WELLMA-DNA End Report

Documentation of data acquisition and conclusions

Dipl.-Ing. Oliver Thronicker and Prof. Dr. rer. nat. Ulrich Szewzyk

TU Berlin 2011

Table of Contents

List of Figures

List of Tables

1 Microbiology of well clogging

Microbiology plays an important part in many technical processes. Water suppliers want to prevent the production of toxins in water reservoirs, the corrosion of distribution pipes or the clogging of pumps and gravel packs. In addition, the elimination of pathogens, which can contaminate the water or hide in biofilms among harmless bacteria is one of the most important tasks.

When dealing with groundwater, it is essential to take into account the chemical and microbiological processes that could compromise this vital resource.
Flow velocity in the deep groundwater can be as slow as several meters a year, temperatures are mostly constant and there is usually no oxygen to promote the degradation of complex organic molecules.

The construction and commission of a drinking water well changes these conditions drastically. The flow velocity in close vicinity to the well increases extremely and through a mixture of different groundwater levels in the filter area, chemical conditions change as well. Oxygen or other electron acceptors can reach the filter area, which can lead to a massive development of iron bacteria, which in addition to their spacious morphology, deposit huge quantities of insoluble iron hydroxides and lead to a steady decline in well performance due to ochre formation.

1.1 The Iron Bacteria

The iron bacteria are a group of bacteria known since the first half of the 19th century, their features nevertheless still remain controversial. They can be found all over the world, were the environmental conditions are suitable. In addition to an appropriate electron acceptor like nitrate or oxygen, ample amounts of bivalent iron have to be available to promote their growth.

In anaerobic ground waters, iron is continuously transformed from its trivalent oxidized, to the water soluble, bivalent form. This happens chemically (e.g. FeOOH (Goethite) + e^- + $3H^+$ —> $Fe2^+$ +H_2O), but also through iron reducing bacteria, which use trivalent iron species as an electron acceptor, to oxidize organic substances (Teutsch et al. 1997).

When exposed to small amounts of oxygen through construction and operation of drinking water abstraction wells, the iron in otherwise anaerobic groundwater, can be chemically and biologically re-oxidized again. Iron bacteria catalyze this process and are found especially in the anaerobic/aerobic border zone. In the presence of ample nutrients provided by strong flow rates, a development of appreciable biomass is possible. Therefore, especially the upper filter area and the suction grid of the pump are impacted (Cullimore, R.1999). Other factors of influence are: temperature, pH-value and redox-potential of the respective water. Some of the best-known representatives of this group of organisms are:
Leptothrix ochracea, Gallionella ferruginea, Thiobacillus ferrooxidans and *Siderocapsa sp.*

Iron bacteria are able to utilize the energy provided by the oxidation of bivalent iron ($4Fe^{2+}$ + $4H^+$ + O_2 = $4Fe^{3+}$ + $2H_2O$) or manganese for their metabolism. In this case reduced iron takes the role of electron donor and oxygen functions as the electron acceptor. The energy provided by this reaction is very small (-29 kj per mol iron for pH 2) (Ehrlich 2002). At circumneutral oxygenated environments, the energy yield can increase to an estimated −90 kJ/ mol iron (Emerson D. et al. 2010, Roden E. et al. 2004).

The downside is the vastly increased abiotic oxidation of Fe(II) in oxygenated water, with a half-life of only few minutes. At microaerobic conditions, as they are often encountered during drinking water abstraction, the half-life of Fe(II) can be up to 300-fold longer though (Roden E. et al. 2004). In addition, the constant influx of new iron rich water into the pump and filter area render this limitation even more negligible.

Of no small concern however is the fact that the precipitated iron is gradually cutting off the bacterial cells from the steady stream of substrates. This is why many iron bacteria developed specific strategies to prevent entrapment. Some relocate the iron oxidation into a long stalk, growing away from the original cell, some develop long sheaths that get incrusted and can serve as an escape tunnel, if environmental conditions change. Some even excrete halos of extra-cellular polymeric substance (EPS) catalyzing the iron precipitation (Chan et al. 2009) apart from the cell.

This taken into account, it is not unusual for Berlin water wells to contain vast amounts of different ferric hydroxides and iron bacteria.

1.2 Iron reducing bacteria

Anaerobic dissimilatory ferric iron-reducing bacteria on the other hand gain energy through reduction of iron minerals and catalyze iron solubilization in many anoxic environments (Straub et al. 2001). Just like the iron precipitating bacteria, they have been isolated from a great number of habitats all around the world, including sediments, soils and the terrestrial subsurface. (Kim et al. 2011, Aklujkar 2010)

At circumneutral conditions, most iron minerals are barely soluble, so the mechanism of electron transfer to the minerals is a challenge.
Humic substances are known to act as electron carriers (Kappler et al. 2004), but some bacteria also use conductive nano structures (Reguera 2005) or extracellular compounds (Nevin and Lovley 2002) for the electron transfer.

The well-known iron reducing bacteria *Geobacter sp.* and *Geothrix sp.* require strict anaerobic conditions and a simple organic electron donor. Hence, they sometimes compete with sulfate-reducing organisms, which live under the same conditions. Their potential for the biological removal of ochreous deposits is one of the questions in this study, since it is already established that iron reducers are able to completely reverse the precipitation of ferryhydrites by iron respiration resulting in huge amounts of soluble Fe(II) in iron-rich groundwater systems (Straub et al. 1998).

1.3 Test Systems: BART

In recent years, several test systems have been developed for the evaluation of bacterial activity in technical systems. One of the most widely used systems is the „Biological Activity Reaction Test" for the detection of iron bacteria and several other groups (Cullimore 1999). This test consists of glass tubes containing different growth media, which are combined with a water sample, to induce bacterial growth.

The main advantage of such a cultivation-based method is the broad range of microbial species, which can be detected (Siqueira and Rôças 2005). An advertised feature of the test system is the complete independence of a chemical or microbiological laboratory, since there is no incubator necessary and the test can be performed on site.

Color changes, turbidity, precipitations and the formation of rings or bubbles serve as indicators for bacterial activity and a reference card allows for quick evaluation of the results. (Hach Company 1995).

