S rRNA gene sequences of the Lake Kasin library were closely affiliated with any known taxa of Fe(II)-oxidizers or Fe(III)-reducers.

Abundance, distribution, and activity of putative Fe(III)-reducers in the sediment

Notably, 16S rRNA gene sequences from our bacterial Fe(III)-reducing enrichments did not occur in the clone library. Even though we tried to limit biases introduced by DNA extraction and PCR by combining DNA extracts from different extraction methods and pooling amplicons

from several independent PCRs, we cannot completely rule out that this phenomenon is due to method-related limitation.

Unfortunately, little is known about the Fe(III)-reducing capability of species that are most closely related to the microorganisms we found in our Fe(III)-reducing enrichment cultures. *Halobaculum gomorrense* described by Oren et al. (1995) has not been tested for its capability to reduce Fe(III), but it could not grow with nitrate as electron acceptor or fermentatively with arginine. *Dehalobacter restrictus* has been described as an anaerobic bacterium that can grow by dehalogenating halogenated phthalides. However, its capability to reduce Fe(III) has not been tested either (Yoshida et al., 2009). *Lactobacillus fabifermentans* has been described as a facultative anaerobe, but its capability to reduce Fe(III) has also not been shown (De Bruyne et al., 2009). *Desulfosporosinus lacus*, on the other hand, can use Fe(III) but also SO_4^{2-} as terminal electron acceptor (Ramamoorthy et al., 2006). The electron acceptor preferences of *Desulfosporosinus* under *in situ* conditions are not know. As mentioned in the introduction Fe(III) reduction is thermodynamically more favorable than sulfate reduction. Even though for exact calculations activities would need to be considered the relatively higher energy yield of Fe(III) reduction over sulfate reduction is not expected to change.

Overall, absolute cell numbers of the enriched putative Fe(III)-reducers affiliated with *Desulfosporosinus* spp., *Bacilli* and *Halobaculum gomorrense* increased in the Fe-rich sediment layer (1.5 to 2.5 cm depth) of Lake Kasin. Also total cell numbers of Bacteria and Archaea were highest at this particular depth. While the relative abundance of *Bacilli* is more or less constant throughout the entire sediment profile, *Halobaculum gomorrense*-related archaea and *Desulfosporosinus* spp.-related bacteria were more abundant in the upper sediment layers. Interestingly, the highest numbers of 16S rRNA gene copies of the later group were found between 1 and 1.5 cm just above the iron-rich layer. It could be that it is particularly the high content of bioavailable Fe(III) that supports growth of *Desulfosporosinus* spp. in this sediment layer.

Table 2: *Comparison of rates of Fe(II) production in Fe(III)-reducing enrichment cultures with Fe(II) concentrations in Lake Kasin sediment.*

Fe(III)-reducing enrichment cultures	Cell numbers [2, 3]	Fe(II) production rates[4] [µmoles Fe(II) mL^{-1} day^{-1}]	µmoles Fe(II)[2]
Halobaculum gomorrense relatives	1.86×10^5	0.01	
Desulfosporosinus spp.	7.13×10^4	0.02	28
Bacilli (containing *Bacillus alkalidiazotrophicus*[1])	5.48×10^5	0.06	

[1]culture originates from sediment of salt Lake Baskunchak, Southern Russia
[2]per g dry sediment of 0-10 cm composite sample from Kasin sediment
[3]determined by qPCR with primers “Hgomorr”, “Desulfosp.” and “Bacilli”
[4] in the enrichment cultures

The cumulative rate of Fe(II) production in all three Fe(III)-reducing enrichment cultures reached 1 µmol mL^{-1} day^{-1} (Table 2). Based on this rate it would take the three enrichment cultures about one month to form the 28 µmoles of Fe(II), the amount of Fe(II) that we measured per g dry weight of sediment in the composite sample from the top 10 cm. Even if we assume that the *in situ* Fe(II) production rates are lower than the once we calculated for our enrichment cultures, our calculations still show that the microorganisms we enriched have the capability to generate the amount of Fe(II) present in Lake Kasin. Our MPNs showed that Fe(III)-reducers were up to an order of magnitude more abundant than anaerobic Fe(II)-oxidizers in the sediment of Lake Kasin (figure 3). Considering that hardly any NO_3^-, but more than 1 mM of acetate was detected in the sediment leacheate, we conclude that only minor amounts of the Fe(II) that was formed by the Fe(III)-reducers in the sediment is readily re-oxidized by anaerobic Fe(II)-oxidizers in the reduced zones of the sediment. Thus, a great amount of the Fe(II) below the oxygen penetration depth in the sediments of Lake Kasin has been formed by microbial Fe(III) reduction. The fact that we did not detect any FeS mineral phases by Mössbauer spectroscopy does seem to argue for a minor contribution of abiotic Fe(III) reduction by reduced sulphur species.

In summary, we showed that: (i) microbial Fe(III) reduction does occur at concentrations of up to 5 M NaCl extending the natural habitat boundaries of this important microbial process; (ii) the microbial community composition of Lake Kasin sediment has distinct features but overall resembles the community composition of other hypersaline habitats; (iii) the Fe mineral phases found in Lake Kasin are likely the product of microbial activity; (iv) the iron reduction rates quantified for the obtained enrichment cultures explain the Fe(II) concentrations found in the Lake sediments; (v) the presence of anaerobic microbial Fe(II)-oxidizers and Fe(III)-reducers suggests an active microbial Fe cycling in Lake Kasin at NaCl concentrations close to the solubility limit. Further, the occurrence of microbial Fe(III) reduction at 5 M NaCl, does have important implications for ancient Earth scenarios and the search for life on other planets, such as Mars where Fe-rich hypersaline brines have been inferred (Möhlmann and Thomsen, 2011; Tosca et al., 2005 and 2011). Comprehensive studies of Fe(III)-reducing and Fe(II)-oxidizing microbial populations in various environments are a prerequisite to systematically unravel the role and intricate interplay of these microbial processes in the biogeochemical cycling of iron.

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6

Abundance, distribution, and activity of Fe(II)-oxidizing and Fe(III)-reducing microorganisms in hypersaline sediments of Lake Kasin, Southern Russia

APPENDIX

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Materials and Methods:

Description of the study site

The Pre-Caspian Depression is part of the continental or semi-arid desert biome, divided by the River Volga that flows into the Earth's largest enclosed water body, the Caspian Sea. Widespread deep-water salt deposits of lower Permian age are prominent in the up to 23 km thick sediment piles. Redistributed by extensive salt tectonics, these salt deposits form more than 1,000 salt domes. Supplemented by thousands of smaller saline dips, these salt affected soils cover an area of 500,000 km^2 on both side the border between Russia and Kazakhstan. Lake Kasin is a typical hypersaline axial depression whose central part spans about 1,000 m × 200 m, with the lowest point at ~22 m below sea level. The surrounding landscape is flat and often covered with a thin salt layer (sulfate, carbonate, halite). Spare sagebrush-dominated vegetation covers the underlying dune formations, starting 100 m from the center, where salt affected soils turn into sloping sand and clay soils. Few halophytic plant species like *Salicornia* spp. can be found at the edges of the small lake. The region is mainly used for livestock; crop production only occurs in the Volga lowlands. Mean annual temperature for the region is 7.5°C (-10°C to +30°C) and annual rainfall averages around ~300 mm.

At the time of sampling in August 2009 Lake Kasin was partially covered with <10 cm saturated water layer (about 50 × 100 m). The air temperature reached 37°C and soil surface temperature increased up to 44°C. Atmospheric conditions were very dry (<40% humidity) with 50% cloud cover and moderate to strong wind.

Most probable number counts and enrichments of anaerobic Fe(II)-oxidizing and Fe(III)-reducing microorganisms

For MPN counts, 1 g of sediment was suspended in 10 mL salt water medium and the suspension was consecutively diluted 1:5 with medium to obtain a master dilution series of 12

dilutions. With each of these 12 dilutions, seven wells per one column of a 96 well plate (900 µL selective growth medium per well) were inoculated (100 µL inoculum per well). The last well of each column served as negative control. All MPN plates were set up in an anoxic chamber (glovebox) under N_2-atmosphere, sealed with a plastic foil, taken out of the anoxic chamber and immediately put into Anaerocult® bags (Merck) together with wetted O_2-indicator strips (Merck) in order to make sure anoxic conditions were maintained during incubation. After 6 weeks of incubation at 20°C in the dark, microbial growth was determined based on the number of parallels per dilution step. The MPN of cells per gram dry soil was calculated with the program "Most Probable Number Calculator" version 4.04 (Environmental Protection Agency, USA). The MPN counts were corrected after Salama as described in Klee (1993). Criteria used to determine microbial growth in the parallels of the dilution series were as follows: Wells were considered positive for growth of anFeOx if they showed a color change from slight green-blue to nearly black. Growth of FeRed was positive if the medium in the wells had turned from red-brown to nearly black. This was in agreement with a reduction of at least twice as much Fe(III) to Fe(II) as in the average of the wells that served as negative control as determined by ferrozine tests (Stookey, 1970). FeRed and anFeOx from selected wells were transferred into fresh media as it had been used in the MPNs every 6 to 8 weeks or as soon as most of the Fe(III) had been reduced or the Fe(II) had been oxidized, respectively. Between 1% and 2% of inoculum were used for the transfers that were carried out under sterile and anoxic conditions. Enrichments were incubated in the dark at 28°C.

Gradient tubes to enumerate microaerophilic Fe(II)-oxidizers

In contrast to Emerson and Floyd (2005) who used modified Wolfe's mineral medium (MWMM), salt water medium with 0.5 M NaCl as described in the section "Most probable number studies" was used both in the top layer and in the bottom layer. The initial concentration of the sodium bicarbonate buffer in this medium was 15 mM. The FeS was only washed twice

with anoxic H_2O and prepared directly before use. In the bottom layer, 1 mL of a 1:1 mix of FeS and salt water medium was used. The final concentration of FeS in the bottom layer was approximately 0.46 M. After addition of the bottom layer, the headspace was exchanged with N_2/CO_2 (90:10), tubes were capped with butyl rubber stoppers and kept at 4°C for 30 minutes. The medium for the top layer was prepared anoxically by taking the medium out of the autoclave at 80°C and flushing the headspace with N_2/CO_2 for 10 minutes. After the top layer medium had cooled down to 40°C, buffer, vitamins and all other additives were added as listed in the section "Most probable number studies and enrichments of Fe(II)-oxidizing and Fe(III)-reducing microorganisms" of the manuscript. In order to stabilize the top layer, the concentration of low melt agarose was increased to 0.5%. 10 mL of top layer were added anoxically on top of the bottom layer with syringes and needles that had been flushed with N_2/CO_2. In total, 21 gradient tubes were prepared and kept overnight to allow them to solidify before inoculation. For inoculation, tenfold dilutions of Lake Kasin sediment from 0-10 cm in SWM up to a dilution of 10^{-5} were prepared as described in the manuscript. From each dilution, four parallel tubes were inoculated with 100 µL of sediment suspension using 1 mL syringes and needles. Tubes were inoculated sterilely under atmospheric conditions in order to bring some O_2 into the system. Inocula were inserted at about 2/3 depth of the top layer. One gradient tube that served as a negative control was also penetrated with an empty needle down to the same depth. After inoculation, tubes were closed again with butyl rubber stoppers and incubated at 28°C for two months.

DGGE

The first amplification reactions of the long archaeal 16S rRNA gene fragments were performed in a final volume of 25 µL containing 1× PCR buffer with 1.5 mM $MgCl_2$ final concentration (Promega), 200 µM dNTP mix (New England Biolabs), 200 nM of each primer, 0.625 U Taq DNA-Polymerase (Promega) and 10 ng of DNA extract as a template in a total volume of 25 µL.

The thermocycler program was as follows: initial denaturation at 94°C for 2 min; 30 cycles of denaturing (94°C for 30 sec), annealing (58°C for 1 min) and elongation (72°C for 1 min 30 sec) and a final elongation at 72°C for 10 min. The second amplification reactions of the short archaeal and the amplification reactions of the bacterial 16S rRNA gene fragments were performed in a final volume of 50 µL. The reaction mix contained 1× PCR buffer (Promega), 4 mM $MgCl_2$, 200 µM dNTP mix (New England Biolabs), 200 nM of each primer, 1.25 U Taq DNA-Polymerase (Promega) and 10 ng of DNA extract as a template. The thermocycler program included: initial denaturation at 94°C for 2 min; 10 cycles of denaturing (94°C for 1 min), annealing (65°C to 56°C with a temperature decrease of 1°C per cycle for 1 min) and elongation (72°C for 1 min) plus 20 cycles of denaturing (94°C for 1 min), annealing (55°C for 1 min) and elongation (72°C for 1 min) and a final elongation at 72°C for 10 min. The concentrations of the PCR products were estimated by comparing the intensities of the resulting bands on an agarose gel to the band intensity of a marker fragment of defined concentration. Depending on the band intensities of the PCR products, between 5 and 20 µL were mixed with 5 µL of loading buffer consisting of 60% glycerol, 1 mM EDTA pH 8.0, 1% bromophenol blue and 0.5% xylem xylol and applied on a DGGE gel. DGGE gels were prepared in a DGGE unit type V20 HCDC from BioRad Laboratories GmbH, Munich, Germany according to the manufacturer's suggested protocol. All DGGE gels had a gradient of denaturant (7 M of urea and 40% v/v of formamide) ranging from 35% in the uppermost part of the gel to 60% in the lowest part of the gel. For bacterial 16S rRNA gene amplicons, 6% polyacrylamide gels were used and run for 16 h at 100 V and 60°C. Archaeal 16S rRNA gene amplicons were applied on 8% polyacrylamide gels which were run for 6 h at 100 V and 60°C. Gels were stained with ethidium bromide and DNA bands were visualized under UV.

Supplementary figure 1:

Figure S1: *Geographic maps showing A) the location of our sampling site in Southern Russia and B) the exact sampling position Kasin at about 50 m away from the water-covered area. C) shows the depth profile of the hypersaline sediment as well as the distribution of the four soil horizons H1-H4. The depth profile was sampled with plastic tubes as illustrated in D).*

Supplementary figure 2:

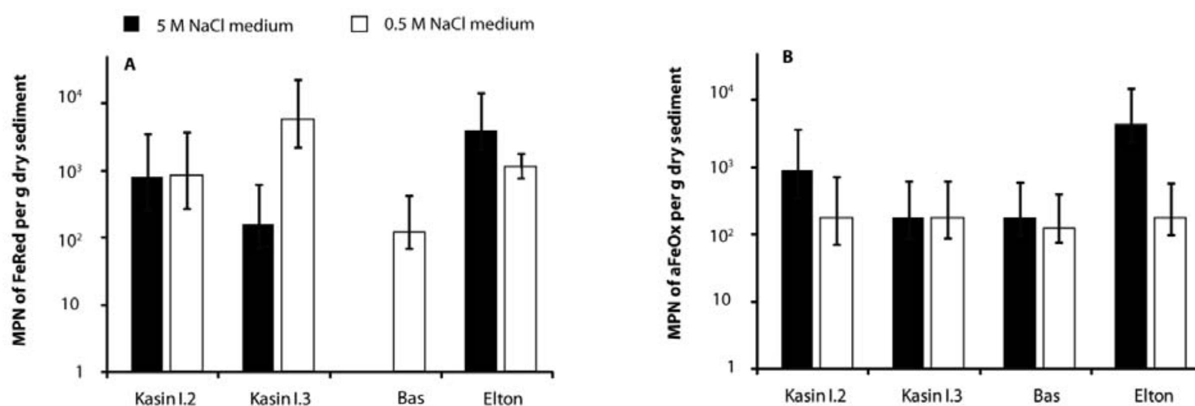

Figure S2: *Most probable numbers of culturable A) Fe(III)-reducing (FeRed) and B) anaerobic Fe(II)-oxidizing (anFeOx) microorganisms from the top 10 cm of different salt lake sediments in mineral medium with 5 M (black bars) or 0.5 M NaCl (white bars), respectively. Medium for Fe(III)-reducer was supplemented with 0.5M ferrihydrite as electron acceptor and 0.5 M of each lactate and acetate as electron donors. For the anaerobic Fe(II)-oxidizer, 1 M $FeCl_2$ was added as electron donor and 0.4 M NO_3^- as electron acceptor. The Fe(II)-oxidizer medium further contained 0.05 M acetate as a C source. Error bars denote 95 % confidence intervals determined from seven replicate samples according to (Klee, 1993).*

Supplementary figure 3:

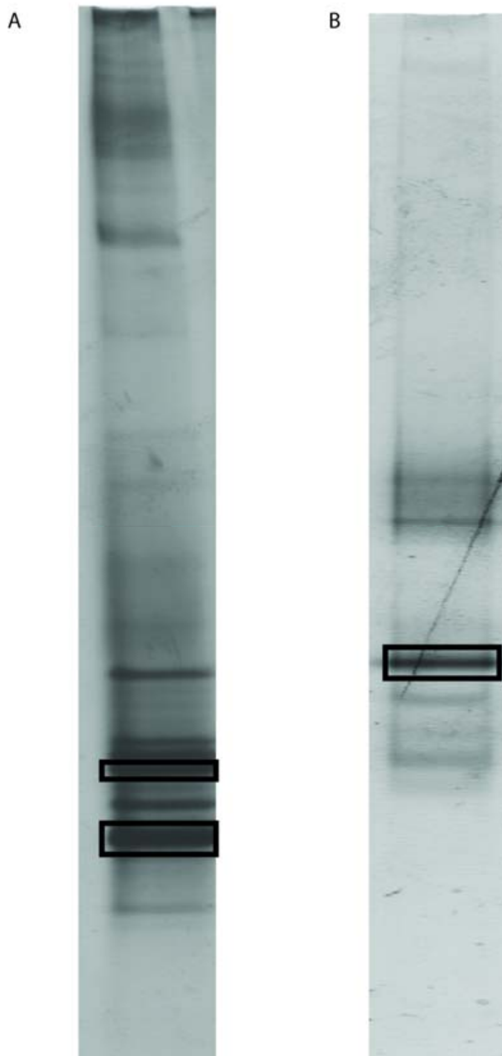

Figure S3: *DGGE gels with PCR amplificates of partial 16S rRNA genes from enrichments from Kasin 0-10 cm that reduce Fe(III) over several transfers. A) shows the banding pattern originating from an archaeal enrichment that reduces Fe(III) at 5 M salinity and B) shows the banding pattern originating from bacterial enrichment that reduces Fe(III) at 0.5 mM salinity. Dominant bands marked with black rectangles were cut out from ethidium bromide-stained gels, reamplified, ligated into pCR4 and transformed into competent Escherichia coli cells (DH5α). Three plasmids with inserts originating from each band were sequenced in order to enable phylogenetic classification of the dominant strains in the Fe(III)-reducing enrichment cultures.*

Supplementary table 1:

Table S1: *Sequences of qPCR primers applied in this study.*

Target	**forward Primer (5'-3')**	**reverse Primer (5'-3')**	**Target group hits of forward/reverse primer***	**References**
General *Bacteria*	341F: CCTACGGGAGGCAGCAG	797R: GGACTACCAGGGTATCTAATCCTGTT	-	(Muyzer et al., 1993; Nadkarni et al., 2002)
General *Archaea*	109F: ACKGCTCAGTAACACGT	915R: GTGCTCCCCCGCCAATTCCT	-	(Grosskopf et al., 1998; Stahl and Amann, 1991)
Desulfosporosinus sp.	Desulfosp.432F: GTACTGTCTTTGGGGAAG	Desulfosp.598R: CCTGATCTTTCACACCGG	84/72	This study
Bacillus sp.	Bacilli354F: GCAGTAGGGAATCTTCCG	Bacilli518R: ATTACCGCGGCTGCTGG	24894/529028	This study, (Muyzer et al., 1993)
Halobaculum gomorrense and close relatives	Hgomorr396F: ACTCCGAGTGCGGAGGCA	Hgomorr656R: CCCTTCGAGTCTCCCTGT	7/7	This study

* Based on a 100 % match using the program TestProbe (SILVA probe match/evaluation tool) and the SILVA SSUr108Ref data set (http://www.arb-silva.de/search/testprobe/).

Supplementary table 2:

Table S2: *Thermal profiles and standards used for qPCR in this study. Vectors pCR2.1® and pCR4® were obtained from Invitrogen (Darmstadt, Germany).*

Target	Thermal profile	Origin of 16S rRNA gene used as standard	Vector	Molecular weight vector + standard
General *Bacteria*	98°C-5s/60°C-12s	*Thiomonas sp.*	pCR2.1®	3.29×10^6 g/mol
General *Archaea*	98°C-5s/52°C-10s/ 72°C-15s	*Halobacterium salinarum*	pCR4®	3.33×10^6 g/mol
Desulfosporosinus spp.	98°C-5s/60°C-12s	DGGE band of Fe(III)-reducing enrichment (HE604952)	pCR4®	2.81×10^6 g/mol
Bacilli	98°C-5s/60°C-12s	Clone library sequence (Kasin-B2-E03; HE604676)	pCR4®	3.28×10^6 g/mol
Halobaculum gomorrense	98°C-5s/60°C-12s	Sequence from Fe(III)-reducing enrichment (HE604942)	pCR4®	3.33×10^6 g/mol

Supplementary table 3:

TABLE S3: *Mössbauer parameters for Lake Kasin sediment spectra of the Fe-rich sediment layer at 1-3 cm depth.*

Site	Mineral	Fe ox. State	δ (mm/s)	ΔE_Q or 2ε (mm/s)	B_{hf} (T)	σ (mm/s or T)	A (%)	Fe(II)/Fe_T
				RT				
D1	Ak/Lp	Fe(III)	0.38	0.55	-	0.2	51	0.11
D2	Akaganéite	Fe(III)	0.37	0.95	-	0.2	20	
D3	Akaganéite	Fe(III)	0.37	1.33	-	0.2	12	
D4	Green rust	Fe(II)	1.05	2.67	-	0.2	3	
D5	Green rust	Fe(II)	0.6	2.2	-	0.2	8	
D6	Green rust	Fe(III)	0.27	0.73	-	0.2	7	
				77 K				
D1	Ak/Lp/GR	Fe(III)	0.46	0.42	-	0.2	24	0.02
D2	Akaganéite	Fe(III)	0.46	0.91	-	0.2	22	
D3	Akaganéite	Fe(III)	0.46	1.51	-	0.2	7	
S1	Akaganéite	Fe(III)	0.45	-0.12	47.7	1.0	7	
S2	Akaganéite	Fe(III)	0.42	-0.41	43.6	1.0	4	
S3	Ak/Lp	Fe(III)	0.47	0.05	0.05	30.5	35	
D4	Green rust	Fe(II)	1.25	2.55	-	0.2	1	
D5	Green rust	Fe(II)	1.27	2.86	-	0.2	1	
				~5 K				
S1	Ak/GR	Fe(III)	0.47	-0.32	48.9	2.2	42	0.05
S2	Akaganéite	Fe(III)	0.48	0.38	48.7	2.3	30	
S3	Ak/Lp	Fe(III)	0.40	0.22	41.6	2.0	11	
D1	Ak/Lp/GR	Fe(III)	0.47	0.55	-	0.3	13	
D2	Green rust	Fe(II)	1.22	2.97	-	0.3	5	

Supplementary table 4:

Table S4: *Phylogenetic affiliation of the 16S rRNA gene sequences from the archaeal and bacterial clone libraries from Lake Kasin sediment including sequences from dominant strains of the Fe(III)-reducing enrichment cultures obtained in this study. "OTU"= operational taxonomic unit; "ACC" = accession number. Sequences that were grouped into one OTU have a 16S rRNA gene sequence similarity of ≥ 97%. "Similarity" refers to the sequence similarity between a respective OTU and its closest cultivated relative.*

Phylogenetic affiliation	OTU	Clone(s)	ACC	Closest cultivated relative	ACC	Similarity (%)
Methanomicrobia_Methanosarcina	OTU1	Kasin-A2-C07	HE604512	*Methanohalobium evestigatum Z-7303*	CP002069	92
Methanomicrobia_Methanosarcina	OTU1	Kasin-A3-E09	HE604615	*Methanohalobium evestigatum Z-7303*	CP002069	97
Thermoplasmata_Marine Benthic Group D and DHVEG-1	OTU1	Kasin-A1-H01	HE604477	n/a		
		Kasin-A1-H02	HE604486			
		Kasin-A2-H09	HE604561			
		Kasin-A1-A04	HE604413			
		Kasin-A2-D12	HE604525			
Thermoplasmata_KTK4A	OTU1	Kasin-A3-D11	HE604605	n/a		
		Kasin-A1-H09	HE604483			
Thermoplasmata	OTU1	Kasin-A1-D08	HE604449	n/a		
		Kasin-A1-F09	HE604467			
		Kasin-A1-G08	HE604473			
		Kasin-A1-F10	HE604468			
		Kasin-A1-C07	HE604438			
		Kasin-A1-H05	HE604479			
	OTU1	Kasin-A2-H03	HE604555			
		Kasin-A2-C10	HE604515			
	OTU1	Kasin-A3-A06	HE604570			
	OTU1	Kasin-A2-G04	HE604545			
		Kasin-A1-H07	HE604481			
Thermoplasmata_CCA47	OTU1	Kasin-A2-C05	HE604510	n/a		
		Kasin-A1-B11	HE604430			
Thermoplasmata_SAGMEG	OTU1	Kasin-A3-A04	HE604568	*Methanocaldococcus fervens*	AF056938	79
Thermoplasmata_SAGMEG	OTU1	Kasin-A3-H03	HE604634	*Methanocaldococcus fervens*	AF056938	80
		Kasin-A1-B05	HE604425			
		Kasin-A2-C04	HE604509			
Halobacteriales_Halobacteriaceae	OTU1	Kasin-A2-A01	HE604487	*Halopelagius inordinatus*	EU887284	88-89
		Kasin-A2-A03	HE604488			
		Kasin-A2-A04	HE604489			
		Kasin-A2-A05	HE604490			
		Kasin-A2-A08	HE604492			

Kasin-A2-A11	HE604494
Kasin-A2-B01	HE604496
Kasin-A2-B03	HE604497
Kasin-A2-B06	HE604500
Kasin-A2-B07	HE604501
Kasin-A2-B08	HE604502
Kasin-A2-B09	HE604503
Kasin-A2-B10	HE604504
Kasin-A2-B11	HE604505
Kasin-A2-B12	HE604506
Kasin-A2-C03	HE604508
Kasin-A2-C06	HE604511
Kasin-A2-C11	HE604516
Kasin-A2-D04	HE604519
Kasin-A2-D08	HE604522
Kasin-A2-D11	HE604524
Kasin-A2-E02	HE604527
Kasin-A2-E03	HE604528
Kasin-A2-E06	HE604530
Kasin-A2-E07	HE604531
Kasin-A2-E08	HE604532
Kasin-A2-F02	HE604536
Kasin-A2-F05	HE604538
Kasin-A2-F06	HE604539
Kasin-A2-F10	HE604540
Kasin-A2-F12	HE604542
Kasin-A2-G02	HE604543
Kasin-A2-G03	HE604544
Kasin-A2-G05	HE604546
Kasin-A2-G06	HE604547
Kasin-A2-G07	HE604548
Kasin-A2-G08	HE604549
Kasin-A2-G12	HE604552
Kasin-A2-H01	HE604553
Kasin-A2-H05	HE604557
Kasin-A2-H07	HE604559
Kasin-A2-H10	HE604562
Kasin-A2-H12	HE604564
Kasin-A3-A01	HE604565
Kasin-A3-A05	HE604569
Kasin-A3-A07	HE604571
Kasin-A3-A08	HE604572
Kasin-A3-A09	HE604573
Kasin-A3-A12	HE604575
Kasin-A3-B01	HE604576
Kasin-A3-B02	HE604577

Kasin-A3-B03	HE604578
Kasin-A3-B04	HE604579
Kasin-A3-B07	HE604581
Kasin-A3-B08	HE604582
Kasin-A3-B09	HE604583
Kasin-A3-C01	HE604585
Kasin-A3-C02	HE604586
Kasin-A3-C03	HE604587
Kasin-A3-C07	HE604590
Kasin-A3-C09	HE604591
Kasin-A3-C12	HE604594
Kasin-A3-D01	HE604595
Kasin-A3-D04	HE604598
Kasin-A3-D06	HE604600
Kasin-A3-D07	HE604601
Kasin-A3-D08	HE604602
Kasin-A3-D09	HE604603
Kasin-A3-D10	HE604604
Kasin-A3-E01	HE604607
Kasin-A3-E03	HE604609
Kasin-A3-E04	HE604610
Kasin-A3-E08	HE604614
Kasin-A3-F01	HE604617
Kasin-A3-F04	HE604619
Kasin-A3-F07	HE604621
Kasin-A3-F10	HE604624
Kasin-A3-F11	HE604625
Kasin-A3-F12	HE604626
Kasin-A3-G04	HE604628
Kasin-A3-G06	HE604630
Kasin-A3-G12	HE604633
Kasin-A3-H04	HE604635
Kasin-A3-H06	HE604637
Kasin-A3-H07	HE604638
Kasin-A3-H12	HE604641
Kasin-A1-A03	HE604412
Kasin-A1-A05	HE604414
Kasin-A1-A08	HE604417
Kasin-A1-A11	HE604419
Kasin-A1-A12	HE604420
Kasin-A1-B01	HE604421
Kasin-A1-B02	HE604422
Kasin-A1-B03	HE604423
Kasin-A1-B04	HE604424
Kasin-A1-B06	HE604426
Kasin-A1-B07	HE604427

		Kasin-A1-B10	HE604429			
		Kasin-A1-B12	HE604431			
		Kasin-A1-C01	HE604432			
		Kasin-A1-C03	HE604434			
		Kasin-A1-C04	HE604435			
		Kasin-A1-C09	HE604440			
		Kasin-A1-C10	HE604441			
		Kasin-A1-D02	HE604444			
		Kasin-A1-D03	HE604445			
		Kasin-A1-D10	HE604451			
		Kasin-A1-D11	HE604452			
		Kasin-A1-E02	HE604455			
		Kasin-A1-E03	HE604456			
		Kasin-A1-E08	HE604459			
		Kasin-A1-E09	HE604460			
		Kasin-A1-F01	HE604464			
		Kasin-A1-F03	HE604466			
		Kasin-A1-G01	HE604470			
		Kasin-A1-G02	HE604471			
		Kasin-A1-G11	HE604475			
		Kasin-A1-G12	HE604476			
		Kasin-A1-H04	HE604478			
		Kasin-A1-H06	HE604480			
Halobacteriales_Halobacteriaceae	OTU1	Fe(III)-red. (5M NaCl) clone 1	HE604940	*Halogranum rubrum*	EU887283	89
Halobacteriales_Halobacteriaceae	OTU1	Kasin-A1-E05	HE604458	*Halopelagius inordinatus*	EU887284	85
Halobacteriales_Halobacteriaceae	OTU1	Kasin-A3-C04	HE604588	*Halopelagius inordinatus*	EU887284	89
		Kasin-A3-G01	HE604627			
Halobacteriales_Halobacteriaceae	OTU1	Kasin-A2-A07	HE604491	*Halogranum rubrum*	EU887283	92-93
		Kasin-A3-B10	HE604584			
		Kasin-A1-D04	HE604446			
		Kasin-A3-F08	HE604622			
		Kasin-A2-D10	HE604523			
Halobacteriales_Halobacteriaceae	OTU1	Kasin-A3-D05	HE604599	*Halalkalicoccus jeotgali* B3	CP002062	89
		Kasin-A3-G05	HE604629			
Halobacteriales_Halobacteriaceae	OTU1	Kasin-A2-H04	HE604556	*Halobaculum gomorrense*	AB477983	97-98
		Kasin-A2-D06	HE604521			
		Kasin-A3-C05	HE604589			
Halobacteriales_Halobacteriaceae	OTU1	Kasin-A2-H08	HE604560	*Halobaculum gomorrense*	AB477983	98-99
		Kasin-A3-E02	HE604608			
		Kasin-A1-C05	HE604436			
Halobacteriales_Halobacteriaceae	OTU1	Fe(III)-red. (5M NaCl) clone 8	HE604947	*Halobaculum gomorrense*	AB477982	97-98
		Fe(III)-red. (5M NaCl) clone 7	HE604946			

