Redox Reactivity and Bioavailability of Iron Oxyhydroxides in the Subsurface

Subproject 2: Structure and Activity of Microbial Iron-Reducing Communities

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SUMMARY:
In the past 6-month period sampling, and subsequent enrichment and isolation of iron reducers, at the Appels & Waarde sites (so-called 'bog iron ore', at the surface) and Banisveld landfill (subsurface samples) has been done. Sub-culturing from the first round enrichments was performed three times for the Appels and Waarde sites. A few iron reducers have been isolated directly from Appels sample, by inoculating agar medium. Molecular methods have been optimized to monitor the iron reducer community in the enrichments. The dominant iron reducers are expected to be recovered from the subcultures. In addition, bottles with agar medium will be applied for isolation as well. Specific iron reducers (*Shewanella*, *Geobacter*) were detected in different layers of sediment cores and the enrichments. The numbers and the diversity of culturable iron reducers at the Appels site is higher than at Waarde. The iron reducing communities in the enrichments differ between the lowest and highest dilutions, for Appels. There is less variation of the iron reducer community in the enrichments with Waarde sample. Shewanella was detected directly in the upper sediment layer from 0-11cm and in some enrichments with iron citrate as electron acceptor acceptor, but was not in other enrichments with insoluble iron oxides, except one (F6L 10^{-2}). Geobacter was detected in all sediment layers and low dilution enrichments (10^{-2} to 10^{-4}) except for the enrichments with Fe citrate. In other words, Shewanella and Geobacter are detected in the environmental samples and enrichments, but do not seem to be the dominant culturable iron reducers.
Samples were also collected from the Banisveld landfill (Boxtel), at 2.75-3.7 m depth below surface. The Banisveld landfill is a landfill leachate polluted site and iron reducers are the major microorganisms present and possibly responsible for BTEX degradation. The enrichment and isolation is ongoing. Because of the specific environmental conditions (subsurface, low temperature, low nutrients concentration, and different

sediment structure-sandy etc.), color changes (indicative of iron reduction) only occurred at low dilutions, 10^{-1} to 10^{-2} , after nearly 4-month incubation. However, iron reduction is more pronounced in the enrichments with polluted sediment than the ones containing clean sediment.

Meanwhile, cultivation of the strict anaerobic microorganism-*Geobacter metallireducens* has been set up, which is used as a reference strain throughout the environmental experiments. The original strain from DSM did contain more species. A pure strain was obtained via plating on agar medium and further serial transfer and culturing. Successful batch cultures with *G. metallireducens* were done to become familiar with cultivation of strict anaerobic bacteria. *G. metallireducens* was also cultured in the retentostat but the methods for this continuous culturing is still being optimized.

Major activities performed during this period:

• Optimizing the incubation conditions for strictly anaerobic bacteria-*Geobacter metallireducens,* and subsequent consortia recovery in retentostat

• Sampling at Appels & Waarde sites (so-called 'bog iron ore') and Banisveld landfill, enrichment and isolation of iron reducers from these samples

• Physiological and molecular monitoring of enrichments, isolates and environmental samples

• *Geobacter metallireducens* (DSM) Purification through agar serum bottle and serial transfer in liquid medium

Materials and Methods

1. Strict anaerobic iron reducers are the major group of microorganisms in our studied sites; so to obtain the suitable growth conditions-strict anaerobic conditions is the first requirement in successful enrichment, isolation and investigation of iron reducers in this project. Here are a few methods used throughout our experiments.

• Anaerobic gas (N_2:CO_2=90:10) flushes the glove box and is also used to bubble the media, which are for batch culture.
• Titanium solution (Zehnder and Wuhrmann 1976; Jones and Pickard 1980) is used to remove the trace oxygen in anaerobic gas, both for glove box and retentostat culturing.
• 0.5mM Fe^{2+} solution in batch culture medium to remove the trace oxygen.

2. Appels and Waarde sites (sampling at 6-11-02):

Site description: the sampling site was chosen by Geochemistry department, Utrecht university based on their previous research. The sites are 'bog iron ores' rich in iron deposit and low oxygen concentration. Duplicate plastic tubes (∅4.5cm x 30cm) were used to collect the samples, aseptically and anaerobically, then transferred to anaerobic jar as soon as possible. The samples were treated immediately once they reached the lab, at the same day as sampling.

Experimental design for wide range isolation of iron reducers

Different types of iron are present naturally, chelated iron, poorly crystalline Fe (III) oxides, and crystalline (HSA goethite, alpha-FeOOH, Hematite, alpha-Fe_2O_3), Lepidocrocite (gamma-FeOOH), Smectite (clay mineral), Magnetite, Montmorillonite, etc. In sediment, crystalline Fe (III) is more abundant (2-10x) than poorly crystalline. It can be degraded considerably (Roden, Leonardo et al. 2002). In past, only poorly crystalline iron oxide was regarded that it can be degraded. Based on these findings, we designed the experiments with different types of iron (Fe citrate, AQDS-anthraquinone 2,6-disulfonate, humic acid analog, six-line ferrihydrite, and hematite prepared according to literature (Schwertmann and Corned),) combined with acetate and lactate as electron donor and carbon source , in order to isolate the iron reducers able to dissimilate a wide range of iron oxides.

Materials and methods

Media: Medium obtained from DSM for Geobacter was slight modified by use different iron sources and concentration instead of iron citrate. Medium composition (per liter) is: Fe(III) citrate 10mM or F6L 5mM or Hematite 5mM, $NaHCO_3$ 2.50g, NH_4Cl 1.50g, NaH_2PO_4 0.60g, KCl 0.10g, acetate 2mM and lactate 1mM, yeast 0.1g, Na_2WO_4 x 2 H_2O 0.25mg, Trace element solution (Nitrilotriacetic acid 1.500g, MgSO4 x 7 H_2O 3.000g, $MnSO_4$ x 2 H_2O 0.500g, NaCl 1.000g, $FeSO_4$ x 7 H_2O 0.100g, $CoSO_4$ x 7 H_2O 0.180g, $CaCl_2$ x 2H_2O 0.100g, $ZnSO_4$ x 7 H_2O 0.180g, $CuSO_4$ x 5 H_2O 0.010g, $KAl(SO_4)_2$ x 12 H_2O 0.020g, H_3BO_3 0.010g, Na_2MoO_4 x 2 H2O 0.010g, $NiCl_2$ x 6 H_2O 0.025g, Na_2SeO_3 x 5 H_2O 0.300mg, per liter)10.00ml

F6L and Hematite were supplied as a colloidal solution, sterilized by filteration, added after medium (modified DSM medium) autoclaved. Media were autoclaved in pressure-serum bottle sealed by rubber stops after anaerobic gas bubbling. 0.5mM ferrous solution was used to remove possible trace of oxygen in media.