In case of the BART system, this method has several pitfalls though. One of the main criticisms is that the system is not suitable for quantitative evaluations, since the incubation is not conducted under standardized conditions. Especially for the application in drinking water wells, recent results have shown that clogging-related bacteria have vastly differing and sometimes very long growth rates. (Schmidt 2008) So, the cultivation could take several days or even weeks, before a reaction is visible.

In addition, several microorganisms may grow in the BART media, who may be unrelated to well clogging or not be the dominant species, while many of the actually important bacteria may not be cultivable at all, due to their specific environmental needs (flow velocity, temperature or special nutrients only available at the sampling site) (Oliver 2005).

1.4 Molecular test systems

To circumvent these long known restrictions and pitfalls of classic cultivation, molecular methods offer an alternative and efficient approach for a broad range of technical applications. In medical and hygienic monitoring, were high specificity and sensitivity is essential, molecular analysis is already an essential and well-established tool (Clarke 2002, Quiagen GmbH 2011).

Most molecular test systems are based on the polymerase chain reaction (PCR), which allows the amplification of specific DNA fragments with the help of custom designed primers. The amplified DNA can be detected during or after the PCR with the help of fluorescent molecules. In the context of bacterial analysis, it is important to calculate appropriate primers of sufficient specificity for the desired group of bacteria. This can be accomplished by utilizing databases containing the target strain as well as other related bacteria.

In this work, we adapt the molecular approach to the subject of microbiologically induced ochre formation. Building upon the data of this study, aim is to develop a molecular indicator system for well monitoring. In order to achieve this, we first analyzed the populations of different wells, gaining sequences of the dominant bacterial species. Phylogenetic analysis and results of simultaneous cultivation trials lead to a selection process, resulting in several primers that can now be applied, improved and further complemented.

2 Methods

2.1 Sampling

To gain the necessary insight into the population structure of the Berlin water wells, samples had to be acquired in various ways. A sampling campaign of more than 21 water wells has been conducted during the project and more than 400 DNA samples along with samples for microscopic analysis or cultivation trials have been collected. The sampling methods had to be continuously refined to suit the needs of the project.

2.1.1 Biofilm samples

Because of their construction characteristics, wells are a unique research environment, which makes sampling a delicate process. In order to ensure the undisturbed growth of biofilms, a suitable sampling device had to be designed. One of the requirements was the ability to take samples without interrupting well operations beyond a reasonable level. Therefore, existing standpipes (interior and exterior) were utilized.

The designed sampling device, made it possible that neither additional construction work, nor opening the wellhead was necessary, which was a great advantage. For safety reasons though, the pump had to be shut down briefly during introduction of the sampling device, but could be restarted shortly afterwards.

The sampling device was redesigned and optimized several times during the project. The final design was composed of a stainless steel wire (2 mm 7x7), because steel was easier to handle, in case the device got slightly jammed.
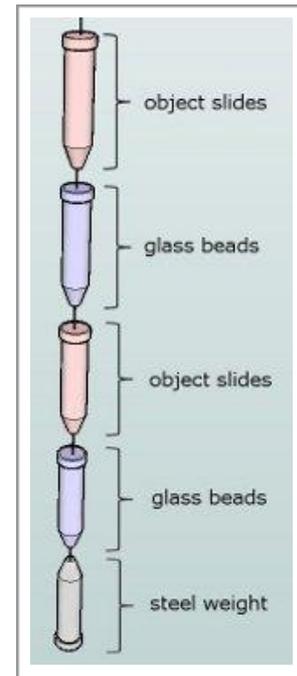
Pic.1: Sampling system

Alongside the steel wire, several perforated containers (15 ml falcon tubes) were attached with simplex clamps. These autoclavable polypropylene containers were equipped with glass beads or microscope slides. Since normal slides wouldn't always fit into the standpipes, an instrument was developed to cut object slides in half.

While the slides were used for microscopic analysis, the glass beads in the containers were used as carrier material for bacteria, from which DNA was to be extracted. The terminal container was filled with a steel weight to keep the device straight.

2.1.2 Water sampling

Environmental sampling is always prone to contamination and degradation, which is especially problematic in quantitative molecular and microbiological analysis.
Collection of water samples for bacterial community analysis poses a great challenge in particular, because large quantities of water need to be collected and since chemical fixatives always pose a severe risk of damaging the bacterial DNA, the water needs to be cooled in order to prevent further bacterial growth, resulting in a change of population. Cooling of large quantities of water, though, is often way too impractical to be employed.

In order to solve this problem and further enhance the sampling method for water wells, a prototype in-field filtration unit was developed in addition to the sampling device described in the previous chapter. With the modified filtration unit, it became possible to collect a large amount of bacterial cells directly at the sampling site, which could subsequently be cooled very easily.

Improving the sampling process was another vital step stone in the development of a molecular detection system. For future sampling campaigns, the device could be further modified, with a small air pressure cartridge, allowing for even faster and more convenient biological sampling on a routine basis.

Instruction sheet for the in-field filtration unit

To be able to use a sartorius filtration unit (SM 162 49; max 10 bar; max 150 °C ; Vol 0,22 l) at sampling campaigns in the field, a high pressure bicycle pump (Fit H2 von Airace) was connected with an adapter (modified bicycle valve and air filter (Millex FG50, Millipore)).

Application:
1) Introduction of filter (isopore membranfilter 0,2 μm, Millipore, GTTP04700)
2) Autoclaving of filtration unit. Because the valve contains rubber components not suitable for high temperatures it is necessary to unscrew the top and autoclave without the vent as well as the "bicycle pump")

In Field:
3) Water is poured in (200 ml).

4) Connection of pump to filtration unit.

5) Pumping, until water is completely removed from filtration unit.
6) Eventually refilling with water, until needed amount of water has been processed (one litre), or filter is no longer permeable.

7) Filtration unit is opened at the bottom end (threaded coupling).