		Fe(III)-red. (5M NaCl) clone 5	HE604944			
		Fe(III)-red. (5M NaCl) clone 4	HE604943			
		Fe(III)-red. (5M NaCl) clone 3	HE604942			
		Fe(III)-red. (5M NaCl) clone 9	HE604948			
Halobacteriales_Halobacteriaceae	OTU1	Kasin-A3-C11	HE604593	*Halobaculum gomorrense*	AB477982	89
		Kasin-A3-F05	HE604620			
		Kasin-A3-E06	HE604612			
		Kasin-A1-H08	HE604482			
Halobacteriales_Halobacteriaceae	OTU1	Kasin-A2-H02	HE604554	*Halobaculum gomorrense*	AB477982	89-90
		Kasin-A1-D12	HE604453			
Halobacteriales_Halobacteriaceae	OTU1	Fe(III)-red. (5M NaCl) clone 2	HE604941	*Halomicrobium katesii*	EF533994	85
Halobacteriales_Halobacteriaceae	OTU1	Fe(III)-red. (5M NaCl) clone 10	HE604949	*Halomicrobium katesii*	EF533994	85
Halobacteriales_Halobacteriaceae	OTU1	Kasin-A3-A02	HE604566	*Halomicrobium katesii*	EF533994	88
		Kasin-A1-A06	HE604415			
Halobacteriales_Halobacteriaceae	OTU1	Kasin-A1-D09	HE604450	*Halomicrobium katesii*	EF533994	86-89
		Kasin-A2-D02	HE604518			
Halobacteriales_Halobacteriaceae	OTU1	Kasin-A1-E10	HE604461	*Halorhabdus utahensis*	AF071880	93
Halobacteriales_Halobacteriaceae	OTU1	Kasin-A3-G07	HE604631	*Halorhabdus utahensis*	AF071880	94
Halobacteriales_Halobacteriaceae	OTU1	Kasin-A2-C09	HE604514	*Halorhabdus utahensis*	AF071880	96
Halobacteriales_Halobacteriaceae	OTU1	Kasin-A2-F01	HE604535	*Halomicrobium mukohataei*	CP001688	89-90
		Kasin-A3-E11	HE604616			
Halobacteriales_Halobacteriaceae	OTU1	Kasin-A1-C11	HE604442	*Halomicrobium mukohataei*	CP001688	90
Halobacteriales_Halobacteriaceae	OTU1	Kasin-A2-C08	HE604513	*Halomicrobium mukohataei*	CP001688	84
		Kasin-A2-D05	HE604520			
Halobacteriales_Halobacteriaceae	OTU1	Kasin-A3-D12	HE604606	*Halobacterium noricense*	AJ548827	98
		Kasin-A3-H10	HE604640			
Halobacteriales_Halobacteriaceae	OTU1	Fe(III)-red. (5M NaCl) clone 6	HE604945	*Halobacterium noricense*	AJ548827	94
Halobacteriales_Halobacteriaceae	OTU1	Kasin-A1-E04	HE604457	*Halobacterium noricense*	AJ548827	94
		Kasin-A2-E01	HE604526			
Halobacteriales_Halobacteriaceae	OTU1	Kasin-A1-C02	HE604433	*Halobacterium noricense*	AJ548827	92-93
		Kasin-A1-E01	HE604453			
		Kasin-A1-E12	HE604463			
		Kasin-A1-E11	HE604462			
		Kasin-A1-G07	HE604472			
		Kasin-A1-F11	HE604469			
		Kasin-A1-G10	HE604474			
		Kasin-A3-A03	HE604567			

		Kasin-A3-B06	HE604580			
		Kasin-A3-F02	HE604618			
		Kasin-A2-F03	HE604537			
		Kasin-A2-E09	HE604533			
		Kasin-A2-D01	HE604517			
		Kasin-A2-H11	HE604563			
		Kasin-A3-A10	HE604574			
		Kasin-A2-A09	HE604493			
Halobacteriales_Halobacteriaceae	OTU1	Kasin-A3-E07	HE604613	*Halobacterium noricense*	AJ548827	92-93
		Kasin-A3-F09	HE604623			
		Kasin-A3-G10	HE604632			
		Kasin-A2-A12	HE604495			
		Kasin-A1-D05	HE604447			
		Kasin-A1-F02	HE604465			
		Kasin-A2-H06	HE604558			
		Kasin-A3-D02	HE604596			
		Kasin-A2-B05	HE604499			
		Kasin-A3-D03	HE604597			
		Kasin-A2-G10	HE604550			
		Kasin-A1-H10	HE604484			
Halobacteriales_Halobacteriaceae	OTU1	Kasin-A3-C10	HE604592	*Halobacterium noricense*	AJ548827	93
		Kasin-A3-H08	HE604639			
		Kasin-A3-H05	HE604636			
Halobacteriales_Halobacteriaceae	OTU1	Kasin-A1-C08	HE604439	*Haloarcula japonica*	EF645685	96
Halobacteriales_Halobacteriaceae	OTU1	Kasin-A1-H12	HE604485	*Natronomonas pharaonis DSM 2160*	CR936257	92
Halobacteriales_Halobacteriaceae	OTU1	Kasin-A1-A07	HE604416	*Halorubrum xinjiangense*	AY510707	99
		Kasin-A1-A10	HE604418			
Halobacteriales_Halobacteriaceae	OTU1	Kasin-A1-A02	HE604411	*Halonotius pteroides*	AY498641	95
		Kasin-A1-C06	HE604437			
		Kasin-A1-D01	HE604443			
		Kasin-A2-E11	HE604534			
		Kasin-A3-E05	HE604611			
		Kasin-A2-F11	HE604541			
		Kasin-A2-G11	HE604551			
Crenarchaeota_Marine Benthic Group A	OTU1	Kasin-A2-C02	HE604507	*Staphylothermus marinus*	X99560	79
Miscellaneous Crenarchaeota Group	OTU1	Kasin-A1-D07	HE604448	*Staphylothermus marinus*	X99560	81
Miscellaneous Crenarchaeota Group	OTU1	Kasin-A2-B04	HE604498	*Staphylothermus marinus*	X99560	82
		Kasin-A2-E04	HE604529			
Firmicutes_Bacilli_Bacillaceae	OTU1	Kasin-B5-C03	HE604893	*Virgibacillus necropolis*	AJ315056	98-99
		Kasin-B5-D01	HE604898			
		Kasin-B5-B04	HE604886			
		Kasin-B1-C09	HE604779			
		Kasin-B1-D10	HE604786			
		Kasin-B3-D07	HE604731			
		Kasin-B5-C08	HE604896			

		Kasin-B5-H08	HE604935			
		Kasin-B5-A09	HE604879			
		Kasin-B5-F11	HE604923			
		Kasin-B4-H06	HE604870			
		Kasin-B4-A11	HE604828			
Firmicutes_Bacilli_Bacillaceae	OTU1	Kasin-B4-F11	HE604860	*Paraliobacillus quinghaiensis*	EU135728	95-98
		Kasin-B4-G11	HE604867			
		Kasin-B2-C05	HE604660			
Firmicutes_Bacilli_Bacillaceae	OTU1	Kasin-B1-G04	HE604800	*Pontibacillus chungwhensis*	AY553296	84-96
		Kasin-B1-G11	HE604804			
		Kasin-B4-G04	HE604862			
		Kasin-B1-F03	HE604793			
		Kasin-B1-G07	HE604801			
		Kasin-B2-E03	HE604676			
		Kasin-B3-B08	HE604715			
		Kasin-B2-D02	HE604666			
		Kasin-B3-C02	HE604719			
		Kasin-B1-F04	HE604794			
		Kasin-B1-C01	HE604776			
Firmicutes_Bacilli_Bacillaceae	OTU2	Kasin-B5-E04	HE604910	*Pontibacillus chungwhensis*	AY553296	98
		Kasin-B5-B03	HE604885			
Firmicutes_Bacilli_Bacillaceae	OTU3	Kasin-B1-A01	HE604814	*Pontibacillus chungwhensis*	AY553296	97
Firmicutes_Bacilli_Bacillaceae	OTU1	Kasin-B2-B05	HE604654	*Bacillus neizhouensis*	EU925618	98
Firmicutes_Bacilli_Lactobacillaceae	OTU1	Fe(III)-red. (0.5M NaCl) clone 2	HE604951	*Lactobacillus fabifermentans*	AM905388	99
Firmicutes_Clostridia_Peptococcaceae	OTU1	Fe(III)-red. (0.5M NaCl) clone 1	HE604950	*Dehalobacter restrictus*	U84497	97
Firmicutes_Clostridia_Peptococcaceae	OTU1	Fe(III)-red. (0.5M NaCl) clone 3	HE604952	*Desulfosporosinus lacus*	AJ582757	97
Firmicutes_Clostridia_Peptococcaceae	OTU1	Kasin-B2-D08	HE604671	*Desulfitibacter alkalitolerans*	AY538171	86
	OTU1	Kasin-B1-B12	HE604775	*Halanaerobium fermentans*	AB023308	88
		Kasin-B2-F08	HE604688			
		Kasin-B3-E02	HE604735			
Firmicutes_Clostridia_Halanaerobiaceae	OTU1	Kasin-B3-F04	HE604746	*Halocella cellulosilytica*	X89072	94
Deferribacteres	OTU1	Kasin-B5-D03	HE604900	*Caldithrix abyssi*	AJ430587	78
	OTU1	Kasin-B3-C09	HE604724	*Caldithrix abyssi*	AJ430587	81-82
		Kasin-B4-G05	HE604863			
Chloroflexi_Anaerolineaceae	OTU1	Kasin-B3-E07	HE604738	n/a		
		Kasin-B3-F12	HE604751			
Chloroflexi_Anaerolineaceae	OTU2	Kasin-B3-A04	HE604705	n/a		
Chloroflexi_Anaerolineaceae	OTU3	Kasin-B4-B06	HE604832	n/a		
Chloroflexi_Anaerolineaceae	OTU4	Kasin-B5-A05	HE604877	n/a		
		Kasin-B5-D11	HE604908			
Chloroflexi_Anaerolineaceae	OTU5	Kasin-B1-E05	HE604789	n/a		
		Kasin-B2-H09	HE604703			

		Kasin-B3-B05	HE604712			
Chloroflexi_Anaerolineaceae	OTU6	Kasin-B1-C07	HE604816	n/a		
		Kasin-B2-C10	HE604663			
		Kasin-B4-A03	HE604821			
Chloroflexi_Anaerolineaceae	OTU7	Kasin-B5-F09	HE604922	n/a		
		Kasin-B2-B07	HE604656			
Candidate division OP11	OTU1	Kasin-B2-C02	HE604658	n/a		
	OTU2	Kasin-B3-F01	HE604744	n/a		
	OTU3	Kasin-B5-D10	HE604907	n/a		
	OTU4	Kasin-B1-D12	HE604788	n/a		
Candidate division OD1	OTU1	Kasin-B5-F03	HE604917	n/a		
		Kasin-B5-G05	HE604928			
Candidate division OD1	OTU2	Kasin-B3-D09	HE604732	n/a		
Candidate division OD1	OTU3	Kasin-B5-G03	HE604926	n/a		
		Kasin-B5-H01	HE604931			
Candidate division OD1	OTU4	Kasin-B5-G01	HE604924	n/a		
Candidate division OD1	OTU5	Kasin-B2-D09	HE604672	n/a		
Candidate division OD1	OTU6	Kasin-B1-G09	HE604802	n/a		
Candidate division OD1	OTU7	Kasin-B5-A01	HE604874	n/a		
		Kasin-B5-B01	HE604883			
Candidate division OD1	OTU8	Kasin-B5-A12	HE604882	n/a		
		Kasin-B2-D11	HE604674			
Candidate division TM7	OTU1	Kasin-B2-B04	HE604653	n/a		
		Kasin-B4-E10	HE604851			
Deionococcus-Thermus	OTU1	Kasin-B1-H11	HE604812	*Truepera radiovictrix*	DQ022076	89
		Kasin-B2-F07	HE604687			
Planctomycetes	OTU1	Kasin-B5-D09	HE604906	n/a		
Planctomycetes	OTU2	Kasin-B5-F01	HE604915	n/a		
Planctomycetes_Brocadiaceae	OTU1	Kasin-B1-B10	HE604774	*n/a*		
TAO6	OTU2	Kasin-B1-D05	HE604783	n/a		
TAO6	OTU3	Kasin-B5-D07	HE604904	n/a		
TAO6	OTU4	Kasin-B2-F05	HE604685	n/a		
Gemmatimonadetes	OTU1	Kasin-B3-G03	HE604752	n/a		
		Kasin-B3-G08	HE604756			
		Kasin-B5-C07	HE604895			
		Kasin-B5-G12	HE604930			
		Kasin-B5-A11	HE604881			
Gemmatimonadetes	OTU2	Kasin-B4-A02	HE604820	n/a		
Gemmatimonadetes	OTU3	Kasin-B4-F08	HE604858	n/a		
Gemmatimonadetes	OTU4	Kasin-B4-F12	HE604861	n/a		
Acidobacteria_RB25	OTU1	Kasin-B5-G02	HE604925	n/a		
		Kasin-B5-H03	HE604933			
		Kasin-B5-D06	HE604903			
		Kasin-B2-D06	HE604669			
		Kasin-B5-F05	HE604919			

Nitrospirae_Nitrospiraceae	OTU1	Kasin-B4-D09	HE604844	*Thermodesulfovibrio hydrogeniphilus*	EF081294	87
Nitrospirae_OPB 95	OTU1	Kasin-B4-B12	HE604835	n/a		
		Kasin-B4-F02	HE604853			
Alphaproteobacteria_Rhodobacteriaceae	OTU1	Kasin-B2-A10	HE604649	*Rubrimonas cliftonensis*	D85834	89
		Kasin-B2-F09	HE604689			
Deltaproteobacteria_Deferribacteriaceae	OTU1	Kasin-B5-D02	HE604899	*Flexistipes sinusarabici*	M59231	99
Deltaproteobacteria_Desulfobacteraceae	OTU1	Kasin-B1-D01	HE604781	*Desulfosalsimonas propionicica*	DQ067422	92-96
		Kasin-B4-C03	HE604837			
Deltaproteobacteria_Desulfobacteraceae	OTU2	Kasin-B5-A04	HE604876	*Desulfosalsimonas propionicica*	DQ067422	96
		Kasin-B5-C01	HE604891			
Deltaproteobacteria_Desulfobacteraceae		Kasin-B1-D08	HE604785	*Desulfotignum phosphitoxidans*	AF420288	96
Deltaproteobacteria_Desulfohalobiaceae	OTU1	Kasin-B5-B09	HE604888	*Desulfohalobium utahense*	DQ067421	83
Deltaproteobacteria_Desulfohalobiaceae	OTU2	Kasin-B3-A12	HE604710	*Desulfohalobium utahense*	DQ067421	90
Deltaproteobacteria_Desulfohalobiaceae	OTU3	Kasin-B5-A10	HE604880	*Desulfohalobium utahense*	DQ067421	88-91
		Kasin-B1-H08	HE604809			
Deltaproteobacteria_DTB120	OTU1	Kasin-B4-F09	HE604859	n/a		
Deltaproteobacteria	OTU1	Kasin-B3-F03	HE604745	*n/a*		
Gammaproteobacteria	OTU1	Kasin-B4-C05	HE604839	*Idiomarina ramblicola*	AY526862	98-100
		Kasin-B3-B07	HE604714			
		Kasin-B1-G02	HE604799			
		Kasin-B2-C11	HE604664			
		Kasin-B3-E09	HE604740			
		Kasin-B2-G12	HE604696			
Gammaproteobacteria_Halothiobacillaceae	OTU1	Kasin-B4-E05	HE604849	*Halothiobacillus halophilus*	U58020	97-100
		Kasin-B4-H08	HE604871			
		Kasin-B4-F04	HE604855			
		Kasin-B4-B02	HE604829			
		Kasin-B4-A04	HE604822			
		Kasin-B4-E09	HE604850			
		Kasin-B4-F07	HE604857			
		Kasin-B4-F03	HE604854			
		Kasin-B2-E01	HE604675			
		Kasin-B2-G03	HE604692			
		Kasin-B3-E12	HE604743			
		Kasin-B1-F06	HE604795			
		Kasin-B4-H09	HE604872			
		Kasin-B4-C04	HE604838			
		Kasin-B4-B04	HE604830			
		Kasin-B4-B09	HE604834			
		Kasin-B2-D03	HE604667			
		Kasin-B4-B07	HE604833			
		Kasin-B4-D01	HE604841			
		Kasin-B4-H01	HE604868			
		Kasin-B4-D07	HE604843			
		Kasin-B4-D12	HE604846			

Kasin-B1-A08	HE604766
Kasin-B1-B08	HE604772
Kasin-B2-H03	HE604699
Kasin-B2-H02	HE604698
Kasin-B2-E07	HE604677
Kasin-B3-C01	HE604718
Kasin-B1-G01	HE604798
Kasin-B3-D06	HE604730
Kasin-B3-B04	HE604711
Kasin-B2-E09	HE604678
Kasin-B3-F11	HE604750
Kasin-B3-H05	HE604761
Kasin-B1-H12	HE604813
Kasin-B3-F10	HE604749
Kasin-B2-D04	HE604668
Kasin-B1-H09	HE604810
Kasin-B2-H01	HE604697
Kasin-B1-C10	HE604780
Kasin-B3-C07	HE604723
Kasin-B1-E08	HE604790
Kasin-B3-H02	HE604759
Kasin-B1-F12	HE604797
Kasin-B2-H08	HE604702
Kasin-B1-F01	HE604792
Kasin-B2-D07	HE604670
Kasin-B1-G12	HE604805
Kasin-B2-G01	HE604691
Kasin-B2-C12	HE604665
Kasin-B3-H07	HE604762
Kasin-B1-D02	HE604782
Kasin-B3-H03	HE604760
Kasin-B3-C05	HE604721
Kasin-B3-C12	HE604727
Kasin-B2-D10	HE604673
Kasin-B5-B12	HE604890
Kasin-B5-E01	HE604909
Kasin-B4-G08	HE604865
Kasin-B1-C12	HE604817
Kasin-B2-B06	HE604655
Kasin-B2-C03	HE604659
Kasin-B3-E01	HE604734
Kasin-B2-F11	HE604690
Kasin-B3-G10	HE604757
Kasin-B2-F02	HE604682
Kasin-B2-A07	HE604646
Kasin-B3-C10	HE604725

		Kasin-B2-B03	HE604652			
		Kasin-B5-H11	HE604938			
		Kasin-B2-C08	HE604662			
		Kasin-B1-A04	HE604765			
		Kasin-B3-D01	HE604728			
		Kasin-B3-B06	HE604713			
		Kasin-B3-D03	HE604729			
		Kasin-B3-B09	HE604716			
		Kasin-B1-D11	HE604787			
		Kasin-B1-H06	HE604808			
		Kasin-B3-A10	HE604708			
		Kasin-B5-H09	HE604936			
		Kasin-B5-E08	HE604913			
		Kasin-B5-E05	HE604911			
		Kasin-B5-A03	HE604875			
		Kasin-B5-C09	HE604897			
		Kasin-B5-A08	HE604878			
		Kasin-B4-A01	HE604819			
		Kasin-B4-C06	HE604840			
		Kasin-B4-A06	HE604824			
		Kasin-B3-E08	HE604739			
		Kasin-B3-G12	HE604758			
		Kasin-B3-C11	HE604726			
		Kasin-B1-B09	HE604773			
		Kasin-B4-E04	HE604848			
		Kasin-B3-A11	HE604709			
		Kasin-B3-C06	HE604722			
		Kasin-B3-D12	HE604733			
		Kasin-B2-C07	HE604661			
		Kasin-B5-D08	HE604905			
		Kasin-B5-G04	HE604927			
		Kasin-B5-E11	HE604914			
		Kasin-B5-F02	HE604916			
		Kasin-B5-D04	HE604901			
		Kasin-B5-H07	HE604934			
		Kasin-B5-H12	HE604939			
		Kasin-B5-B06	HE604887			
		Kasin-B4-G09	HE604866			
		Kasin-B1-E11	HE604791			
		Kasin-B4-D06	HE604842			
		Kasin-B4-A07	HE604825			
		Kasin-B5-C05	HE604894			
		Kasin-B5-F04	HE604918			
		Kasin-B3-E10	HE604741			
		Kasin-B4-D11	HE604845			
Gammaproteobacteria _Halomonadaceae	OTU1	Kasin-B5-D05	HE604902	*Halomonas andesensis*	EF622233	95

		Kasin-B4-E02	HE604847			
Gammaproteobacteria _Halomonadaceae	OTU2	Kasin-B1-D09	HE604818	*Halomonas sulfidaeris*	AF212204	96-98
		Kasin-B2-A08	HE604647			
		Kasin-B5-E06	HE604912			
		Kasin-B5-F06	HE604920			
		Kasin-B1-C04	HE604778			
		Kasin-B1-F10	HE604796			
		Kasin-B1-G10	HE604803			
Gammaproteobacteria _Halomonadaceae	OTU3	Kasin-B5-B11	HE604889	*Halomonas kenyensis*	AY962237	94
Gammaproteobacteria _Halomonadaceae	OTU4	Kasin-B2-H07	HE604701	*Halomonas cerina*	EF613112	97
Gammaproteobacteria _Halomonadaceae	OTU5	Kasin-B1-A06	HE604815			
		Kasin-B1-H01	HE604806	*Halomonas cerina*	EF613112	95-96
Gammaproteobacteria _Halomonadaceae	OTU1	Kasin-B1-B05	HE604770	*Chromohalobacter nigrandesensis*	AJ277205	99
Gammaproteobacteria _Alteromonadaceae	OTU1	Kasin-B1-B02	HE604768	*Marinobacter flavimaris*	AY517632	98
		Kasin-B1-B01	HE604767			
		Kasin-B4-F06	HE604856			
		Kasin-B2-E10	HE604679			
		Kasin-B2-F01	HE604681			
		Kasin-B4-A10	HE604827			
Gammaproteobacteria _Alteromonadaceae	OTU2	Kasin-B2-A05	HE604645	*Marinobacter guineae*	AM503093	95-96
		Kasin-B4-G06	HE604864			
		Kasin-B2-B02	HE604651			
		Kasin-B2-A12	HE604650			
Gammaproteobacteria _Alteromonadaceae	OTU3	Kasin-B2-H04	HE604700	*Marinobacter guineae*	AM503093	96-97
		Kasin-B4-C02	HE604836			
		Kasin-B3-E06	HE604737			
		Kasin-B1-B04	HE604769			
		Kasin-B3-B12	HE604717			
Gammaproteobacteria _Alteromonadaceae	OTU4	Kasin-B2-G10	HE604694	*Marinobacter salicampi*	EF486354	96-97
		Kasin-B3-H10	HE604763			
		Kasin-B3-G04	HE604753			
		Kasin-B1-C03	HE604777			
		Kasin-B3-E11	HE604742			
Gammaproteobacteria _Alteromonadaceae	OTU5	Kasin-B2-F06	HE604686	*Marinobacter lacisalsi*	EU047505	99
		Kasin-B1-D07	HE604784	*Halospina dentitrificans*	DQ072719	97-98
		Kasin-B4-B05	HE604831			
		Kasin-B4-A09	HE604826			
		Kasin-B4-E11	HE604852			
		Kasin-B3-E05	HE604736			
		Kasin-B3-G05	HE604754			
		Kasin-B3-G06	HE604755			
		Kasin-B2-A03	HE604643			
		Kasin-B3-C03	HE604720			
		Kasin-B1-H10	HE604811			
		Kasin-B4-H10	HE604873			
		Kasin-B2-A02	HE604642			

		Kasin-B3-F09	HE604748			
		Kasin-B3-H11	HE604764			
		Kasin-B1-H04	HE604807			
		Kasin-B5-B02	HE604884	*Mariprofundus ferrooxydans*	EF493243	83
		Kasin-B5-C02	HE604892			
Candidate division EM19	OTU1	Kasin-B2-C01	HE604657	n/a		
		Kasin-B3-A05	HE604706			
		Kasin-B5-F07	HE604921			
Candidate division EM19	OTU2	Kasin-B5-H02	HE604932	n/a		
		Kasin-B3-F05	HE604747			
		Kasin-B2-F04	HE604684			
Candidate division EM19	OTU3	Kasin-B2-F03	HE604683	n/a		
Candidate division EM19	OTU4	Kasin-B5-H10	HE604937	n/a		
Candidate division EM19	OTU5	Kasin-B4-A05	HE604823	n/a		
		Kasin-B4-H02	HE604869			
Candidate division EM19	OTU6	Kasin-B5-G06	HE604929	n/a		
Candidate division EM19	OTU7	Kasin-B2-G04	HE604693	n/a		
Candidate division EM19	OTU8	Kasin-B3-A09	HE604707	n/a		
Candidate division EM19	OTU9	Kasin-B2-A09	HE604648	n/a		
		Kasin-B2-H11	HE604704			
Spirochaetes_Spirochaetaceae	OTU1	Kasin-B2-A04	HE604644	*Spirochaeta halophila*	M88722	89
Spirochaetes_Spirochaetaceae	OTU2	Kasin-B2-E11	HE604680	*Spirochaeta halophila*	M88722	86
		Kasin-B2-G11	HE604695			
		Kasin-B1-B07	HE604771			

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7

Enrichment of Fe(III)-reducing and Fe(II)-oxidizing microorganisms from Russian and South African salt lake sediments and salt pans at up to 5 M salinity

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Abstract

While several studies have investigated Fe-cycling in freshwater lakes and sediments, knowledge about microbial Fe(III) reduction and Fe(II) oxidation in hypersaline environments is scarce. We quantified culturable Fe(III)-reducing, NO_3^--reducing Fe(II)-oxidizers and microaerophilic Fe(II)-oxidizers in 12 different salt pans and salt lake sediments and characterized subsequent enrichment cultures morphologically and phylogenetically. While Fe(III)-reducing microorganisms could be enriched from all of the samples, NO_3^--reducing Fe(II)-oxidizers were only found in eight and microaerophilic Fe(II)-oxidizers in only one of the samples. Unlike Fe(II)-oxidizing enrichments, several Fe(III)-reducing cultures remained stable over several transfers even though attempts to obtain a single strain by agar shake dilution series were not successful. Many of the Fe(III)-reducing enrichments were dominated by different types of

Bacilli. In several cases, phylogenetically very closely related strains were enriched from geographically distant places if the same culturing conditions were chosen. While both Bacteria and Archaea were detected in Fe(III)-reducing enrichments cultured in medium with 0.5 M NaCl, one culture that reduced Fe(III) at 5 M NaCl only consisted of Archaea. Addition of small amounts of a yeast extract/cofactor solution significantly accelerated Fe(III) reduction rates of otherwise very slow-growing Fe(III)-reducing enrichments. In summary, this study showed that both microbial Fe(III) reduction and at least NO_3^--dependent Fe(II)-oxidation can take place in hypersaline environments. Consequently, the potential for microbial Fe cycling under hypersaline conditions does exist.

Introduction

With salinities ranging from below seawater salinity (i.e. 35 g/L^{-1} dissolved salt) to NaCl saturation (304 g/L^{-1} or 5.2 M) (Oren, 2001), salt lakes represent extreme habitats for microbial life (Litchfield and Gillevet, 2002; Rothschild and Mancinelli, 2001). However, high primary productivity rates were often measured showing that salt-adapted species thrive regardless of the extreme environmental conditions (Sorokin et al., 2007).

One motivation for studying the microbiology of salt lakes comes from the facts that primordial life on earth might have started in hypersaline environments (Dundas, 1998; Knauth, 1998), which can also be found on Mars (Langevin et al., 2005; Mancinelli et al., 2004; Squyres et al., 2004). Thus, a better understanding of microbial diversity and metabolic activity in hypersaline environments might help to understand how life on our planet developed as well as to judge if and how extinct or extant life on Mars might be shaped.

In order to adapt to the hyperosmotic conditions prevailing in saline environments, the majority of halophilic microorganisms follow the so-called “low salt-in” strategy of accumulating osmoactive organic molecules (= “compatible solutes”). This adaptation strategy can be found in methanogenic archaea and the majority of halophilic bacteria except for *Haloanaerobiales* (Oren,

2001). This group of bacteria as well as halophilic Archaea pursue the alternative "high salt-in" strategy of accumulating KCl in their cytoplasm (Oren, 2001).

The production of compatible solutes is highly energy-expensive (30 to 109 ATP molecules are needed to produce one molecule of a compatible solute (Oren, 2001)). This fact has been used to explain the often observed decrease in metabolic diversity with increasing salinity and lead to the hypothesis of an upper limit of salinity for every metabolic process being determined by thermodynamic constraints (Oren, 1999; Oren, 2001; Oren, 2010). In other words, metabolic processes from which a lot of energy is gained such as nitrate reduction where 2872 kJ are released per 4.8 mol of nitrate reduced with 1 mol of glucose are expected to occur up relatively high environmental salt concentrations. Processes from which less metabolic energy can be derived such as methanogenesis from H_2 and HCO_3^- (- 34 kJ/mol of HCO_3^-, (Oren, 2001)), on the other hand, can only be maintained in environments with lower salt concentrations. So far, the majority of the observations made in hypersaline environments strengthen the aforementioned hypothesis (Oren, 2010).

While metabolic processes of microorganisms involved in the biogeochemical cycling of sulfur including dissimilatory SO_4^{2-} reduction in salt lake water and sediments (Kjeldsen et al., 2007; Kulp et al., 2006; Sorokin et al., 2010) have been studied extensively, knowledge about the use of oxidized metal ions such as iron (Fe) in the form of Fe(III) as electron acceptor in anaerobic hypersaline environments is scarce (Oren, 2010). For thermodynamic reasons, the use of Fe(III) in the form of $Fe(OH)_3$ as electron acceptor is more favorable than the use of SO_4^{2-} : $\Delta G = -48$ kJ are released per mole of electrons transferred from acetate to Fe(III) at pH=7 in comparison to -7 kJ per mole of electrons electron transferred from acetate to SO_4^{2-} under the same conditions (Madigan and Martinko, 2006). Consequently, from a thermodynamic point of view, wherever bioavailable Fe(III) is not limiting, microbial Fe(III) reduction should dominate over SO_4^{2-} reduction. With typical concentrations of 1-5 % of sediment dry matter, Fe(III)hydroxides have indeed been shown to represent important electron acceptors in freshwater sediments (Schink

and Benz, 1999). In hypersaline sediments, similar amounts of Fe-bearing minerals have been found and identified (de Koff et al., 2008; Long et al., 1992; Qi et al., 1992). However, these studies only consider abiotic aspects of Fe geochemistry in hypersaline sediments and neglect the possible role of microorganisms on Fe mineral transformations. Rate, extent and product identity arising from microbial Fe mineral transformations are influenced by a plethora of factors. Among others, aggregate size, crystallinity and surface area of the starting Fe(III) minerals (Bonneville et al., 2004; Bosch et al., 2009; Roden and Zachara, 1996), the presence of counterions such as phosphate and carbonate (Fredrickson et al., 1998) as well as the concentration of humic material in the respective environment (Jiang and Kappler, 2008), [Amstaetter and Kappler, submitted to GCA] are known to control microbial Fe(II) oxidation and Fe(III) reduction. In contrast to these factors, the effect of varying ionic strength on microbial Fe(II) oxidation and Fe(III) reduction processes has not been subject to any systematic study has so far.

Focusing on microbiology, a few isolates from hypersaline environments have been reported to be capable of Fe(III) reduction. Unfortunately, in several cases the isolates were shown to reduce dissolved Fe(III) only and not tested for the reduction of any solid Fe(III) phases (Blum et al., 1998; Blum et al., 2009; Pollock et al., 2007) which represent the dominant form of Fe(III) at the neutral or even alkaline pH values of most salt lake environments (Stumm and Morgan, 1996). In other cases, halotolerant rather than halophilic bacteria were isolated from moderately saline environments: one example is *Geoalkalibacter ferrihydriticus* which can only grow and reduce Fe(III) at up to 50 g/L^{-1} NaCl (Zavarzina et al., 2006). The same has been observed for several *Bacillus* strains (Boone et al., 1995; Kanso et al., 2002) as well as for an Fe(III)-reducing isolate from the sediment of the hypersaline lake Chaka in China. This strain originated from 880 cm depth where salinity was only slightly higher than in freshwater (Jiang et al., 2007). SO_4^{2-}-reducing bacteria have been shown being capable of using H_2 produced from fermenting microorganisms as an electron donor in medium containing 10 % NaCl. This demonstrates the potential for interspecies H_2-transfer coupled to SO_4^{2-} reduction in hypersaline environments

(Cayol et al., 2002). In this context, it would be very interesting to know whether this potential expands to Fe(III) reduction as well. However, no halophilic Fe(III)-reducer has been identified so far that can use H_2 as an electron donor.