Under anaerobic conditions, 1g mixed sediment (from 5 to 15cm, highest [Fe] was found in this layer.) was dissolved in 0.1%NaPP solution (anaerobic autoclaved) first, then a decimal serial dilution from 10^{-2} to 10^{-8} in 10ml serum bottle was made for different types of media. These inoculated bottles were incubated at 30°C, dark conditions. In addition to batch culture, agar serum bottle was used to isolate single iron reducer.

Results and Discussion

1. Enrichment and isolation

The enrichments were checked regularly. The color change due to Fe^{3+} reduction over time can be observed. The first visible color change occurred after about 5 day incubation in low dilution enrichments. The microbial activities or amount of iron reducers were higher in samples collected from the Appels site than at the Waarde site, based on the color change in the highest dilution enrichments (see table below and **Fig.1**). Iron reduction will be confirmed by measuring Fe^{2+}, Fe^{3+} conversion later on.

Appels	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Hematite	+	+	+	±			
Hematite+AQDS	+	+	+	+	+	+	
F6L	+	+	+	+	±		
Fe citrate	+	+	+	+	+		
Waarde							
Hematite	+	+	±				
Hematite+AQDS	+	+	+	±		80 days incubation, 30°C, dark	
F6L	+	+	+	+			
Fe citrate	+	+	+	+			

+: color change due to iron reduction; ±: slight color change

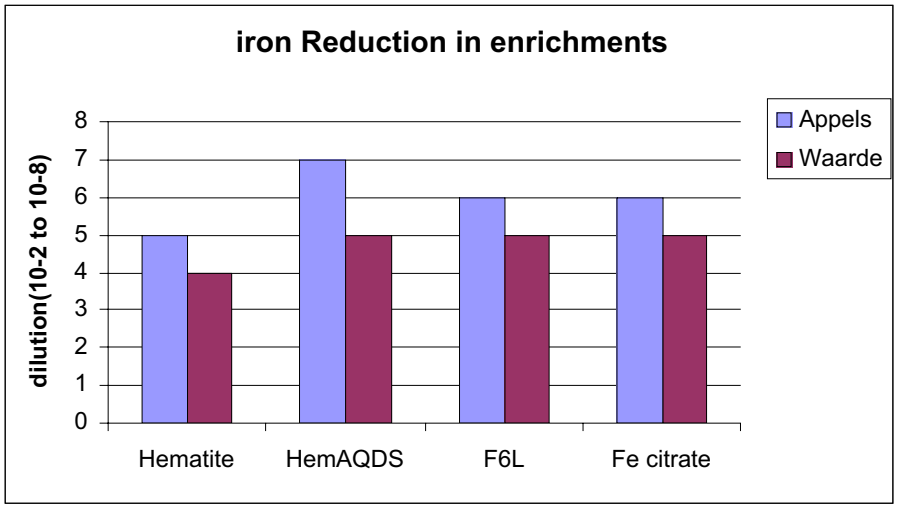


Fig 1. The color change (iron reducing) in highest dilution enrichments is associated with the different types of irons

The lowest and highest dilution with positive reaction were transferred and sub-cultured a few times. The subcultures were checked by DGGE of 16S rRNA amplified products with universal primers for eubacteria. The single species of microorganism (iron reducer) will be recovered after sufficient subculture-transfer. Meanwhile, the agar medium prepared in serum bottle (detail see Page 4. *2. Single colony recovery)* was used to obtain the single colony.

The iron reduction happened early with the easier usable Fe-citrate, and HemAQDS (hematite plus AQDS), slower with the relatively difficult usable F6L and hematite. After a few day incubation, more positive reactions were observed in the enrichment with Fe citrate and HemAQDS. Fe in iron citrate is chelated and it is easier to be utilized by iron reducers. AQDS is helpful to Hematite dissimilation as the electron shuttle between iron reducer and iron oxides. Hematite is most difficult to be used because of its crystal structure, while F6L is relatively easy compared to Hematite due to its poorly crystal structure.

Precipitate was always present in all enrichments, because Fe^{2+}, Fe^{3+} can form different chemical compounds, such as Siderite ($FeCO_3$), vivianite ($Fe_3(PO_4)2.8H_2O$) white; green rust ([FeII(6-x)FeIIIx$(OH)_{12}$]x+[(A2-)x/2.yH_2O]x-, magnetite (Fe_3O_4), 2FeIII + 1FeII black, etc.. We have observed these different color precipitates in our enrichments. There are many factors influencing the precipitate formation, such as iron reducers, iron forms, temperature, pH, gas composition etc.[Roh, 2002].

2. Single colony recovery

To isolate single iron reducers, we used the modified Hungate technique [Miller, 1974] with addition of ferrous iron to remove possible trace oxygen in agar medium (10mM Fe citrate, 2mM acetate, 1mM lactate, 0.1gyest extract/l etc.). There are three types of colonies picked up from the wall of serum bottle. One is white with transparent halo (∅ 2mm), the other is black, no halo, ∅1-2mm, the third is red with small halo, ∅0.5-1mm. These colonies were picked up and transferred into liquid medium and they are growing with iron reduction, forming black, white-gray precipitate respectively. A preliminary experiment indicated they are able to grow on lactate but not acetate as sole E-donor. Further work has to be performed to get more information from these isolates.

3. Molecular approaches to monitor the composition of iron reducer communities.

• Which is better, isolated DNA or cells from the enrichment as template for PCR?