8) Washer and filter are removed with a flame treated tweezer. afterwards stored in a sterile 50 ml Falcontube.
9) Multiple flushing of filtration unit with new sampling water and insertion of new filter.
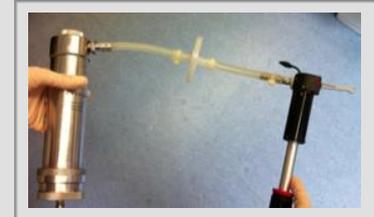
Filter: Pic.2: Filtration unit
Description: Isopore Membrane, polycarbonate, hydrophilic, 0.22 μm, 47 mm, white, plain. Trade Name: IsoporeFilter. Material: Polycarbonate. Media brand name: Isopore. Refractive index: 1.6. Water flow rate, mL/min x cm²: 6. Bubble point at 23 °C: ≥ 3.5 bar, air with water filter type: Screen filter pore size (μm): 0.22. Wettability: Hydrophilic. Filter diameter, mm: 47. Filter Code: GTTP Filtration device and accessory type: Filter Discs/Sheets. Filter color: white. Filter surface: plain. Thickness, μm: 7–22. Gravimetric extractables, %: < 1. Air flow rate, L/min x cm²: 1. Porosity %: 5–20

2.2 Transport and storage

In order to prevent changes in structure or composition of the samples (Stotzky et al. 1962), appropriate methods for transport and storage had to be applied. Depending on their final purpose, the samples were processed in different ways. While the glass beads for DNA analysis were transferred in a sterile 50 ml falcon tube, samples for the microscopic analysis were transported in environmental water or phosphate buffered saline.

All samples were transported in a cooling container filled with cooling bags for the duration of the transport (2-3 hours). After initial microscopic analysis in the lab, the slides were either dehydrated with an ascending ethanol series or frozen at −20 °C like the glass beads (for long term storage).

Samples for electron microscopic analysis have been stored in 3,5 % formaldehyde (for transport). In the lab, a multiple step ascending acetone or ethanol series was followed by critical point drying and subsequent gold coating. The samples were stored dust free at room temperature.

Samples for cultivation trials were either transferred into the growth medium directly at the sampling site or also stored at 4 degrees and then transferred into growth media in the lab.

2.3 Microscopic analysis

In order to gain an overview of the dominant iron bacterial communities in the wells, the slides were microscopically analyzed applying bright field and phase contrast technique. In addition, DNA binding fluorescent dyes were used to greatly enhance the visualization of bacterial cells. Since the staining and visualization of encrusted bacteria proved to be difficult with some samples, methods for solubilizing the incrustations have been tested in addition. These methods include the application of oxalic acid in low concentrations, which chelates the iron and reveals subjacent structures.
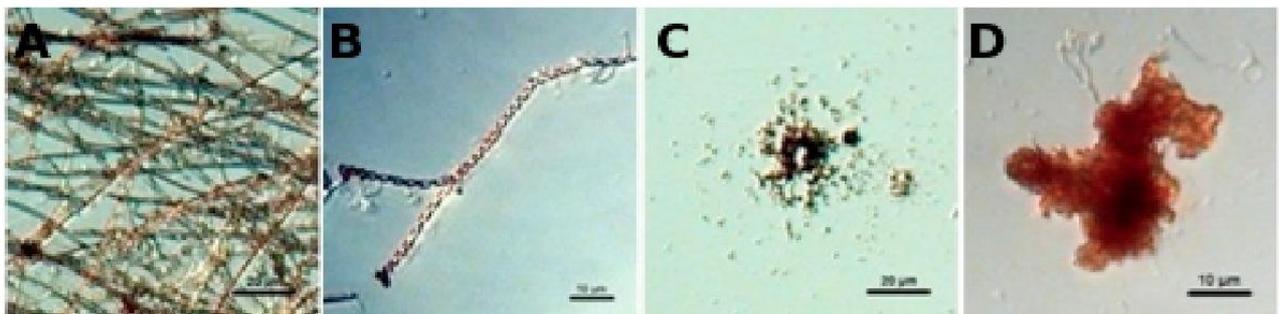
Fig.3: Morphotypes

2.3.1 Results of the microscopic analysis

In order to allow a first microscopic evaluation of the chosen sampling sites and to see first differences in the dominant species of iron bacteria, object slides were exposed in 21 different Berlin wells, for several weeks.

Four major morphotypes of iron bacteria could be identified (Fig. 3): Type A: sheath forming (e.g. *Leptothrix spec.*, *Sphaerotilus spec.*), type B: stalk forming (*Gallionella spec.*, *Toxothrix spec.*), type C: corona forming (*Siderocapsa spec.*) and type D: cell agglomerates (e.g. Actinomycetes).

The results of this microscopic characterization are summarized in the chart below.

Tab.1: Morphotypes in different wells

Well	Morphotype
TEGhzk-13-/73V	A,B
TEGhzk-22-/71V	D
SPAnord01-/04V	B (many),D (many)
SPAnord02-/82V	C (some)
SPAnord03-/99V	A,B,D
TEGsaat19-/71V	C (many)
TEGsaat20-/71V	D
TEGwest12-/84V	D
TEGwest13-/85V	A,B,C,D
TEGwest14-/85V	B,C,D
KAUnord05-/95V	A (some),C,D
KAUnord06-/95V	A, C (many)
KAUnord07-/95V	A,C,D(many)
STOborg14-/90V	-
STOborg15-/90V	A (many),B,D (many)
STOborg19-/90V	-
STOborg20-/90V	A,B,D
BEEgrfe05-/93V	C (some)
BEElind01-/95V	C
BEElind23-/02V	A, B
TIEsued19-/60V	-

2.3.2 Implications of microscopic analyis

The different morphotypes represent at the same time the different strategies in dealing with the precipitation of iron hydroxides (see chapter 1.1). The microstructures of these morphologies may directly impact the macroscopic structure and rigidity of the resulting ochreous deposits (Schenker et al. 2008). So, it would be very important to know which type of iron bacteria occur predominantly at a certain well.

During this study, no in-depth comparative experiments have been conducted between the four major morphotypes, but some results (see chapter 6) indicate that especially stalk forming and sheath forming bacteria seem to be more vulnerable to treatments with hydrogen peroxide than the corona and agglomerate forming species.

2.4 Flow cell

In order to assess the growth characteristics of the bacterial isolates, a flow chamber was designed and constructed based on earlier designs by Szewzyk (Szewzyk & Schink 1988) and Wendlandt (Wendlandt 2001). The new design was continuously revised and is now applicable for a multitude of experimental outlines.