Among other groups of microorganisms, all neutrophilic Fe(III)-reducers tested so far have also turned out capable of transferring electrons to humic substances or to the humic model quinone anthraquinone-2,6-disulfonate (AQDS) (Coates et al., 1998; Finneran et al., 2002; Francis et al., 2000; He and Sanford, 2003; Lovley et al., 1996; Nevin and Lovley, 2000). Humic substances can further enhance the rate of microbial Fe(III) reduction by functioning as electron shuttles between cells and Fe(III) minerals (Jiang and Kappler, 2008; Lovley et al., 1998). Therefore, information about the role of humic substances as electron acceptors in hypersaline environments, which has not been studied so far, would be desirable.

While knowledge about microbial Fe(III) reduction in hypersaline environments is already scarce, even less is known about Fe(II)-oxidizers in these habitats. Previous reports indicated an inhibitory effect of seawater concentrations of Cl^- on microaerophilic (Razzell and Trussell, 1963) (Cameron et al., 1984) and phototrophic (Newman and Poulain, 2009) Fe(II) oxidation. McBeth et al. [2011] recently presented the first study of a microaerophilic Fe(II)-oxidizing strain associated with the *Zetaproteobacteria* isolated from a microbial mat in the Great Salt Bay where salinity ranges between 0 and 2.5 % (McBeth et al., 2011).

To sum up, too little information is available about microbial Fe redox processes in hypersaline environments in order to judge their significance for these ecosystems. This knowledge gap further renders it impossible to estimate whether a cycle between microbial Fe(II) oxidation and Fe(III) reduction might be active in salt lake sediments in a similar way as it has been shown for freshwater sediments (Bruun et al., 2010; Coby et al., 2009; Sobolev and Roden, 2002; Straub et al., 2004).

To elucidate the role and identity of Fe(III)-reducing and Fe(II)-oxidizing microorganisms in hypersaline environments, the goals of this study were:

- to quantify most probable numbers of Fe(III)-reducers, humic substance-reducers, NO_3^--reducing Fe(II)-oxidizers and microaerophilic Fe(II)-oxidizers in different salt lake sediments,
- to enrich and if possible isolate representative strains of these metabolic groups of microorganisms,
- to find out if it is rather bacteria or archaea that dominate the active enrichment cultures by diagnostic PCR and to morphologically characterize the respective microorganisms by microscopy, and
- to measure Fe(III) reduction (and Fe(II) oxidation) rates of selected enrichment cultures under different conditions such as varying electron donors and presence or absence of a yeast extract/cofactor solution.

Materials & Methods

Study sites and sampling

If not indicated otherwise, composite samples of the top 10 centimetres of sediment from different salt lakes in southeast Russia as well as from salt pans in Botswana and Namibia were taken with a spatula and transported in UV-sterilized plastic bags to Tübingen, Germany. Table 1 lists the exact geographical location of the different sampling sites.

During transportation, samples were maintained at 4°C. In Tübingen, samples were sieved (2 mm diameter) and stored for up to two months either at 4°C in the dark (for most probable number experiments) or frozen at -20°C (for DNA extraction).

Table 1: *Location of the salt lake sediments from which the samples used in this study were taken. "Sua" samples originate from Botswana, "WBSR" samples originate from Namibia, "Kasin" as well as "Bas(kunchak)" and "Elton" samples came from Russia.*

Sample name	GPS coordinates	Description sampling site
Sua Pan 3I	S 20°28.953´ E 26°03.690´	Artificial trench next to water pump in salt pan
Sua Pan 3II	S 20°28.953´ E 26°03.690´	Natural water-logged site 50 m north of river inflow; 0-10 cm vertical sample
Sua Pan 1II 0-4	S 20°27.200´ E 25°55.600´	0-4 cm of salt pan; ca. 5 m away from water pump discharge
Sua Pan 1II 20-24	S 20°27.200´ E 25°55.600´	20-24 cm of salt pan; ca. 5 m away from water pump discharge
Sua Pan3bII	S 20°28.953´ E 26°03.690´	Natural water-logged site 50 m north of river inflow; 0-10 cm horizontal sample
Sua2	S 20°26.256´ E 25°54.597´	Artificial trench next to water pump in salt pan
WBSR5	S 23°01.329´ E 14°27.186´	Sea water lagoon in vicinity to salt refinery
WBSR6	S 23°02.785´ E 14°27.905´	Randomly flooded and salty Kuiseb-riverbed
KasinI.1	N47°36.165’ E047°27.129’	Exposed lakebed sediment; ca. 50 m away from flooded area
KasinI.2	N47°36.161' E047°27.119'	Sample taken below the water surface
KasinI.3	N47°36.074' E047°27.286'	Exposed lakebed sediment; ca. 100 m away from flooded area
Bas(kunchak)	N48°23.751' E046°49.543'	Exposed lakebed sediment; ca. 200 m away from flooded area
Elton	N49°09.057' E046°48.001'	Exposed lakebed sediment; ca. 700 m away from flooded area

Laboratory chemical analysis of sediment samples

All geochemical analyses of sediment samples were performed in duplicates. pH was analyzed in 0.01 M $CaCl_2$ according to DIN ISO 10390. Dried samples were milled to fine powder using a planetary mill and weight % content of 30 elements was determined by X-ray fluorescence analysis (XRF) using a Bruker AXS S4 Pioneer X-ray diffractometer. Total inorganic carbon (TIC) content was determined by acidification of dried samples with HCl until no more release of CO_2 could be observed and titration with NaOH to neutral. Total organic carbon (TOC) and nitrogen content (TNC) of decalcified samples were quantified with a "VarioEL" T/N analyzer (Elementar).

Most probable number (MPN) studies and enrichments of anaerobic Fe(II)-oxidizing and Fe(III)-reducing microorganisms

Selective growth media (see below) targeting anaerobic NO_3^--reducing Fe(II)-oxidizers (anFeOx) as well as Fe(III)-reducers (FeRed) and humic substance-reducers (HSRed) were used for MPN counts of the salt pan and sediment samples. Dilution series of sediment suspensions were set up in 96 deep well plates. In order to match the geochemical conditions at the different field sites as well as to test the influence of varying ionic strength on the activity of anFeOx, FeRed and HSRed, four different basic growth media were used in various MPN experiments. Table 2 lists the composition and buffer systems of the individual basic media and clarifies which media were used for which samples.

Mineral media were autoclaved in a Widdel flask and cooled under an N_2 stream. When the media had reached room temperature, 100 mL L^{-1} of 10-fold concentrated buffer solution (see table 2 for information about the different buffer systems used for the different media) that had been autoclaved separately was added as well as 1 mL each from the following 1000 x sterile stock solutions: 7-vitamin solution (Widdel and Pfennig, 1981), trace element solution SL-9 (Tschesch, 1984), 0.02 mM selenate-tungstate solution (Widdel, 1980), 1 M $CaCl_2$, 1 M $NaHPO_4$, 214 µM NH_4VO_3 and 500 mM $Na_2S_2O_3$. If necessary, the pH was adjusted to the

final pH value of the respective medium with sterile and anoxic 1 M NaOH or 1 M HCl after adding all other ingredients.

Table 2: *Composition of basic media used in MPN experiments. All concentrations of ingredients are given in millimoles per liter final concentration.*

Medium name	**SW pH = 9.8**	**SW pH = 8.0**	**Kasin 0.5 M**	**Kasin 5 M**
Samples incubated with this medium	Sua Pan 3I Sua Pan 3II Sua Pan 1II 0-4 Sua Pan 1II 20-24 Sua Pan3bII Sua2	WBSR5 WBSR6	KasinI.1 KasinI.2 Bas(kunchak) Elton	KasinI.1 KasinI.2 Bas(kunchak) Elton
Final pH	9.7 - 9.9	7.8 - 8.0	7.2 - 7.4	7.2 - 7.4
Buffer system	HCO_3^-/CO_3^{2-} (15 mM)	HEPES (10 mM)	H_2CO_3/HCO_3^- (15 mM)	H_2CO_3/HCO_3^- (15 mM)
Composition	**SW pH = 9.8**	**SW pH = 8.0**	**Kasin 0.5 M**	**Kasin 5 M**
NaCl	880	880	500	5 000
$MgCl_2$ x $6H_2O$	55.3	55.3	1.9	1.9
$MgSO_4$ x $7H_2O$	0.1	0.1	0.1	0.1
KCl	9	9	3	3
KBr	0.8	0.8	0.1	0.1
NH_4Cl	4.7	4.7	5	5

The following electron donors and acceptors were added to the media from 100 x stock solutions: For anFeOx the final media contained 4 mM $NaNO_3$ and 10 mM $FeCl_2$ as well as 0.5 mM sodium acetate to allow growth of mixotrophic Fe(II)-oxidizers. For FeRed the media contained 5 mM 2-line ferrihydrite prepared according to (Straub et al., 2005) and a mix of 5 mM sodium acetate and 5 mM sodium lactate. Media for HSRed had final concentrations of 5 mM sodium acetate and 5 mM sodium lactate each as well as 1 g L^{-1} humic acid. For samples from Botwsana

and Namibia, Pahokee Peat humic acid (PPHA, International Humic Substance Society, St. Paul, Minnesota, USA) was used and for samples from Russia, MPNs were set up with humic acid from Aldrich.

For setting up the MPN plates, 1 g of sediment was suspended in 10 mL of basic medium and the suspension was consecutively diluted 1:5 or 1:10 with medium to obtain a so-called master dilution series of 12 dilutions. With each of these 12 dilutions, seven wells of one column of the 96 well plate (900 µL of basic medium amended with selective electron donors and electron acceptors) were inoculated (100 µL inoculum). The last (8th) well of a column served as negative control. All MPN plates were set up in an anoxic chamber (glovebox) under N_2-atmosphere, sealed with a polyester plastic foil, taken out of the anoxic chamber and immediately put into anaercult bags (Merck) together with wetted O_2-indicator stripes (Merck). The anaerocult bags were filled with a silica gel-containing, O_2-consuming mixture, which made sure anoxic conditions were maintained during incubation. Based on the number of parallels per dilution step for which microbial growth was determined after 6 weeks of incubation at 20°C in the dark, the MPN of cells per gram dry soil was calculated with the program "Most Probable Number Calculator" version 4.04 (Environmental Protection Agency, USA). Obtained MPN counts were corrected after Salama as described by (Klee, 1993b). Criteria used to determine microbial growth in the parallels of the dilution series were as follows: wells were considered positive for growth of anFeOx if they showed a color change from slight green-blue to nearly black. Growth of FeRed was stated if the medium in the wells had turned from red-brown to nearly black. For the Fe(III)-reducers, this was in agreement with a reduction of at least twice as much Fe(III) to Fe(II) compared to the average of the wells that served as negative control as determined by ferrozine test (Stookey, 1970).

For determination of the reduction state of humic substances and thus activity of HSRed in the MPN wells, a ferric citrate assay was used (modified from (Lovley et al., 1996)). In brief, 100 µL from each MPN well was mixed with 100 µL of 5 mM anoxic ferric citrate in the glovebox. After

incubation for 20 minutes in the dark, 200 µL of 1 M HCl were added in order to stabilize the Fe(II) that had formed and samples were taken out of the glovebox. Samples were centrifuged for 5 minutes at 14,000 g, and Fe(II) in the supernatant was quantified with the ferrozine assay (Stookey, 1970). HS were considered as microbially reduced, if the Fe(II) measured exceeded the sum of the average Fe(II) measured in the samples from the uninoculated wells plus the confidence value determined for these samples.

FeRed, HSRed and FeOx from selected positive wells were transferred into fresh media of the same kind that had been used in the MPN experiment every 6 to 8 weeks or as soon as most of the Fe(III) had been reduced or the Fe(II) had been oxidized, respectively. Between 1 % and 2 % of inoculum were used for the transfers that were carried out under sterile and anoxic conditions. Enrichments were incubated in the dark at 28°C. To enhance growth of slow-growing enrichment cultures, 2 % (v/v) of both fresh yeast extract and cofactor solution (Breznak et al., 1999) was added to selective enrichments prior to monitoring Fe(II) oxidation and Fe(III) reduction rates.

Gradient tubes to enumerate microaerophilic Fe(II)-oxidizers

Gradient tubes for enumeration and enrichment of microaerophilic Fe(II)-oxidizers were prepared as described by Emerson and Floyd (Emerson and Floyd, 2005) with the following exceptions: instead of modified Wolfe's mineral medium (MWMM), Kasin medium with 0.5 M NaCl as described in the section "Most probable number studies" was used both in the top layer and in the bottom layer. The initial concentration of the sodium bicarbonate buffer in this medium was 15 mM. The FeS was only washed twice with anoxic H_2O and prepared directly before use. In the bottom layer, 1 mL of a 1:1 mix of FeS and Kasin medium with 0.5 M NaCl was used. The final concentration of FeS in the bottom layer was approximately 0.46 M. After addition of the bottom layer, the headspace was exchanged with N_2/CO_2 (90:10), tubes were capped with butyl rubber stoppers and kept at 4°C for 30 minutes. The medium for the top layer was prepared in an anoxic way by taking the medium out of the autoclave at 80°C and flushing

the headspace with N_2/CO_2 for 10 minutes. After the top layer medium had cooled down to 40°C, buffer, vitamins and all other additives listed in the section "Most probable number studies" were added. In order to stabilize the top layer, the concentration of low melt agarose was increased to 0.5 %. 10 mL of top layer were added anoxically on top of the bottom layer with syringes and needles that had been flushed with N_2/CO_2. Gradient tubes were prepared and let stand overnight to allow them to solidify before inoculation. For the inoculates, tenfold dilutions of KasinI.1 and Elton sediment in Kasin medium with 0.5 M NaCl were prepared as described in the section "Most probable number studies" up to a dilution of 10^{-5}. From each dilution, four parallel tubes were inoculated with 100 µL of sediment suspension using 1 mL syringes and needles. Tubes were inoculated sterilely under atmospheric conditions in order to bring some O_2 into the system. Inocula were inserted at about 2/3 depth of the top layer. One gradient tube that served as a negative control was also penetrated with an empty needle down to the same depth. After inoculation, tubes were closed again with butyl rubber stoppers and incubated in the dark at 28°C for two months.

Agar shakes

In order to isolate pure strains of the different metabolic types of microorganisms that were quantified in the MPN experiments, dilution series with medium in agarose were performed with selected enrichments after at least two transfers into fresh media. Agar shakes were prepared with low melt agarose (Carl Roth, Karlsruhe, Germany) which was washed three times with distilled water by mixing a 1.1 % (w/v) agarose solution on a magnetic stirrer for 10 minutes, letting it stand for 10 minutes, removing the supernatant water with a water ejector pump and replacing it with fresh water. After the third washing step, distilled water was added to prepare a 3 % agarose solution. The agarose was liquefied in the microwave and transferred into 15 mL-reagent tubes in 3 mL aliquots. Reagent tubes were covered with aluminum caps and autoclaved. Tubes were taken out of the autoclave while the agar was still liquid and directly transferred to a water bath that was kept at 60°C. In order to prevent water evaporation, aluminum caps were replaced by

sterile butyl rubber stoppers in the vicinity of a flame. For preparing the agar shakes, the same media and additives were used as in the MPN and enrichments with the exception that humic acid was replaced by 200 μM AQDS for isolation of HSRed in order to simplify visualization of colonies in the agar tubes. Bottles with the appropriate media were kept in a second water bath at 40 °C. Dilution series were prepared in the vicinity of a flame and under constant stream of N_2 into open media bottles to maintain sterile and anoxic conditions. In order to prepare one dilution series, 6 mL of pre-warmed medium were added to 7 agarose-containing tubes each. The first tube was then inoculated with 50 μL to 0.5 mL of enrichment culture and mixed by inverting the tube once. One mL of this suspension was transferred into a second tube, mixed immediately by inverting as with the first tube, and so on. After transfer to the next tube, each tube was placed on ice to harden the agar. After hardening, the air above the agar was replaced with N_2 by gassing each tube for 30 seconds. Finally, the tubes were incubated upside-down at 20°C in the dark for up to 2 months. After incubation, colonies that had grown in the agar were analyzed morphologically under a dissection scope. Promising colonies were extracted from the agar matrix by sucking them into a sterile pasteur pipette that was connected to a rubber tube and a cut 1 mL plastic pipette tip as a mouthpiece. Cotton plugs at both ends of the rubber tube prevented sucking the colonies too far. Colonies that were successfully extracted from the agar matrix were transferred into a 1.5 mL microcentrifuge tube that contained 1 mL of basic mineral medium and mixed. The morphology and viability of the microorganisms extracted from the agar was examined microscopically. 500 μL of samples that were considered promising were then used as inocula for a further round of agar shakes and additionally inoculated into fresh liquid media.

DNA extractions

For DNA extractions from sediment samples, 0.3 g of each sample that had been stored at -20°C was allowed to thaw and washed thrice with 1.5 mL of 10 mM Tris-EDTA buffer (pH = 7.0) by vortexing the sediment-buffer slurry and centrifuging it for 10 minutes at 7,200 g. Supernatant from each washing step was combined and filtered through a 0.22 μm cellulose ester filter

(Millipore). Washed sediment samples and filters were taken into separate bead tubes of the PowerSoil DNA isolation kit® (MoBio Laboratories, Carlsbad, CA) and DNA was extracted according to the protocol of the manufacturer. Finally, the DNA extracted from filter and sediment originating from the same sample was eluted subsequently in 2 x 25 µL elution buffer into a single microcentrifuge tube.

For DNA extractions from enrichments, 5 mL of liquid culture were centrifuged for 10 minutes at 7,200 g and pellets were washed thrice with 10 mL of 10 mM Tris-EDTA buffer (pH = 7.0) as described above. Similar as for the sediment samples, supernatant from each washing step was combined and filtered through a 0.22 µm cellulose ester filter. For DNA extractions from HSRed enrichments, washed pellets and filters were taken into separate bead tubes of the PowerSoil DNA isolation kit® (MoBio Laboratories, Carlsbad, CA) and DNA was extracted according to the protocol of the manufacturer. For DNA extractions from anFeOx and FeRed enrichments, the UltraClean DNA isolation kit® (MoBio Laboratories, Carlsbad, CA) was applied, also starting off with two different tubes for pellet and filter samples. Finally, the DNA extracted from filter and pellet originating from the same sample was eluted subsequently in 2 x 25 µL elution buffer into a single microcentrifuge tube.

PCR and DGGE

Partial 16S rRNA genes from the DNA extracts of the sediments, salt pans and enrichments were amplified using the primer pairs 341GCF (5'-CGCCCGCCGCGCGGCGGGCGGGGCGGGGGCACGGGGGCCTACGGGAG GCAGCAG-3') (Muyzer et al., 1993) and mod. 907R (5'-CCGTCAATTCMTTTRAGTTT-3') (DeLong et al., 2006) for amplification of general bacterial 16S rRNA gene fragments. For amplification of general archaeal 16S rRNA gene fragments, primers 20F (5'- TTCCGGTTGATCCYGCCRG-3') (Hallam et al., 2003) and 1392R (5'-ACGGGCGGTGTGTRC-3') (Lane, 1991) were used. Amplified archaeal 16S rRNA gene fragments were used as templates for a nested PCR with primers 344GCF (5'-CGCCCGCCGCGCGGCGGGCGGGGCGGGGGCACGGGGGACGGGGYGCA GCAGGCGCGA-3') (Stahl and Amann, 1991) and 519R (5'-GWATTACCGCGGCKGCTG-3') (Lane, 1991). It was necessary it apply nested PCR on archaeal 16S rRNA genes in order to get sufficient amplificates for denaturing gradient gel electrophoresis (DGGE). If PCRs were set up using primers 344GCF and 519R directly on the DNA extracts, too little DNA was amplified to allow visualization of any bands on an ethidiumbromide-stained agarose gel. The first amplification reactions of the long archaeal 16S rRNA gene fragments were performed in a final volume of 25 µL consisting 1× PCR buffer with 1.5 mM $MgCl_2$ final concentration (Promega), 200 µM dNTP mix (New England Biolabs), 200 nM of each primer, 0.625 U Taq DNA-Polymerase (Promega) and 1 µL of DNA extract as a template in a total volume of 25 µL. The thermocycler program was as follows: initial denaturation at 94°C for 2 min; 30 cycles of denaturing (94°C for 30 sec), annealing (58°C for 1 min) and elongation (72°C for 1 min 30 sec) and a final elongation at 72°C for 10 min. The second amplification reactions of the short archaeal and the amplification reactions of the bacterial 16S rRNA gene fragments were performed in a final volume of 50 µL. Reaction mixes consisted of 1× PCR buffer (Promega), 4 mM $MgCl_2$, 200 µM dNTP mix (New England Biolabs), 200 nM of each primer, 1.25 U Taq DNA-Polymerase (Promega) and 2 µL of DNA

extract as a template. The thermocycler program included: initial denaturation at 94°C for 2 min; 10 cycles of denaturing (94°C for 1 min), annealing (65°C to 56°C with a temperature decrease of 1°C per cycle for 1 min) and elongation (72°C for 1 min) plus 20 cycles of denaturing (94°C for 1 min), annealing (55°C for 1 min) and elongation (72°C for 1 min) and a final elongation at 72°C for 10 min. The concentrations of the resulting PCR products were estimated by eye by comparing the intensities of the resulting bands on an agarose gel to the band intensity of a marker fragment of defined concentration. Depending on the band intensities of the PCR products, between 5 and 20 µL were mixed with 5 µL of loading buffer consisting of 60 % glycerol, 1 mM EDTA pH 8.0, 1 % bromphenol blue and 0.5 % xylenxyanol and applied on a DGGE gel. DGGE gels were prepared in a DGGE unit type V20 HCDC from BioRad Laboratories GmbH, Munich, Germany according to the manufacturer's suggested protocol. All DGGE gels had a gradient of denaturant (7 M of urea and 40 % v/v of formamide) ranging from 35 % in the uppermost part of the gel to 60 % in the lowest part of the gel. For both bacterial 16S rRNA gene amplicons, 6 % polyacrylamide gels were used and run for 16 h at 100 V and 60°C. Archaeal 16S rRNA gene amplicons were applied on 8 % polyacrylamide gels which were run for 6 h at 100 V and 60°C. For visualization of DNA bands, silver staining was used. When bands had to be cut out for later cloning and sequencing, gels were stained with ethidium bromide and DNA bands were visualized under UV. To facilitate later comparison between different gels, at least one lane of each gel was loaded with a marker consisting of 16S rRNA gene amplicons from DNA extracts of four to six defined strains.

Cloning and sequencing

Selective DGGE bands were cut out from ethidiumbromide-stained gels. Gel slices with excised DGGE bands were transferred into 50 µL of nuclease-free water (Carl Roth, Karlsruhe) and incubated at 4°C for 2 h in order to allow diffusion of DNA into the water. 2 µL of this water were then used as template for reamplification of the DNA by PCR under the same conditions as described above with the exception that a 341 forward primer without a GC-clamp was used.

PCR products were then gelpurified with a Wizard® SV Gel and PCR Clean-Up System (Promega laboratories) according to the manufacturer's suggested protocol. Purified PCR products were ligated into a pCR4® cloning vector (Invitrogen) and transformed into *Escherichia coli* TOP10 competent cells according to the protocol suggested by the manufacturer. Colonies that arose from the transformations were then transferred into 5 mL of 50 mg/L ampicillin-containing liquid lysogeny broth (LB) medium with sterile toothpicks and incubated over night at 37°C. From these cultures, plasmids were purified via the QIAGEN® Plasmid Purification kit according to the manufacturer's instructions. DNA was eluted in 50 µL Tris-HCl. One to four clones per excised DGGE band were then sent for sequencing with primers M13F (GTAAAACGACGGCCAG) (Invitrogen) and M13R (CAGGAAACAGCTATGAC) (Invitrogen). Sequences were determined with an ABI 3730 xl automated sequencer at GATC Biotech (Konstanz, Germany).

Microscopy

Microscopy was performed at a Leica DM5500B epifluorescence microscope. In order to make it easier to differentiate between cells and Fe or HS particles in the enrichment cultures, 12 µL of culture was incubated with 3 µL of dye mixture from the Dead/Live® BacLight™ Bacterial Viability Kit (Molecular Probes) for 5 minutes prior to microscopic examination. Under the microscope, samples were analyzed using band pass filters Y3 (excitation maximum: 545 nm; emission maximum: 610 nm) and L5 (excitation maximum 480 nm; emission maximum: 527 nm).

Quantification of Fe(II) and Fe(III) in the enrichments

In order to monitor the activity of NO_3^--reducing Fe(II)-oxidizing and Fe(III)-reducing enrichment cultures, they were sampled every two weeks followed by quantification of Fe(II) and Fe(total) with the ferrozine assay (Stookey, 1970).

In detail, bottles were shaken manually before 0.2 mL of liquid culture were sterilely removed with a N_2-flushed 1 mL syringe and needle. Samples were immediately mixed with 0.2 mL of 1 M HCl, vortexed and centrifuged for 10 minutes at 14,000 g in order to exclude precipitates from further analysis. If concentrations of Fe(II) or Fe(total) were expected to exceed 500 µM, samples were serially diluted 1:10 or 1:100 in 1 M HCl. 50 µL of sample were then added to 50 µL of 1 M HCl (for quantification of Fe(II)) or 10 % (w/v) hydroxylamine hydrochloride in 1 M HCl (for quantification of Fe(total)) in 96 well-plates. Plates for quantification of Fe(total) were incubated for 20 minutes in the dark. 100 µL of 0.1 % (w/v) ferrozine in 50 % (w/v) ammonium acetate were added to each well. After incubation for 5 minutes in the dark, absorption at 562 nanometers was quantified with a spectrophotometer. The absorption values were used to calculate the amount of Fe(II) or Fe(total) with the help of a calibration curve. Calibration curves were calculated using absorption values from standards of known Fe(II) concentration (between 0 and 1 000 µM $FeCl_2$ in 1 M HCl). Standards were treated in the same way as the samples, i.e. they were diluted 1:1 in 1 M HCl before a second 1:1 mixture with 1 M HCl or hydroxylamine hydrochloride in 1 M HCl in a microtiter plate in order to facilitate calculations of Fe(II) and Fe(total) concentrations in the samples. New calibration curves were prepared every 2 weeks or whenever any of the solutions used for the analysis was changed. Exemplary calibration curves for quantification of Fe(II) and Fe(total) can be found in the appendix.

Results and discussion

Table 3 lists the most important geochemical properties of the field samples that were used in this study. The media used throughout the most probable number experiments and enrichments were designed according to these properties in order to provide the microorganisms with environmental conditions as close as possible to the ones they encounter in their natural environment. In our most probable number experiments, we found culturable Fe(III)-reducing microorganisms in all field samples tested except for the sediment of salt lake Baskunchak in the 5 M NaCl-containing medium (figure 1). However, when the same medium with 0.5 M NaCl was used, 1.31 x 10^2 Fe(III)-reducers per g dry sediment were quantified for this field site (figure S2 in chapter 6). Humic substance-reducers grew in all MPN plates apart from the ones set up with KasinI.3 sediment in medium with 5 M NaCl (figure 1). As expected, at least as many HSRed as FeRed were found in most samples. This does not hold true for the Russian samples. However, since the determination of activity of HSRed in the MPN plates involves several experimental steps and an arbitrarily set limit of Fe(II) formed in the analytical assay, this does not necessarily mean that there are less potential HSRed than FeRed in these sediments. Evidence for Fe(II) oxidation by NO_3^--reducing Fe(II)-oxidizers was observed in all MPN plates set up with the Russian samples at both NaCl concentrations. In the African samples, on the contrary, this type of microorganisms was only found in WBSR5, WBSR6 and Sua Pan 3I (figure 1). However, it has to be mentioned that for the Russian samples, activity of NO_3^--reducing Fe(II)-oxidizers, this statement was based on the observation of a color change of the respective wells from bright bluegreen to black and not on the detection of Fe(III). HCl (0.5 M)-extractable Fe(II) and Fe(total) concentrations were found to be about equal in all wells with values ranging from 4.5 to 8 mM.