To get sufficient template for PCR is the first step to obtain efficient DGGE profile, DNA isolation procedures will result in the lose of some DNA, especially when the biomass is low, for instance, in my current enrichments. We did the comparison by using different template-cells and isolated DNA through DGGE profiling analysis. The results showed less bands when PCR was performed with isolated DNA as template than cells as template under the same conditions (see **fig.2**.lane 2,4). On the other hand, there is no big difference between cells and isolated DNA as template (see **fig.2** lane1 and 3, 2 and 4**. fig.3** lane 1,2,4,5 and 7,8). However, the bands of 1ml culture on filter used for DNA isolation are fewer than the ones, which are 2ml and 4ml culture for DNA isolation (see **fig.3** lane 3 and 4,5)

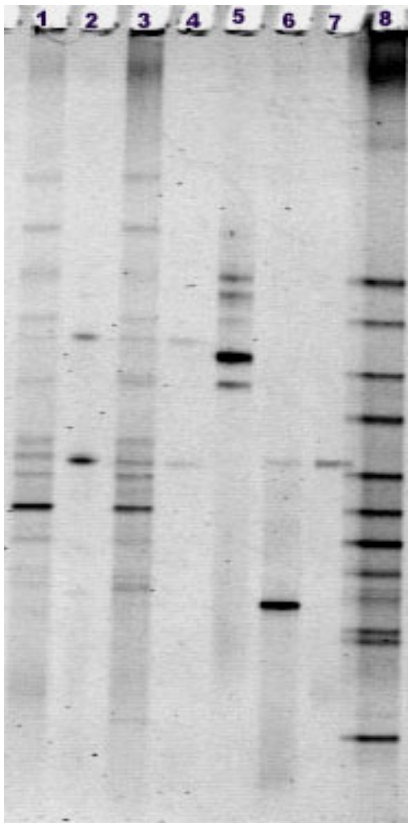


Fig.2 DGGE profiling comparison between the different templates, cells and isolated DNA as template for PCR. Samples were the enrichments from Appels site. Lane1, 3: 10^{-2} dilution of enrichment (Fe citrate as E-acceptor), Lane 2,4: 10^{-6} dilution of enrichment (Fe citrate as E-acceptor); Lane5: Shewanella; Lane6: Geobacter; Lane7: negative control; Lane8: Marker; Lane 1,2 cells as template: Lane 3,4,5,6:

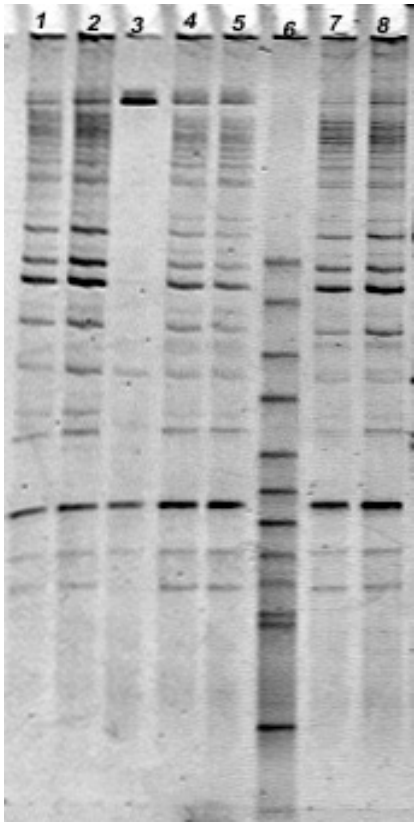


Fig. 3 Influence of different type of templates on DGGE pattern. The sample is the mixture of *G. metallireducens* culturing on Fe NTA. Different ways to prepare the template for PCR with universal primers: Lane1:0.5ml culture isolated by kit; Lane2:0.5ml culture spun down, then washed by 0.85%NaCl, and isolated by by FastDNA SPIN Kit; lane3,4,5: 1, 2, 4ml culture collected on a filter, then isolated by kit; Lane6: marker; Lane7: culture; Lane8: cells washed and suspended by 0.85%NaCl

Based on previous results, culture (cells) can be directly used as template for PCR. Further more, concentrating the cells by centrifuging and removing possible inhibitors in culture (e.g. iron) by washing steps (oxalate solution and 0.85%NaCl) will be helpful to obtain good PCR products.

