Iron Reduction in Freshwater and Saltwater Environments

Nicole Tobler*

*nicole.tobler@eawag.ch

Swiss Federal Institute of Technology Zurich and Swiss Federal Institute for Environmental Science and Technology, Switzerland

Microbial Diversity Summer Course 2003
Woods Hole

Introduction

Iron is one of the most abundant elements in Earth's crust and it naturally exists in two oxidation states, ferrous (Fe^{2+}) and ferric (Fe^{3+}) iron. Ferrous iron forms a variety of different minerals under anoxygenic conditions (for example siderite, vivianite, iron sulfide and pyrite), but if oxygen is present, ferrous iron is stable only under acidic conditions. At neutral pH it is rapidly oxidized to ferric iron (Schwertmann and Fitzpatrick, 1992). Ferric iron forms a variety of different minerals which are hardly soluble under any environmental relevant conditions.

Nonenzymatic processes were generally considered to account for most of the Fe(III) reduction in subsurface environments until recently. The importance of dissimilatory ferric iron-reducing bacteria in the biogeochemical cycling of iron was recognized in the last decade (Lovley, 1997). These organisms gain energy by coupling the oxidation of organic compounds or hydrogen to the reduction of ferric iron oxides. In sediment and soils ferric iron is one of the most important electron acceptors. Only in marine sediments it is counterbalanced by the high concentrations of sulfate (28 mM).

Fe(III) reducing microorganisms show a wide diversity and fall into a number of different phylogenetic groups. They have been isolated from a variety of different marine and freshwater environments (Lovley, 1997; Thamdrup, 2000). The most intensely studied genera are Geobacter and Shewanella.

Although these organisms have been studied for a long time, the mechanisms by which they reduce Fe(III) are not well understood. Their primary electron acceptors are iron oxides which are highly insoluble. Consequently the major question is how they access and reduce them (Lovley, 1997). The most obvious strategy is to live in close association with iron particles and they often are observed in that way (Lovley, 1991). Another strategy is the use of a ferric iron chelator in order to solubilize Fe(III) or the use of an external electron shuttle, which is reduced by the bacteria and then shuttles the electrons to the Fe(III) and gets recycled. There is some evidence that iron reducing bacteria are producing electron shuttles. Shewanella putrefaciens was shown to release a menaquinone-related redox active small molecule into the medium (Newman and Kolter, 2000) and in cultures of Geobacter sulfureducens substantial concentrations of a small periplasmic c-type cytochrome are detectable. This cytochrome is rapidly oxidized in the presence of iron oxides (Seeliger et al. 1998). The intentional production of electron shuttles by bacteria is heavily under debate as there are a lot of uncertainties in experimental set-ups. However, it has been shown that iron reducing bacteria as well as fermenting bacteria can use naturally occurring humic acids as electron shuttle to reduce ferric iron (Lovley et al., 1996; Benz et al., 1998). This is especially important in sediments with high concentration of natural organic matter.

The goal of this study was to compare microbial iron reduction in freshwater and saltwater environments. Experiments were set-up to get an idea of abundance, diversity and activity of iron reducers in these environments. A new approach using an agar plug assay was tested on enrichment cultures in order to test them for the production of electron shuttling compounds.

Materials and Methods

Sampling sites

A freshwater and a saltwater environment were sampled for this project. The freshwater sampling site was School Street Marsh, located near Eel Pond in Woods Hole, MA. The sample was taken from the deeply red colored sediments forming on the pavement of School Street. The iron which gives these sediments their indicative red color is leaking down from a corroding staircase located next to the street.

The saltwater sampling site was Great Sippewissett Saltmarsh, Woods Hole, MA, a tidal marsh area, which is renowned for its microbial mats. A piece of microbial mat was sampled and taken back to the laboratory. The pink layer of this mat was then used for all further experiments with this sediment. The pink layer was chosen because it seems to be the place most likely to harbor iron reducers. They are certainly found above the sulfate reducing zone because of the higher redox potential and when assuming an iron cycle with phototrophic iron oxidizers the iron cycling will take place in the phototrophic zone of the sediment.

Medium composition

Freshwater and saltwater basal mineral medium for iron reducing bacteria was prepared according to the course handouts. A 30 mM bicarbonate buffer is used. The freshwater medium contained 1.0 g/l NaCl, 0.4 g/l $MgCl_2*6H_2O$, 0.15 g/l $CaCl_2*2H_2O$, 0.2 g/l KH_2PO_4, 0.5 g/l KCl and 0.25 g/l NH_4Cl. The saltwater medium was the same except for the concentration of NaCl (20 g/l) and $MgCl_2*6H_2O$ (3.0 g/l). Both media were amended with 0.1% of the 7-Vitamin solution and 0.1% of the trace element solution SL12 which contains EDTA.