Table 3: *Geochemical properties of the salt pan and salt lake sediment samples used in this study.*

Sample	Origin	pH[a]	Cl-content[b]	Fe-content[b]	TOC [%][c]	TIC [%][d]
Sua Pan 3I	Botswana	9.8	3.2	1.53	0.34	22.47
Sua Pan 3II	Botswana	9.5	1.4	1.21	0.79	24.82
Sua Pan 1II 0-4	Botswana	10.0	3.8	0.69	0.05	24.77
Sua Pan 1II 20-24	Botswana	9.9	6.3	1.08	1.17	22.77
Sua Pan3bII	Botswana	9.5	1.8	1.16	0.82	24.26
Sua2	Botswana	9.8	5.8	0.78	1.20	31.16
WBSR5	Namibia	7.9	4.6	3.19	0.40	5.84
WBSR6	Namibia	8.5	2.4	3.63	0.47	4.96
KasinI.1	Russia	7.9	2.5	1.13	0.11	1.85
KasinI.2	Russia	7.7	nd	1.43	4.17	4.30
KasinI.3	Russia	7.6	1.8	0.81	0.18	2.93
Bas(kunchak)	Russia	7.7	4.7	0.70	0.05	2.20
Elton	Russia	7.4	12.1	0.86	0.19	6.74

[a]determined with 0.01 M $CaCl_2$ [b][weight % of dry sediment] quantified by XRF [c]determination via weight loss at titration with HCl [d]quantification with C/N-analyzer of HCl-titrated sample

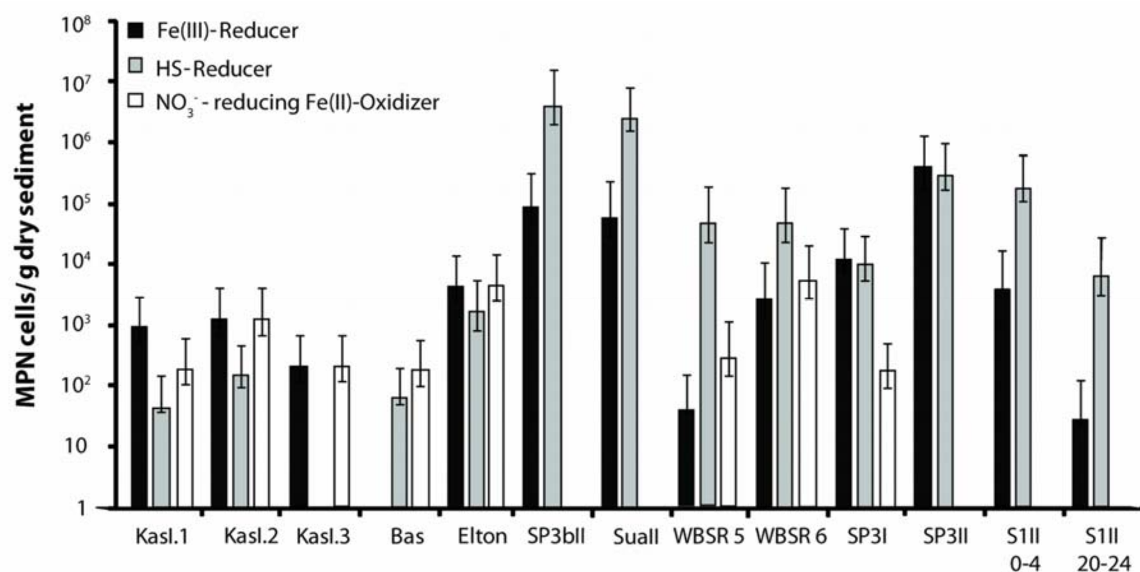


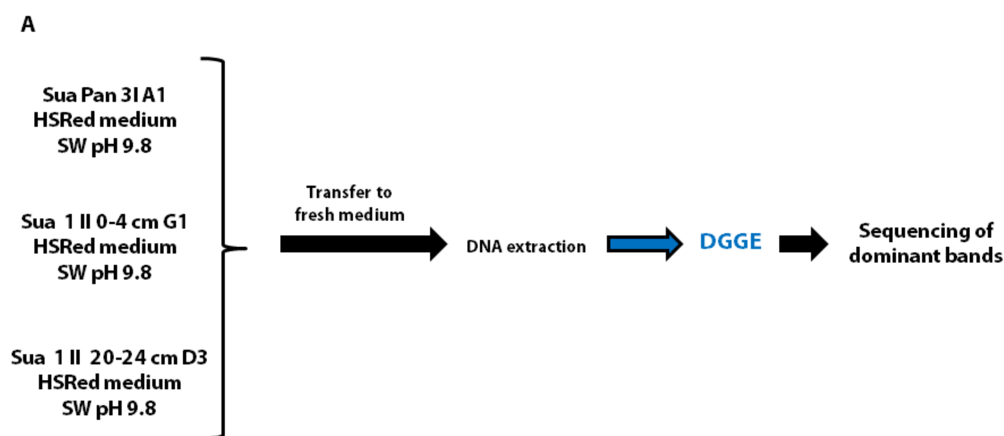
Figure 1: *Most probable numbers of culturable Fe(III)-reducing (black bars, humic substance-reducing (grey bars) and NO_3^--reducing Fe(II)-oxidizing (white bars) microorganisms from different salt lake sediments and salt pans. If no bar in one of the three colors is shown for a given sample, no microorganisms of the respective kind were found. KasI.1 = KasinI.1, KasI.2=KasinI.2, KasI.3=KasinI.3, SP3bII=Sua Pan 3bII, SP3I=Sua Pan 3I, SIII 0-4=Sua 1II 0-4 cm, S1II 20-24=Sua1II 20-24 cm. For samples KasinI.1 through I.3, Bas and Elton, MPN results with medium Kasin 5M are shown. For samples WBSR5 and WBSR6, medium SW pH=8 was used and all other MPNs were set up with medium SW pH=9.8 as basic medium. Media for Fe(III)-reducer was supplemented with 5 mM ferrihydrite as electron acceptor and 5 mM of each lactate and acetate as electron donors. The same electron donors were added to the media for the humic substance reducer, which contained 1 mg/mL of humic acid as electron acceptor. For the anaerobic Fe(II)-oxidizer, 10 mM $FeCl_2$ was added as electron donor and 4 mM NO_3^- as electron acceptor. The Fe(II)-oxidizer media further contained 0.5 mM acetate as a carbon source. Error bars denote 95 % confidence intervals determined from seven replicate samples according to (Klee, 1993a).*

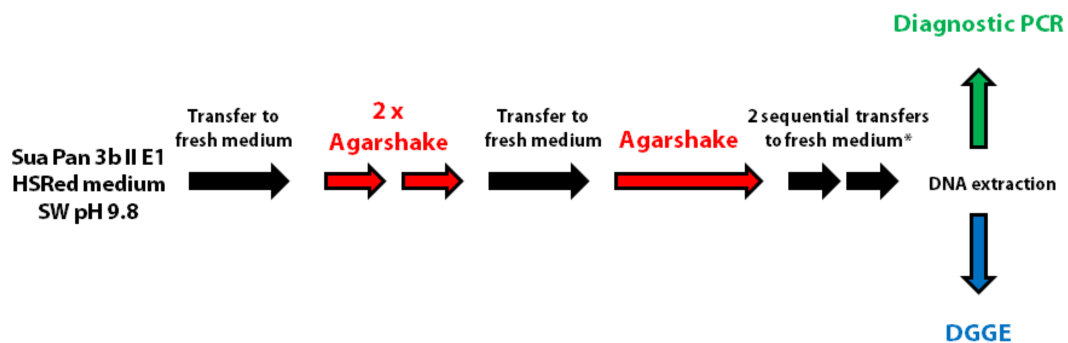
No black precipitate was formed in any of the uninoculated control wells. A possible explanation for this observation would be that Fe(III)-reducers, which were more abundant in the inoculated material, immediately reduced all Fe(III) that was formed by the Fe(II)-oxidizers using acetate as electron donor. Assuming complete oxidation of acetate to CO_2, 4 mM of Fe(II) could be oxidized with the 0.5 mM of acetate that was present in the medium.
Some Fe(III)-reducing bacteria such as the neutrophile *Geobacter metallireducens* (Lovley et al., 1993) as well as several acidophiles (Hallberg et al., 2010; Hallberg et al., 2011; Pronk et al., 1992) are able to oxidize Fe(II) as well. This means that Fe(II) oxidation and Fe(III) reduction could even be carried out by the same bacteria. In MPNs with Russian samples that were set up in 0.5 M NaCl-medium and in 5 M NaCl-medium, similar numbers of active Fe(III)-reducers, anaerobic Fe(II)-oxidizers & HS-reducers were enumerated. This aspect, which implies that the respective microorganisms are well-adapted to the high salinity in their environment, is discussed in detail in chapter 6.

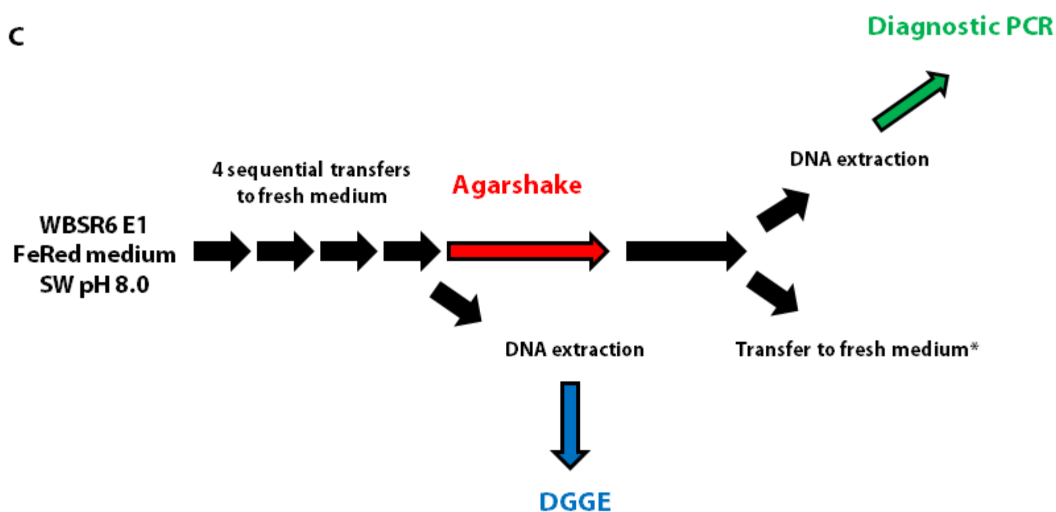
Sequencing of a DGGE band from an Fe(II)-oxidizing enrichment culture that was obtained from one of the MPN wells inoculated with KasinI.1 sediment identified the parent strain as 98 % identical to *Geoalkalibacter ferrihydriticus* on the basis of the 16S rRNA gene (figure 3, table 4). *Geoalkalibacter ferrihydriticus* is known to be able to reduce Fe(III) with acetate (Zavarzina et al., 2006), which strengthens the first hypothesis mentioned above.

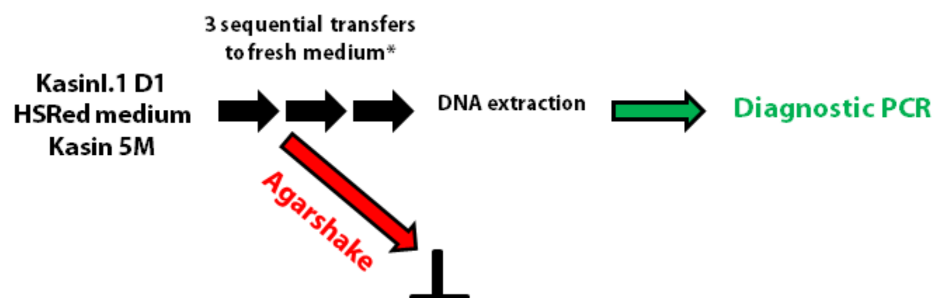
Unfortunately, no NO_3^--reducing Fe(II)-oxidizing enrichment culture could be maintained after several transfers.

In gradient tubes set up with Kasin medium with 0.5 mM NaCl, no microaerophilic Fe(II)-oxidizers grew in tubes that were inoculated with material from KasinI.1. The appearance of a band in one out of four gradient tubes inoculated with undiluted Elton sediment suspended in medium corresponds to 25 microaerophilic Fe(II)-oxidizers per g dry Elton sediment according to (Klee, 1993a). The poor growth of microaerophilic Fe(II)-oxidizers from our field sites confirms previous observations that this metabolic group of microorganisms is very sensitive towards salt (Razzell and Trussell, 1963) (Cameron et al., 1984). Figure 2 provides an overview about what was done with the enrichments started from selected MPN wells in order to further enrich dominant strains as well as to characterize them phylogenetically as well as their Fe(III)-reducing activities.

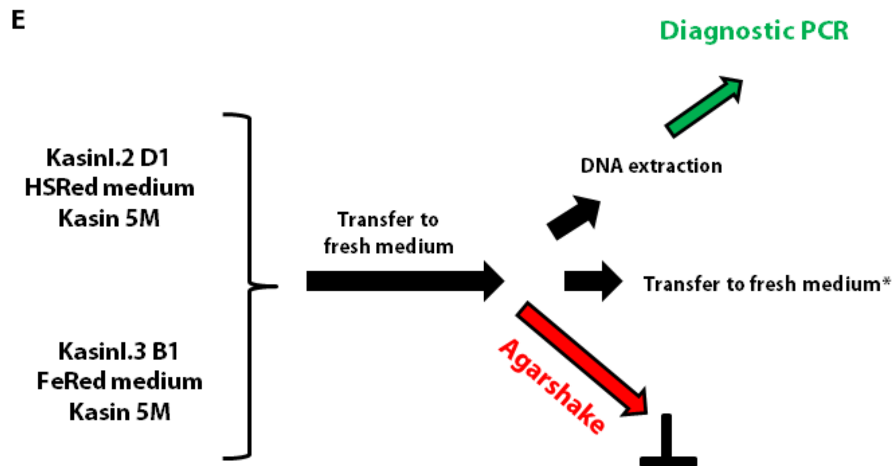



B
Diagnostic PCR
Transfer to fresh medium
2 x Agarshake
Transfer to fresh medium
Agarshake
2 sequential transfers to fresh medium*
Sua Pan 3b II E1
HSRed medium
SW pH 9.8
DNA extraction
DGGE

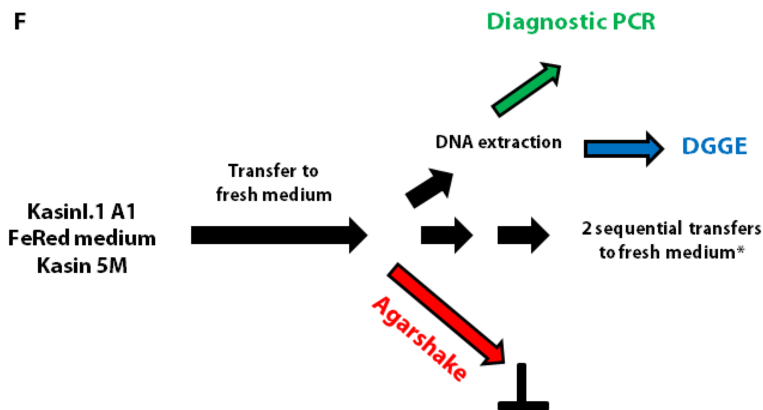

C
Diagnostic PCR
DNA extraction
4 sequential transfers to fresh medium
Agarshake
WBSR6 E1
FeRed medium
SW pH 8.0
DNA extraction
Transfer to fresh medium*
DGGE


D
3 sequential transfers to fresh medium*
KasinI.1 D1
HSRed medium
Kasin 5M
DNA extraction
Diagnostic PCR
Agarshake

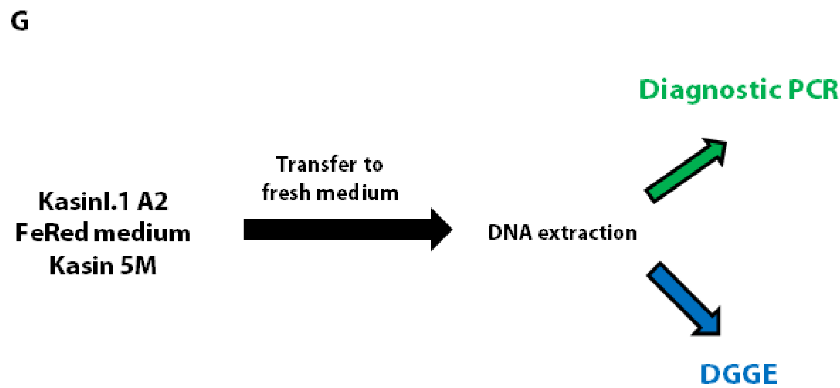
E

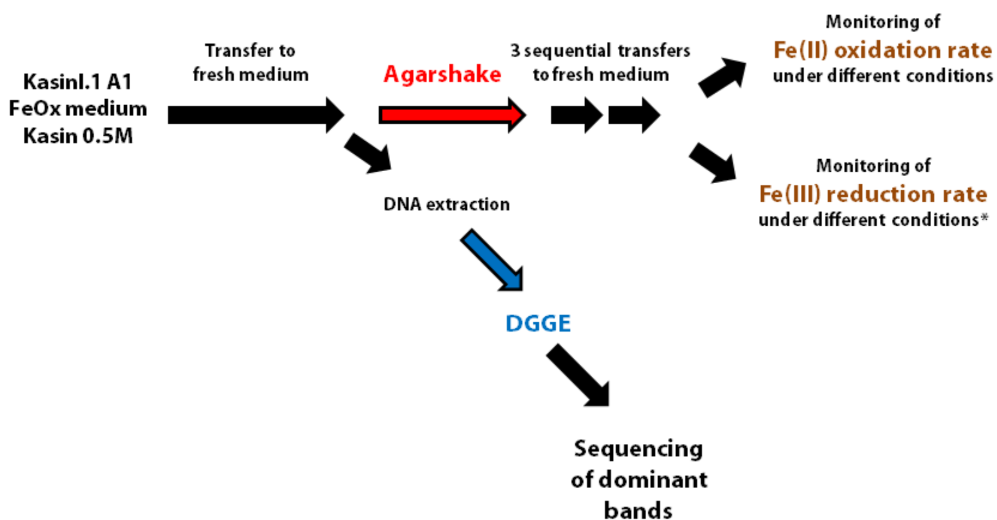


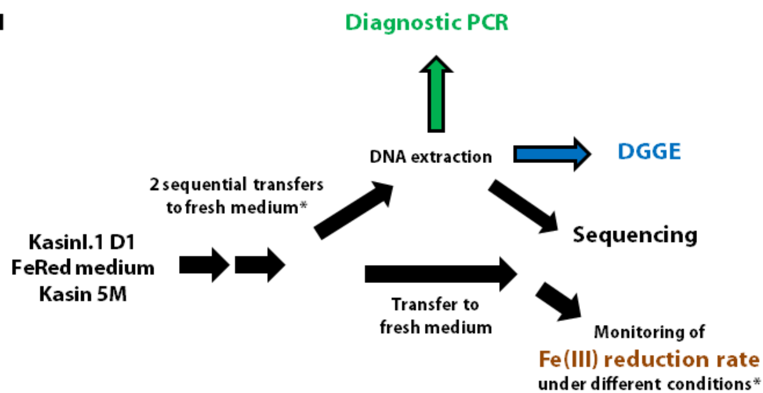
F



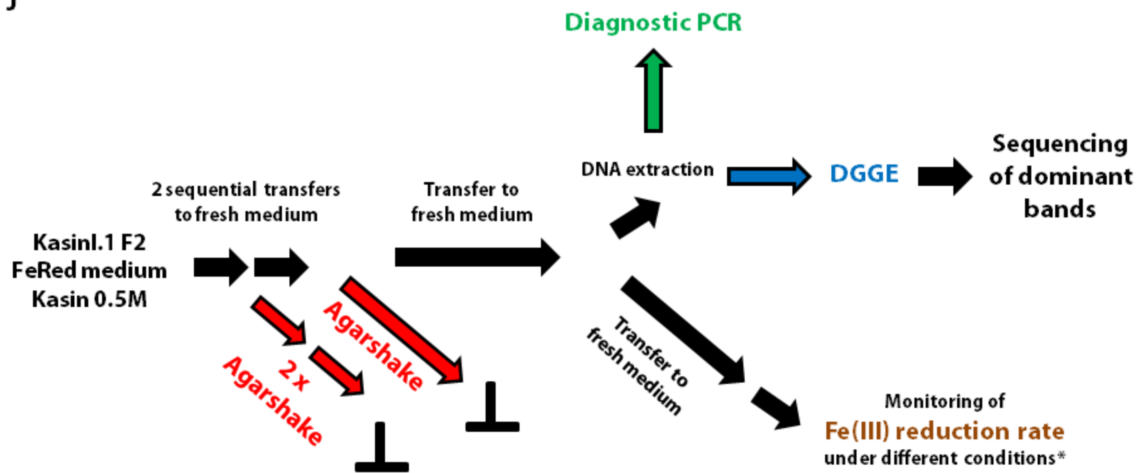
G



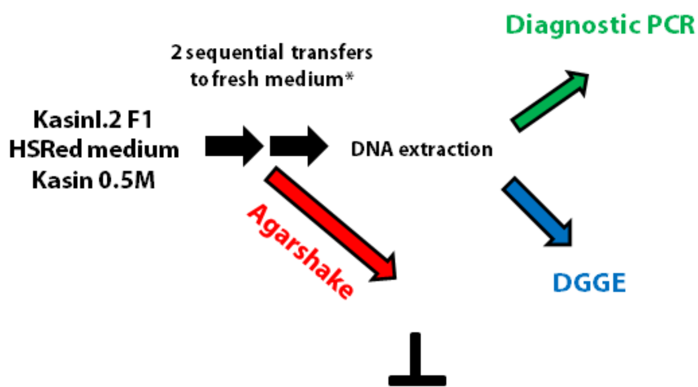

H
KasinI.1 A1
FeOx medium
Kasin 0.5M
Transfer to
fresh medium
Agarshake
3 sequential transfers
to fresh medium
Monitoring of
Fe(II) oxidation rate
under different conditions
Monitoring of
Fe(III) reduction rate
under different conditions*
DNA extraction
DGGE
Sequencing
of dominant
bands


I
Diagnostic PCR
DNA extraction
DGGE
2 sequential transfers
to fresh medium*
Sequencing
KasinI.1 D1
FeRed medium
Kasin 5M
Transfer to
fresh medium
Monitoring of
Fe(III) reduction rate
under different conditions*

J



K



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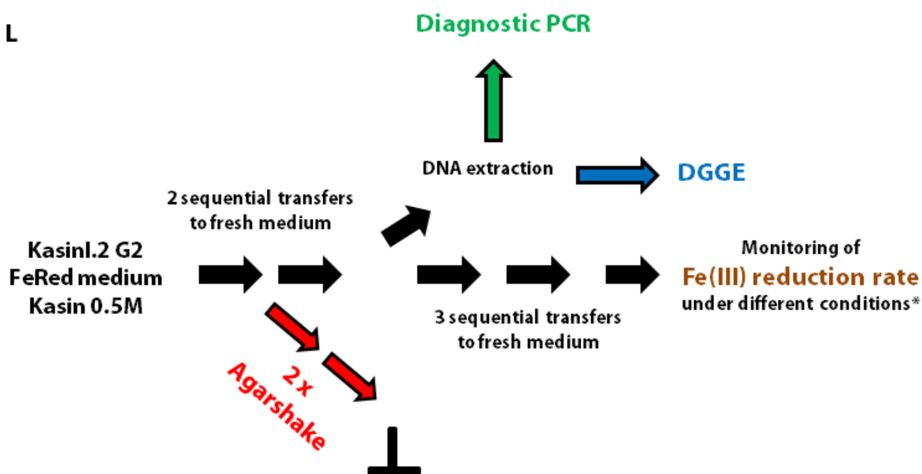

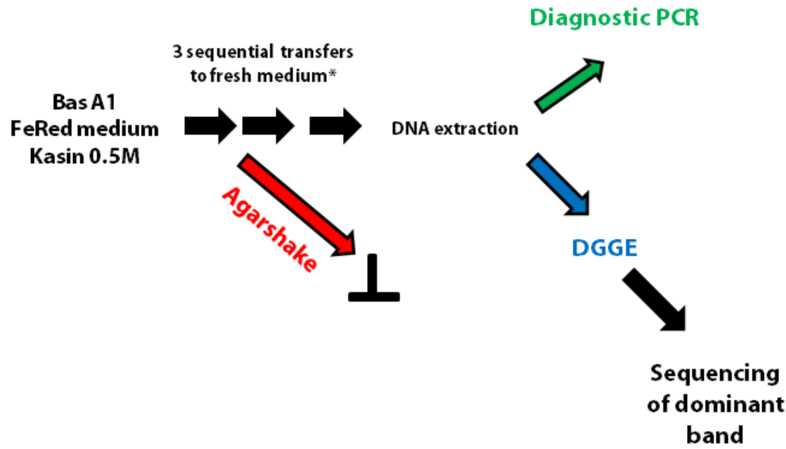

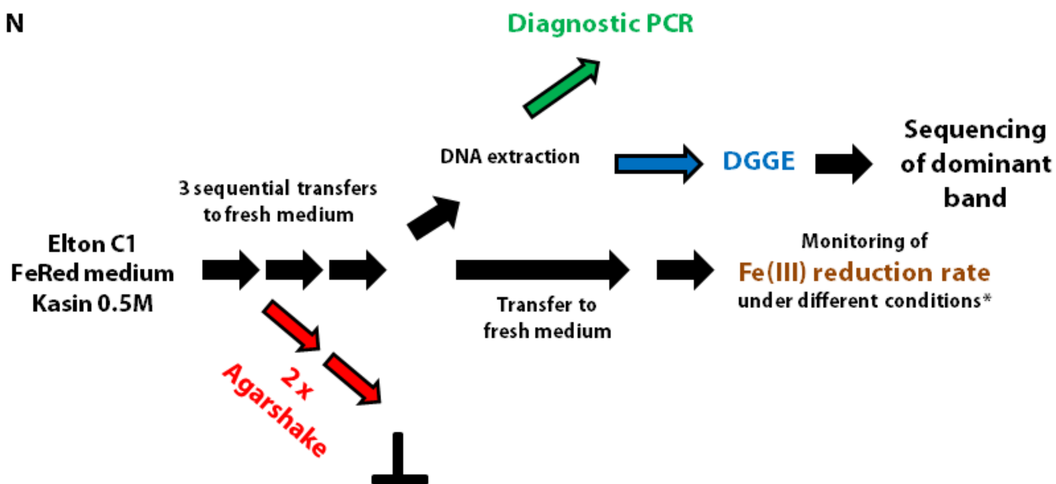

Figure 2: *Overview of the destiny of a selection of enrichment cultures that had been transferred from MPN wells into fresh media. The letter + number code that appears behind the name of the sediment or salt pan sample indicates the parallel and the dilution in the MPN experiment the enrichment was obtained from. Letters from A through G denote parallels of the same dilution. "1" corresponds to the lowest dilution, number "12" to the highest dilution. Numbers of arrows indicate how often one step (e.g. transfer to fresh medium) was repeated. Arrows followed by crosses (⊥) indicate that a given procedure (e.g. agar shakes) did not lead to discernible cell growth. Asterisks mark stages of the enrichments at which microscopic analysis showed the morphologies listed in table 6.*

In the first series of agar shakes, black colonies grew in many of the initially orange FeRed shakes. In the AQDS-containing shakes that were set up to isolate HSRed, white, red and brown colonies were detected in various tubes. Hardly any colony-like patches were seen in FeOx shakes. In most cases when single colonies were picked from any tube for further isolation, these were taken from the third, fourth, fifth or sixth tube of the dilution series. One problem that occurred in some shakes and that made it impossible to pick single colonies was that entire shakes turned black (Fe(III)-reducers) or orange (AQDS-reducers), but no defined colonies could be observed. The suspensions with the colonies that were picked from agar shakes and transferred to mineral medium were subsequently examined by microscopy. In between 50 and 80 % of these suspensions, intact cells were found. However, when new agar shakes were made using these suspensions as inocula, there were only very few cases in which colonies grew in the second round of agar shakes. Cultures that had gone through a series of agar shakes (e.g. SP3bII E1 HSRed) did not necessarily show a higher degree of purification in DGGE (figure 3B). Taken together, this means that the goal of the agar shake method to isolate pure strains of FeRed, HSRed and anFeOx was not achieved. Possible reasons why no growth of colonies was observed in sequential agar shake series include I) that essential micronutrients the respective microbes need for growth got diluted out and II) obligate anaerobes got exposed to too much oxygen during the transfer procedure. Concerning the latter explanation, colonies that were picked from one tube were kept in suspensions with mineral medium under atmospheric conditions for several hours before being inoculated into fresh agar shakes. Some microorganisms do not grow in or on solid medium at all and require liquid medium (Overmann, 2006), which could explain why some of the enrichments did not form colonies even in the first round of agar shakes.

Table 4: *Results from diagnostic PCRs showing whether archaeal and/or bacterial 16S rRNA genes could be successfully amplified by PCR from different enrichment cultures. All enrichment cultures were started from MPN experiments.*

Sample origin	**Incubation conditions**	**Archaeal 16S amplified?**	**Bacterial 16S amplified?**
Sua Pan II E1	HSRed in medium SW pH = 9.8	+	+
KasinI.1 A1	FeRed in medium Kasin 5 M	+	-
KasinI.1 A2	FeRed in medium Kasin 5 M	+	-
KasinI.1 D1	FeRed in medium Kasin 5 M	+	-
KasinI.1 F2	FeRed in medium Kasin 0.5 M	-	+
KasinI.2 F1	HSRed in medium Kasin 0.5 M	+	+
KasinI.2 G2	FeRed in medium Kasin 0.5 M	+	+
KasinI.3 B1	FeRed in medium Kasin 5 M	+	-
Bas A1	FeRed in medium Kasin 0.5 M	+	+
Elton C1	FeRed in medium Kasin 0.5 M	-	+

Table 4 lists the results from diagnostic PCRs to check for the presence of Bacteria and Archaea in different enrichment cultures. It can be seen that most enrichments have both Bacteria and Archaea. Only the enrichments grown in medium with 5 M NaCl are exclusively composed of Archaea. This is in line with the general understanding that Archaea can tolerate more extreme conditions than Bacteria (Jarrell et al., 2006).

Figure 3 shows DGGE gels of partial A) bacterial and B) archaeal 16S rRNA gene fragments amplified from DNA extracts of original field samples and enrichment cultures. All banding patterns originating from field samples appear relatively complex and thus suggest that both bacterial and archaeal diversities are relatively high in the salt pans and salt lake sediments under study. Concerning the samples from the African salt pans, banding patterns that belong to the bacterial amplificates from Sua Pan and WBSR appear very similar among the different samples of these two groups, but both groups seem to be pretty distinct from each other. This clustering reflects the geographic distances which were in the range of several meters between the different Sua Pan and WBSR sites, whereas Sua Pan and WBSR were several 1,000 km apart and the distance between Southern Africa and Russia exceeds 10,000 km. With regard to the bacterial

amplificates of the Russian salt lake sediments, one very strong band can be spotted in the sample from lake Elton. This could indicate that unlike the other field sites, the bacterial community in the sediment of this particular salt lake is dominated by one single bacterial species. However, this assumption has to be taken with caution since one band in a DGGE gel can also be composed of amplificates from different strains or species (Muyzer et al., 1993). In general, Archaea seem to produce more bands than Bacteria both in environmental samples and in enrichments even though according to rRNDB (http://rrndb.mmg.msu.edu/search.php). Archaea only have an average copy number of 1.72 16S rRNA genes versus an average of 4.18 gene copies for Bacteria. In this respect, it has to be kept in mind that DGGE is of limited resolution and rare taxa might not produce a band but rather a smear. For this reason, it cannot be said from DGGE results that Archaea are more diverse than Bacteria in the Russian samples. Clone library data from KasinI.1 indicate a much higher bacterial than archaeal diversity in this environment, for example (Chapter 6). One clear trend in the DGGE data is a decrease in complexity of the microbial communities from original environmental samples to enrichments which can already be seen by eye. This trend appears to be much stronger for the FeOx and FeRed than for the HSRed enrichments. A higher microbial diversity in HSRed compared to FeRed enrichments is consistent with the notion that FeRed represent only one out of several metabolic groups of microorganisms which can reduce humic substances (Lovley et al., 1996), being complemented by fermenting bacteria (Benz et al., 1998), toluene degraders, sulfate-reducers and methanogens (Cervantes et al., 2002). It is notable that none of our enrichments produce only a single DGGE band. This strongly suggests that nothing was enriched to purity. One interesting observation was that phylogenetically closely related Fe(III)-reducers and humic substance-reducers became dominant in enrichments from geographically different salt lake sediments (e.g. the same Bacilli species were found in FeRed enrichments from Bas and Elton, table 5). Since the sediments of Bas and Elton show considerable differences in terms of their elemental composition and ion content (table 3), this result can be attributed to an obviously very

selective effect of the culturing conditions, even if they do not seem to be selective enough to achieve pure isolates.