• DGGE profiling of different enrichments

A **B**

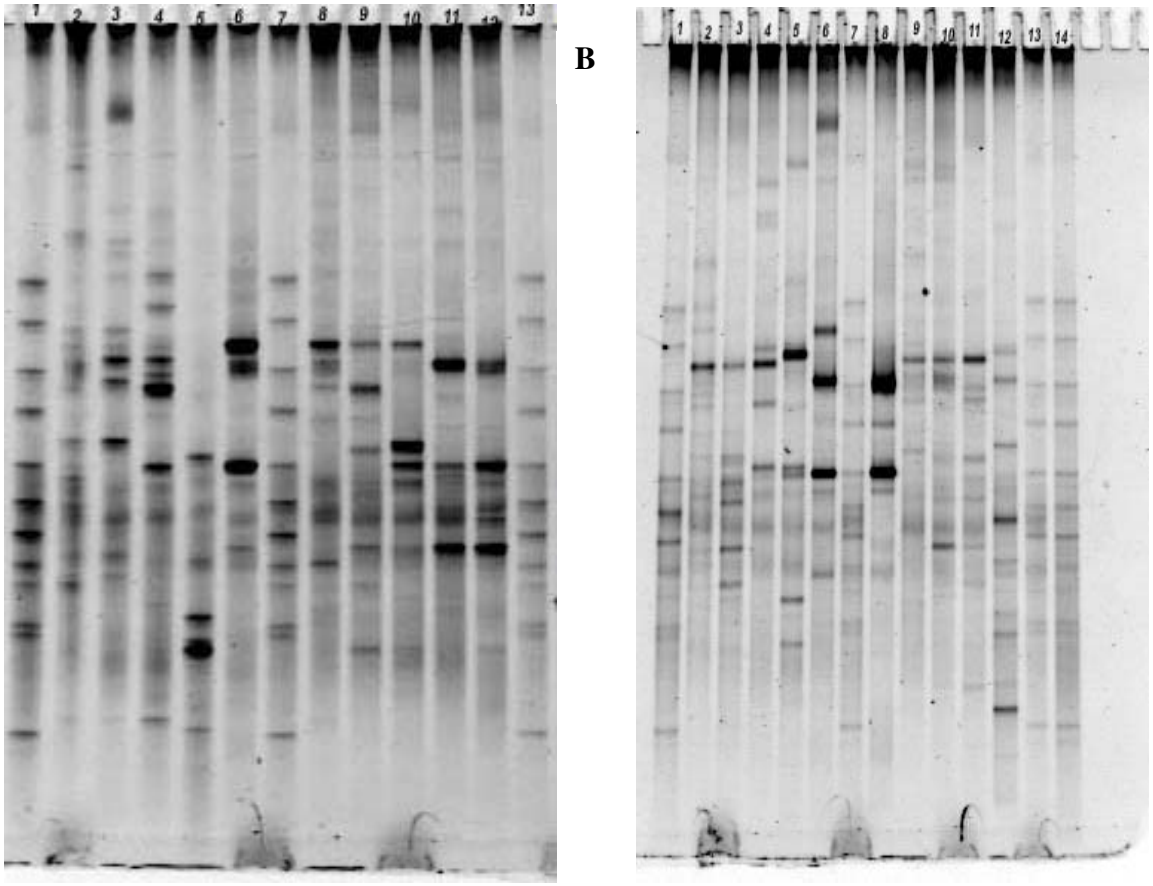
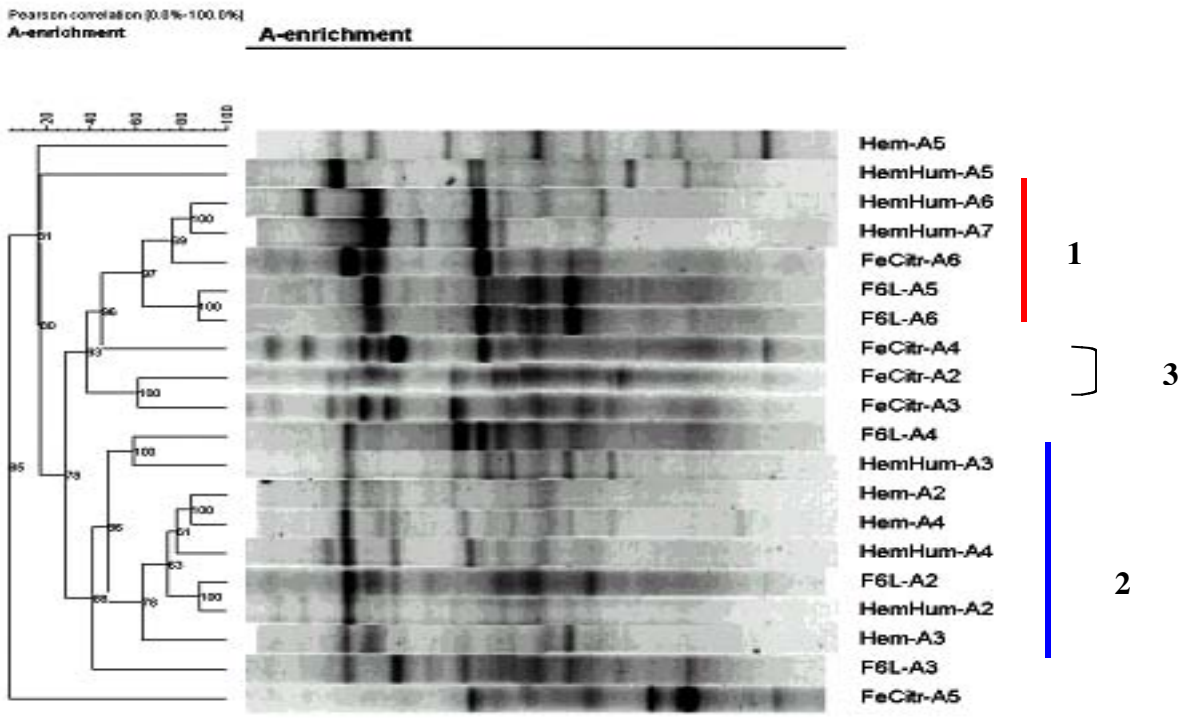


Fig.4. DGGE profiling of 16S rRNA amplified products of enrichments (1st round) from Appels samples. **A**: Lane 1,7,13:Marker; lane2, 3,4,5,6:dilution 10^{-2} to 10^{-6} enrichments with Fe citrate as iron source; lane 8,9,10,11,12: dilution 10^{-2} to 10^{-6} enrichment with F6L as iron source; **B**: Lane1, 7,13:Marker; lane2,3,4,5,6,8: serial dilution 10^{-2} to 10^{-7} enrichments with Hematite and AQDS as iron source; lane,9,10,11,12: dilution 10^{-2} to 10^{-5} enrichment with hematite as iron source

Higher diversity of iron reducers is present in low dilution enrichments except 10^{-5} one with hematite as iron source (**Fig.4B** lane12), and lower diversity in high dilution enrichments. On the other hand, more stronger bands, or so-called dominant species can be seen on the gels and these iron reducers will be isolated from highest dilution, for

instance, **Fig.4 A**: lane 6, 12; and **B**: lane 8. Cluster analysis (Cluster analysis of DGGE gels by Gelcompar 4.0 software, Pearson correlation and UPGMA clustering, see below) showed there are three major groups separately, high dilution enrichments (group 1), low dilution enrichment (group 2) and enrichment with iron citrate (group 3).