Freshwater and saltwater buffer, used in the iron reduction activity assay consisted for freshwater of 1.0 g/l NaCl, 0.4 g/l $MgCl_2*6H_2O$ and 0.5 g/l KCl, which is basically the above described basal mineral medium without nitrogen and phosphate source. The saltwater buffer consisted of 20.0 g/l NaCl, 3.0 g/l $MgCl_2*6H_2O$ and 0.5 g/l KCl. No additional trace elements or vitamins were added.

Amorphous iron(III) hydroxide was prepared according to the course handouts. The concentration of iron in the used stock solution was roughly 670 mM Fe(III).

Enumeration procedures (direct counting and most probable number assay)

For direct counting 0.5 g of freshly collected sediment was immediately fixed in 1.5 ml of 1.5% formaldehyde in PBS for up to 24 hours at 4°C in the dark. Then the sample was washed three times with PBS by centrifuging the sample for 5 minutes at 10'000 rpm, discarding the supernatant and adding another 1.5 ml of PBS. After the third washing step the pellet was stored at –20°C by adding 1.5 ml of 1:1 ethanol/PBS until further processing. Then the sample was resuspended and a 100 fold dilution was made in 1:1 ethanol/PBS. This dilution was then sonicated for 20 seconds at low intensity using 1-second sonication pulses. A quarter of this dilution was then added to roughly 15 ml of PBS and then filtered through a 0.2 μm GTTP Isopore membranefilter (Millipore, Bedford, MA). The filter was air-dried and then parts of it were stained with 1 μg/ml DAPI for 3 minutes, destained for another 3 minutes in destilled water, shortly incubated in 80% ethanol and then air-dried. Afterwards the bacteria on the filter pieces were counted under the fluorescent microscope. The counting was done for four different pieces of the filter. Around 500 microorganisms were counted per filter piece and then the average was taken.

The most probable number approach chosen for this experiment is depicted in Figure 1. First 1.0 g of sediment was tenfold diluted in basal mineral medium. 1 ml of this suspension was given to another 9 ml of basal mineral medium supplemented with 40 mM amorphous ferric hydroxide and 5 mM acetate as well as 5 mM lactate. The cells were diluted out up to a dilution factor of 10^{-9}. The tubes were sealed with rubber stoppers and gassed with 90%/10% N_2 to CO_2. The tubes were incubated at 30°C in the dark. After two weeks the tubes were sampled with needle and syringe for totally produce ferrous iron using the ferrozine assay (see analytical procedures). The MPNs were run in duplicates per environment.
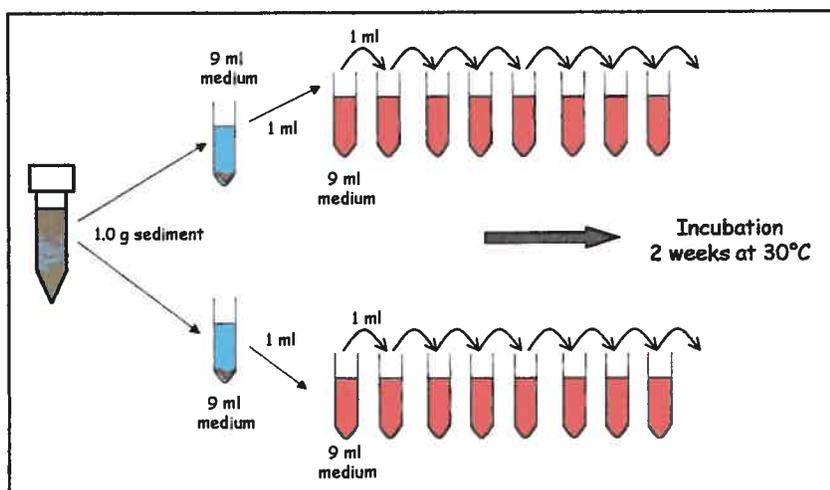
Figure 1 Experimental setup of MPN assay