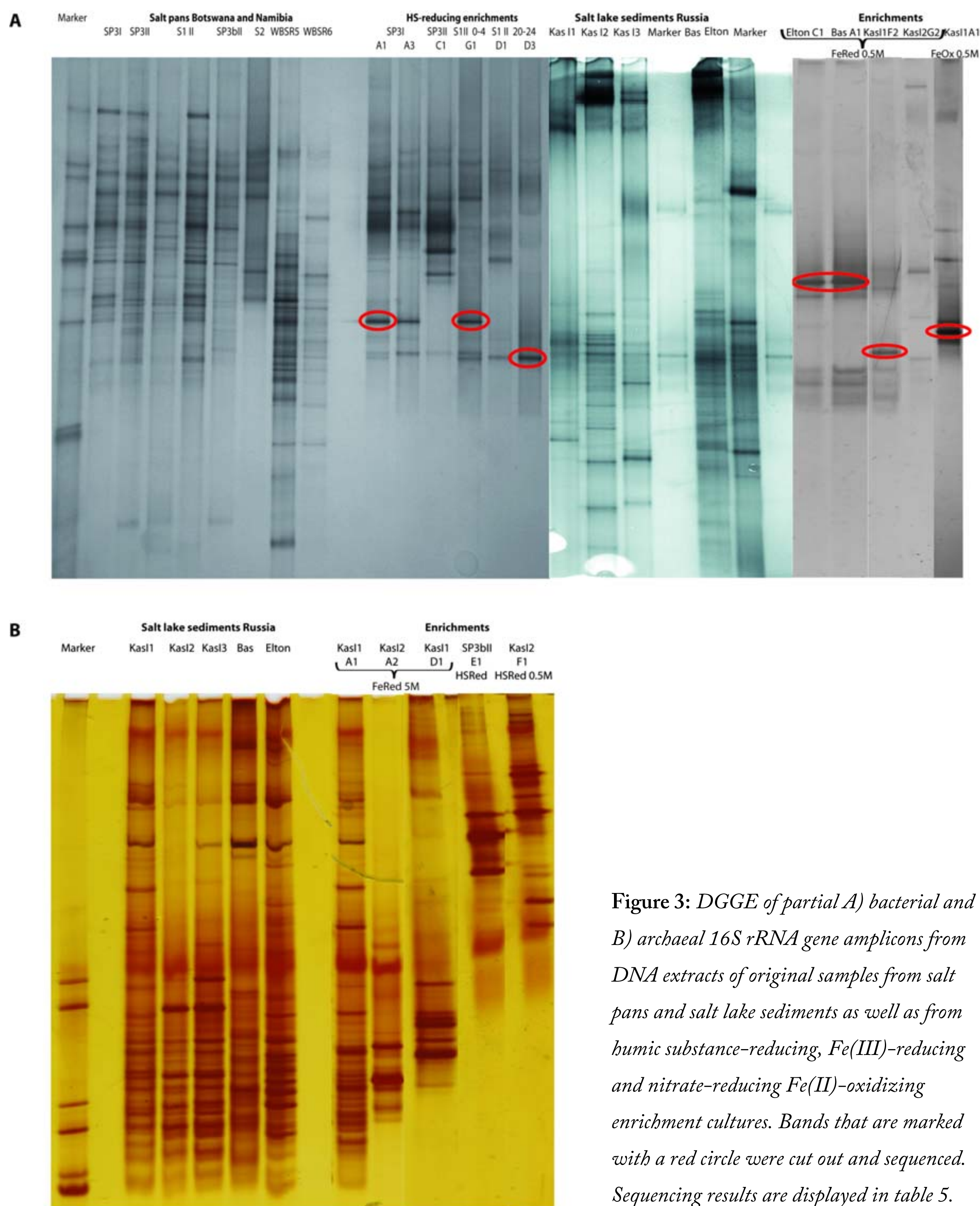

Figure 3: *DGGE of partial A) bacterial and B) archaeal 16S rRNA gene amplicons from DNA extracts of original samples from salt pans and salt lake sediments as well as from humic substance-reducing, Fe(III)-reducing and nitrate-reducing Fe(II)-oxidizing enrichment cultures. Bands that are marked with a red circle were cut out and sequenced. Sequencing results are displayed in table 5.*

Table 5: *BLASTN hits for the most closely related cultivated strains to the clones bearing the partial 16S rRNA gene sequences that were cut out from the DGGE gels shown in figure 3. "Number of hits" refers to the number of clones for which the indicated result was found. Numbers in brackets indicate the total number of clones sequenced. In case of the archaeal Fe(III)-reducing enrichment from KasinI.1 MPN well D1 that was grown in Kasin medium with 5 M NaCl, ten clones with 20F-1392R-amplicons were sequenced.*

Sample origin	Incubation conditions	Most closely related cultivated strains	% sequence identity	Number of hits
Sua Pan 3I A1	HSRed; SW pH=9.8	*Desulfonatronum thiosulfatophilum*[1]	98	4(4)
Sua1II 0-4 cm G1	HSRed; SW pH=9.8	*Halomonas* sp. FIB162[2]	99	3(4)
Sua1 II 20-24 cm G3	HSRed; SW pH=9.8	*Halomonas* sp. FIB162[2]	99	3(4)
KasinI.1 A1	FeOx; Kasin 0.5 M	*Geoalkalibacter ferrihydriticus*[3]	98	1(1)
KasinI.1 D1	FeRed; Kasin 5 M	*Halobaculum gomorrense*[4]	97	6(10)
KasinI.1 F2	FeRed; Kasin 0.5 M	*Dehalobacter restrictus* [5] *Lactobacillus fabifermentans*[6] *Desulfosporosinus lacus*[7]	97 98 97	1(3) 1(3) 1 (3)
Bas A1	FeRed; Kasin 0.5 M	*Bacillus alkalidiazotrophicus*[8] *Anaerobacillus alkalilacustre*[9]	98 98	3(3) 3(3)
Elton C1	FeRed; Kasin 0.5 M	*Bacillus alkalidiazotrophicus*[8] *Anaerobacillus alkalilacustre*[9]	98 98	3(3) 3(3)

[1] (Sorokin et al.), [2] (Sorokin et al., 2008a) , [3] (Zavarzina et al., 2006), [4] (Oren et al., 1995), [5] (Yoshida et al., 2009), [6] (De Bruyne et al., 2009), [7] (Ramamoorthy et al., 2006), [8] (Sorokin et al., 2008b), [9] (Zavarzina et al., 2009)

DGGE bands that were clearly dominant in the banding patterns of selective enrichments are marked by red circles in figure 3. These bands were cut out of the gels followed by sequencing of three or more clones. The archaeal 16S rRNA amplificates used for DGGE only had a length of 175 bp, which was too short to allow any phylogenetic analysis. This is why in order to identify dominant strain(s) in the archaeal FeRed culture originating from KasinI.1 D1 grown in medium with 5 M NaCl, ten clones that were prepared from amplificates with primers 20F and 1392R were sequenced instead of any dominant bands in the respective DGGE gel. The most closely related cultivated strains to the sequences that belong to the highlighted bands

are listed in table 5. Notably, six out of the nine most closely related cultivated strains listed in this table have been isolated from hypersaline environments, all of which except for *Halobaculum gomorrense* orginating from soda lakes or soda soil. *Geoalkalibacter ferrihydriticus* and *Desulfosporosinus lacus* can grow by anaerobic respiration of Fe(III) (Zavarzina et al., 2006), (Ramamoorthy et al., 2006) whereas *Anaerobacillus alkalilacustre* can reduce AQDS, but its capability to reduce Fe(III) has not been tested (Zavarzina et al., 2009). This is also the case for most other closely related cultivated strains apart from *Halobaculum gomorrense. Halobaculum gomorrense* is the only strain of the ones mentioned above that has been tested negative for its capability to reduce Fe(III) (Oren et al., 1995).

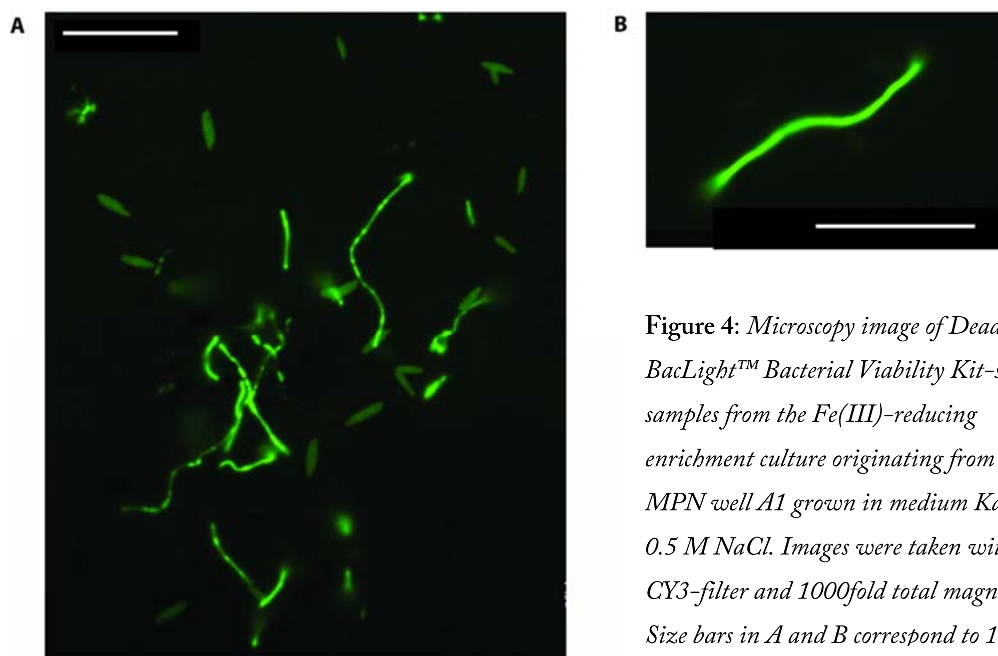

Figure 4: *Microscopy image of Dead/Live® BacLight™ Bacterial Viability Kit-stained samples from the Fe(III)-reducing enrichment culture originating from Bas MPN well A1 grown in medium Kasin with 0.5 M NaCl. Images were taken with a CY3-filter and 1000fold total magnification. Size bars in A and B correspond to 10 µm.*

Figure 4 shows microscopic images of a sample from FeRed enrichment Bas A1 grown in medium with 0.5 M NaCl after staining with the Dead/Live® BacLight™ Bacterial Viability Kit. This enrichment is dominated by very long *Bacillus*-like cells, as they could be observed in many other Fe(III)-reducing enrichments (table 6). The microscopic observation of *Bacillus*-like cells in these enrichments corresponds to results from sequencing of dominant DGGE bands in Bas A1 FeRed and Elton C1 FeRed and is well in line with previous studies of *Bacilli* that can reduce

Fe(III) at moderate salinities (Boone et al., 1995; Kanso et al., 2002).

Table 6: *Morphology of enrichment cultures determined by microscopy using a 1,000 x total magnification. The description of the morphology refers to enrichments at the stages that are marked with an asterisk in figure 2. All samples were stained with the Dead/Live® BacLight™ Bacterial Viability Kit prior to microscopy.*

Sample origin	Incubation conditions	morphology
Sua Pan II E1	HSRed medium SW pH=9.8	hardly any recognizable cells
WBSR6 E1	FeRed medium SW pH=8.0	coccoid and *Bacillus*-like cells
KasinI.1 D1	HSRed medium Kasin 5M	small dot-like cells
KasinI.2 D1	HSRed medium Kasin 5M	hardly any recognizable cells
KasinI.3 B1	FeRed medium Kasin 5M	small coccoid and rod-shaped cells
KasinI.1 A1	FeRed medium Kasin 5M	small non-motile cells
KasinI.1 A1	FeRed medium Kasin 0.5M	very small coccoid cells
KasinI.1 D1	FeRed medium Kasin 5M	rod-shaped cells, some of which are motile
KasinI.1 F2	FeRed medium Kasin 0.5M	rod-shaped cells
KasinI.2 F1	HSRed medum Kasin 0.5M	mostly *Bacillus*-like cells
KasinI.2 G2	FeRed medium Kasin 0.5M	very long *Bacillus*-like cells
Bas A1	FeRed medium Kasin 0.5M	very long *Bacillus*-like cells + rods
Elton C1	FeRed medium Kasin 0.5M	very long *Bacillus*-like cells + some rods and cocci

Since the dominant strain in the initially FeOx enrichment from KasinI.1 A1 was found to be very closely related to the known Fe(III)-reducer *Geoalkalibacter ferrihydriticus* (table 5), this enrichment was grown under Fe(III)-reducing conditions as well.
As it can be seen in figure 5, Fe(III) reduction rates showed strong variations among the different enrichments. The most effective Fe(III)-reducing enrichment was found to be KasinI.2 G2 in medium Kasin with 0.5 M NaCl in presence of yeast extract/cofactor solution. This enrichment reduced 100 % of initially 5 mM $Fe(OH)_3$ within 30 days both with lactate and acetate and with H_2 as electron donors.

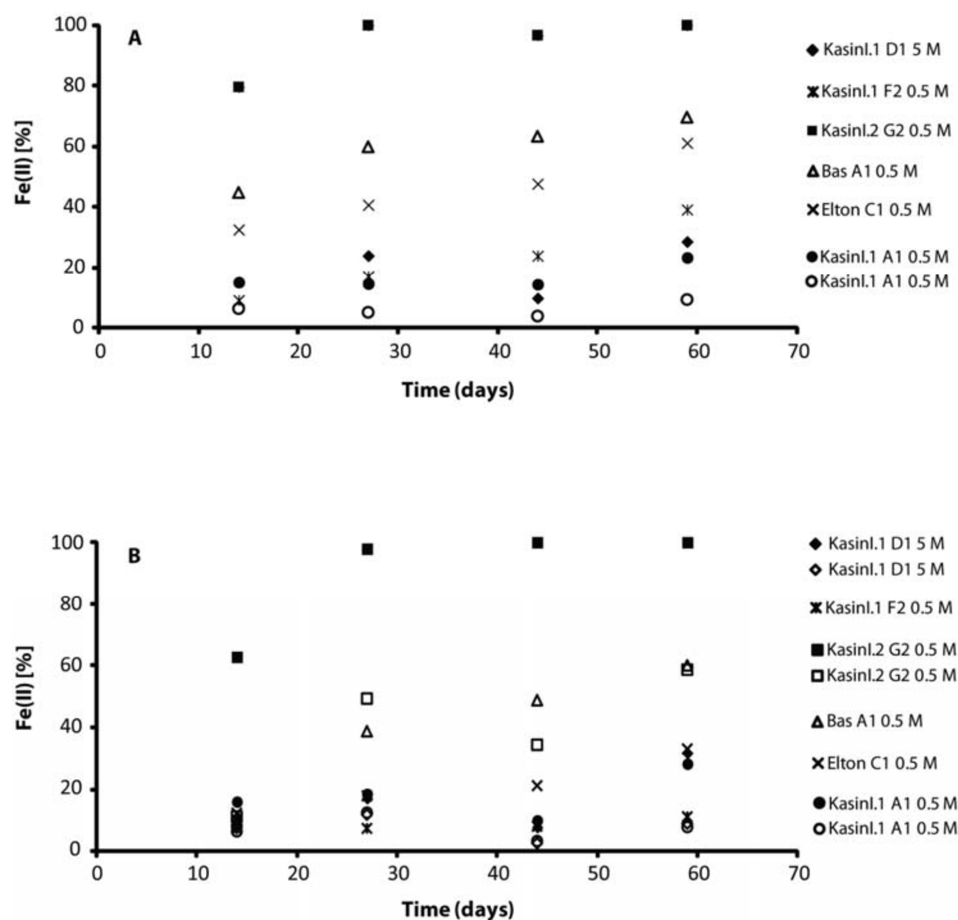

Figure 5: *Fe(III) reduction over time in selected enrichments using 5 mM ferrihydrite as electron acceptor and A) 5 mM lactate and acetate and B) H_2 as electron donor. From the total amount of 0.09 mmoles of H_2 that were added to the serum bottles, estimated final concentrations were about 2.8 µM of H_2(aq) in solution and 1.8 mM or 8 % of H_2 in the headspace. Open symbols and stars represent enrichments to which no yeast extract and cofactor solutions were added. Filled symbols represent cultures to which 2 % (v/v) of both yeast extract and cofactor solution were added at t=0.*

The only FeRed enrichment that remained active in medium with 5 M NaCl over time, namely the one stemming from KasinI.1 D1, showed very low rates of Fe(III) reduction: only 30 % (with lactate/acetate) or 23 % (with H_2) of 5 mM $Fe(OH)_3$ were reduced by this enrichment within 60 days. Without the addition of yeast extract and cofactor solutions, no Fe(III) was reduced at all by this enrichment.

In general, an enhancement of Fe(III)-reduction rates of the different cultures can be seen by the addition of of yeast extract/cofactor solution. This effect could be observed in enrichments KasinI.1 A1 with lactate/acetate as well as with H_2 and KasinI.1 D1 and KasinI.2 G2 with H_2. Based on microscopic examination, the composition of the enrichment cultures did not seem to change a lot upon addition of these solutions. Even if this observation still requires validation by more sophisticated methods, it is well feasible that the conditions under which these cultures were grown initially both during the MPN experiment and during the first transfers were already selective enough to starve most microorganisms unable to grow on Fe(III) reduction. This would mean that one does not need to fear that other types of microorganisms such as fermenters start to overgrow enrichments of the kind as they were prepared in this study if yeast extract/cofactor solution is added after three or more transfers to selective medium. However, it has to be conceded that the organic material that is added with the yeast extract could also stimulate the rate of Fe(III) reduction indirectly by serving as an electron shuttle. Thus, enrichment of Fe(III)-reducers in present of yeast extract could lead to the enrichment of microorganisms that do not reduce the Fe(III) itself, but the organic material within the yeast extract and the reduced organic material reduces the Fe(III) chemically.

According to figure 5B, all enrichments that were tested under both conditions can reduce Fe(III) with H_2 almost at the same rate as with lactate/acetate. However, it has to be considered that the enrichments grown with H_2 were inoculated with 4 % (v/v) of enrichments grown with 5 mM of both lactate and acetate. Consequently, 200 µM of both lactate and acetate were co-transferred to the new cultures. Assuming oxidation of all lactate to acetate and complete oxidation of all acetate to CO_2, this concentration of organic acids would enable reduction of 3.4 mM Fe(III) to Fe(II). Thus, in all FeRed cultures examined under H_2 apart from KasinI.2 G2 where 5 mM of Fe(III) were reduced, it cannot be excluded that residual lactate and acetate and not H_2 were used as electron donors. From a thermodynamic point of view, H_2 is a better electron donor than both organic acids since the 2 H^+/H_2 redox couple has a potential of -420 mV at pH = 7 while the potentials for CO_2/acetate and pyruvate/lactate at the same pH are -280 and -190 mV, respectively (Madigan and Martinko, 2006).

The fact that at least this one culture could definitely reduce Fe(III) with H_2 suggests that the potential for the use of H_2 produced by other groups of microorganisms by Fe(III)-reducers in

salt lake sediments does exist. However, this hypothesis still awaits validation e.g. one would need to test whether a stoichiometric decrease in H_2 concentration goes along with Fe(III) reduction in the cultures under study.

Conclusions & Outlook

As described in the "Results and discussion" section, a number of relatively stable Fe(III)-reducing enrichment cultures could be gained from various salt pans and salt lake sediments of different geochemical properties. However, none of these cultures could be enriched to purity. The main reason for this was that no colonies could be maintained through sequential agar shake dilution series. This fact might either be due to I) essential micronutrients the respective microbes need for growth being diluted out or II) obligate anaerobes being exposed to too much oxygen during the transfer procedure. Case I could be approached by adding small amounts of yeast extract/cofactor solution to the medium that is used in the agar shakes even though it would bear the danger of growing undesired fermenting microorganisms. However, at least Fe(III)-reducing colonies could still be distinguished by the black color that appears around them due to the formation of mixed-valent Fe(II)/Fe(III) (hydr)oxides. In order to approach case II, the method would need to be repeated under totally anoxic conditions. This would be possible by transferring the colonies that are picked from one agar tube into anoxic medium that is kept under N_2 or N_2/CO_2-atmosphere. The colonies would then be suspended and kept within this medium until inoculation into the next series of agar shakes. The only reason why it was not done this way in the present study is that the possibly deleterious effect of exposing the colonies to low amounts of oxygen for some hours was underestimated.

Another suggestion to improve the agar shake method for the isolation of Fe(III)-reducers would be to use ferric citrate instead of ferrihydrite as an electron acceptor. In contrast to the Fe(III) in ferrihydrite, the Fe(III) in ferric citrate is kept in solution by the complexing organic acid, rendering it more easily accessible to microbial reduction.

In addition to intensified efforts to obtain real isolates, a number of interesting further experiments could be done either with the isolates or with the enrichments in order to shed more light on Fe(III) reduction under hypersaline conditions. One question one could address concerns the influence of ionic strength on Fe mineral formation. In order to test this, one would first need to determine the minimum and maximum concentrations of NaCl at which the different enrichments or isolates still grow. The next step would be to incubate the enrichments or isolates in media spanning a gradient of NaCl between these threshold concentrations together with $Fe(OH)_3$ or ferric citrate and lactate/acetate and follow Fe(III) reduction over time. In addition to examining whether the rates of Fe(III) reduction depend on the ionic strength of the medium, mineral products of Fe(III) reduction would be identified by Mössbauer spectroscopy and XRD and characterized by SEM. According to the "energy hypothesis" stated in the introduction, one would expect that for the enrichments where "low salt-in" strategists catalyze the Fe(III) reduction, the rate of this process decreases with increasing salinity since the respective microbes need to spend more energy on increased compatible solute production the more hyperosmotic conditions they live in. In the enrichments where "high salt-in" strategists are responsible for Fe(III) reduction, as it is most likely the case in culture KasinI.1 D1 kept in medium with 5 M NaCl that is dominated by a close relative of *Halobaculum gomorrense*, such a correlation would not necessarily be expected since the accumulation of increased concentrations of KCl in the cytoplasm does not require high amounts of energy (Oren, 2001). Following this line of thinking, one would expect that for "low salt-in" strategists, the variety of Fe(III) phases usable as electron acceptors depends on the ionic strength of the medium they are surrounded by. The reason behind this expectation is the standard redox potential, or the affinity to accept electrons, decreases from poorly crystalline ferrihydrite ($E_H^{0'}$ = - 67 mV (Stumm and Sulzberger, 1992)) to Fe(III) (hydr)oxides such as goethite ($E_H^{0'}$ = - 266 mV (Stumm and Sulzberger, 1992)) and magnetite ($E_H^{0'}$ = - 427 mV (Stumm and Sulzberger, 1992)). This means that with the same electron donor, more energy is gained by the reduction of ferrihydrite than by reduction of magnetite or goethite. Consequently, there might be certain levels of salinity where a

"low salt-in" strategist could still gain enough energy for compatible solute production by reducing ferrihydrite, but not by reducing more crystalline Fe(III) minerals. On the other side of the Fe cycle, it could be shown in this study that mixotrophic NO_3^--reducing Fe(II)-oxidizers exist in hypersaline environments and that they can also be active in medium with up to 5 M NaCl. However, maintenance of stable Fe(II)-oxidizing enrichment cultures over several transfers turned out to be more difficult than in the case of the Fe(III)-reducers. This could be explained by NO_3^--reducing Fe(II)-oxidizers being more specific in terms of their needs for particular growth conditions. Consequently, more studies are needed in order to define conditions under which this kind of microorganisms can be cultured. Only then will it be possible to study the dependence of microbial Fe(II) oxidation on ionic strength in a similar way as described above for the reductive part of the Fe cycle.

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7

Enrichment of Fe(III)-reducing and Fe(II)-oxidizing microorganisms from Russian and South African salt lake sediments and salt pans at up to 5 M salinity

Maren Emmerich, Ankita Bhansali, Odeta Shuti, Sebastian Behrens & Andreas Kappler

APPENDIX

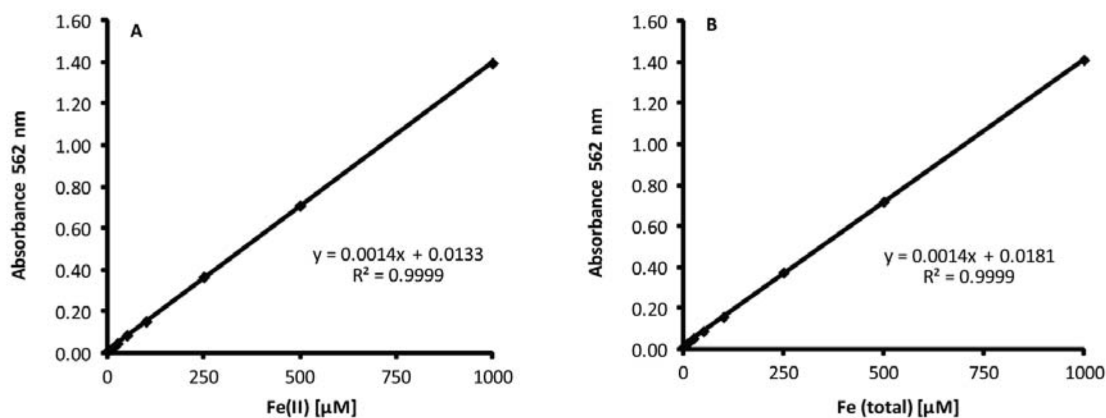


Figure A1: *Exemplary calibration curves and linear regression used for the calculations of A) Fe(II) and B) Fe(total) in Fe(II)-oxidizing and Fe(III)-reducing enrichment cultures.*

8

Assessing the potential for direct microbial contribution to VOX formation through biohalogenation reactions catalyzed by halogenating enzymes in salt lake sediments

Maren Emmerich, Andreas Kappler & Sebastian Behrens

Abstract

A number of important microbially mediated processes such as methanogenesis, sulfate and nitrate reduction as well as ammonia oxidation can already be tracked in the environment by phylogenetic or functional marker genes. Formation of organohalogens by incorporation of halogen ions into organic material has been shown to be mediated by microbial haloperoxidase enzymes. Since there are still missing sources to account for a vast amount of naturally produced organohalogens, haloperoxidases could serve as a valuable target for a functional marker gene assay to chase microbial halogenations processes in the environment. We tested this hypothesis with a PCR-based approach using degenerate haloperoxidase-specific primer pairs.

Our major findings were that I) the sequence similarity of haloperoxidases from different microorganisms is too large to allow the design of any degenerate primers to tackle all of them at the same time. II) Even though we obtained promising PCR products with some of the primers we designed to amplify haloperoxidases from enzyme-specific subgroups of different microbial taxa, none of the obtained PCR products could be verified as haloperoxidase by DNA sequencing. This means that an extended PCR screening approach combined with other methods is needed in order to locate and identify responsible enzymes for microbially mediated halogenation reactions in the environment.

Introduction

Chlorinated hydrocarbons are abundant in natural environments including atmosphere and soil (Oberg, 2003). This group of organic compounds was disreputed because some of its representatives were shown to contribute to stratospheric ozone depletion (Molina, 1974), have high global warming potentials (e.g CCl_4 has a 1400 fold higher global warming potential than CO_2; (WMO, 2007)), and promote cancer (*IARC*, 1997; Maltoni et al., 1974). These problems mainly apply to short-chain and thus volatile organohalogens (VOX). After anthropogenic emission of these compounds was restricted and relatively high concentrations e.g. of methyl halides continued to be measured in the atmosphere (Butler, 2000), it became clear that natural sources must also exist. The detection of mixing ratios of several ppt of ClO and other halogen oxides over salt lakes such as the Dead Sea (Smoydzin and von Glasow, 2009), the Great Salt Lake in Utah (Stutz et al., 2002), Salar de Uyuni in Bolivia (Honninger et al., 2004) and the Caspian Sea (Wagner et al., 2001) rendered these environments suspects of contributing significantly to global VOX emissions. This hypothesis was strengthened by a microcosm study of Weissflog and coworkers, who measured considerable formation of highly chlorinated C1 and C2 hydrocarbons in setups where salt lake sediments were incubated under conditions that

permitted the activity of halophilic microorganisms, but not in sterile controls (Weissflog et al., 2005). Incubation studies of ^{36}Cl with forest soil strongly suggested that an enzyme-dependent biotic process is responsible for the incorporation of ^{36}Cl into organochlorines (Bastviken et al., 2007; Bastviken et al., 2009; Rohlenova et al., 2009). This goes in line with the previous finding of Asplund et al., (1993) who could prove the presence of a haloperoxidase-like catalyst that stimulated the chlorination of organic matter in eight out of nine different soil extracts tested. Bringing these findings together, the question arises whether microbial haloperoxidases could actively halogenate organic matter in salt lake sediments. The degradation products of this halogenated organic matter could then be released as VOX. Haloperoxidases form one out of three groups of halogenating enzymes produced by microorganisms. An overview of the classification of microbial halogenating enzymes can be found in table 1. Haloperoxidases are particularly interesting with regard to VOX formation since their reaction mechanism includes the formation of freely diffusible intermediates such as hypochloric acid (HOCl) which explains why haloperoxidases mediate rather unspecific halogenation reactions (Manoj, 2006). The first enzyme of this class, the chloroperoxidase from the fungus *Leptoxyphium fumago*, has been described as early as 1959 (Shaw, 1959). 40 years later, the crystallographic structure of this enzyme could be resolved (Sundaramoorthy et al., 1998). The availability of an enzymatic assay that allows the detection of unspecific halogenation activity (Morris and Hager, 1966) alleviated the discovery of a series of further enzymes similar to the *L. fumago* chloroperoxidase. These discoveries included enzymes in which the oxidation of a heme-Fe to the +IV state in the catalytic center by H_2O_2 is crucial for the formation of the halogenating agent in the form of hypohalous acid (e.g. HOCl) to occur.

Table 1: *Groups of halogenating enzymes within microorganisms*

	(Sub)group	Organisms	Environmental relevance
Haloperoxidases (non-substrate-specific)	Heme-type haloperoxidases	fungi & bacteria	soils
	Vanadium-dependent haloperoxidases	algae, fungi & bacteria	mainly marine environments
	Perhydrolases	bacteria	probably no halogenating activity under natural conditions
	(Sub)group	**Organisms**	**Environmental relevance**
Halogenases (substrate-specific & regioselective)	Flavin-dependent halogenases	bacteria	most important enzymes for production of halogenated metabolites in microorganisms (mainly antibiotics); chlorination of aromatic rings
	α-ketoglutarate-dependent halogenases	bacteria	chlorination of aliphats during antibiotic synthesis
Methyltransferases		marine red algae, fungi, plants, bacteria	marine and soil environments

Besides the chloroperoxidase from *Leptoxyphium fumago* (Shaw, 1959), a bromoperoxidase from *Pseudomonas aureofaciens* (van Pee and Lingens, 1985) and two haloperoxidases from *Streptomyces toyocaensis* (Marshall and Wright, 1996) serve as examples of this type of heme-dependent haloperoxidases.

H_2O_2-dependent oxidation of the halide substrate to the hypohalite state (an oxyanion with a halogen in an oxidation state of +1) does also take place in the second type of haloperoxidases, the vanadium (V)-dependent haloperoxidases. Representatives of these enzymes have mainly been found in seaweed (Butler and Carter-Franklin, 2004). Even though V-dependent haloperoxidases seem to be more widespread in the marine environment, V-dependent chloroperoxidases from

the terrestrial fungus *Curvularia inaequalis* (Vanschijndel et al., 1993) as well as from two *Streptomyces* strains (Winter et al., 2007) have been described, too. The existence of a third group of haloperoxidase-like enzymes, the perhydrolases, which do not harbor any metal ions has been hypothesized based on in vitro experiments (Wiesner et al., 1988). However, it has so far not been shown that these enzymes can also perform halogenating activity under in vivo-conditions (van Pee, 2003). Although a number of haloperoxidases have been found within microorganisms that produce halogen-containing antibiotics, it has never been proven so far for any haloperoxidase to be involved in the synthesis of any halogenated metabolite by studying knockout mutants of the respective genes. Instead, it has been proposed that the HOCl generated by these enzymes plays a role in decomposing plant material and in harming organisms competing for the same resources within terrestrial environments (Bengtson et al., 2009).

For flavin- and α-ketoglutarate-dependent halogenases, on the contrary, their involvement in the formation of halometabolites has been shown by the inability of deletion mutants to produce these metabolites for a number of examples (van Pee and Patallo, 2006). The first one concerned the formation of the tryptophan-derived Cl-containing antibiotic pyrrolnitrin, for which two halogenase genes with different substrate specificities were found in the biosynthetic gene cluster (Hammer et al., 1997). One of these halogenase gene products, PrnA, mediates the specific chlorination of tryptophan at C7. Tryptophan 7-halogenases were found to be involved in the formation of further halometabolites by other strains, including rebeccamycin synthesis by *Lechevalerieria aerocolonigenes* (Sanchez et al., 2002). However, as it was shown later, all tryptophan 7-halogenases need the additional presence and activity of flavin-reductases that transfer electrons from NADH to FAD for the halogenation reaction to occur (Keller et al., 2000). During the reaction cycle, the halogenase binds $FADH_2$, which then gets oxidized to FADH-OOH by O_2. This can then easily react with chloride giving rise to FAD-OCl and finally HOCl, which, in contrast to haloperoxidases, cannot leave the active site due to blockage by flavin and is directed to a tryptophan that is also bound at the active site and whose

electrophilicity is enhanced by adjacent amino residues enabling chlorination by HOCl at C7, which does not occur if free hypochlorite is used in solution (van Pee et al., 2006). α-ketoglutarate-dependent halogenases, on the other hand, whose role in halometabolite formation have been proven on the example of SyrB2 involved in syringomycin E synthesis by *Pseudomonas syringae* by an *in vitro* halogenation assay (Vaillancourt et al., 2005), use a different mechanistic strategy for halogenations of aliphates: in these enzymes, an Fe^{IV}=O intermediate is formed assisted by Fe complexation by α-ketoglutarate, which is prone to nucleophilic attack by Cl^-. The reactive Cl-Fe^{IV}=O species that arises from this reaction then absorbs a H-radical from the aliphatic substrate, which thus gets in turn activated to absorb a Cl-radical from the now existent Fe^{III}-OH intermediate (Vaillancourt et al., 2006).

In comparison to haloperoxidases and halogenases, knowledge about the third group of microbial halogenating enzymes, the methyltransferases, is very scarce. In contrast to all other halogenating enzymes, they pursue a nucleophilic strategy for their catalytic activity using S-adenosyl-methionine as a substrate (Blasiak and Drennan, 2009). Methylation of halogenide ions by S-adenosylmethionine has been detected in marine red algae, fungi, ice plant (Harper et al., 1989; Wuosmaa and Hager, 1990) as well as in several terrestrial and marine bacteria (Amachi et al., 2001; Eustaquio et al., 2008). The only fluorinating enzyme detected so far that has been isolated from *Streptomyces cattleya* and described in crystallographic detail (Dong et al., 2004) is also a methyltransferase.