• Specific iron reducer group detection-Shewanella, Geobacter, and Archaea in enrichments and sediments

Shewanella is present in sediment at depth 0-11cm, as well as in enrichment containing Fe citrate, but non-detectable level in more deep layer of sediment (11-15cm) and enrichment containing insoluble iron oxides, such as enrichments containing F6L and Hematite with or without AQDS except one (F6L 10^{-2} dilution enrichment). In comparison, Geobacter was found in all layers of sediments and enrichments containing insoluble iron oxide (F6L, hematite+AQDS and hematite) but not in the enrichment containing iron citrate. The table shows that iron reduction occurred in the highest dilution enrichments (Fe-citrate 10^{-6}, F6L 10^{-6}, Hem+AQDS 10^{-7}, Hem 10^{-5}). **Fig.5 B** shows that Shewanella was detected in the highest dilution enrichments (Fe citrate 10^{-4}, and F6L 10^{-2}) and Geobacter was detected in the highest dilution enrichments of F6L 10^{-4}, Hem+AQDS 10^{-4}, Hem 10^{-4}. These results indicate that there was no iron reducer belonged to Shewanella or Geobacter groups present in higher dilution enrichments. The cluster analysis of DGGE gels demonstrated the pattern is different between high dilution enrichments and low dilution enrichments.

Some Archaea [Kashefi, 2001] are also capable of growing by metal reduction. PCR targeting Archaea showed that Archaea were present in all layers of sediment cores, whereas only detected in the enrichments containing iron citrate (dilution $10^{-2,3,4}$). However, methane was detected in all enrichments and it means that methanogenesis happened in these enrichments.

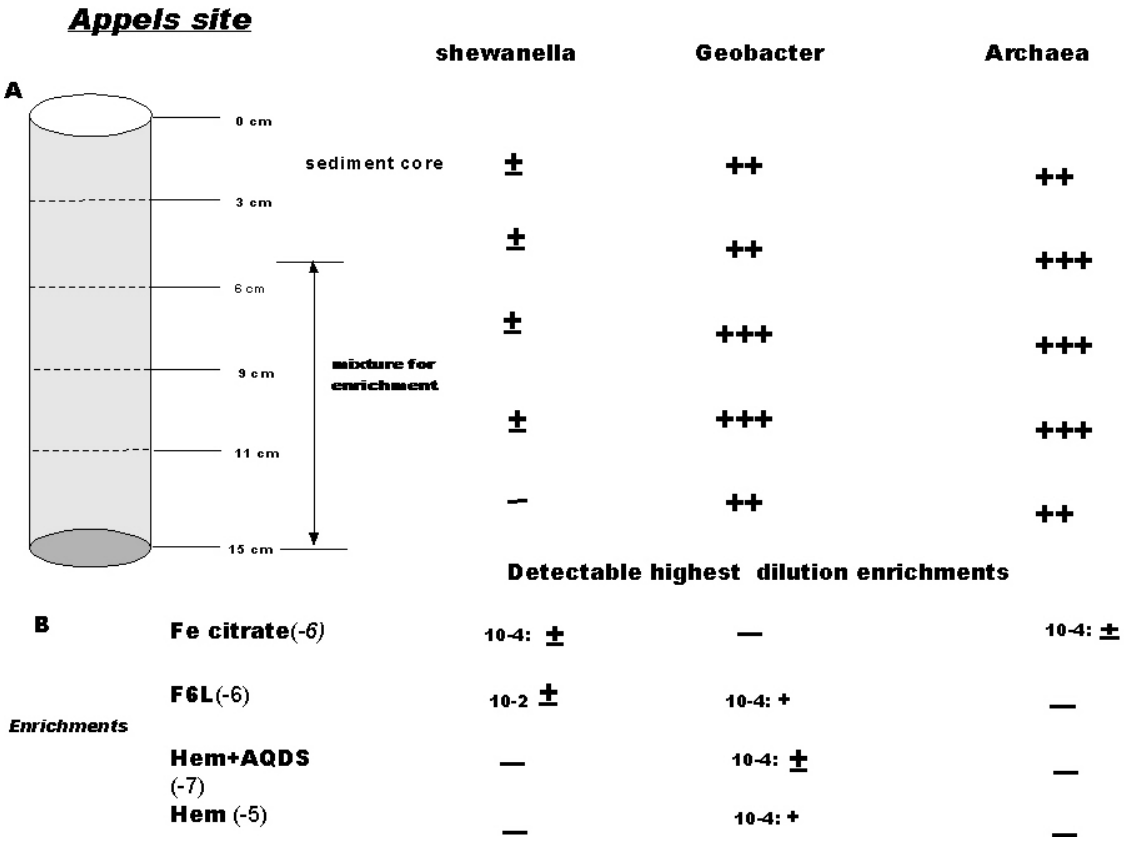
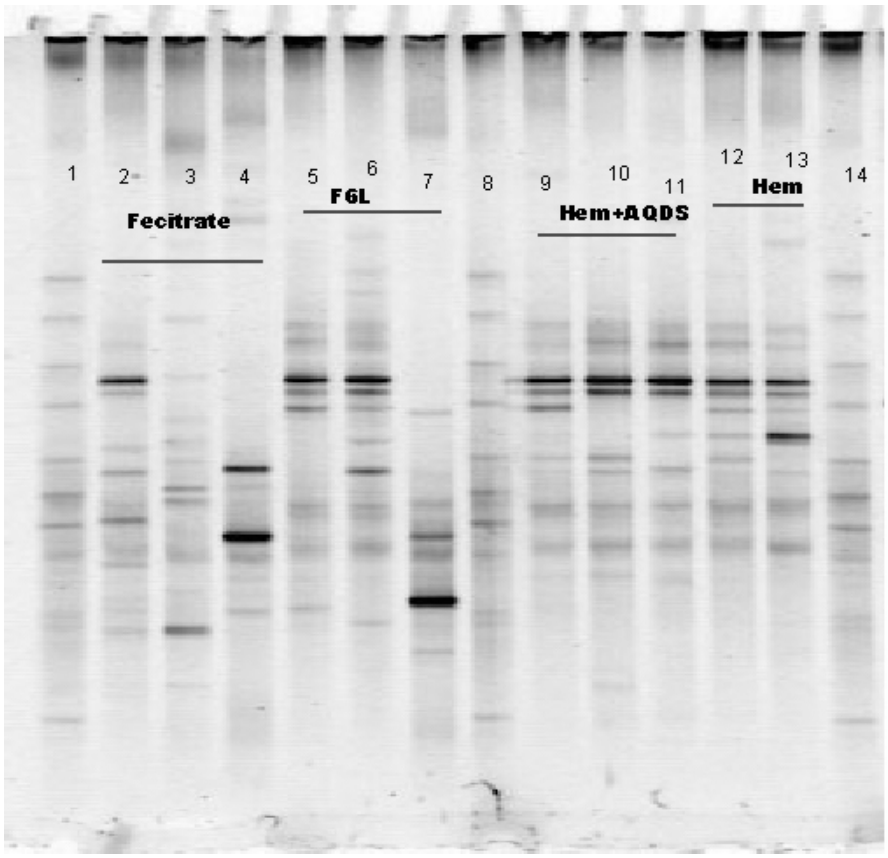