The cell consists of two glass cones, an object slide and a cover slip combined with silicone (Fig. 4). The two glass cones serve as an access point for the attachment of tubes, which allow the operational mode as a continuous flow reactor. To prevent the diffusion of oxygen into the flow cell, iso versinic tubings can be used and the splices can be coated with an impermeable varnish.

One mayor advantage of this layout is the option to autoclave the whole setup and the easy and cost effective reconstruction.
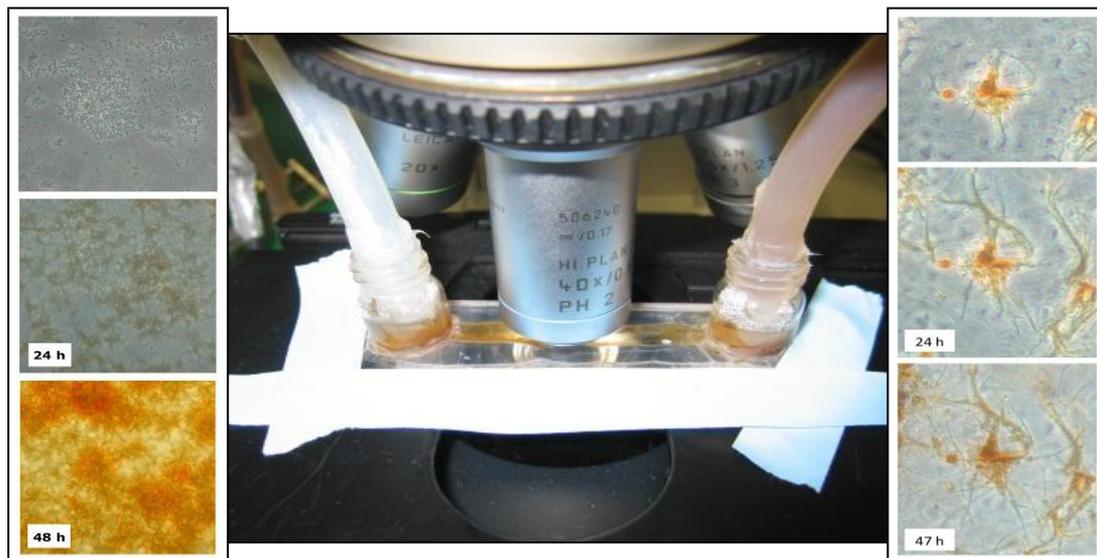
Fig.4: Flow cell. Left: Isolate 126 Right: Isolate 151

2.4.1 Implications of flow cell experiments

With the help of the flow cell, it was possible to gain deeper insight into the growth characteristics of different types of ochre forming bacteria (Jäger 2010). In figure 4, it can be seen that the cluster forming isolate 126 had a completely different pattern of ochre formation than isolate 151. So, estimates about the rigidity of ochre formations, depending on the dominant species could be possible, if a molecular detection method exists.

2.5 Scanning electron microscopy

Scanning electron microscopy permitted an even deeper insight into the structure of the collected environmental samples and pure-culture biofilms. The results of the classic microscopic trials could be validated and growth characteristics of certain bacteria could be observed more clearly. When assessing the clogging potential of different iron oxidizing bacteria, the ultra-structure can help to evaluate the physical properties of the resulting biofilms. In addition, in some cases even the visualization of morphological changes during different growth phases was possible.

2.5.1 Results of scanning electron microscopy

Scanning electron microscopy (SEM) of pure cultures of several different isolated bacteria revealed distinct differences in growth and morphology.
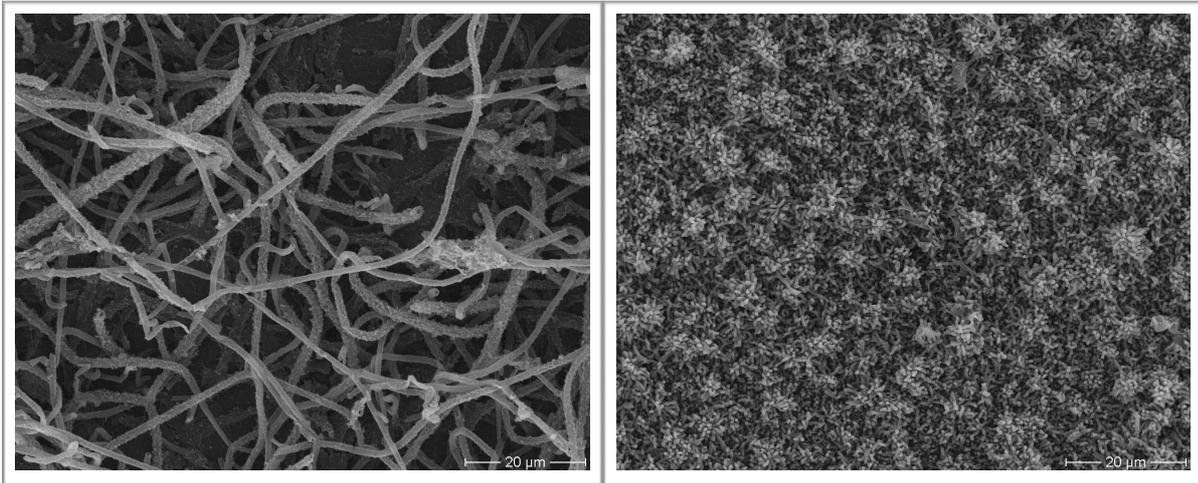
Fig.5: SEM picture (20 µm) of *Leptothrix sp.* (left) and *Kineosporia sp.* (right)

While Lepthothrix strains grow in a broad meshed filamentous network, *Kineosporia* strains grow in a dense carpet coating the entire surface.