To sum up, we currently have both I) mechanistic knowledge of haloperoxidase functioning and II) insights about halogenations activities in the environment gained from bulk chlorination studies. However, these two lines of research have never been combined in order to draw conclusions about diversity, abundance, distribution and activity of the individual groups of halogenating enzymes from different organisms in the environment. As a consequence, a knowledge gap exists concerning the identification of halogenating enzymes that are active in

different environments as well as the quantification of their activities under varying conditions. This knowledge gap is depicted in figure 1.

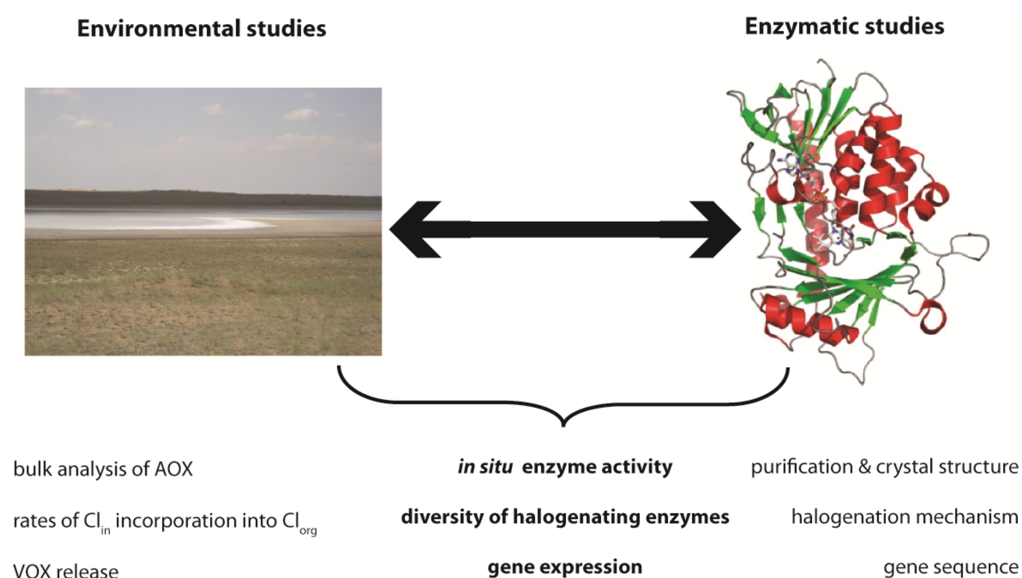


Figure 1: *Scheme illustrating the knowledge gap tackled in this study: while both bulk chlorination of organic material (left part of figure) and mechanisms of halogenating enzymes (right part of figure) have been studied extensively, links are missing between these two kinds of experimental approaches. Determining in-situ enzyme activity, studying diversity of halogenating enzymes and quantifying gene expression will allow to determine to which extent which enzymes contribute to the formation of chlorinated hydrocarbons observed in the field. (The picture in the right was adopted from Anderson & Chapman, Molecular BioSystems, 2006 and shows the FAD-binding domain of the tryptophan 7-halogenase PrnA).*

The goal of this study was to bridge this knowledge gap by developing a quantitative PCR assay with degenerate primer pairs to identify genes for different types of haloperoxidases in salt lake sediments. This will help us in the future to determine the diversity, abundance and distribution of these enzymes, which could account for a major fraction of naturally produced organohalogens and prove their role in the formation of natural organohalogens.

Materials & Methods

Bacterial and fungal reference strains

Bacillus pseudomycoides DSM No.: 12442 (Nakamura, 1998); *Streptomyces aculeolatus* DSM No.: 41644 (Shomura et al., 1987) and *Leptoxyphium fumago* DSM No.: 1256 (Pickard, 1981) were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). *B. pseudomycoides* and *S. aculeolatus* were grown in liquid lysogeny broth medium at 37°C for 25 days. *C. fumago* was already delivered by the DSMZ as actively growing culture which was used directly for DNA extraction.

DNA extractions

Sediment samples from various salt pans, salt lake sediments and a seawater-flooded slough (see table 3 for detailed information about sampling sites) were kept at 4°C until arrival in Tübingen, Germany, where they were sieved through a 2 mm mesh and frozen at -20°C until further analysis. For DNA extractions, samples were thawed and 0.3 g of each sample were washed three times with 1.5 mL of 10 mM Tris-EDTA buffer of pH = 7.0 by vortexing the sediment-buffer slurry and centrifuging it for 10 minutes at 7200 g. Supernatants from the three washing steps were combined and filtered through a 0.22 µm cellulose ester filter. Washed sediment samples and filters were transferred into separate bead tubes of the PowerSoil DNA isolation kit® (MoBio Laboratories, Carlsbad, CA) and DNA was extracted according to the protocol of the manufacturer. In the end, DNA extracted from filter and sediment originating from the same sample was eluted sequentially in 2 x 25 µL elution buffer (C6) into one microfuge tube. DNA from *L. fumago* was extracted using the peqGOLD Fungal DNA Mini Kit® (PEQLAB Biotechnologie GmbH, Erlangen, Germany) from about 100 mg of fungal tissue that had been shock-frozen in liquid N_2. The kit was applied according to the instructions of the manufacturer,

and DNA was eluted in 100 µL of DEPC-treated H_2O (Carl Roth GmbH, Karlsruhe, Germany).

DNA from *B. pseudomycoides* and *S. aculeolatus* was extracted from 2 x 25 mL of densely grown liquid culture each. Liquid cultures were centrifuged for 10 minutes at 7200 g, pellets were washed with 25 mL of 10 mM Tris-HCL, pH = 8 and resuspended in 0.5 mL of Tris-HCL. 1 µL of 0.5 M EDTA, pH = 8 and 25 µL of 10 mg/mL lysozyme in 10 mM Tris-HCL were added and the mixture was incubated at 37°C for 55 minutes. The mixtures were then transferred into 2 mL Phase Lock Gel Heavy microfuge tubes (5 PRIME GmbH, Hamburg, Germany) to enhance phase separation of DNA from other cell components together with 0.5 mL of phenol:chloroform:isoamylalcohol (25:24:1). After 5 minutes of shaking at room temperature and centrifugation at 7200 g for 5 minutes, the DNA-containing aqueous phase was transferred into fresh Phase Lock tubes and the phenol:chloroform:isoamylalcohol-extraction step was repeated. DNA was then precipitated from the aqueous phase with 0.1 volume of 3 M sodium acetate (pH 5.5) and 2.5 volumes icecold ethanol at -80°C for 30 minutes. After centrifugation at 20 000 g for 30 minutes, pellets were washed with 70 % ethanol, centrifuged again and dried. Finally, pellets were dissolved in 50 µL of nuclease-free H_2O.

Strategy to design haloperoxidase-specific primer pairs

Since known haloperoxidases revealed a high sequence diversity on the DNA level, we started our design of haloperoxidase-specific primers with a BLASTP search of proteins with high structural similarities to the well-annotated heme-type haloperoxidase from the fungus *Leptoxyphium fumago* (gi|11322380; (Conesa et al., 2001)) and vanadium-dependent haloperoxidases from *Clostridium botulinum* (gi|168182149), *Rhodospirillum baltica* (gi|32471931) and *Pyrenophora tritici repentis* (gi|187979729). This allowed us to detect at least some sequence motifs that were conserved within several hits.

The bacterial and eukaryote sequences that were found by this search were then aligned separately using the Geneious.Pro software (Biomatters Ltd., Auckland, New Zealand) and

disposed all complete outliners as well as all sequences with unknown parent organism. For the remaining protein sequences, we searched for the corresponding DNA sequences. After addition of three further gene sequences from vanadium-dependent haloperoxidases from two *Streptomyces* strains (Winter et al., 2007) and removal of redundant DNA sequences, another alignment was made on the basis of the resulting DNA sequences. According to sequence similarities, these sequences were put into 7 groups for bacterial and 2 groups for eukaryotic enzymes comprising between 1 and 7 sequences each. In the following, we designed degenerate primer pairs for each group and ordered these together with reference strains known to harbour one of the target sequences in their genome (see table 2 for primer sequences and table 3 for further information about the groups we assigned to our sequences).

Further two additional primer pairs were designed on the basis of DNA alignments of vanadium-dependent haloperoxidase genes of *Clostridia* and *Bacilli* strains as well as for cofactor-free haloperoxidases (= perhydrolases) from all bacteria except for *Mycobacteria.* Sequences of these primers can also be found in table 1.

All primer pairs were designed in a way that the expected product size was between 650 and 1200 bp. All primer sequences were undergone a "BLASTN" search for "somewhat similar sequences" against "Others". This was done in order to check the specificities of the primers as well as to identify possible unwanted target sequences the primers might bind to.

PCR reactions

All PCRs were performed in a reaction volume of 50 µL. Reaction mixes consisted of 1x PCR buffer with 1.5 mM final concentration of $MgCl_2$ (Promega), 200 µM dNTP mix (New England Biolabs), 500 nM of each primer, 1 M betain, 250 ng of BSA, 1.25 U Taq DNA-Polymerase (Promega) and 2 µL of DNA extract as a template. Reactions with primer pair Bac1 contained 500 ng of BSA. For reactions with primer pairs HaloCB, betain was omitted. For reactions with primer pair Lfum, only 250 nM of each primer was used.

Thermocycler programs were as follows: initial denaturation at 94°C for 2 min; 30 cycles of denaturing (95°C for 30 s), annealing (see table 1 for temperatures used for the individual primer pairs) and elongation (72°C for 90s for primer pair HaloCB and 60 s for all other primer pairs) and a final elongation at 72°C for 10 min.

Cloning and sequencing

PCR amplificates of hypothetic haloperoxidase genes were purified with a Wizard® SV Gel and PCR Clean-Up System (Promega laboratories) according to the manufacturer's suggested protocol. Eluted amplificates were re-amplified using the same primers and PCR protocol as before and products were again purified with a Wizard® SV Gel and PCR Clean-Up System and eluted into 50 µL of DEPC-treated H_2O. The purified PCR products were ligated into a pCR4® cloning vector (Invitrogen, Darmstadt, Germany) and transformed into Escherichia coli TOP10 chemocompetent cells according to the protocol suggested by the manufacturer.

Table 2: *Sequences of haloperoxidase-specific primers designed in this study and annealing temperatures used for PCRs with these primer pairs.*

Primer pair		Primer sequence	Annealing temperature	product length (bp)
Bac1	fw	5'-TACCTGGTGGTCTGATAGG-3'	54°C	827
	rev	5'-GCCAATGAACACCAGCTG-3'		
Strep1	fw	5'-TCGGCCACCAACCGGAAC-3'	59°C	808
	rev	5'-CGCGGTGATCGGCCGC-3'		
Lfum	fw	5'-GCATTGGCTACCCATACGAC-3'	54.3°C	846
	rev	5'-TGGTGGCGAGGAGGAATG-3'		
Cbot 1	fw	5'-CGGAAACACTYTTCCG-3'	45°C	940
	rev	5'-TCTTCATTGTAGGTATTTCTAT-3'		
Cyano1	fw	5'-CCGCCATAMAATCGAGA-3'	49°C	1037
	rev	5'-GACGGTTCWAACCAACTC-3'		
HaloCB	fw	5'- AAGGCT TTACCWCATAAT-3'	47°C	1194
	rev	5'- ATATTGGYAGCAAGCTTA-3'		
HaloNH	fw	5'- GTTTCTMCAYGGCTGGCC-3'	57°C	662
	rev	5'- GGAACRAYCTKRTCGTCA-3'		

The transformed cells were plated onto Luria-Bertani medium (LB) plates containing 50 mg/L of ampicillin, 40 mg/L X-gal and 100 µM IPTG and incubated overnight at 37°C. Colonies of white color indicating successful transformants were transferred with a toothpick into 100 µL of liquid LB medium containing 50 mg/L of ampicillin in 96 well-plates and incubated for 4 h at 37°C. After growth of little colonies could be observed on the bottom of the wells, 100 µL of a 1:1 (v:v) mixture of phosphate buffer saline and glycerol was added and the plates were transferred to -80°C for storage. Three clones of each transformation reaction were transferred from these glycerol stocks into 5 mL of LB medium containing 50 mg/L of ampicillin and incubated overnight at 37°C. The overnight cultures were then subjected to plasmid extractions using the peqGOLD Plasmid Miniprep Kit® according to the manufacturer's instructions. Plasmid concentrations were determined with a NanoDrop (Thermo Scientific) and plasmids

were diluted with nuclease-free H_2O to concentrations between 20 and 60 ng DNA/µL before they were sent for sequencing.

Sequencing reactions were carried out at GATC Biotech (Konstanz, Germany) with primer M13R (CAGGAAACAGCTATGAC) (Invitrogen). The sequences of the inserts were determined with an ABI 3730 xl automated sequencer. Sequences were typically 900-1000 bp long. Remaining vector as well as bad quality parts of the sequences were eliminated with the Geneious.Pro software before sequences were submitted to BLASTN (settings: "somewhat similar sequences" against "Others") for search of similar sequences based on pairwise sequence alignments.

Results

Our initial search for enzymes with stated or hypothesized haloperoxidase activity resulted in 163 bacterial and 94 eukaryote sequences. After disposing of complete outliners and sequences with unknown parent organism, 13 sequences of eukaryotic and 51 sequences of bacterial origin were left. The corresponding DNA sequences were grouped as shown in table 3.

Table 3: *Groups of haloperoxidase sequences and primers designed for these groups. Primers printed in bold were actually ordered to start the screening with. Strain species printed in bold were ordered from the DSMZ and their DNA extracts were used as positive controls in the respective PCRs.*

Group name	Organisms with target (putative) haloperoxidase genes	Accession numbers of target gene products	Primer sequences	Expected product length (bp)
Cbot1	*Clostridium botulinum* A2 strain Kyoto, B1 strain Okra, F strain Langeland	YP_002802554.1 YP_001779876.1 YP_001389606	**5'-CGGAAACACTYTTCCG-3'** **5'-TCTTCATTGTAGGTATTTCTAT-3'**	940
Cbot2	*Clostridium botulinum* Bf, *Clostridium sporangenes*	ZP_02616813 ZP_02993631	5'- CTATAATCCTGAAGTAATCC-3' 5'- GCTCTATCTTTGAGTAATC-3'	651
Bac1	*Bacillus mycoides* Rock 3-17 ***Bacillus pseudomycoides* DSM 12442**	ZP_04155349 ZP_04149477	**5'-TACCTGGTGGTCTGATAGG-3'** **5'-GCCAATGAACACCAGCTG-3'**	827
Strep1	*Streptomyces avermitilis* MA-4680 ***Streptomyces aculeolatus*** (napH3 and napH4 genes)	NP_821923 EF397639	**5'-TCGGCCACCAACCGGAAC-3'** **5'-CGCGGTGATCGGCCGC-3'**	808
Strep2	*Streptomyces aculeolatus, Micromonospora*	EF397639 CAJ34370	5'- GCCSSWGGGGTACTCGGG-3' 5'-GCSSWGCACGGKGTGYKGAAG-3'	882
Cyano1	*Lyngbya* sp. PCC 8106 *Cyanothece* sp. ATCC 51142 *Trichodesmium erythraeum* IMS 101	ZP_01621346 YP_001803460 YP_723199	**5'- CCGCCATAMAATCGAGA-3'** **5'- GACGGTTCWAACCAACTC-3'**	< 1 037
Cyano2	*Microcystis aeruginosa* PC 7806 *Nostoc* sp. PCC 7120 *Synechococcus* sp. PCC 7335 *Anabaena variabilis* ATC 29413 *Microluteus chthonoplastes* PCC 7420	YP_001655960 NP_484716 ZP_05040132.1 YP_325496.1 EDX78799.1 ZP_01629010	5'- AGCCTTGWAAAGWGGVAA-3' 5'- CCAARCTGCTTTTAMGGC-3'	< 1 111

Nodolaria spumigena CCY9414

Group name	Organisms with target (putative) haloperoxidase genes	Accession numbers of target gene products	Primer sequences fw rev	Expected product length (bp)
HaloCB	*Clostridium botulinum* (7 sequences) ***Bacillus (pseudo)mycoides*** (3 sequences)		**5'- AAGGCTTTACCWCATAAT-3'** **5'- ATATTGGYAGCAAGCTTA-3'**	1 194
Others (Bacteria)	*Chlamydomonas reinhardtii* strain CC503 *Nitrosospira multiformis* ATCC 25196 *Sagittula stellata* E-37 *Phaeobacter gallaeciensis* BS 107 *Bradyrhizobium* sp. ORS 278 *Bradyrhizobium japonicum* USDA 110 *Rhodopseudomonas palustris* HaA2 *Rhodospirellula baltica* SH1 *Sorangium cellulosum* 'So ce-56'	XP_001703431.1 YP_412388.1 ZP_01744290 ZP_02149712 YP_001203397 NP_773645 YP_487110 NP_864925 YP_001615269	5'- CCCNAGGCGCCGATGC-3' 5'- TGSAGTGGCCGGAGG-3'	< 1 159
Lfum	***Leptoxyphium fumago***	AAA33026	**5'-GCATTGGCTACCCATACGAC-3'** **5'-TGGTGGCGAGGAGGAATG-3'**	846
HaloNH	all bacterial perhydrolases except for sequences from *Mycobacteria*		**5'-GTTTCTMCAYGGCTGGCC-3'** **5'-GGAACRAYCTKRTCGTCA-3'**	662
Others (Fungi)	*Magnaporthe grisea* *Curvularia inaequalis* *Chaetomium globosum* CBS *Coprinopsis cinerea okayana*	XP_365508 X85369 XP_001220510 XM_001835341	5'- GASAYGGGCTACTTCTGG-3' 5'- ACRCCGACAAAGACGCG-3'	< 677

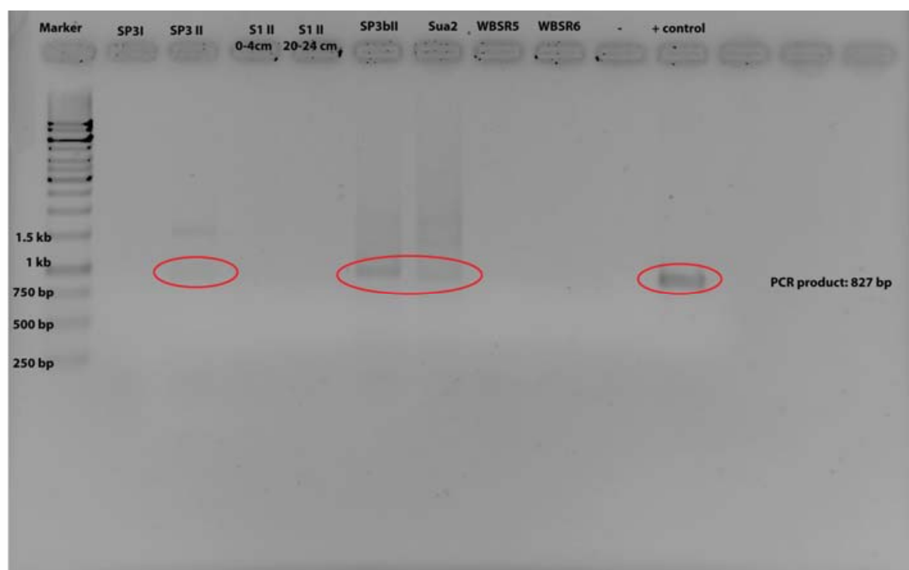
After optimization of PCR conditions using the respective primers on DNA extracts from the reference strains, PCRs were carried out with the DNA extracts from different salt pans and salt lake sediments listed in table 4.

Table 4: *Salt pans, salt lake sediments and slough samples DNA extracts of which were used as templates for PCR reactions with haloperoxidase-gene-specific primers designed in this study.*

Name of salt pan or salt lake	Geographic location	Sampling depth
Sua Pan 3I	S 20°28.953´ E 26°03.690´	0-4 cm
Sua Pan 3II	S 20°28.953´ E 26°03.690´	0-4 cm
Sua Pan 1II 0-4	S 20°27.200´ E 25°55.600´	0-4 cm
Sua Pan 1II 20-24	S 20°27.200´ E 25°55.600´	20-24 cm
Sua Pan3bII	S 20°28.953´ E 26°03.690´	0-10 cm
Sua2	S 20°26.256´ E 25°54.597´	0-10 cm
WBSR5	S 23°01.329´ E 14°27.186´	0-10 cm
WBSR6	S 23°02.785´ E 14°27.905´	0-10 cm
KasinI.1	N47°36.165' E047°27.129'	0-10 cm
KasinI.2	N47°36.161' E047°27.119'	0-10 cm
KasinI.3	N47°36.074' E047°27.286'	0-10 cm
Baskunchak	N48°23.751' E046°49.543'	0-10 cm
Elton	N49°09.057' E046°48.001'	0-10 cm
Iwik II	N19°54'09.2"; W16°17'15.6"	0-10 cm
Iwik III	N19°54'24.9"; W16°16'39.5"	0-10 cm
Sebkha N'Drameha 0-10	N18°51'17.3"; W15°38'24.5"	10-20 cm
Sebkha N'Drameha 10-20	N18°51'17.3"; W15°38'24.5"	10-20 cm
Elkhorn Slough	California	0-5 cm

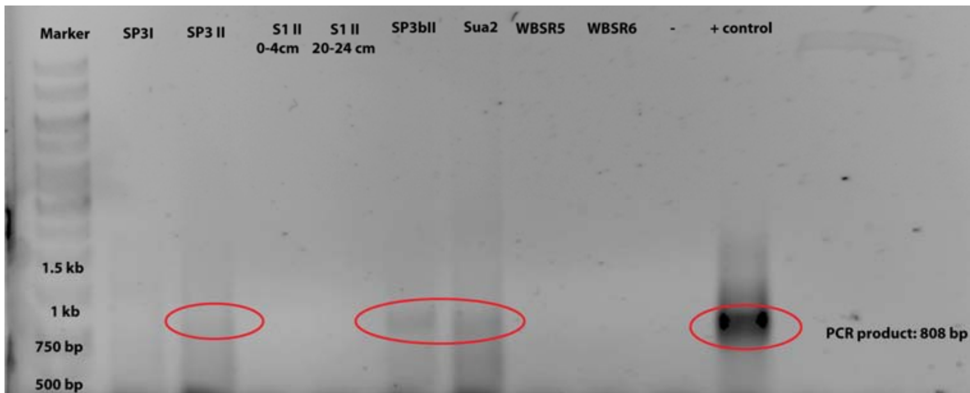
Figure 2 shows 4 different agarose gels (A to D) with the PCR products from diverse sets of PCRs using the different degenerate primers and DNA templates.

A)



B)

C)



D)

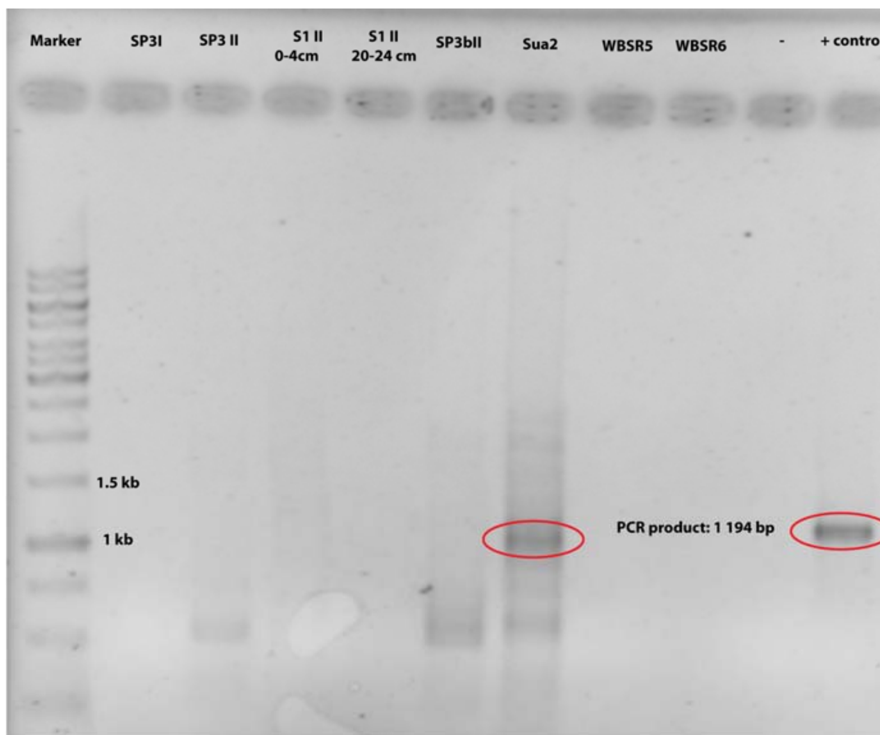


Figure2: *PCR products obtained with primers designed for haloperoxidase gene groups from A) Bac(illus) 1, B) L(eptoxyphium) fum(ago) , C) Strep(tomyces) 1 and D) HaloCB on DNA extracts from diverse salt lake sediments and salt pans.*

With primer pairs Cyano1, Cbot1 and HaloNH, no PCR products were obtained with any of the DNA extracts. For the DNA extracts from Mauretannian salt pans and Russian salt lake sediments, no PCR products were obtained with any of the haloperoxidase-specific primer pairs tested. Table 5 summarizes which putative haloperoxidase PCR products were cut out of preparative agarose gels, reamplified, purified again, ligated into pCR4 and cloned into *E. coli* TOP10 cells:

Table 5: *Clone libraries of putative haloperoxidase gene sequences amplified from salt pan, salt lake sediment and slough DNA extracts."Number of clones" refers to the number of white colonies that were transferred into 96-well-plates and stored as glycerol stocks.*

Origin of the DNA extract	Primer pair	Number of clones
Sua2	Bac1	16
Elkhorn Slough	Bac1	96
Sua2	Strep1	8
Sua Pan3bII	Strep1	30
Sua Pan 1II 0-4	Lfum	32
Sua2	HaloCB	96

3 clones from each library were sent for sequencing, and obtained sequences were analyzed by Nucleotide BLAST search. Table 6 lists the BLAST output obtained for the individual clones.

Table 6: *BLAST output of the best hits for the putative haloperoxidase gene sequences in the clone libraries listed in table 5. Hit sequence length and % of identical sites refer to BLASTX hits. Letter and number codes at the end of the clone names refer to the respective microtiter plates and positions therein in which these clones are kept as glycerol stocks.*

Clone	Best hit by BLASTN	Best hit by BLASTX (accession number)	Hit sequence length (amino acids)	% Identical sites
Sua 2_Bac1_IA1	lentiviral transfer vector			
Sua 2_Bac1_IB1	no significant similarity found	DNA-binding transcriptional repressor FabR [Marinobacter aquaeoli VT8] (YP_958979)	168	77.5
Sua 2_Bac1_IC1	no significant similarity found	DNA-binding transcriptional repressor FabR [Marinobacter aquaeoli VT8] (YP_958979)	164	78.2
Sua2_Strep1_IA4	no significant similarity found	phosholipid/glycerol acyltransferase [Alkalilimnicola ehrlichii MLHE-1] (YP_740858)	168	85.2
Sua2_Strep1_IB4	no significant similarity found	phosholipid/glycerol acyltransferase [Alkalilimnicola ehrlichii MLHE-1] (YP_740858)	173	84.0
Sua2_Strep1_IC4	lentiviral transfer vector			
Sua Pan3bII_Strep1_IA5	lentiviral transfer vector			
Sua Pan3bII_Strep1_IB5	no significant similarity found	Von Willebrand factor type A domain protein, precursor [Deionococcus deserti VCD 115]	282	45.6

		(YP_002787539)		
Sua Pan3bII_Strep1_IC5	no significant similarity found	Von Willebrand factor type A domain protein, precursor [Deionococcus deserti VCD 115] (YP_002787539)	124	44.9
Sua Pan 1II 0-4_Lfum1_IA9	no significant similarity found	Hypothetical protein NP6158A [Natronomonas pharaonis DSM 2160] (YP_327706)	151	90.7
Sua Pan 1II 0-4_Lfum1_IB9	no significant similarity found	no significant similarity found		
Sua Pan 1II 0-4_Lfum1_IC9	*Natromonas pharaonis* DSM 2160 plasmid PL131	Hypothetical protein NP6158A [Natronomonas pharaonis DSM 2160] (YP_327706)	151	90.1
Elkhorn Slough_Bac1_IIA1	no significant similarity found	Hypothetical protein CLONEX_01825 [Clostridium nexile DSM 1787] (ZP_03289623)	86	90.8
Elkhorn Slough_Bac1_IIB1	no significant similarity found	histidinol dehydrogenase [gamma proteobacterium NOR51-B] (ZP_04957580)	253	83.8
Elkhorn Slough_Bac1_IIC1	no significant similarity found	Hypothetical protein CLONEX_01825 [Clostridium nexile DSM 1787] (ZP_03289623)	171	100
Sua2_HaloCB_IIIA1	no significant similarity found	putative signal transduction histidine kinase [Haliscomenobacter hydrossis DSM 110] (YP_004448362)	275	26.9

Sua2_HaloCB_IIIB1	lentiviral transfer vector			
Sua2_HaloCB_IIIC1	no significant similarity found	putative signal transduction histidine kinase [Haliscomenobacter hydrossis DSM 110] (YP_004448362)	284	30.6

Unfortunately, none of the putative haloperoxidase sequences from our clone libraries tested could be identified as haloperoxidase sequence based on a BLASTN/BLASTX search nor did a BLASTP search of the BLASTX hits hit any putative haloperoxidase.

Discussion

The fact that we could not identify any haloperoxidase genes in our DNA extracts even though we had promising PCR products can be due to several reasons: First, we only sequenced three out of up to 96 clones obtained with each PCR product. Out of these 18 sequences, five turned out to be empty plasmid vector, which points to a methodological weak point of this study: by including a screening PCR with vector primers after cloning, these empty vectors could have been excluded from sequencing beforehand in favour of a higher number of clones that actually carry an insert. Sequencing a higher number of PCR products would have increased our chances to find haloperoxidase genes. However, it cannot be denied that our PCRs had produced a lot of unspecific amplificates even though surprisingly, these unspecific products were often very similar in length to the actually wanted amplificates. Considering this, we have to concede that the primers we had designed were too unspecific for amplifying haloperoxidase genes even though a BLASTN search of the primer sequences had been performed beforehand in order to exclude possible unwanted target sequences. According to this search, the only primer pair where both primers yielded identical non-target sequences among the 100 best hits was primer pair Strep1.

A second reason why we did not find any haloperoxidases probably is most likely due to the great sequence diversity of known haloperoxidases. Even on the amino acid level, both heme-dependent and vanadium-dependent haloperoxidases share less than 50 % sequence identity to the same types of enzymes produced by other species (van Pee, 2003). This is why we had to restrict our screening to an extremely limited number of haloperoxidases from about 30 bacterial strains and fungal species in total. Most microbial haloperoxidases which have been identified so far cannot be amplified with any of the primer pairs we used. Thus, the fact that we did not identify any haloperoxidase gene sequences in our environmental DNA extracts does not necessarily mean that there are no haloperoxidase genes present or active in the salt pans and salt lake sediments we studied.
Concerning sequence similarities, the case is different for the specifically halogenating $FADH_2$-dependent halogenases. Enzymes of this class share two conserved peptide motifs (Chen and van Pee, 2008) the corresponding DNA sequences of which could be used as possible targets for degenerate primers. As for α-ketoglutarate-dependent halogenases and methyltransferases, not enough enzymes are known to allow any general statement concerning sequence similarities within these groups.