Iron reduction activity assay

The iron reduction activity assay is depicted in Figure 2. Roughly 1.0 gram of sediment was added to medium as well as to the buffer. The additional carbon source sample was run in duplicates; one replicate for the carbon source blank and for the water blank was setup. The additional carbon source was 5 mM acetate and 5 mM lactate, the provided ferric iron hydroxide had a concentration of 40 mM. After inoculation the samples were gassed for 5 min with 90%/10% N_2 to CO_2 and then incubated at 30°C in the dark. The totally produced ferrous iron was determined by the ferrozine assay (see analytical methods) every other day. The samples were taken with syringes and needles, which were flushed with 90%/10% N_2 to CO_2 prior to sampling.
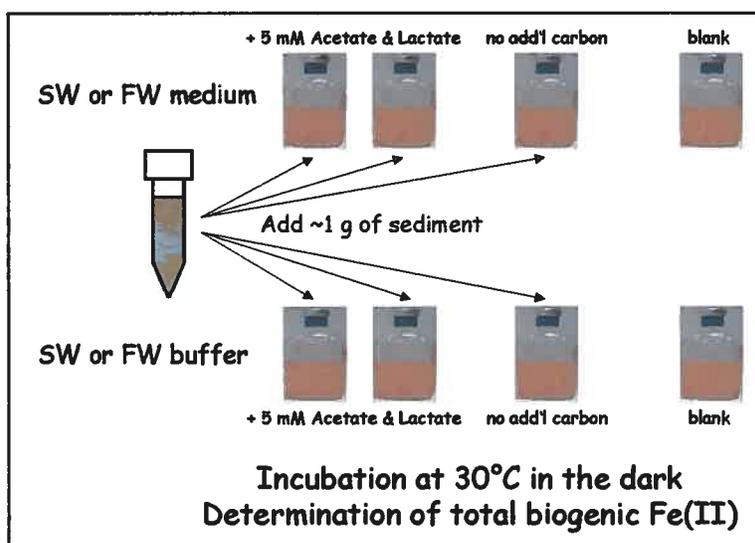

Figure 2 Experimental setup of iron reduction activity assay

Analytical methods

Totally produced ferrous iron was quantitatively determined for the MPNs and the iron reduction activity assay using the ferrozine assay (Stookey,1970). 0.1 ml of sample was added to 0.9 ml of 0.5 M HCl and mixed. After centrifugation at 13'000 to 14'000 rpm (15 min), 0.1 ml of the supernatant was added to 0.9 ml of 0.5 M HCl in a cuvette. 1 ml of ferrozine solution was added, the sample was mixed and the absorbance was measured at 562 nm after 10 minutes.

For the quantitative assessment of ferrous iron in the samples a calibration curve was prepared in the range from 0.5 mM to 15 mM Fe(II). The standards were prepared with $(NH4)_2Fe(SO_4)_2*6H_2O$ in 1 M HCl. Standards were treated completely the same as the samples.

Agar plug assay

The agar plug assay was set up according to the paper by Straub and Schink (2003), who used this assay for the first time in order to check for the production of electron shuttling compounds by iron reducers. The experimental setup for this assay is shown in Figure 3. Agar plugs containing 14 mM poorly crystalline ferric iron hydroxide were produced in the same way as described by Straub and Schink (2003).

3 ml of a washed and 6 times concentrated cell culture from class enrichments for iron reducers was added on top of the agar plug. The inoculum for the initial enrichment was taken from School Street Marsh. The cultures were grown in a freshwater medium containing 5 mM acetate or 10 mM ethanol, respectively, and 40 mM of poorly crystalline ferric iron hydroxide. After 7 days the grown cultures were transferred into fresh medium with iron hydroxide and after 7 more days to medium without ferric hydroxide but with 5 mM of fumarate as electron acceptor.

5 mM acetate or ethanol were added to the dense cell cultures as well as 1 mM of the crystalline ferric iron hydroxide in order to stimulate initial iron reduction. Two agar plugs were prepared for every enrichment. One of them was without AQDS and the other with 200 μM AQDS in the supernatant. The headspace of the tubes was exchanged with 90%/10% of N_2/CO_2 during five minutes and the cultures were incubated at 30°C in the dark.
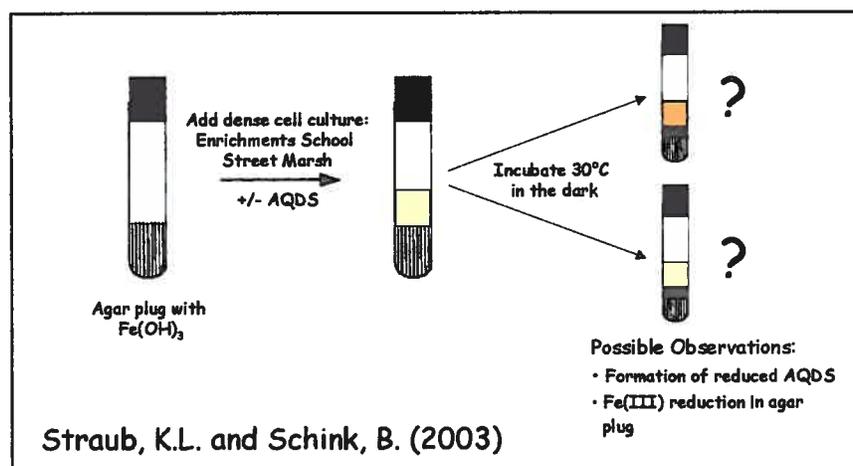

Figure 3 Experimental setup of agar plug assay

Isolation of iron reducers

Half of the microbial diversity class did agar shakes according to the course handouts for the iron reducing enrichments that have been described above (enrichments from School Street Marsh, grown on acetate/ethanol with 5 mM fumarate as the terminal electron acceptor). Five of these agar shakes were used to pick colonies and to isolate iron reducing bacteria from School Street Marsh. One of these agar shakes was with fumarate as the electron acceptor, the other four were with 40 mM poorly crystalline ferric iron hydroxide.