Closer observation reveals a budding behavior of *Kineosporia*, resulting in distinct patterns, which at a certain stage of colony density seem to result in excessive iron oxidation (chapter 2.4).
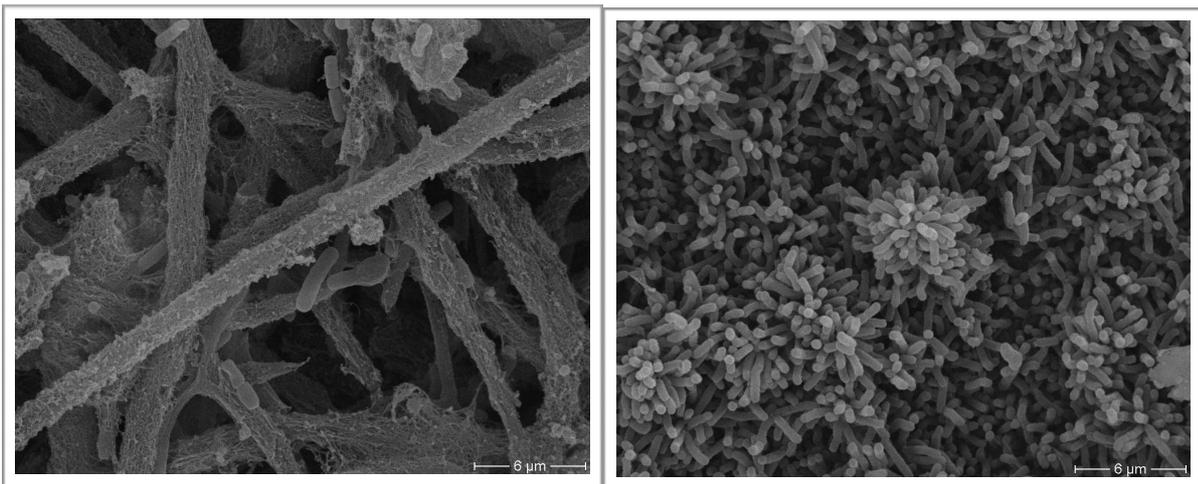
Fig.6: SEM picture (6 µm) of *Leptothrix sp.* (left) and *Kineosporia sp.* (right)

2.5.2 Discussion of SEM

The sheath forming species are colonized themselves by different other bacteria living within the iron incrustations in deeper layers of the colonies. The sheath serves as a very fast growing, three-dimensional extension of the biofilm. By precipitating iron hydroxides and serving as surface for secondary colonization (chapter 3.1) it promotes a very fast thickening of the incrustations. By recrystalization of hydroxides, further hardening could also be possible, if left untreated for longer periods of time.

3 Molecular Methods

3.1 FISH

Applying the FISH (Fluoresence In-Situ Hybridization) method, it is possible to specifically visualize target organisms in a community. The method utilizes the fact that bacterial cells contain a multitude of ribosomes, partly composed of ribonucleic acid (RNA). Since ribosomal RNA contains very specific regions, genetic probes labeled with fluorescent dyes can bind selectively to bacteria that contain the target sequence. With the help of suitable light sources, these signals can be visualized.

Many iron bacteria are part of the group of the Beta-proteobacteria, a class in the bacterial phylogenetic system that groups bacteria according to base sequences of ribosomal 16S-ribonucleic acid (16S-rRNA). Important representatives of the Beta-proteobacteria can be found in the genera Sphaerotilus, Nitrosomonas, Spirillum, Thibacillus and Gallionella.

During the first trials, gene probes specific for the Beta- and Gamma-proteobacteria were tested. In later stages of the project, a gene probe specific for an iron bacterium in close relation to *Leptothrix cholodnii* was developed (Lehmann 2009) and established (Rönsch 2011) during the course of two diploma theses, which have been supervised by the author. This gene probe later was applied during a third diploma thesis (Papke 2011), which focused on the effect of hydrogen peroxide on the ochreous biofilms.

3.1.2 Results of hybridization trials

Initial experiments were performed to evaluate hybridization of gene probes to native biofilms on exposed glass slide. Since encrustation of bacteria by iron hydroxides could interfere with hybridization, selected slides were pre-treated with oxalic acid to dissolve precipitates. Probes for the group of Gamma- and Beta-proteobacteria were used. It turned out that with glass slides exposed for one or two weeks, hybridization was possible without problems. Glass slides that were exposed for more than seven weeks had to be pre-treated to improve hybridization results.

With some slides exposed in non-operating (OFF) wells, it was noted that the ratio of Beta-proteobacteria to the total cell count appeared to be lower compared to those exposed in operating (ON) wells. In addition, differences in total cell count and ratio of Beta-proteobacteria could be observed in the respective wells.

Detailed analysis of mixed populations in model biofilms granted deeper insights into the interactions of iron bacteria with the biofilm community in respect of structure and nutrients.