In this study, we restricted ourselves to screen for microbial haloperoxidase genes in hypersaline environments and neglected all other groups of halogenating enzymes because we considered haloperoxidases the most promising candidates for the production of short-chain halogenated hydrocarbons due to their characteristic of performing non-substrate specific halogenations. However, theoretically short-chain halogenated hydrocarbons could also arise from the degradation of larger halogenated organic molecules such as antibiotics where halogenases are the agents responsible for the introduction of halogen atoms into the molecules. Even though they might only account for potential sources of methylhalides and no other VOX, it might be equally worth to extend the screen and analyze diversity and distribution of methyltransferases. To sum up, a far more extended PCR screen needs to be done in order to

cover a representative fraction of genes of halogenating enzymes before any statement can be made concerning their distribution, diversity and potential environmental significance in salt lake sediments. However, even if such a screen can help us to identify some haloperoxidase, halogenase or methyltransferase genes at some sites, studies with mRNA and enzyme activity assays will be needed before we can draw any conclusions about how microorganisms might contribute to VOX formation by the activity of their halogenating enzymes. Some facts considering the potential activities of the different kinds of halogenating enzymes in the environments: a prerequisite for the activity of haloperoxidases is the presence of hydrogen peroxide which occurs naturally up to concentrations of 60 µM and is mainly introduced into soils and sediments by rain water, even though it can also be produced by some microbial enzyme systems (Bengtson et al., 2009). Salt lakes including the ones our sediment samples have been retrieved from are usually located in arid regions with little precipitation (Hammer, 1986). This means that the concentration of hydrogen peroxide should be rather low in these environments and is thus likely to represent one limiting factor for haloperoxidase acitivity. Another limiting factor for haloperoxidase activity in neutral or slightly alkaline salt lake sediments could be pH. The pH optimum of the bromoperoxidase activity of the enzyme isolated from *Pseudomonas aureofaciens* by van Pee and Lingens (1985) is pH = 5.5 with about 10 % of its maximal activity left at pH = 7.5 (van Pee and Lingens, 1985). The vanadium-dependent haloperoxidase from *Curvularia inaequalis* also has a pH optimum of pH = 5.5 (Vanschijndel et al., 1993) while pH optima of other haloperoxidases are even lower e.g. with a value of pH = 4.0 for a heme-dependent chloroperoxidase from *Streptomyces toyocaensis* (Marshall and Wright, 1996). Both $FADH_2$- and α-ketoglutarate-dependent halogenases as well as methyl transferases, on the contrary, have been shown to display substantial or even optimal activity at circumneutral pH (Keller et al., 2000; Vaillancourt et al., 2005; Wuosmaa and Hager, 1990).

A further point to consider in order to estimate the potential activity of halogenating enzymes in salt lake sediments concerns the availability of substrates to be halogenated. In the case of

halogenases and methyltransferases, metabolites of the microbial metabolism such as α-ketoglutarate, tryptophane or S-adenosylmethionine are needed, which can be assumed to be produced directly by the microorganisms that express the halogenating enzymes. Haloperoxidases, on the other hand, produce free hypohalous acids. Consequently, the identity of the halogenated products that arise from the activity of haloperoxidases entirely depends on the identity of the molecules that surround them. If a lot of organic material is available in the proximity of active haloperoxidases, one can expect that substantial amounts of organohalogens will be formed. Unfortunately, the salt lake sediments and salt pans our samples came from are extremely poor in organic content (TOC often < 1 % of dry weight).

Considering these aspects, even if it seems to be an intriguing idea at the first glance, it is extremely challenging to find out whether the microbe-dependent VOX-release of pH-neutral, organic-poor and mainly anoxic salt-lake sediments can be explained by haloperoxidase activity. Since the activity of halogenases depends on oxic conditions, (Vaillancourt et al., 2006), methyltransferases, which do not necessarily need O_2 or even H_2O_2 for their activity seem to be a promising group of halogenating enzymes that should be considered as potential key catalysts for VOX formation in hypersaline environments.

Conclusions & Outlook

The discussion section illustrates two main tasks that need to be worked on in order to allow conclusions concerning the influence of microorganisms to natural VOX formation by enzymatic halogenations:

1) An improved method is needed in order to specifically target haloperoxidase, halogenase and methyl transferase genes in the environment. The most straightforward idea would be to perform an extended PCR screen including all three groups of halogenating enzymes expressed by microorganisms. Since we do already have 16S rRNA-based clone libraries for Bacteria and

Archaea from one of the field sites (Chapter 6), this does already provide some information about the phylogenetic groups of microorganisms that are present at this site. It might happen that the screen for halogenating enzymes gives us too many results in the first place. In this case, it would be a good idea to first only design primers specific for sequences that belong to groups of microorganisms of which we know that representatives are present in the environment we are looking at.

If, on the other hand, no haloperoxidase, halogenase or methyl transferase genes could be tracked by an extended PCR screen, a metagenomic approach might be more successful. This would include next-generation sequencing of total DNA extracted from a VOX-emitting field site. The resulting metagenome could then be checked not only for the presence of haloperoxidase gene-like sequences but also for genetic environments that are known to occur in proximity to halogenating enzymes. The latter information might help to judge whether a gene could encode a halogenating enzyme or not. At least for halogenases, a number of examples are known where the genes for these enzymes are embedded in antibiotic synthesis gene clusters (van Pee, 2003). Whether a candidate gene does indeed encode a halogenating enzyme would always need to be verified by cloning the sequence into an appropriate vector, overexpressing the gene of interest in a host organism and performing activity assays with the purified protein.

A completely different approach would be to look for the halogenating enzymes themselves instead of looking for their genes. This could be done by applying antibodies that bind specifically to microbial enzymes which are known to possess high halogenation activity. The way to do it would be a Western Blot against environmental protein extracts.

The advantage of this strategy would be that a positive result from a protein-based method would already imply that the genes of interest are actually expressed and thus include more information than any proof of presence of a gene encoding a halogenating enzyme. On the other side, the main disadvantage of using antibodies clearly lies in their complex and costly way of production and their lack of specificity in environmental protein extracts.

2) The key question that follows the detection of genes encoding halogenating enzymes in the

environment naturally is whether these genes are actually expressed and functionally active. If the proteins themselves were detected with antibodies, the second question would still need to be answered.

In order to quantify expression levels of haloperoxidase, halogenase or methyl transferase genes detected in environmental samples, one would need to extract RNA, reversely transcribe it to cDNA and determine the number of the transcripts of interest by qPCR. This method could even be used to find out under which conditions the genes of the halogenating enzymes are preferentially expressed by incubating the environmental samples e.g. at different temperatures, levels of water saturation etc. prior to RNA extraction.

An alternative to the RNA approach would be to assess the activity of unspecifically halogenating enzymes in different environments. In order to do this, one would have to prepare a protein extract from the environmental samples of interest. This extract would then have to be used in the monochlorodimedone (MCD)-assay which had been developed to detect unspecific halogenations activity (Morris and Hager, 1966) and (Hager et al., 1966). As shown by (Asplund et al., 1993), this assay is also applicable to extracts from environmental samples. An interesting continuative experiment in order to bridge the gap between knowledge about organisms and function with relation to haloperoxidases would be to add varying amounts of the strains from which haloperoxidase genes had been identified by PCR to the original environmental samples. These would then be incubated under conditions under which haloperoxidases had been active in the previous experiments and undergone protein extraction followed by a MCD assay. Finally, levels of haloperoxidase activity in the different samples could then be correlated with the amount of haloperoxidase gene-harboring cells that had been added. In the best case, this would allow to assess the relative contribution of the tested strains to unspecific halogenation processes in the particular environment the samples came from.

Unfortunately, the MCD assay is only suited to quantify the activity of unspecifically halogenating enzymes such as haloperoxidases since it relies on the halogenation of the MCD

substrate that causes a loss in absorbance at 278 nm (Hager et al., 1966; Morris and Hager, 1966). In order to measure the activity of specifically halogenating enzymes such as halogenases, it would be necessary to use their natural substrates (van Pee and Patallo, 2006) which are not known in most cases. This means that for an evaluation of the extent to which these enzymes contribute to VOX release from particular environmental samples, one would have to perform microcosm studies and incubate these samples under a set of different conditions. Alongside these incubations, GC-MS- or GC-ECD-based VOX-measurements should be performed over time. If MCD-assays were made for samples from the same timepoints as the GC-ECD-measurements, one could subtract the amount of VOX that are probably due to haloperoxidase activity from the total amount measured by GC-MS or GC-ECD. The residual amount of VOX could then be assumed to originate from degradation products of specifically halogenated organic compounds and thus to be due to the activity of halogenases and methyl transferases or arise from abiotic processes. Some final remarks should be made concerning the environments that are considered in search of active halogenating enzymes. Even though microbe-dependent VOX-formation has so far only been shown directly to occur in salt lake sediments (Weissflog et al., 2005), the low organic carbon content of many of these environments implies that substrates for unspecific halogenations by haloperoxidases might be lacking in salt lake sediments. Since, on the other hand, haloperoxidases are probably the best candidates to explain microbe-dependent VOX formation under environmental conditions that are beneficial for their activity, it seems reasonable to look for their presence and activity in environments that provide these conditions. These would include organic-rich and low-pH coniferous forest soils where first studies suggesting the activity of haloperoxidases have already been made (Asplund et al., 1993; Bastviken et al., 2009; Rohlenova et al., 2009) as well as organic- and Cl^--rich environments such as mangroves. Even though it remains a long way to go, following these ideas will undoubtedly bring us closer to our final goal, namely to link knowledge from bulk chlorination studies to insights about the mechanistics, activity and distribution of the individual halogenating enzymes.

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9

Evaluation of the potential of microbes in Russian salt lake sediments to reductively dechlorinate chlorinated ethenes

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Abstract

Chlorinated ethenes are widespread groundwater pollutants mainly of industrial origin. Some microorganisms can degrade these compounds by using chlorinated ethenes or other organohalogens as terminal electron acceptors for respiration in a process called reductive dehalogenation. For the most physiologically restricted genus of reductive dehalogenators, *Dehalococcoides*, no other metabolism is known they can grow on besides reductive dehalogenation. Since 1) these bacteria have also been found in pristine environments, 2) homologues of reductive dehalogenase genes from *Dehalococcoides* are abundant in seafloor sediments and 3) a vast number of organohalogens are produced naturally, also haloorganic compounds of natural origin might have served as original substrates for reductively

dehalogenating microbes such as *Dehalococcoides*. Salt lake sediments represent one environment where extensive release of highly chlorinated short-chain hydrocarbons has been observed. Therefore, we tested 1) whether *Dehalococcoides*-specific 16S rRNA genes can be detected in salt lake sediments from Southern Russia and 2) if reductive dechlorination of chlorinated ethenes takes place in microcosm experiments with lactate and acetate as electron donors. Both experiments gave negative results, which might either be due to the absence of reductive dechlorinators in the sediment studied or due to the lack of suitable conditions that stimulate the activity of dechlorinators. Consequently, further studies are needed before it can be ruled out that reductive dechlorination takes place in organohalogen-emitting salt lake sediments.

Introduction

Tri- (TCE) and tetrachloroethene (PCE) are highly lipophilic and chemically resistant chemicals, which makes them well suited compounds for many industrial applications such as degreasing, flame retardation and dry cleaning (Häggblom and Bossert, 2003). Their chemical recalcitrance has rendered PCE and TCE among the most common groundwater contaminants (Fawell and Nieuwenhuijsen, 2003). The solubility of PCE and TCE in water is relatively low (4.46 and 1.07 g/L^{-1} (Kirk and Othmer, 1979; Schwarzenbach et al., 2003)), while the density of these compounds simultaneously exceeds the density of water. Consequently, PCE and TCE tend to sink to the deeper layers of the aquifers and accumulate in anoxic aquifer regions. Due to the high toxicity and carcinogenicity of PCE and its degradation (by)products (Stevens and Eisenmann, 1997), extensive efforts have been made to decontaminate environments that are polluted with chlorinated ethenes. One of the strategies applied in this context is bioremediation. This implies either the addition (bioaugmentation) or the (bio-)stimulation of the indigenous chloroethene-degrading microorganisms at the contaminated site. Till date microbial degradation of chlorinated ethenes have found to occur by metabolic, cometabolic and other indirect processes initiated by microbial activity. (Smidt and de Vos, 2004). One way of metabolic degradation of

chlorinated ethenes that has been subject to extensive investigation comprises the use of these compounds as electron acceptors for anaerobic respiration, the so-called reductive dechlorination or halorespiration (Diekert et al., 1998; Furukawa, 2003). As shown in Figure.1, PCE is dechlorinated to TCE, 1,2 cis-dichloroethene and via vinyl chloride to harmless ethene by stepwise microbial reduction (Distefano et al., 1991; Vogel and Mccarty, 1985).

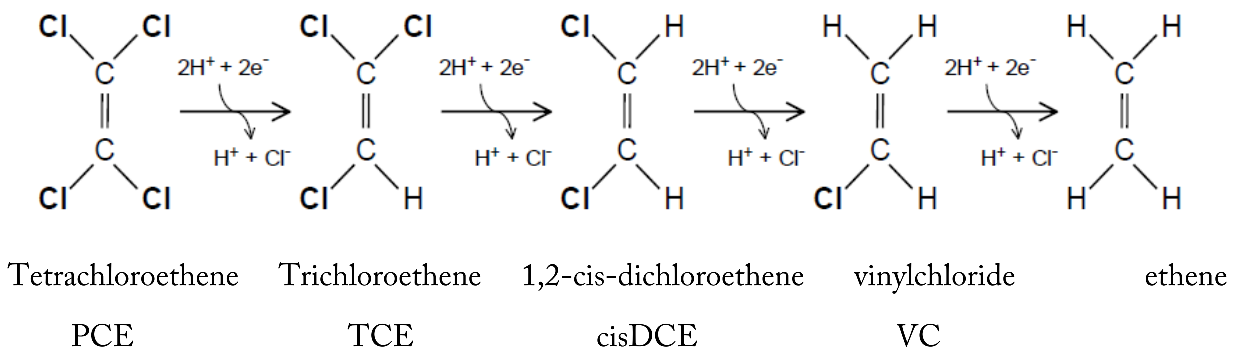

Figure 1: *Stepwise Reductive dechlorination of PCE to ethene.*

However, most bacteria that are capable of reductive dechlorination can only perform a subset of the reactions shown in figure 1. While a phylogenetically widely distributed variety of bacteria can reduce PCE to TCE (Deweerd et al., 1990; Fathepure and Boyd, 1988; Holliger et al., 1993) or one step further to cisDCE (Krumholz, 1997; Miller et al., 1997; Scholzmuramatsu et al., 1995), till date only few members of the genus *Dehalococcoides* have been identified that are capable of dechlorinating cisDCE to VC and ethene (Cupples et al., 2003; He et al., 2003; Loffler et al., 2003a; Loffler et al., 2003b; Maymo-Gatell et al., 1997). One explanation for this phenomenon is given by the decrease in standard redox potentials of chlorinated ethenes with decreasing number of chlorine residues, which renders their use as electron acceptors thermodynamically less and less favorable. Using H_2 as an electron donor, 163.57 kJ are released per mole of PCE reduced under standard conditions at pH=7, while this value decreases to 161.17 kJ mol^{-1} for the reduction of TCE and further to 141.17 kJ mol^{-1} for cisDCE under otherwise identical conditions (He et al., 2002).

The only genus known so far of which representatives can catalyze the two final reduction reactions and transform cisDCE into ethene is *Dehalococcoides*. This genus is associated with the deeply branching bacterial phylum Chloroflexi (McMurdie et al., 2009). The only organisms identified so far that can perform all reduction steps from PCE to ethene belong to this genus as well (Maymo-Gatell et al., 1997; McMurdie et al., 2009; Smidt et al., 2010). Members of the *Dehalococcoides* have a variety of *reductive dehalogenase homologous* (*rdh*) genes in their genomes (McMurdie et al., 2009) (Smidt et al., 2010). Within the four *Dehalococcoides* genomes that have been sequenced so far, between 11 and 36 different *rdh* genes have been identified (McMurdie et al., 2009; Wagner et al., 2009). The reductive dehalogenases (RDs) are heterodimeric membrane-anchored proteins. They are substrate-specific enzymes that catalyze the reductive dehalogenation of halogenated organic compounds (Smidt and de Vos, 2004). With the plethora of RDs that *Dehalococcoides* contain, their ability to dechlorinate is not just restricted to chlorinated ethenes but also includes polychlorinated dibenzo-p-dioxins (Fennell et al., 2004), PCB congeners (Bedard et al., 2007), chloroethanes (Edwards and Grostern, 2006) and chlorinated benzenes (Adrian et al., 2000).

Notably, the majority of *rhdA* genes of the sequenced *Dehalococcoides* strains cluster within highly unstructured or so-called "high plasticity regions" of the genome (McMurdie et al., 2009). Among the *rhd* genes, vinyl chloride-reductases and associated genes show distinct codon usage bias from the rest of the respective genomes (Spormann et al., 2007). Together with further findings, these observations have given rise to the assumption that *Dehalococcoides* might have acquired *rdh* genes by horizontal gene transfer (McMurdie et al., 2009). This is particularly intriguing since according to current knowledge, *Dehalococcoides* seems to be metabolically restricted to organohalide respiration with hydrogen as electron donor (Adrian et al., 2000; Loffler et al., 2005; Maymo-Gatell et al., 1997; Zinder et al., 1999). The obvious question that follows is what kind of metabolism did *Dehalococcoides* thrived on before notable amounts of anthropogenic organohalides entered the environment. Besides their presence which has been

observed and studied at many contaminated sites (Hendrickson et al., 2002), *Dehalococcoides*-like *Chloroflexi* have also been detected in pristine river (Loffler et al., 2000), tidal flat (Kittelmann and Friedrich, 2008) and hypersaline lakebed (Swan et al., 2010) sediments. Further, *rdh* genes have been identified in marine subsurface sediments by gene-specific PCR (Inagaki et al., 2009). The latter study was complemented by microcosm experiments which could prove that active dehalogenation of 2,4,6-tribromophenol and TCE took place in a slurry from a Japanese sediment sample that had been amended with these haloorganic compounds. Considering that 1) there are more than 4700 halogenated organic compounds for which natural sources have been identified, many of which with marine origin (Gribble, 2010) and 2) even substances that had been believed for a long time to be true xenobiotics such as vinyl chloride are produced naturally by terrestrial ecosystems (Keppler et al., 2002) and volcanic eruption (Jordan et al., 2000), it is tempting to speculate that haloorganic compounds of natural origin have served as ancestral substrates for microbial RDs (Smidt and de Vos, 2004). If this holds true, one would expect *Dehalococcoides* and other dehalogenating microorganisms to be present in environments where these compounds are produced in high concentrations, such as in salt lake sediments (Weissflog et al., 2005). Therefore, the goals of this study were to 1) test if *Dehalococcoides* can be detected in salt lake sediments from Southern Russia, 2) perform microcosm experiments where different chlorinated ethenes are added to sediment slurry and monitor reductive dechlorination and 3) analyze by a fingerprinting method such as denaturing gradient gel electrophoresis (DGGE) which strains increase in abundance in microcosms in which reductive dechlorination occurs.

Materials & Methods

Chemicals and media

All chemicals used were of analytical grade or higher. The medium used in the microcosm experiments contained 5 M NaCl, 100 µM $MgSO_4$ x 7 H_2O, 3 mM KCl, 100 µM KBr, 5 mM

NH_4Cl and 1.9 mM $MgCl_2$. Medium was prepared in a Widdel flask, autoclaved and cooled under a stream of N_2. When the medium reached room temperature, 15 mL L^{-1} of 1 M $NaHCO_3$ that had been autoclaved separately was added as a buffer as well as 1 mL each from the following 1000 x sterile stock solutions: 7-vitamin solution (Widdel and Pfennig, 1981), trace element solution SL-9 (Tschesch, 1984), 0.02 mM selenate-tungstate solution (Widdel, 1980), 1 M $CaCl_2$, 1 M $NaHPO_4$, 214 µM NH_4VO_3 and 500 mM $Na_2S_2O_3$. If necessary, pH was adjusted to a value between pH=7.2 and pH=7.4 with sterile and anoxic 1 M NaOH or 1 M HCl after adding all other ingredients.

Sampling and DNA extraction

Composite samples of the top 10 centimetres of sediment from three different salt lakes in southeast Russia (oblast Astrachan) were taken with a spatula and transported in UV-sterilized plastic bags to Tübingen, Germany. Table 1 shows the exact geographical location of the different sampling sites.

Table 1: *Location of the salt lake sediments the samples used in this study came from.*

Sample name	GPS coordinates	Description sampling site
KasinI.1	N47°36.165’ E047°27.129’	exposed lakebed sediment; ca. 50 m away from flooded area
KasinI.2	N47°36.161' E047°27.119'	sample taken under the water surface
KasinI.3	N47°36.074' E047°27.286'	exposed lakebed sediment; ca. 100 m away from flooded area
Bas(kunchak)	N48°23.751' E046°49.543'	exposed lakebed sediment; ca. 200 m away from flooded area
Elton	N49°09.057' E046°48.001'	exposed lakebed sediment; ca. 700 m away from flooded area

During transportation, samples were maintained at 4°C. In Tübingen, samples were sieved (2 mm diameter) and stored further either at 4°C in the dark (for the microcosm experiments) or frozen at -20°C (for DNA extraction).

For DNA extractions, 0.3 g of each sample was allowed to thaw and washed thrice with 1.5 mL of 10 mM Tris-EDTA buffer (pH = 7.0) by vortexing the sediment-buffer slurry and

centrifuging for 10 minutes at 7200 g. Supernatant from each washing step was combined and filtered through a 0.22 µm cellulose ester filter. Washed sediment samples and filters were taken into separate bead tubes of the PowerSoil DNA isolation kit® (MoBio Laboratories, Carlsbad, CA) and DNA was extracted according to the protocol of the manufacturer. Finally, the DNA extracted from filter and sediment originating from the same sample was eluted subsequently in 2 x 25 µL elution buffer provided in the kit into a single microcentrifuge tube.

PCRs

Diagnostic PCRs with 16S rRNA primers specific for *Dehalococcoides* sp. (DHC 1 fw, 5'-GATGAACGCTAGCGGCG-3', and DHC 1377 rev, 5'-GGTTGGCACATCGACTTCAA-3', (Hendrickson et al., 2002)) were set up as follows: 1x PCR buffer with 1.5 mM $MgCl_2$ final concentration (Promega), 200 µM dNTP mix (New England Biolabs), 400 nM of each primer, 2.5 U Taq DNA-Polymerase (Promega) and 2 µL of DNA extract as template were mixed with DNase-free water in a total reaction volume of 50 µL. DNA extracts from the *Dehalococcoides*-containing coculture VS (Cupples et al., 2003) served as a positive control. The following thermocycler program was used: initial denaturation at 95°C for 1 min; 30 cycles of denaturing (95°C for 1 min), annealing (55°C for 1 min) and elongation (72°C for 1 min) and a final elongation at 72°C for 10 min.

Setup of PCE- and cis-DCE-spiked microcosms

Table 2 gives an overview about the different sets of microcosms that were prepared for this study. For all sediment-containing setups, 19.5 g of 2 mm-sieved KasinI.1 sediment was filled into 100 mL serum bottles, flushed with N_2 for 30 minutes and closed with sterile butyl rubber stoppers. All further steps were performed in an anoxic glovebox using sterilized equipment and solutions. 54.5 mL of anoxic medium was added to the sediment-containing setups while the bottles for the PCE and cisDCE sorption control setups received 63.35 mL of medium in order to fill all bottles with an equal volume.

Table 2: *Overview about cisDCE- and PCE-spiked microcosms prepared in this study.*

Name setup series	Ingredients	Number of parallels
PCE sorption control*	medium, PCE	3
PCE abiotic control	medium, PCE, autoclaved sediment, sodium azide	4
PCE experiment	medium, PCE, sediment	4
cisDCE sorption control	medium, cisDCE	3
cisDCE abiotic control	medium, cisDCE, autoclaved sediment, sodium azide	4
cisDCE experiment	medium, cisDCE, sediment	4

*for quantification of sorption to glass and stopper

0.65 mL of an electron donor solution containing 500 mM of both lactate and acetate was added to every setup ensuring a final concentration of 5 mM of both organic acids. Abiotic controls were additionally amended with 0.26 g of sodium azide, giving a final concentration of 60 mM. Finally, 1 mL of 13 mM (PCE) or 26 mM (cisDCE) from stock solutions of chlorinated ethenes that were assumed to be completedly dissolved in water was added the respective culture flasks with a gastight glass syringe. After addition of the chlorinated ethenes, the bottles were immediately sealed with butyl rubber stoppers, crimped and gently mixed manually. Setups were taken out of the glovebox and incubated at 28°C in the dark. They were incubated for a day to obtain equilibrium before the first measurements were made. Figure 2 shows how the microcosms looked like after set up.

Figure 2: *Microcosms spiked with cisDCE (top row) or PCE (bottom row). The left bottles display(glass and stopper) sorption control setups. Setups in the center contain sterilized KasinI.1 sediment and setups in the right are microbially active.*

GC-MS-measurements

A gas chromatograph (TraceGC 2000, Thermo Finnigan, Milan, Italy), connected to a single-quadrupole-mass-spectrometer (Trace DSQ, Thermo Finnigan, Austin, TX, USA) was used for quantification of chlorinated ethenes (PCE, TCE, DCE, VC). The GC was equipped with a RTX-VMS column (0.32 mm by 60 m, film 1.8 µm; Restek corp, Bellefonte, USA) and a programmable temperature vaporizer (PTV) injector (Thermoquest, Austin, TX, USA). Bottles were slightly shaken before sampling. 100 µl of the headspace was manually sampled with a 1 mL luer lock syringe (Hamilton, Bonaduz, Switzerland) and manually injected into the gas chromatograph which was set to the following conditions: the injector temperature was 250°C. The injector was run at a split ratio of 1:10. Helium 5.0 (Air Liquide, Duesseldorf, Germany) was used as a carrier gas and run at a flow rate of 1.5 mL/min. The temperature of the transfer

line and the ion source were set to 250°C and 220°C, respectively. The temperature program started with an initial temperature of 40°C which was held for 5 min. This was followed by a steady temperature increase of 12°C per minute until a final temperature of 150°C was reached which was held for 2 minutes. The MS was run at a measuring frequency of 3.86 scan per second and a mass range of 943.1 amu. Xcalibur data system V 1.3 (Thermo Finnigan, Austin, TX, USA) was used for data analysis.

Calculation of chlorinated ethene concentrations

The concentrations of chlorinated ethenes in the samples were calculated based on standard calibrations obtained from known dilutions of PCE, TCE, cisDCE and VC. Diagrams showing the standard calibrations and the resulting formulas that were used for the calculations is included in the appendix. The solutions used as standards were prepared from a methanolic standard mixture that contained 500 mg/L of PCE, TCE and cisDCE and 25 mg/L of VC. In order to create a series of standards with increasing concentrations of chlorinated ethenes, different volumes of the methanolic standard mixture ranging from 0 to 1 ml were added to a total volume of 14 mL of medium in 20 mL glass vials which were sealed with Teflon-layered butyl rubber septa and metal crimp caps. By filling 20 mL vials with a total liquid volume of 14 mL it was ensured that the same liquid:headspace ratio was obtained in the standard solutions as in the experimental setups. Further, the same medium was used as the matrix in both cases ensuring equal ionic strength in all solutions. Thus, the same partitioning of the chlorinated ethenes between liquid and gas phase should be expected both in experimental samples and in standard solutions. Based on this expectation, it was not considered necessary to determine the exact amount of any of the chlorinated ethenes that was injected into the GC from the 100 µL headspace samples. Instead, calculations were performed based on signal intensities resulting from known (theoretical) total concentrations of PCE, TCE, cisDCE and VC in the standards that would prevail if these compounds were all completely dissolved in the liquid phase. Standard solutions were allowed to stand to obtain equilibrium for at least 1 h after preparation

and before measuring. Measurements of the standards were carried out in the same way as measurements of the experimental setups, i.e. by shaking the bottle before sampling 100 µl of the headspace was injected into the GC with a gas-tight syringe.
One complete measurement of 9 standards with different chlorinated ethene concentrations was performed at the beginning of the experiment. Calibration curves for all chlorinated ethenes were created based on results from this measurement and used for the calculations of PCE, TCE, cisDCE and VC concentrations in all samples measured throughout the duration of the experiment.

In order to correct for temporal fluctuations of the detector sensitivity, standards with 0 µL, 50 µL (corresponding to theoretical final concentrations of 37.5 µM VC and 14 µM PCE) and 100 µL (corresponding to theoretical final concentrations of 75 µM VC and 28 µM PCE) of methanolic mix standard in 14 mL total volume of medium were measured together with the samples from the experimental setups at every sampling time. Signal intensities resulting from these standard subsets were compared to signal intensities from the same standard concentrations that had been measured at the first standard measurement series. This resulted in the output of a daily variation factor. Signal intensities resulting from the measurements of samples from experimental setups were divided by this factor before respective concentrations of chlorinated ethenes were calculated. For the final calculations, the calibration curves that are shown in the appendix were used.

Results

Polymerase chain reactions with *Dehalococcoides* sp.-specific 16S rRNA gene primers did not result in the amplification of any PCR products from DNA extracts of five different salt lake sediments from Southern Russia (figure 3). Since PCR products could be obtained from these DNA extracts with primer pairs targeting general bacterial and archaeal 16S rRNA genes (Chapter 6) and a DNA extract from the *Dehalococcoides*-containing enrichment culture VS did

give a product using the *Dehalococcoides*-specific primers, this means that the amount of *Dehalococcoides* cells in the sediments under investigation was below the detection limit of this particular PCR.

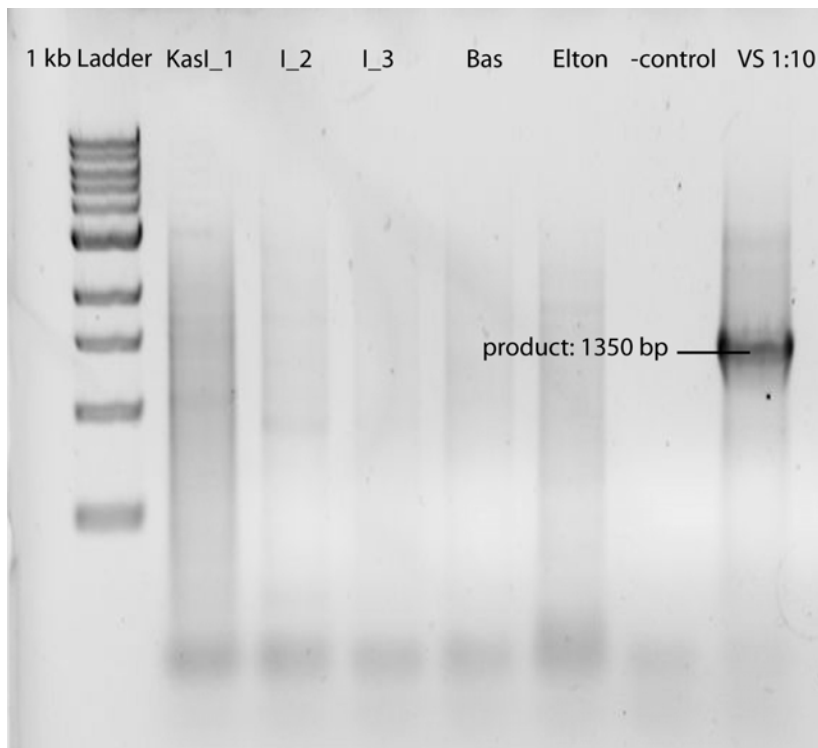

Figure 3: *Agarose gel showing PCR products obtained with Dehalococcoides-16S-specific primers on DNA extracts from Russian salt lake sediments.*

Figure 4 shows the concentration of PCE measured in the PCE-spiked microcosms over time. It is striking that only about 40 µM of PCE was measured at t=1d even though a calculated concentration of 200 µM was intended. The concentration of PCE measured in the microcosms decreases to below 10 µM within 50 days in all the live parallels. However, both controls with sterilized sediment and sorption controls without any sediment show exactly the same behaviour. Formation of TCE, cis-DCE or VC was not observed in any setup at any sampling time (data not shown).