In order to pick the colonies from the agar shake, the agar plug with the embedded colonies was extracted from the balch tube by first cutting the glass below the top were it forms a bottleneck. The agar plug was taken out by driving a sterile Pasteur pipette to the bottom of the agar plug and then using a very low gas pressure in order to press it out of the tube onto a sterile petri dish. Using a razor blade the agar plug was cut into thin layers exposing the position of the colonies in the agar plug. The colonies were picked with Pasteur pipettes which contained a little bit of medium and immediately transferred the picked colony into new medium with 40 mM poorly crystalline ferric iron hydroxide, 5 mM acetate or ethanol and 100 µM AQDS. The headspace was exchanged with 90%/10% N_2/CO_2. The cultures were then incubated at 30°C in the dark.

Terminal restriction fragment length polymorphism (t-RFLP)

DNA was extracted from environmental samples using the Mo Bio ultra clean soil DNA isolation kit for School Street Marsh sediments and the plant DNA isolation kid for Sippewissett sediments (Mo Bio Laboratories, Solana Beach, CA) according to the modified protocol by Brian Wade.

Our strategy for the t-RFLP was to use specific forward primers for Shewanella 783F (5'-AAAGACTGACGCTCAKGCA-3') (Snoeyenbos-West et al., 2000), Geothrix 182F (5'-AGACCTTCGGCTGGGGATGCT-3') (Snoeyenbos-West et al., 2000) and Geobacter 561F (5'-GCGTGTAGGCGGTTTCTTAA-3') (Stults et al., 2001), as well as the general eubacterial primer 8F (5'-AGAGTTTGATCCTGGCTCAG-5') and for all of the sample the FAM-labeled 1492-R (3'-GGTTACCTTGTTACGACTT-5'). The used annealing temperature was 55°C for all different primer sets. Following this approach it should be possible to dissect the community in the sediment into different specific iron reducer groups, to get an idea of the diversity in the group of specific iron reducers and additional information on abundance of iron reducers in the chosen environment. Afterwards a digest was run on the PCR products with Hha1. The digest products were sent to Michigan State University for t-RFLP analysis.

Results and Discussion

Direct counts and most probable number assay (MPN)

By direct counting of DAPI-stained cells an average of 1.6×10^{10} and 1.3×10^{10} cells per gram of sediment were determined for the collected samples from School Street Marsh and from Sippewissett Salt Marsh, respectively. The error associated with the chosen technique for assessing the cell density tends to be roughly 10% of total cell counts. Therefore I conclude that there is no significant difference in the microbial cell density between School Street Marsh and Sippewissett Salt Marsh.

Table 1 shows the concentrations of total biogenic Fe(II) measured in the MPNs after two weeks of incubation. For School Street Marsh iron reduction up to a dilution of 10^{-8} and 10^{-9}, respectively, where found in two parallels which indicates the presence of up to 1 billion of microorganisms capable of iron reduction in this environment. There seem to be less iron reducers present in the samples from Sippewissett Salt Marsh. Iron reduction was found up to a dilution of 10^{-5} respectively 10^{-7}. The positive measurement for the 10^{-8} dilution of replicate 1 is probably due to a transfer of one single sediment particle up to this dilution and is not taken into account.

There is much heterogeneity in the Fe(II) concentrations obtained for the duplicates for both environments. To make a more valid assessment for the presence of iron reducers in these environments one should perform the MPN tubes at least in triplicates and incubate them for at least four weeks or even longer. A longer incubation time would probably also lead to more homogenous iron(II) concentrations.

Table 1 MPN results for sediment samples taken from School Street Marsh and Sippewissett Salt Marsh. Shown are the measured total biogenic Fe(II) concentrations in mM, nd denotes not detected, cells/g stands for cells per gram of sediment capable of Fe(III) reduction.

School Street	10^2 cells/g	10^3 cells/g	10^4 cells/g	10^5 cells/g	10^6 cells/g	10^7 cells/g	10^8 cells/g	10^9 cells/g
Replicate 1	4.42	3.37	4.44	4.48	1.67	4.13	0.07	0.21
Replicate 2	4.41	4.11	4.74	2.99	1.98	0.14	0.08	nd
Analysis	+	+	+	+	+	+	+	(+)
Sippewissett	10^2 cells/g	10^3 cells/g	10^4 cells/g	10^5 cells/g	10^6 cells/g	10^7 cells/g	10^8 cells/g	10^9 cells/g
Replicate 1	3.89	1.63	0.52	0.35	nd	nd	0.34	nd
Replicate 2	3.85	1.00	0.31	0.30	0.35	0.34	nd	nd
Analysis	+	+	+	+	(+)	(+)	(-)	-

Combining the information from the direct counts and the MPNs it appears that 1 to 10% of the total microbial population in School Street Marsh is able to use ferric iron as an electron acceptor. In sediment samples from Sippewissett Salt Marsh, however, iron reducing bacteria only account for 0.1% or even less of the total microbial population.