Fig.5. Scheme of iron reducer detection in sediment layers and enrichments. Number in bracket of B is the highest dilution enrichment having iron reduction. Symbols, +++, ++, +, ±, - represent the density of PCR products on agarose gel from highest to no signal.

There is less analysis on Waarde site samples till now. However, A DGGE gel (see below) was run for the enrichments for iron reducer community comparison. It shows there is less diversity of iron reducers in Waarde sample enrichments compared to Appels sample enrichments. There is one same dominant band in all enrichments except lane 3, 4 and 7.



DGGE profiling is in comparison with the different enrichments (1st round) of Waarde sample. Lane2, 3,4: Enrichment with iron citrate provided, $10^{-2,3,4}$; lane 5,6,7:$10^{-2,3,4}$ F6L provided, lane 9,10,11: hem and AQDS provided, $10^{-2,3,4}$; lane 12,13: hematite provide, $10^{-2,3}$

The subcultures of the lowest and highest dilution enrichments (3 times) for Appels and Waarde sites have been done and DGGE profiling is going to be analyzed as well. The data will be compared with the 1st round enrichments in order to decide which enrichments should be used for isolation to obtain wide range iron reducers.

3. Banisveld landfill (Sampling date: 16-11-02):

• *Enrichment and isolation by using different forms of iron oxide provided in media*

Sampling site description: Banisveld landfill is known as a polluted area by BTEX. Iron reducing microorganism is the dominant group coupled to pollutant degradation, in which is strictly anaerobic conditions. Thus, to avoid oxygen contaminant and microbial containment from surface is the most important factor to obtain intact environmental samples. The subsurface sample was collected with the help of geology department, VU. Sample was immediately and anaerobically stored in a stainless container. Samples from mixture of 2.75m-3.6m, polluted site, coded Pol01 and mixture of 2.3m-3.2m, clear site, coded Col01 were used for enrichment and isolation.

Considering subsurface conditions, the media used for enrichment and isolation were slight different compared to the ones for Appels and Waarde sites. Most of the components are the same, but less yeast extract (10mg/l) was added. Iron pyrophosphate instead of iron citrate was used as soluble iron source in combination with lactate or

acetate or benzoate as electron donor and carbon source. Medium preparation and inoculation procedures were the same as the ones for Appels and Waarde site samples, but incubation temperature is different, 25ºC, dark for Banisveld landfill samples. Meanwhile, MPN experiment was also done in microtiter-plate with iron pyrophosphate and acetate medium.

The amount of iron reducer is much low compared to Appels and Waarde samples, because of different environmental conditions, such as subsurface location, low temperature, low nutrients concentration, and different sediment structure-sandy etc. The color change (iron reduction) in the enrichments only occurred in low dilution, 10^{-1} to 10^{-2} after nearly 4-month incubation. However, iron reduction is more active in the enrichments with polluted sediment than the one containing clean sediment. Further work (PCR detection of shewanella, geobacter, geothrix, archaea, DGGE, isolation, [Fe] measurement, MPN to count the number) will be carried out on these enrichments.

4. Retentostat culturing of *Geobacter metallireducens*

-Medium selection: use the same medium as the one in batch culture but without Fe^{2+} solution added
We use *Geobacter metallireducens* (from DSM) as a reference strain to get familiar with retentostat cultivation. However, the strain is a mixture instead of 'pure culture' checked by DGGE gel with universal primers (see **Fig.6** lane 1,4). Thus, agar-serum-bottle and serial transfer were applied to purify the culture.

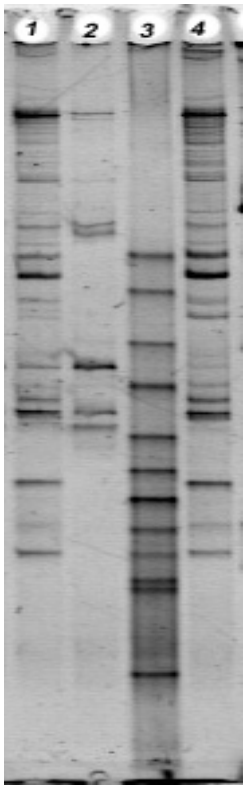


Fig.6 DGGE profile of PCR products with universal primers for Geobacter culture
lane1: *Geobacter metallireducens* culture from TNO; lane2: negative control; lane3: Marker; lane4: active culture of *Geobacter metallireducens* from DSM

Single colonies were obtained through agar serum bottle (see **Fig.7**). It was performed in glove box, and all the operations were carried out anaerobically. Although the anaerobic plates were also tried, there was rarely colony formation visible.

The tiny colonies, ø 1-2mm, white with small halo were picked up and transferred to liquid medium. Afterwards, the culture was made a serial transfer over time in an iron NTA, acetate, and vitamin medium. The purification was checked on DGGE gel of PCR products with universal primers. The single band on DGGE gel indicated the culture was pure(see **Fig.8**).