Figure 5 shows a fluorescence microscopic image of a mixed culture of a sheath-forming iron bacterium and bacteria from one of the well sampling sites. The FISH-Probe (green signal) specifically established for an isolate in close relationship to *Leptothrix cholodnii*, made it possible to visualize the interactions of this iron bacterium with other bacteria in the biofilm. While probe signals (green signal) clearly show Leptothrix cells only inside the sheaths, total cell stains with DAPI (blue signal) reveal multiple other bacteria in very close proximity around the sheath-forming bacteria. Additional hybridization with probes for iron-reducing bacteria (red signal) also verified the presence of *Geobacter sp.* in these cell agglomerates.
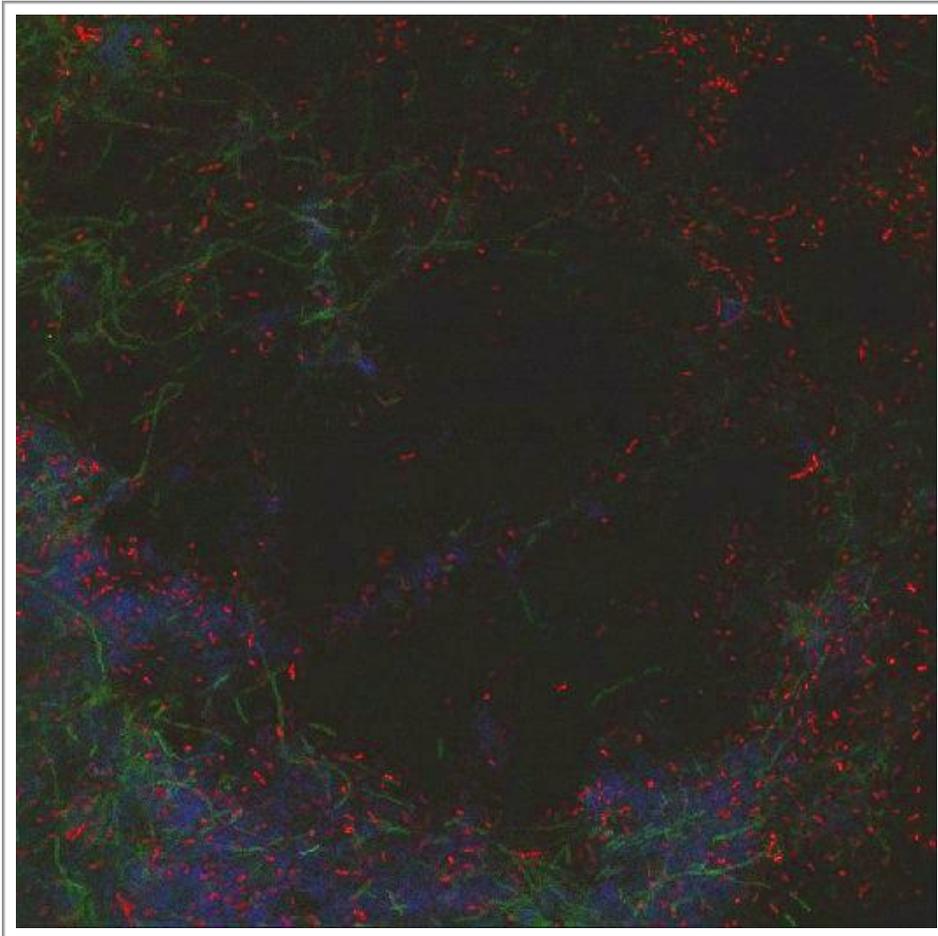
Fig.7: CLSM picture of *Leptothrix sp.* (green), *Geobacter sp.* (red) and other bacteria (blue) in a biofilm reactor

3.1.3 Discussion of FISH

The results of fluorescence in situ hybridizations indicate that especially sheath-forming and probably stalk-forming bacteria serve as an important structural element in the thick ochreous deposits (see also chapter 2.5) and can act as a surface for secondary colonization. This clearly shows that the iron bacteria are key organisms and can have a tremendous impact on the structure and composition of the biofilm. If it is possible to detect them before mass development occurs, counter measures could be much more effective.

The fact that iron-reducing bacteria have also been detected inside the incrustations that surround the sheaths can be of great advantage, if a change in environmental conditions can be accomplished. The iron-reducing bacteria could potentially destabilize the deposits and greatly increase the effect of shear force or H_2O_2 treatments on the biofilms.

4 The molecular biological experiments

4.1 DNA Extraction

In order to extract bacterial DNA from the biofilms, glass beads (Ø 3 mm) were exposed in the wells in addition to the object slides. After recovery from the well, these glass beads were stored at – 20°C and the attached biofilm was detached with the help of shaking and washing steps.

Protocol for DNA extraction of water samples

1) A 50 ml tube filled with a filter recovered from the filtration unit is filled with 10 flame treated (DNA free)glass beads and 5 ml PCR water.
2) The tube is treated on a vortex mixer for 1 minute, until the filter is clean.
3) The resulting liquid is divided into three 2 ml Eppendorf reaction tubes and centrifuged (3000g, 3 min).
4) After discarding the supernatant, the remaining pellets are resuspended in 150 µl PCR water and reunited into the spin column of the FastDNA® SPIN KIT FOR SOIL.

During test trials, it was determined which extraction method was the most suitable for our samples. The important impact of the extraction method on the DNA recovery has already been discussed in several research papers (Martin-Laurent et al. 2001, Stach et al. 2001). For this study, we decided on the FastDNA® SPIN KIT FOR SOIL, by MP Biomedicals, which combines chemical lysis, with mechanical bead beating, as recommended for community analysis (Krsek 1999).

During the progress of the project, more than 400 samples from different sampling locations have been collected, including both, water and biofilm samples. The samples have been processed and the bacterial DNA has been extracted, generating enough material for follow-up analyses. DNA amplification, cloning and DGGE trials have been conducted. It was observed that many of the biofilm samples yielded only low amounts of DNA compared to the water samples. In addition, certain substances accumulated in the biofilms and appeared to interfere with the PCR. PCR, conditions could be optimized to a sufficient degree to resolve these problems.

4.2 DNA Cleanup

After extraction, the DNA was still contaminated with chemical soil components, which were not eliminated during the extraction process (Conradi 2011). Since no suitable method existed at the beginning of the project to identify the nature of the inhibiting components, the DNA extracts were further diluted with PCR water, in an attempt to also dilute the contaminations to a point, where the subsequent amplification was no longer inhibited. In later stages of the project, a "NanoDrop DNA Analyser" became available at the department, which allowed for precise quality control and led to further cleaning steps, which enabled us to process the water samples in an optimized way. Unprocessed DNA extracts still contained PCR-inhibiting components like humic acids, which absorb at wavelengths of 230 nm, while the cleaned DNA samples only showed absorption around 260 nm. We used the Wizard® SV Gel and PCR Clean-Up System (Promega) for processing the DNA extract.

4.3 PCR

Collected DNA was amplified with PCR (polymerase chain reaction), following the optimization of PCR parameters. For amplification of the 16s rDNA for sequencing we used the primer set 63f, 1387r (Marchesi et al. 1998) and for the DGGE experiments the primer set by Muyzer (Muyzer et al. 1993) and a modified touchdown approach as presented by Don (Don et al.1991).

4.4 PCR enhancement

In order to increase the yield or in some cases even allow for a product in the first place, an enhancer solution (Ralser et al. 2006) was tested and applied in later stages of the project. It has the potential to stabilize the polymerase enzyme and to neutralize inhibitory contaminants, which bypass the cleaning

Fig.8: Phases of PCR optimization

procedure. Additional advantages include its low cost and the option to further fine-tune it for specific applications. The application of the enhancer solution was a major breakthrough in the effort to generate a standardized PCR protocol.