Determination of iron reduction activity

The results for the iron reduction activity assay for School Street Marsh are shown in Figure 4. The left graph is for the freshwater medium and the right graph for the freshwater buffer, which contained no trace elements, vitamins, nitrogen and no phosphate.

7

A first rough observation shows that surprisingly there was iron reduction in all of the incubations (with and without additional carbon source, i.e. acetate and lactate) except in the water blank which was not inoculated. This result clearly indicates the presence and microbial utilization of organic carbon – probably stemming from the inoculum.
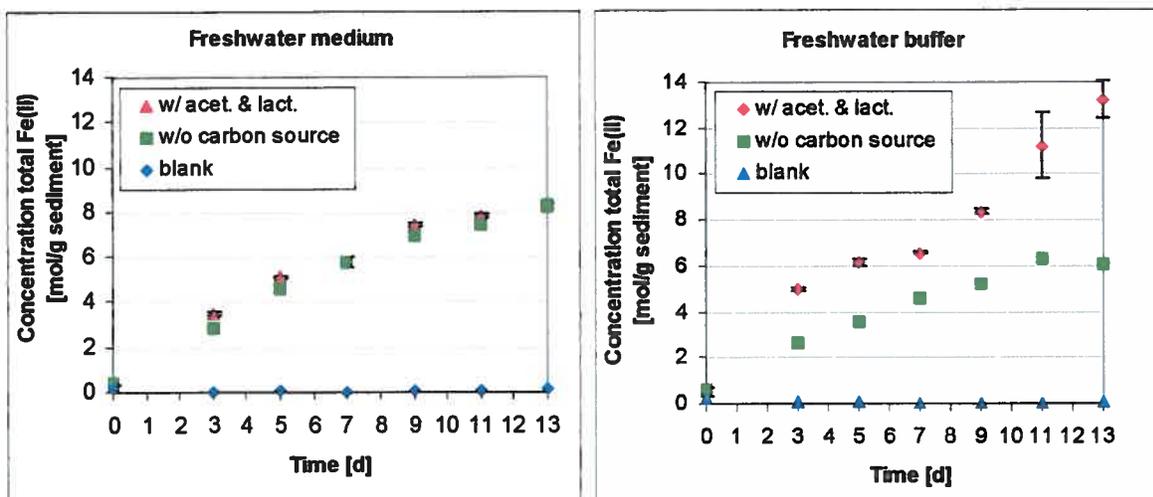

Figure 4 Iron reducing activity measurements for School Street Marsh. w/ acet. & lact means addition of 5 mM acetate and 5 mM lactate. The error bars for the treatment with acetate and lactate denotes the standard deviation of the duplicate.

Having a closer look at the iron reduction in the freshwater medium we see no differences when we compare the samples with additional carbon source (5 mM acetate and 5 mM lactate) and without. Obviously the carbon source is not limiting under these conditions. This is not the case in the incubations done in freshwater buffer (where trace elements, vitamins, etc. are missing). In these incubations iron reduction stops after eleven days without additional carbon source, whereas the samples with acetate and lactate still produces ferrous iron.

The comparison of the freshwater medium and the freshwater buffer indicates that iron reduction in the freshwater buffer with additional carbon source produces much more ferrous iron than in the freshwater medium. One possible explanation for this could be that the phosphate which is present in the freshwater medium but not in the buffer passivates the surface of the iron hydroxide by complexing the Fe(III). The consequence would be that the iron hydroxide would be less available for microbial reduction. Both bottles incubated without additional carbon source showed iron reduction, probably indicating the presence of carbon coming from the inoculum. However, the iron reduction in the buffer seems to be limited compared to the medium. This could be due to the lack of nitrogen, phosphate, trace elements and vitamins. It is conceivable that without this additional nutrients microorganisms are not able to degrade more complex energy sources which could be present due to the inocculum of sediments at the beginning.

The results for the iron reducing acitivity measurements for Sippewissett Marsh samples (Figure 5), showed different patterns compared to the ones from School Street Marsh, but again it was obvious that there is bioavailable carbon present in the inocculum because both of the samples where no additional carbon was added are reducing iron. Furthermore, we saw in all incubations a plateau formation at around 3 to 4 days. At this point, iron reduction stops for a day and reassumes again afterwards. It could either be that the microorganisms are changing their carbon source and therefore have to adapt before they can grow on a new substrate. Another possibility could be that there are different iron reducers present, for example one group that produces Fe(II) at the beginning of the incubation and is then overtaken by another group of organisms.