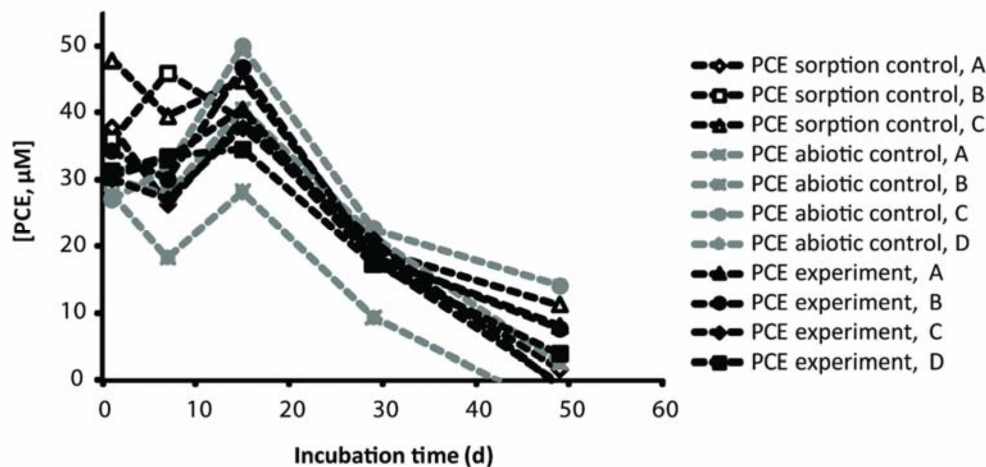

Figure 4: *Concentration of PCE in PCE-spiked microcosms with KasinI.1 sediment over time. Filled black symbols represent actual experimental setups. Open black symbols display sediment-free sorption controls and grey symbols show data from setups with sterilzed sediment. All setups were prepared in 5 M NaCl-containing medium and were amended with 0.5 M of both lactate and acetate. A, B, C and D refer to independent parallels of the same kind of setup.*

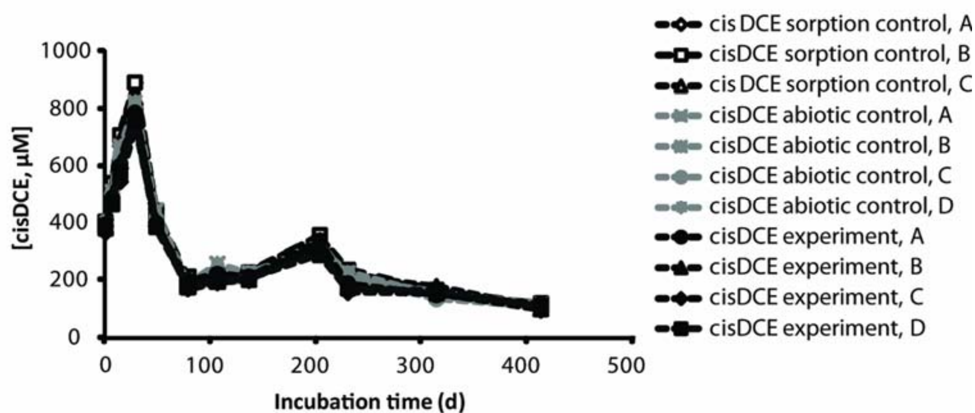

Figure 5: *Concentration of cisDCE in cisDCE-spiked microcosms with KasinI.1 sediment over time. Filled black symbols represent actual experimental setups. Open black symbols display sediment-free sorption controls and grey symbols show data from setups with sterilzed sediment. All setups were prepared in 5 M NaCl-containing medium and were amended with 0.5 M of both lactate and acetate. A, B, C and D refer to independent parallels of the same kind of setup.*

The microcosms spiked with cisDCE show a similar trend as the PCE-spiked bottles. cis-DCE concentrations measured over time are shown in figure 5. At t=1d, the expected concentration of

400 µM cisDCE could be measured in all setups. Apart from an increase until t=15d, the amount of cisDCE measured over time decreased constantly in all setups including abiotic controls. Formation of VC was not observed in any setup at any sampling time (data not shown). Interestingly, all biotically active microcosms but not the abiotic controls started to turn dark brown within a few weeks until they reached an almost blackish color. This color change was similar to the color change observed in Fe(III)-reducing enrichment cultures (Chapter 7).

Discussion

The concentrations of PCE and cisDCE in the microcosms decreased over time. This was observed in the experimental setups and sterilized as well as sediment-free controls. Thus, it can be concluded that microbial reductive dechlorination did not occur in our microcosms. This could be due to two main reasons: 1) either there were no microorganisms capable of reductive dechlorination in KasinI.1 sediment or 2) the conditions provided during the experiment were not favorable for the dechlorinators to be active or survive. Concerning the first scenario, we know from the results of our diagnostic PCR that *Dehalococcoides* is not present in KasinI.1 in significant numbers (figure 3). Unfortunately, (Hendrickson et al., 2002) did not provide exact detection limits of minimum numbers of *Dehalococcoides* 16S rRNA gene copies that need to be present in the reaction mixture in order to get a PCR product that is visible on a gel with the primer set we used. However, it was stated that detection limits of the different primer sets these authors used to detect *Dehalococcoides* varied between 10 and 1000 copies per reaction mixture (Hendrickson et al., 2002). This would correspond to a number of between 975 and 9.75×10^4 copies of *Dehalococcoides* 16S rRNA gene copies extracted per g dry sediment. Considering that the total number of cells is only about 4×10^7 per g dry sediment in the composite sample of the top 10 centimeters of KasinI.1 (Chapter 6), a detection limit of almost 10^5 *Dehalococcoides* cells (according to http://rrndb.mmg.msu.edu/search.php, all *Dehalococcoides* strains sequenced so far have only a single16S rRNA gene copy) seems pretty high and by no means a sufficient basis to

exclude the presence of *Dehalococcoides* in this environment. In a bacterial 16S rRNA gene-based clone library of KasinI.1, 15 out of 313 sequences were affiliated with the Dehalococcoidetes subphylum of the Chloroflexi (Chapter 6). The closest cultivated relative of all these clones is *Dehalogenimonas lykanthroporepellens*. Members of this species have been described as strictly anaerobic reductive dehalogenators of polychlorinated aliphatic alkanes which can only use H_2 as an electron donor. However, *Dehalogenimonas* is unable to use chlorinated ethenes as electron acceptors (Moe et al., 2009). In the light of these insights, it seems more rational to use polychlorinated aliphatic alkanes instead of or in addition to chlorinated ethenes as electron acceptors in the microcosm experiments. Unfortunately, the results of the clone library were only available after the completion of the microcosm experiments. In addition to the electron acceptors used, one might equally question the choice of lactate and acetate as electron donors. The idea behind the use of lactate and acetate was to induce fermenting bacteria in the soil that could release H_2 by fermentation (Fennell et al., 1997) (He et al., 2002). Even though cisDCE and VC are so far only known to be reductively dechlorinated by strictly hydrogenotrophic *Dehalococcoides* species (Cupples et al., 2003; Loffler et al., 2003a; Loffler et al., 2003b; Maymo-Gatell et al., 1997), PCE dechlorination turned out to be about five times more effective with acetate than with H_2 in a microcosm study where different electron donors for reductive dechlorination were compared (He et al., 2002). However, since we also had one series of microcosms set up with cisDCE, we could have added H_2 directly as an electron donor to one set of parallels in order to create more selective conditions to induce the growth of hydrogenotrophic cisDCE-dehalogenators. A further idea would have been to use propionate or butyrate. These volatile fatty acids are known to produce H_2 slowly and at low partial pressures (He et al., 2002). The advantage of a slow and steady release of H_2 would be the creation of a chemical environment under which reductive dechlorination outperforms other hydrogenotrophic processes such as methanogenesis and acetogenesis for thermodynamic reasons (McCarty and Yang, 1998; Sanford et al., 1999). In addition to the choice of the right electron donors and acceptors to stimulate the activity of potential reductively dechlorinating bacteria in the sediments

further geochemical conditions needs to be considered as well. The most crucial factor in our case is most certainly the high salinity of 5 M NaCl prevalent both at the field site and in our microcosms. To our knowledge, no information exists so far concerning the salinity tolerance of microorganisms that are capable of reductive dechlorination and the ability of these microorganisms to retain their metabolic activity. Concerning our specific field site, it has to be noted that no production of VOX has been measured from Kasin sediments (Chapter 4). This means that at least no chlorinated methanes, ethanes and ethens were available for reductively dehalogenating microorganisms. If this information had been available when the microcosm experiment was started, it would have made more sense to use the sediments from lake Elton instead of KasinI.1 since sediment from lake Elton could be shown to release considerable amounts of methyl chloride (Chapter 4).

Some questions concerning the quantification of chlorinated ethenes in our microcosms remain. Since the same loss of PCE and cisDCE over time was observed both in sediment-containing setups and in sediment-free controls, it can be excluded that these compounds adsorbed to the sediments to a large extent. However, since the same loss was observed both in experimental setups and in the sediment-free sorption controls, a considerable amount of the loss might be explained by sorption to the glass or to the butyl stoppers, even though this has not been a problem in previous studies where butyl stoppers were used as well (Loganathan, 2009). In addition, some abiotic degradation must have taken place. (Gorby et al., 2000) have shown with the example of tetrachlorocarbon that Fe(II) sorbed to Fe-minerals can reductively dechlorinate chlorinated hydrocarbons by abiotic methods. Since both poorly crystalline and crystalline Fe(II) and Fe(III) are present and together constitute almost 1 weight percent of KasinI.1 (Chapter 6) it is well feasible that PCE and cisDCE were abiotically degraded by a similar mechanism. However, this would not explain why the sediment-free microcosms also showed a decrease in chlorinated ethane concentrations. The color change to dark brown in the microbially active setups suggests that Fe(III)-reducing microorganisms were probably active in the respective bottles.

The concentration of PCE measured at t=1d was about 5-fold lower than expected. This was due to a wrong estimation of the amount of PCE that is soluble in water. Since methanol is toxic to many bacteria, we added the chlorinated ethenes from stock solutions prepared with water as the solvent. However, the water solubility of PCE is only 4.46 g or 0.74 mmol L^{-1} (Schwarzenbach et al., 2003), resulting in stock solutions that contained much less PCE as the intended 13 mM. Consequently, less PCE was added to the microcosm setups than planned.
In contrast to the trend of decreasing PCE and cisDCE concentrations over time, the concentrations determined for t=15 d exceed the concentrations of these compounds that were initially added, which can obviously not be the case. These high values can be linked to a particular small daily variation factor measured on this day, namely 0.39 for PCE and 0.6 for cisDCE. Dividing the peak areas recorded for PCE and cisDCE from the microcosm setup samples by these low values before calculating their respective concentrations resulted in extraordinarily high values for the latter. Even though this strongly suggests that something went wrong with the preparation of the standards on this particular day, I preferred to keep the normalized value in our dataset in order to be consistent with our quantification of chlorinated ethene concentrations.

Conclusions & Outlook

As it becomes clear from the previous sections, only the first two goals stated in the introduction could be fulfilled with this study. 1) *Dehalococcoides* sp. were not detected in salt lake sediments from Southern Russia and 2) neither cisDCE nor PCE could be used as electron acceptors in microcosm experiments with KasinI.1 sediment slurries. Since no microbial reductive dechlorination took place in the microcosms, it was not possible to determine which bacteria increase in abundance in microcosms in which this process occurs.
However, it is always easier to draw conclusions from a process that could be observed in an experiment rather than to conclude from not having observed a particular process that this

particular process cannot take place at all. Consequently, the fact that we did not observe microbial reductive dechlorination in our microcosms does not necessarily mean that this kind of metabolism does not occur in salt lake sediments. As stated several times in the discussion section, if certain information about KasinI.1 that were obtained later had been available when the microcosms were set up, it would have been possible to choose some experimental conditions slightly differently. Starting the microcosms with sediments that are known to emit volatile organohalogens (VOX) would have provided a much better basis for testing the hypothesis whether reductively dehalogenating microbes are present and active in environments where these compounds are produced in high concentration.

Consequently, the use of VOX-emitting sediment would be the most important factor to consider in case these experiments were to be repeated in the future. Further, one would need to make sure the electron acceptor added is indeed available in sufficient concentration. In addition, it would be interesting to expand the range of electron acceptors tested and include e.g. chlorinated methanes. The same holds true for possible electron donors. In a microcosm study with contaminated subsurface sediment where different electron donors were tested, molasses turned out to be a very efficient electron donor to stimulate reductive dechlorination of PCE (Loganathan, 2009). Besides molasses, molecular H_2, propionate and butyrate might be good candidates to create conditions under which reductive dehalogenators of chlorinated ethenes can be active, as it has been discussed in the previous section. Further, an interesting experiment would be to stepwise increase the salinity in the media used for reductively dehalogenating enrichment cultures. The goal of such an experiment would be to determine the respective limits in ionic strength reductively dechlorinating microorganisms can tolerate. This information would then provide valuable hints concerning the estimation of how active reductive dehalogenators might be in salt lake sediments.

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9

Evaluation of the potential of microbes in Russian salt lake sediments to reductively dechlorinate chlorinated ethenes

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APPENDIX

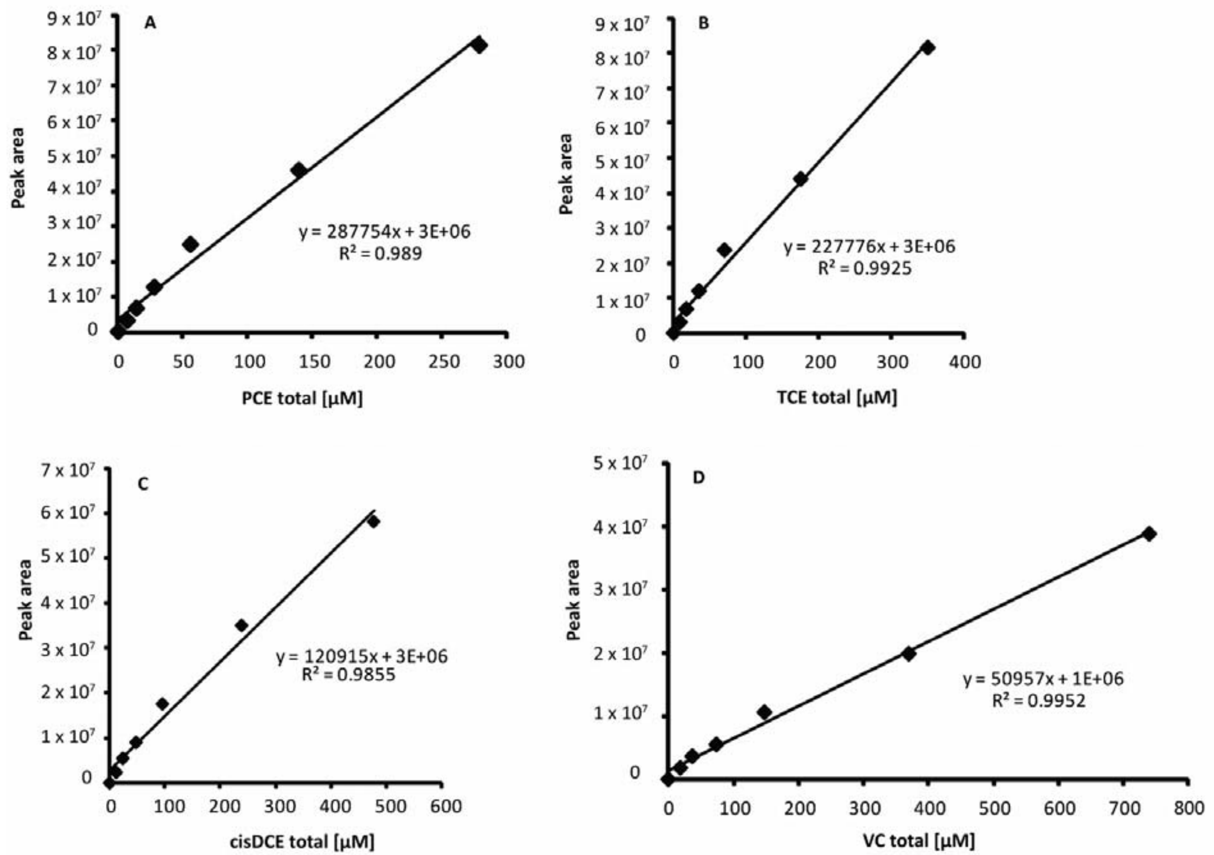

Figure A1: *Calibration curves and linear regression used for the calculations of total concentrations of A) PCE, B) TCE, C) cisDCE and D) VC in the microcosm setups.*

Conclusions and outlook

It was the goal of this study to evaluate both direct and indirect ways how microorganisms could potentially contribute to natural VOX formation. This process has repeatedly been observed in soils of low pH (Huber et al., 2009; Keppler et al., 2006; Keppler et al., 2002) as well as in salt lake sediments (Weissflog et al., 2005). One conceivable way how microorganisms could indirectly stimulate VOX release from these environments is by contributing reactive Fe phases and organic radicals that could then undergo abiotic reactions with halides leading to the formation of VOX. Therefore, a lot of experiments were carried out with acidophilic and halophilic Fe(III)-reducing microorganisms. The main achievements that resulted from these experiments were as follows:

- it was shown that *Acidiphilium* SJH which was used as an example of an acidophilic Fe(III)-reducing strain can neither reduce humic substances nor induce the formation of semiquinone radicals. From this it can be concluded that the general understanding that "all Fe(III)-reducing microorganisms can also reduce humic substances" is not universally true (Chapter 5).

- the presence of anaerobic Fe(II)-oxidizing and Fe(III)-reducing as well as HS-reducing microorganisms in different salt lake sediments was demonstrated and these groups of microorganisms were proven to be active in highly saline medium (Chapters 6 and 7). This implies that there could be an active microbial cycling of Fe taking place in environments with up to 5 M NaCl salinity.

- the most abundant strains (*Halobaculum gomorrense*, *Desulfosporosinus lacus*, *Bacillus alkalidiazotrophicus* and *Anaerobacillus alkalilacustre*) in Fe(III)-reducing enrichment cultures from salt pans and salt lake sediments that were incubated at different salinities were identified and their abundance in their natural environment was quantified (Chapter 6).

- it was shown that the addition of the neutrophilic Fe(III)-reducing strain *Shewanella oneidensis* MR1 to methyl chloride-emitting sediment from salt lake Elton does not increase the amount of methyl chloride that is emitted from freeze-dried and milled sediment (Chapter 4). From this it can be concluded that the Fe(II) and organic radicals formed by this Fe(III)-reducer do not stimulate methyl chloride formation in lake Elton sediment.

From the results of further experiments that were performed in the scope of this study

- the presence and activity of chlorinated ethene-respiring microorganisms in the sediment of salt lake Kasin could be excluded with a very high probability, suggesting that at least dehalorespirers who use this group of VOX as their natural substrate do not play any role in this environment (Chapter 9), and
- the presence of some groups of halogenating enzyme-encoding genes in 18 salt lake sediments tested could almost certainly be excluded (Chapter 8).

Regarding the last point, it has to be admitted that the genetic diversity of these groups of genes turned out to be too high to investigate more than a small subset of them in the scope of a subproject of this PhD thesis. Consequently, it will be the focus of a new PhD thesis to examine the presence and activity of genes for halogenating enzymes in salt lake sediments more closely.

With regard to the possible ways in which microorganisms could influence natural VOX formation in the terrestrial environment illustrated in figure I.4, the results obtained in this study suggest that it is not very likely that acidophilic Fe(III)-reducers stimulate this process indirectly by creating reactive Fe phases and organic radicals (Chapter 5). In previous batch experiments, the addition of dissolved Fe(III) led to the formation of abiotic VOX (Keppler et al., 2006; Keppler et al., 2002). When Fe(III) is provided in dissolved form, the Fe^{2+} that results from microbial reduction of Fe^{3+} at acidic pH will exist either as a free or complexed ion in solution, but there will not be any Fe minerals available for Fe^{2+} to sorb to. However, it had been shown in the past that particularly the surface of Fe(III) or to mixed-valent Fe(hydr)oxides serves as a

catalyst that stimulates sorption of Fe(II) which acts as a strong reductant (Amonette et al., 2000; Buchholz et al., 2011; Elsner et al., 2004). Since these kinds of reactive Fe phases do not form when an acidophilic Fe(III)-reducing bacterium is incubated with dissolved $Fe(III)_2(SO_4)_3$, and since at least the strain we used (*Acidiphilium SJH*) can additionally not transfer any electrons to humic substance molecules and cause the formation of semiquinone radicals, there is no possibility for microbial stimulation of natural VOX formation in such a system. However, it might be worth testing whether the reduction of Fe(III) minerals that remain stable at pH 4 and below, such as goethite and magnetite (Heron et al., 1994; Poulton and Canfield, 2005), is possible by other strains such as *Acidiphilium* SJH as demonstrated before (Bridge and Johnson, 2000), and whether this leads to the formation of reactive Fe phases that can stimulate VOX formation in batch setups.

In chapter 4 it was shown that the addition of very high cell numbers of the neutrophilic Fe(III)-reducer *Shewanella oneidensis* MR1 to methyl chloride-emitting salt lake sediment did not stimulate further emission thereof (Chapter 4). That is why the hypothesis of the indirect way of microbial stimulation of natural VOX formation in pH-neutral salt lake sediments is challenged. It has to be pointed out, though, that it is always more difficult, if not impossible, to rule out that a particular process can take place under any conditions than to show that a process does take place if one is lucky enough to find suitable conditions for the process to occur. For this reason, we cannot exclude that microorganisms can indirectly stimulate natural VOX formation by providing reactive Fe phases and organic radicals under any conditions just because this did not happen under the conditions we applied. Consequently, more experiments need to be done in which the influence of different factors (e.g. adding different kinds of Fe(III) phases, organic material in various forms, different bacterial strains etc.) is tested systematically to allow a more precise estimation under which conditions such a process would be feasible and under which conditions it can be excluded. For the direct contribution of microorganisms to VOX emissions via the activity of halogenating enzymes, the case is different, since the release of methyl iodide,

methyl bromide and methyl chloride by methyl transferase-expressing fungi (Harper, 1985; Wuosmaa and Hager, 1990), algae (Wuosmaa and Hager, 1990), bacteria (Amachi et al., 2001) and archaea (Amachi et al., 2001) has already been demonstrated in a number of studies. So it is known that this process does take place, even though great uncertainty exists concerning its significance on a global scale. Unfortunately, it was not possible to create much more clarity in this question in the scope of this study. However, it was still an achievement of this study to set the basis for a comprehensive investigation of the influence of the activity of different classes of microbial halogenating enzymes on global VOX emissions by tackling some groups of genes for these enzymes in an exemplary way using a PCR screen with degenerate primers.

Major drawbacks of this study included I) analytical challenges and II) the lack of samples from a field site that is known to emit VOX. Concerning I), the GC-MS system that was used for identification and quantification of VOX throughout this study was not available due to technical problems for almost a year in the beginning of this PhD work. Further, the detection limits of this device for different VOX were significantly higher than those of the instrument used by (Keppler et al., 2002) and (Keppler et al., 2006), which was probably one of the reasons why the results from abiotic formation of e.g. vinyl chloride achieved by (Keppler et al., 2002) could not be reproduced in this study (Chapter 4). In absence of a reliable analytical system that allows tracing of VOX in the relatively low concentrations in which they are produced by natural processes including biotically stimulated ones, it is simply not possible to define any conditions under which microbially stimulated natural VOX formation takes place. Concerning II), at the timepoint this work started, it was known from one study of sediments from six different salt lakes in the same geographical area that microbially dependent release of VOX can take place in this kind of environment (Weissflog et al., 2005). Even though one of our sampling campaigns led us to a region that was geographically close to these salt lakes in Southern Russia, we chose to examine previously uninvestigated field sites and sampled sediments from different salt lakes in the same area. There was no analytical possibility available in the field to check if the sediments

we sampled emitted VOX. The only way a pre-screening of sediments for VOX-emission could be performed would require the vicinity of a highly sensitive GC-MS at a research institution close to the potential sampling site(s). Since this was not available in the Kalmykian steppe, we had to decide which sampling sites to focus on based on parameters that could be determined in the field, e.g. a high Fe content. The obviously high Fe content together with the distinguishable vertical heterogeneity of the sediment profile of KasinI.1 made me decide for this sediment as my main sampling site. In order to enable various kinds of analyses, I took about 2 kg of sample from KasinI.1 sediment which diminished my capacity to take similar amounts of sample from other sites due to limitations in transportation capacities. Unfortunately, when KasinI.1 sediment was later analyzed in the lab, no VOX emissions could be detected by GC-MS (Chapter 4). This means that samples from this sediment, which I mainly worked with during this study, were not suited to investigate the influence of microorganisms on VOX formation. A second sampling campaign to the salt lakes that had been found to emit VOX was not possible in the scope of this PhD thesis due to logistic and time constraints. However, the availability of field samples that do emit VOX is a fundamental prerequisite in order to be able to analyze how microorganisms influence this process in the environment in any way. This is why, in order to save resources, I would strongly recommend to first I) invest in setting up a reliable GC-MS system with an extremely low detection limit for VOX comparable to the one (Keppler et al., 2002) used and II) screen different salt lake sediments and maybe also other environments until one or several sites are found in which VOX-release is clearly measurable before focusing further on the role of microorganisms in natural VOX formation. Further environments that might be worth considering in this context include forest soils of low pH as well as mangroves. In contrast to the salt lake sediments investigated here with an extremely low organic carbon content of mostly below 1% (Chapter 7), the organic carbon content of both forest soils (Buringh, 1984) and mangroves as they occur along ocean coastlines throughout the tropics (Donato et al., 2011) is significantly higher, ensuring the availability of sufficient carbon substrate for chlorination. I would recommend investigating forest soil of low pH, since haloperoxidases have been shown to

be most active at pH values between pH 4 and 5.5 [*Marshall and Wright*, 1996; *van Pee and Lingens*, 1985; *Vanschijndel et al.*, 1993], and extracts with haloperoxidase-like activity have been obtained from spruce forest soil in the past (Asplund et al., 1993). Since several recent studies have strongly suggested that the transformation of Cl_{in} to Cl_{org} that takes place in forest soil is mediated by microbial enzymes (Bastviken et al., 2009; Rohlenova et al., 2009), I would find it very interesting to follow up on this path and try to identify both microorganisms and enzymes that are responsible for this process. Mangrove forests are more favorable for this study compared to salt lakes, since they also represent highly saline systems such as salt lakes, but in contrast to the latter ones, a high organic carbon content is ensured. Since mangroves can be found along the tropical coasts of all major oceans (Donato et al., 2011), there should be no shortage in possible field sites to test.

After a decision for a VOX-emitting field site of focus for a future investigation has been made, I would further recommend doing a 16S rRNA gene-based clone library analysis first to get an idea about which kinds of microorganisms are present at this particular site. This knowledge could then be of valuable help during the planning of further experiments or analyses such as PCR screens for different groups of halogenating enzymes since there would already be some basic information available as to enzymes of which phylogenetic groups of microorganisms would be more or less likely to be found in this particular environment.

Overall, this study represented pioneer work in the field of microbial influence on natural formation of volatile organohalogens. Three different groups of microorganisms (acidophilic Fe(III)-reducers, neutrophilic Fe(III)-reducers and halogenating enzyme-expressing microorganisms) were investigated with respect to this feature, and both groups of Fe(III)-reducers were found to be rather unlikely to indirectly stimulate VOX formation in the environment. However, further studies need to be done to draw clearer conclusions. This thesis includes several suggestions of directions to go in order to bring more light into the question of the (micro)biotic sources of this fascinating class of compounds.

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Statement of personal contribution

The work described in this thesis was funded by the research unit 763, “Natural Halogenation Processes - Atmosphere, Soil” funded by the German Research Foundation (Deutsche Forschungsgesellschaft, DFG), whose aim it is to elucidate mechanisms of natural formation of halogenated products. This thesis constitutes a subproject within this scientific frame assessing the direct and indirect influence of microorganisms on the formation of volatile organohalogens. The conceptual background to this project was designed by Prof. Dr. Andreas Kappler, who was the main advisor throughout this project. Dr. Sebastian Behrens acted as an additional scientific advisor in some parts of this project. Unless otherwise stated, experiments were either designed together with Prof. Dr. Andreas Kappler, Dr. Sebastian Behrens or by me alone and carried out by me. The final analysis and discussion of the results, as well as all manuscripts, were completed in cooperation with Prof. Dr. Andreas Kappler and Dr. Sebastian Behrens, as indicated in the headers of the individual chapters of this thesis.

The main exceptions are as follows:

In chapter 4, gas chromatography-mass spectrometry (GC-MS) measurements and data analysis thereof were performed partly in collaboration with Dr. Stefan Huber, Ines Mulder and Torsten Krause and partly by Dr. Stefan Huber alone. Methane measurements and analysis thereof were performed in collaboration with Annegret Walz.

In chapter 5, GC-MS measurements and data analysis thereof were performed by Dr. Stefan Huber. Electro spin resonance (ESR) spectroscopy measurements were carried out by Dr. Andrea Paul. Figure 4A was developed from a sketch designed by Dr. Jie Jiang.

The most probable number experiment and first transfers to fresh media of enrichment cultures from Russian salt lake sediments grown in medium with 0.5 M NaCl as well as the molecular work underlying the construction of the 16S rRNA gene-based clone library described in chapter

6 were carried out as Master thesis project of Ankita Bhansali, to whom I acted as a co-supervisor. Mössbauer spectroscopy and analysis of the spectra were performed by Dr. Christian Schröder. Sequential Fe extractions and measurements of Fe concentrations in the respective samples were performed by Eduardo Marquez who did this under my supervision. Karin Stögerer contributed with considerable help during routine molecular work and Dr. Tina Lösekann-Behrens helped me with the software analysis of the clone library data. Dr. Karsten Kotte was the main organizer of the sampling campaign to Russia, where the samples analyzed in this chapter came from.

The most probable number experiment and first transfers to fresh media of enrichment cultures from Russian salt lake sediments grown in medium with 0.5 M NaCl described in chapter 7 as well as molecular characterization of these enrichments were carried out by Ankita Bhansali in the scope of her Master thesis. Odeta Shuti helped me with some Fe measurements for the determination of Fe(III) reduction rates of selected enrichment cultures. Dr. Karsten Kotte was the main organizer of the sampling campaign to Russia, where some of the samples analyzed in this chapter came from. Samples from Namibia and Botswana were taken by Dr. Karsten Kotte.

Dr. Christine Laskov, Dr. Thomas Wendl and Daniel Buchner helped me to design the microcosm experiment described in chapter 9.

Curriculum vitae

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Education

2008-2011	Ph.D. at the Eberhard Karls Universität Tübingen, supervisor: Prof. Dr. Andreas Kappler
2010	Hopkins Microbiology Course, Stanford University in Monterey, CA, USA
2002-2008	Diploma in biology at the Ruprecht Karls Universität Heidelberg Thesis: "Analysis and modification of specificity of the chemotactic pathway of *Escherichia coli*", supervisor: Prof. Dr. Victor Sourjik
2002	Abitur

Publications

Emmerich, M., Kappler, A. (2011)	Absence of microbial humic substance reduction at acidic pH: implications for stimulation of natural organohalogen formation and for the mechanism of acidophilic Fe(III) reduction. Biogeochemistry, in press.
Emmerich, M. et al.	Phylogeny and distribution of Fe(II)-oxidizing and Fe(III)-reducing microorganisms in salt lake sediments of Southern Russia, submitted.

Presentations and posters

2011 International Symposium of Subsurface Microbiology (ISSM), presentation: “*Diversity and distribution of Fe(II)-oxidizing and Fe(III)-reducing microorganisms in salt lake sediments of Southern Russia*”

2011 Jahrestagung der Vereinigung für Allgemeine und Angewandte Mikrobiologie (VAAM), poster: “*Diversity and distribution of Fe(II)-oxidizing and Fe(III)-reducing microorganisms in salt lake sediments of Southern Russia*”

2010 Symposium at the soil chemistry group at the Eidgenössische Technische Hochschule Zürich, presentation: *“Fe biogeochemistry and organohalogen (trans-)formation in Russian salt lake sediments”*

2009 Goldschmidt conference, poster: *“Stimulation of natural organohalogen formation by microorganisms”*

2009 Biannual congress of the Federation of European Microbiological Societies (FEMS), poster: *“Characterization of microbial communities in organohalogen-emitting South African salt lake sediments”*

2008 RAISEBIO summerschool “Chemicals in soil”, poster: *“Formation of volatile organohalogens stimulated by microbially activated Fe minerals and humic substances”*

Teaching

2011 Teaching assistant at the Hopkins Microbiology Course, Stanford University in Monterey, CA, USA

2010 Supervision of a Master student

2009-2010 Tutorial „Biology for Geoscientists“

2008-2009 Supervision of students during a „Lab course for Geoscientists”