4.5 DGGE

Denaturing gradient gel electrophoresis is a powerful and cost effective tool to assess similarities between different bacterial populations. With the help of specifically designed primers (Muyzer et al. 1993), a very heterogeneous fragment of the 16s rDNA was amplified (V3 region) and subsequently applied on a gel containing a denaturing gradient. Exploiting the different denaturation properties of different code fragments of DNA, this resulted in the fragments being separated according to their genetic code, creating unique band patterns.

4.5.1 Results of DGGE

The results of the the DGGE trials (Popiol 2009) show similarities, but also differences between populations of different wells (Fig.9). Even neighbouring wells show different population compositions. Some bands though appear in many of the analyzed wells and the diversity of observable bands differs strongly between the habitats. DGGE analysis of the monitoring wells at STOborg15-/90V for example revealed distinct differences between the populations at 6 m and the populations at 13 and 16 m depth. Two additional clusters emerged, illustrating the differences between the river facing side and the land facing side (Rauch 2010).
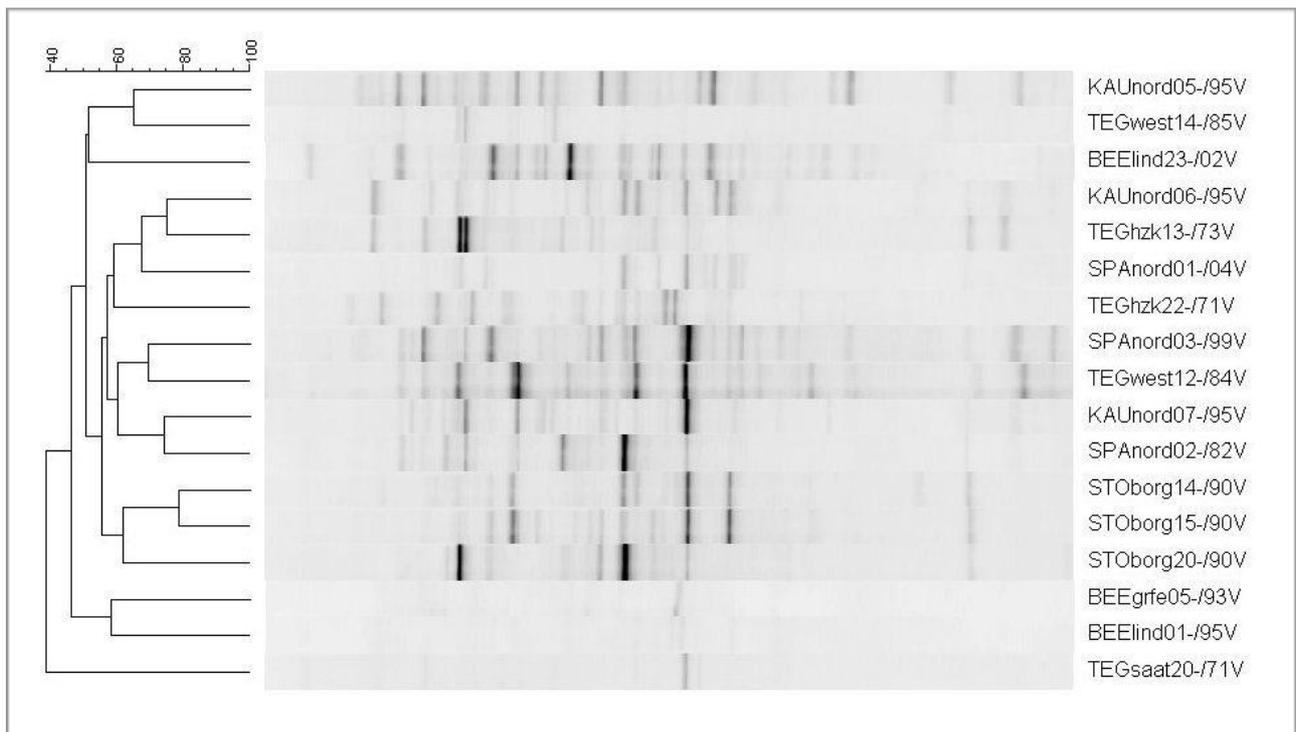
Fig.9: DGGE patterns of different wells

4.6 Sequencing

In order to identify the dominating species of iron bacteria in the wells, cloning and sequencing of multiple samples has been performed.
DNA fragments of the 16s rDNA have been inserted into a cloning vector and subsequently introduced into *E.coli sp.* for amplification. The plasmids, containing the fragments of 16s rDNA have been retrieved, sequenced and stored at −80 °C.

The resulting data has been used for phylogenetic analysis, primer and probe design. The plasmid stock can serve as future reference and standard for quantitative real-time PCR.

5 Cultivation based methods

5.1 Isolation of pure cultures

During the sampling campaigns, several isolation attempts have been made to gain pure cultures of iron precipitating bacteria. In this process, samples from different wells (see Tab.1) and different locations within the well (water, pump coating and biofilms on glass beads) have been collected.

Decimal dilutions (up to 10^{-4}, depending on inoculum) of biofilm suspensions were prepared with PBS (phosphate buffered saline). The last two dilution steps were spread on agar plates (media: modified Lepthothrix medium and groundwater), two replicates per medium. The primary plate was sealed with parafilm and incubated at room temperature for up to two months. The growth of colonies was checked regularly. Depending on the respective media and sample, several weeks were necessary for development of colonies. Brown or black colonies were assumed to be the results of iron or manganese precipitation. Such colonies were partly collected with sterile toothpicks and streaked on new agar plates for sub-cultivation.

Attempts were made to pick as many different morphologically different colonies as possible. The primary plate was incubated and slower growing colonies that developed were subsequently sub-cultivated for an additional period of time. Through repeated dilution, streaking contaminations were gradually reduced. The time required for isolation of a pure culture varied. In general, 3-10 transfers, each after 2-6 weeks, were needed. These cultures were morphologically consistent and stayed consistent after repeated streaking.

5.2 Cryopreservation

After isolation, the cultures had to be transferred into cryoconservation. Even though this method is one of the most effective for long-term preservation, with many environmental isolates this can be a very intricate process. In general, mesophilic bacteria do not react very favorable (Harrison 1955) when exposed to freezing. This is partly attributed to the formation of ice crystals damaging the cell walls and outer membranes (Calcott and MacLeod 1975). This is why most modern cryopreservation utilizes a wide range of chemical protectants (Hubalek 2003). Most protocols are optimized for fast growing planktic cells. During WELLMA-DNA, an alternative protocol was designed to suit the demands of biofilm-forming iron bacteria.