In the buffer as well as in the medium the sample without additional carbon source produces less ferrous iron after the above mentioned plateau. The incubations with medium starts to produce Fe(II) but not at the same levels as the one with additional carbon. The sample in buffer shows the same pattern as the one in School Street: reduction stops after 11 days.
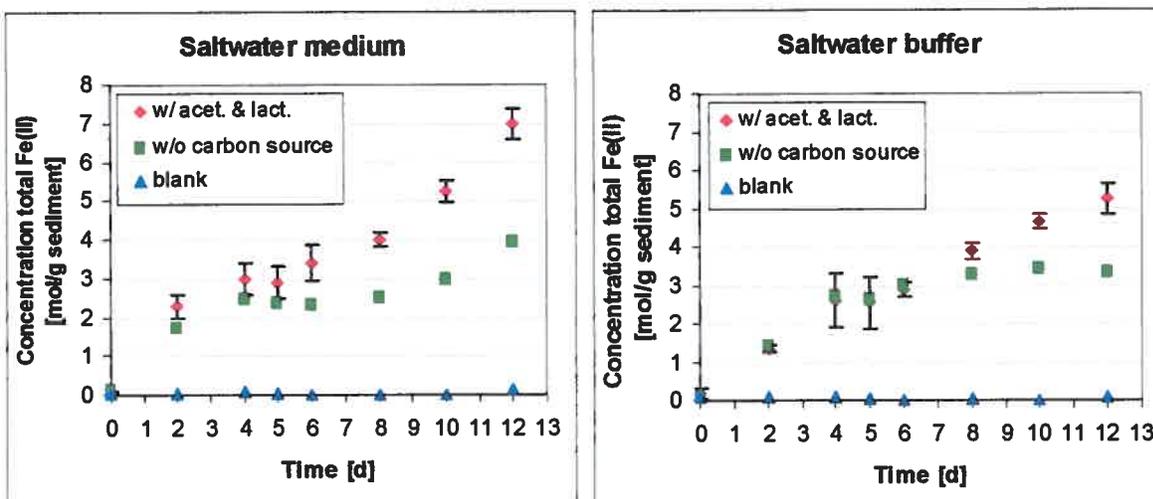

Figure 5 Iron reducing activity measurements for samples from Sippewissett Marsh. w/ acet. & lact means addition of 5 mM acetate and 5 mM lactate. The error bars for the treatment with acetate and lactate denotes the standard deviation of the duplicate.

To compare the activities of iron reduction in School Street and Sippewissett we look at a short term activity within the first 3 days and at the long term activity within 12 days. Interestingly, the short term activity seems to be more or less the same for the different treatments in both environments. Within this period of time about 3 mM of ferrous iron is produced. In addition, the fact that we see no differences within this period of time between samples with and without additional carbon source is an indicator that we measure the inherent iron reduction activity of the cultures.

The long term activity within 12 days shows huge differences between both environments and between the different treatments. In School Street samples up to 13 mM of Fe(II) are detected compared to incubations with samples from Sippewissett Salt Marsh where only 8 mM was detected. It has to be noted that in contrast to the short term activity the long term activity is not a suitable indicator for the inherent iron-reducing capability because enriching/growth effects superimposing the activity measurements are very likely.

Evaluation of electron shuttle mechanism: agar plug assay

For the agar plug assay six different cultures from our class enrichments for iron reducing bacteria obtained with poorly crystalline ferric iron hydroxide as electron acceptor were used, three of them were grown on 10 mM ethanol and the other three on 5 mM acetate. The cultures were incubated for five days at 30°C prior to analysis of the agar plug for iron reduction.

The results for this assay are summarized in Table 2. In the presence of AQDS five of the six strains could reduce the AQDS in the supernatant, which was obvious due to the typical color change in the supernatant from colorless to yellowish/orange. In all of these five cultures the formation of a black ring in the uppermost part of the agar plug was observable indicating Fe(III) reduction, magnetite formation and thus clearly showing diffusion of reduced AQDS into the agar. Interestingly, also the assays without the addition of AQDS showed a black precipitate at or even within the the agar layer. However, it was difficult to tell exactly if there was magnetite present in the agar plug because of the formation of blackish precipitates at the interface of cell suspension to the agar plug due to the initial reduction of 1 mM ferric hydroxide which had to be added to the overlaying cell suspension to initiate their activity.