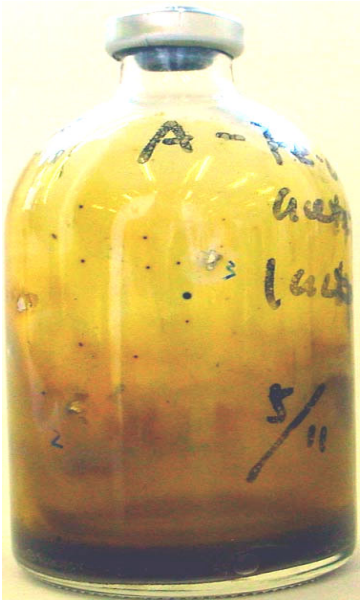


Fig.7. Agar serum bottle for isolating single iron Reducers (colonies seen in the wall, Appels sample)

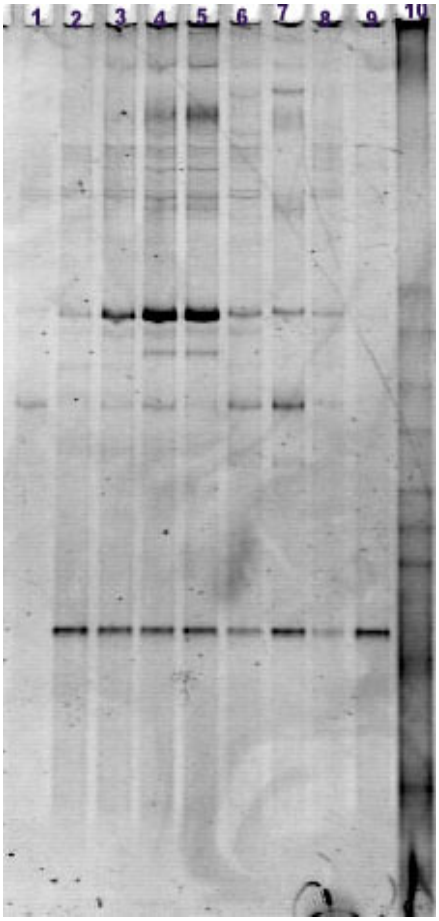


Fig.8. DGGE comparison of single isolate over 8 times transfer in a liquid medium. Lane1: negative control; lane2 to lane7: decimal serial dilution; lane8: 100 x dilution from lane7

-Retentostat setup & *Geobacter metallireducens* cultivation:

• *Strict anaerobic:* there are two major ways to keep the fermentor anaerobic. One is that anaerobic gas (N_2:CO_2=90:10) goes through Titanium solution to remove trace oxygen; the other is to keep the vessel under pressure.

• *Precipitate formation during culturing*: The precipitate accumulated over time and finally it blocked the filter of fermentor, three weeks in this case. Culture and filtrate sample were collected daily and anaerobically. Sample for Fe analysis was stored in HCl immediately. The color of culture was dark-red and the color of filtrate was much light (see **Fig.9**).

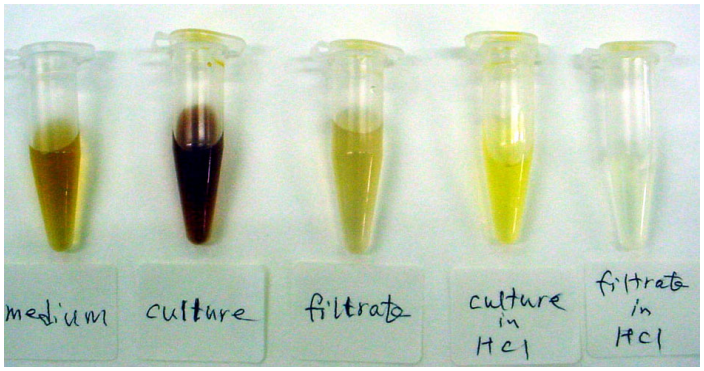


Fig. 9. Different colors were shown for medium, culture, filtrate, culture in HCl, and filtrate in HCl (from left to right)

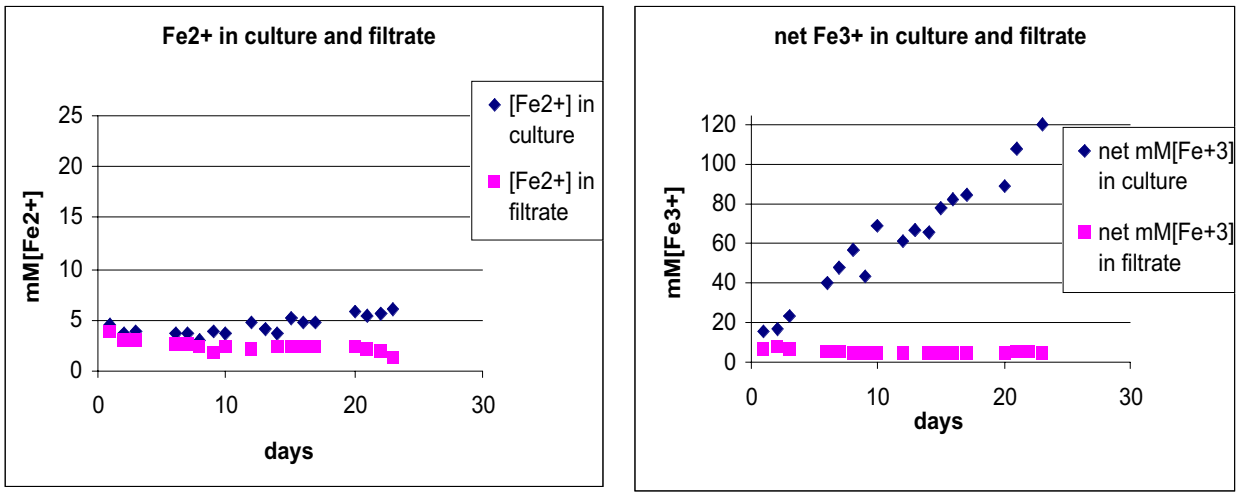


Fig.10 Fe^{3+} and Fe^{2+} conversion in retentostat cultivation of *Geobacter metallireducens*

From the retentostat cultivation of *Geobacter metallireducens*, we knew that Geobacter grew in the fermentor by iron reduction. When we cultivated *G. metallireducens*, the culture color was red (including cells and precipitate) and different from batch culture-colorless with light-yellow precipitate. That indicated there was still a lot of Fe^{3+} remained, and this is in agreement with the measurement (see **Fig.10**). [Fe^{2+}] and [Fe^{3+}] are both accumulating during the cultivation in the vessel. Especially, [Fe^{3+}] is