Table 2 Observation of iron reduction in agar plug in six different cultures grown on ethanol or acetate with and without the addition of AQDS. + denotes iron reduction in the agar plug, - denotes no observed iron reduction, red denotes the presence of reduced AQDS in the supernatant

	EtOH 1	EtOH 2	EtOH3	Acetate 1	Acetate 2	Acetate 3
No AQDS	-	-	-	(+)	-	-
Plus AQDS	+/red	+/red	+/red	+/red	-	+/red

To get an idea if the iron(III) in the agar plug was really reduced in the upper most part the Acetate 1 culture, which looked the most promising, the tube was sacrificed. It was cut open, the cell suspension was removed and the agar plug was taken out by driving a sterile Pasteur pipette to the bottom of the agar plug and then using a very low gas pressure in order to press it out of the tube. Then the agar plug was observed for the formation of a reduced layer of Fe(III).
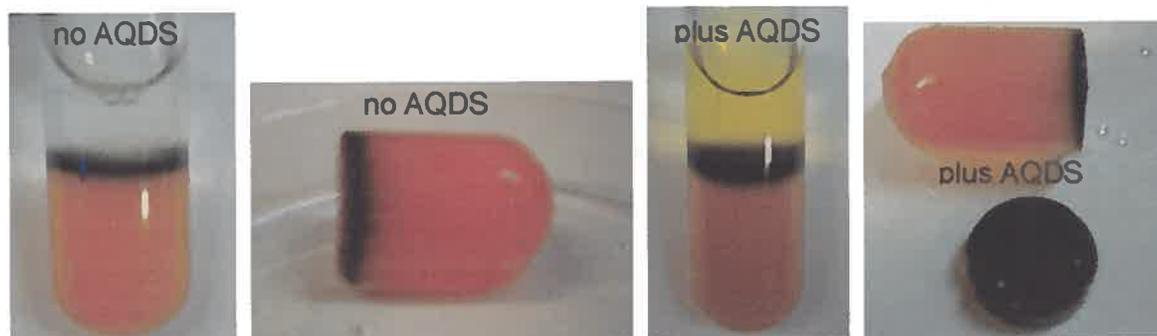

Figure 6 Pictures of agar plug assay and extracted agar plug from culture Acetate 1

Figure 6 depicts the agar plug assays still intact after five days of incubation on the most left picture and on the second picture from the left. Both Agar plugs indicate a possible iron reduction but it is much more pronounced in the assay with AQDS (right side of figure). Both extracted agar plugs show a zone of black iron reduction. In the case were AQDS was added, the sliced agar piece was indicating a clear reduction (magnetite formation) - much more pronounced than in the assay where no AQDS was added. The formation of reduced iron was either still very minor or an artefact of the above mentioned ferrous iron precipitation.

By using this assay, Straub and Schink (2003) showed the production of electron shuttling compounds by two enrichment cultures. The excretion of electron shuttles by iron-reducing microorganisms is heavily debated in the literature but it is not unlikely that also the enrichments obtained in this study were producing electron shuttling compounds. But in order to really tell the difference between iron precipitation at the interface or real reduction of the iron in the agar plug it would be necessary to incubate these assays for a longer time, especially the ones without AQDS in order to see a clear formation of magnetite indicating the formation of Fe(II).

Isolation of iron reducers and sequencing

From the 20 picked colonies of the five different agar shakes nine showed growth after five days of incubation either in the form of reduced AQDS, the transformation of the red ferric hydroxide into brown-blackish precipitates or both of them. The other eleven cultures looked more or less like the control. We found no growth for the cultures which were picked from the fumarate/acetate agar shake. Maybe this is due to a long log phase in order to adapt to the new medium (in particular to the solid electron acceptor, i.e. ferric iron hydroxide).

10

DNA was extracted from six different cultures, one of each initial agar shake and an additional one from a positive Fe(OH)$_3$/EtOH shake. Two of these extracts were made from cultures without obvious signs of growth. We were able to extract DNA even from these cultures, which indicates that there were enough cells although there were no obvious signs of growth. The DNA extracts were PCR amplified with the eubacterial primer set for rRNA genes, 8F and 1492R, positively checked for PCR product on an agarose gel and then sent to Michigan State University for sequencing. Unfortunately, we did not get sequences from these samples.

t-RFLP

In order to get the FAM-labeled 1492R-primer we had to demonstrate that we were able to extract DNA from our samples, in my case DNA from School Street Marsh and Sippewissett. Furthermore, we had to test our specific primers for *Geobacter*, Shewanella and Geothrix to be sure that they were specific. The last test was to demonstrate that we get a product when digesting PCR product from our specific primers.

We could demonstrate that DNA from these two environments is extractable and amplifiable with the general eubacterial primer set (8F/1492R). We tested the specific forward primers for Shewanella (477F), Geobacter (561F) in combination with 1492R on pure cultures of Shewanella oneidensis, Geobacter metallireducens, mixtures of these two organisms with a Ralstonia species and on the Ralstonia as a negative control. The Shewanella and the Geobacter specific primer seemed to be specific under the given circumstances. The digest with Hha1 of PCR product from amplification of Geobacter metallireducens with the Geobacter specific primer yielded several digest products, which was another important indicator that a t-RFLP on the chosen environmental samples with the specific primers could work.

The FAM-labeled 1492R primer was ordered and we set up a PCR with the specific primers and this 1492R for the environmental samples in order to get product for the following digest and the t-RFLP analysis. This PCR yielded no product at all. Even the positive control did not work which was a strong indicator of a primer problem with the FAM-labeled 1492R. This was indeed the case. The FAM-label was wrongly placed on the 3 prime end instead of the 5 prime end. The primer could not anneal.

We set up another attempt to get a t-RFLP by using the FAM-labelled 8F-primer and the Geobacteraceae specific 825R (Snoeyenbos-West et al., 2000), which we had not used until then. We got PCR product, cleaned it up and digested it with Hha1. The samples were then sent to Michigan State University for the t-RFLP analysis. Although the samples were analysed, the corresponding data files of my environmental samples from School Street Marsh and Sippewissett were not downloadable from the ftp-server.

Conclusions

The abundance of iron reducing bacteria is according to the determined cell densities and the results from the M PNs 1 00 t imes h igher i n S chool S treet M arsh c ompared t o S ippewissett. T he sampled r egion i n School Street Marsh is an extremely well suited environment for iron reducers because of the iron corrosion on the staircase, leading to a constant high input of iron. Furthermore, this environment has a high input of organic matter. Sippewissett has a much lower abundance of iron reducers but it is conceivable that this is still one of the most comfortable places for iron reducers in saltwater environments due to the high input of organic matter and a steady input of iron from the landmass. A lot of other saltwater environments are iron limited.

The activity levels of iron reducers in these two environments are very different. Although the same levels of short term activity were determined for both environments, one has to keep in mind, that there are 100 times less iron reducers in Sippewissett present meaning that the activity of iron reducers in Sippewissett is indeed a hundredfold higher than the activity for the ones from School street Marsh. This could be due to a higher turnover rate in Sippewissett because of lower available iron concentrations and the enhanced precipitation of iron species due to the high concentration of different sulfur species.

In both environments different metabolic groups of iron reducing bacteria seem to be involved in the iron reduction process. As there are supposedly a lot of humic acids present in School Street Marsh as well as in Sippewisset, fermenting bacteria able to reduce iron indirectly by reduction of electron shuttles such as humic acids which in turn react chemically with the iron(III) could play a major role in iron reduction. Furthermore, iron reducing bacteria which produce electron shuttles themselves and which consequently do not have to rely on external electron shuttling compounds could be involved.

Acknowledgments

References

Benz, M., Schink B. and Brune A. (1998) Humic acid reduction by *Propionibacterium freudenreichii* and other fermenting bacteria. Appl. and Environ. Microbiol. 64, 4507-4512

Lovley, D.R. (1991) Dissimilatory Fe(III) and Mn(IV) reduction. Microbiol. Rev. 55, 259-287

Lovley, D.R., Coates, J.D., Blunt-Harris, E.L., Phillips, E.J.P. and Woodward, J.C. (1996) Humic substances as electron acceptors for microbial respiration. Nature 382, 445-448

Lovley, D.R. (1997) Microbial Fe(III) reduction in subsurface environments. FEMS Microbiol. Rev. 20, 305-313

Newman, D.K and Kolter, R. (2000) A role for excreted quinones in extracellular electron transfer. Nature 405, 94-97

Schwertmann, U. and Fitzpatrick, R.W. (1992) Iron minerals in surface environments. In: Biomineralization Processes of Iron and Manganese (Skinner, H.C.W. and Fitzpatrick, R.W., Eds.). pp. 7-31. Catena, Cremlingen.

Seeliger, S., Cord-Ruwisch, R. and Schink, B. (1998) A periplasmic and extracellular *c*-type cytochrome of *Geobacter sulfurreducens* acts as ferric iron reductase and as an electron carrier to other acceptors or to partner bacteria. J. Bacteriol. 180, 3686-3691

Snoeyenbos-West, O.L., Nevin, K.P., Anderson, R.T. and Lovley, D.R.(2000) Enrichment of Geobacter species in response to stimulation of Fe(III) reduction in sandy aquifer sediments. Microbial Ecol. 39, 153-167

Stookey, L.L. (1970) Ferrozine - A new spectrophotometric reagent for iron. Anal. Chem. 42, 779-781

Straub, K.L. and Schink, B. (2003) Evaluation of electron-shuttling compounds in microbial ferric iron reduction. FEMS Microbiol. Lett. 220, 229-233

Stults, J.R., Snoeyenbos-West, O.L., Methe, B., Lovley, D.R. and Chandler, D.P. (2001) Application of the 5 ' fluorogenic exonuclease assay (TaqMan) for quantitative ribosomal DNA and rRNA analysis in sediments. Appl. and Environ. Microbiol. 67, 2781-2789

Thamdrup, B. (2000) Bacterial manganese and iron reduction in aquatic sediments. In: Adv. Microb. Ecol., Vol. 16 (Schink, B., Ed.), pp. 41-84. Kluwer Academic/Plenum Publishers, New York.