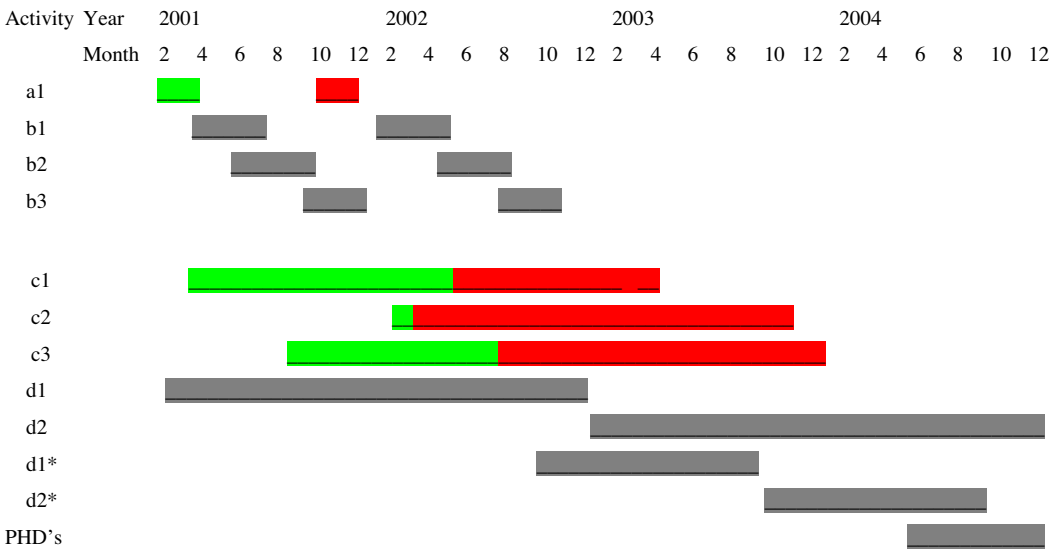
continuously increasing and Fe^{2+} in filtrate may inhibit the Fe^{3+} dissimilatory. However, the precipitate needs to be identified, and also why so much Fe^{3+} goes to precipitate. In filtrate, $[Fe^{2+}]$ and $[Fe^{3+}]$ became more or less stable from the third sampling day. The increasing volume in last three days due to vessel filter-blocked may be the reason for $[Fe^{2+}]$ to go down.

However, problems still exist for parameter measurement-biomass related (e.g. protein) due to precipitate, which entraps the cells. This precipitate will prevent the protein release from the cells. It may affect the precise determination of the physiological characteristics.

PROGRESS: WHERE AM I IN THE SCHEDULE.

A detailed research timetable as given in the original research application is given below:

Green : done. Red: to be done



Activities:

A: all participants; b: project 1; c: project 2; d: project 3

a1 Sampling of selected sites

b1 determination of surface chemical properties

b2 determination of the (abiotic) redox reactivity and reductive dissolution kinetics

b3 determination of microbial redox reactivity using well characterized organisms

c1 estabishing the kinetics of natural consortia from sampled environments in different fermenter types

c2 combining retentostat experiments with Fe(II) adsorption as measured in project 1

c3 establishing the presence and changes in microbial communities, using standard and molecular methods

bc1 joint study of reduction of synthetic and natural Fe(II) solids with different Fe(III) reducers

d1 modeling and development of ECA in cooperation with WFM Röling and HV Westerhoff

d2 testing the generality of developed models

PHD's Writing papers and thesis

Planning for next period:

• Subculture of enrichments (Appels, Waarde and Banisveld landfill sample) in order to isolate single species (iron reducer) able to utilize different types of iron oxides as electron acceptor

• Identify the isolates via physiological and molecular methods

• Clone library buildup for enrichments

• Optimize the parameter measurement, such as measurements related to biomass, protein, TOC, or cell counting, and acetate, lactate

• Culturing Geobacter and consortia establishment of environmental samples in retentostat

Time schedule for activities from March to September

Items	***Activities***	***Periods***
1	Optimize the parameter measurement for retentostat cultivation, e.g. protein, TOC, acetate	March-early April, 03
2	Molecular and physiological analysis of enrichments from Appels, Waarde and Banisveld samples	March – June, 03
3	Isolation and identification from enrichments	May – July, 03
4	Clone library buildup of enrichments	April – July, 03
5	Geobacter culturing & consortia (Banisveld landfill sample) establishment in retentostat	Middle, April – September, 03

Reference

Kashefi, K.Tor, J. M.Nevin, K. P.Lovley, D. R.(2001) “Reductive precipitation of gold by dissimilatory Fe(III)- Reducing Bacteria and Archaea”. Applied and Environmental Microbiology. Vol. 67: 3275-3279

Jones, G. A. and M. D. Pickard (1980). "Effect of Titanium citrate as reducing agent on growth of rumen bacteria." Applied and Environmental microbiology Vol.39 (No.6): 1144-1147.

Miller T.L, Wolin. M.J. (1974). A serum bottle modification of the Hungate technique for cultivating obligate anaerobes Vol.27 N0.5.

Roden, E. E., M. R. Leonardo, et al. (2002). "Immobilization of strontium during iron biomineralization coupled to dissimilatory hydrous ferric oxide reduction." Geochimica Et Cosmochimica Acta 66(16): 2823-2839.

Roh, Yul,Liu, Shi V, Li, Guangshan, Huang, Heshu, Phelps, Tommy J and Zhou, Jizhong.(2002). Isolation and Characterization of Metal-Reducing Thermoanaerobacter Strains from Deep Subsurface Environments of the Piceance Basin, Colorado. Appl. Environ. Microbiol..Vol. 68. 6013-6020

Schwertmann, U. and R. M. Corned "'Iron oxides in the laboratory preparation and characterization'." Zehnder, A. J. B. and K. Wuhrmann (1976). "Titanium (III) citrate as a Nontoxic oxidation-reduction buffering system for the culture of obligate anaerobes." Science 194: 1165-1166.