Protocol for cryopreservation of iron bacteria

1) A cryo tube filled with glass beads and 400 µl of groundwater medium is inoculated with a pure culture.

2) The tube is then put inside a petrie dish and placed on a shaker for several weeks, until a brown color change of the beginning iron oxidation, signals the successful biofilm formation.

3) 100 µl of glycerin are added and the tube is placed back on the shaker for 30 minutes.

4) Subsequently, the medium is removed from the beads and the tubes are placed in a cryo unit with liquid nitrogen, and placed in a −80°C freezer.

5.3 Gradient tube technique

In addition to the preservation of heterotrophic iron bacteria, attempts have been made to cultivate autotrophic iron oxidizers (Jäger 2010). For that reason, we applied the gradient tube technique described by Emerson (Emerson & Moyer 1997, Picardal et al. 2011). This technique is based on the formation of gradients of iron and oxygen inside an organic-free mineral medium. Iron bacteria can grow in a specific microaerophilic zone between these gradients (Fig.10).

5.3.2 Results of gradient tube trials

After performing a DGGE with several of the inoculated tubes, many distinct band patterns could be observed (Fig.11).
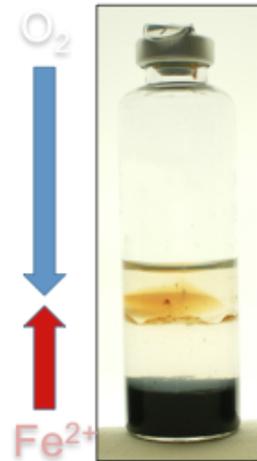
Fig.10: Gradient tube
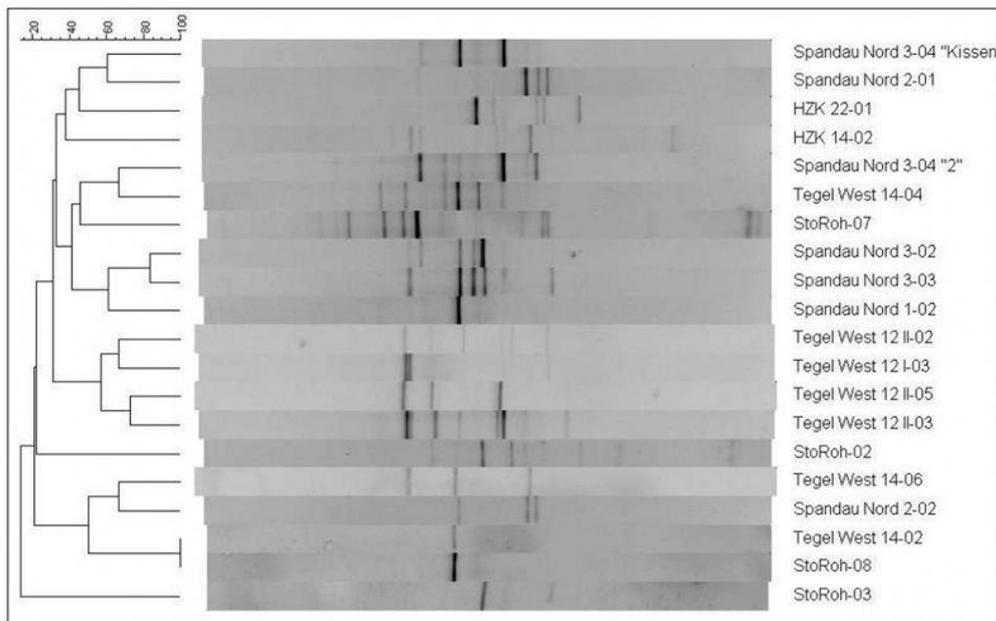
Fig.11: DGGE patterns of different tubes

5.3.3 Discussion of gradient tube trials

The gradient tube technique, much like the BART test, is a cultivation-based method and can only target a fraction of the analyzed populations. In addition, bands that are visually observed can contain multiple different strains of bacteria and without sufficient dilution trials, there is a high risk of carrying heterotrophic bacteria over, who can either be supported by exudates of the autotrophic target population or directly feed on organic matter introduced during inoculation.

Nevertheless, the combination with molecular methods like DGGE analysis adds an additional dimension and may even allow for careful comparisons of the iron oxidizing populations of the different wells. Unlike the general DGGE comparisons of the complete bacterial populations, we obtain a great reduction in observable bands. Also, the diversity of different bands is reduced, narrowing down to 8 to 10 different bands, many of which can be found in several different wells.

Since the gradient tube technique is a cultivation-based approach, the DGGE cannot tell if the observed species are dominating the habitats or merely exist subordinate to other groups.

5.4 Isolates

During the project, nearly 200 iron-precipitating bacteria have been isolated. Many of them belonged to the same species though and some had to be abandoned because of accompanying bacteria or a decrease in viability after continuous inoculation. The isolates that have been kept in cryopreservation include relatives of the following species:
Hydrogenophaga sp., Pseudomonas sp., Kineosporia sp., Brevundimonas sp., Caulobacter sp. and *Leptothrix sp.*

6 Evaluation of hydrogen peroxide treatment

Biofilms in drinking water wells can contain a multitude of different bacterial species. Bacteria in biofilm communities are usually already very resistant to antimicrobial treatments (Mah & O'Toole 2001). Well-based biofilms posses a multitude of additional protection mechanisms, which make them very hard to deal with in well regeneration. Due to trace amounts of oxygen entering the gravel pack and the filter area during well operation, the bacteria in direct contact with the liquid phase need methods to deal with the oxidative stress. Just like most bacteria located on the surface, they posses specific enzymes, so-called catalases, to prevent damage to vital cell components by reactive oxygen species (Elkins et al 1999). Obligate anaerobic groundwater bacteria usually do not need to produce these enzymes, since their habitat contains no free oxygen. But when it comes to preventive treatment with hydrogen peroxide, these catalases play a vital role in the decomposition and detoxification of the H_2O_2.

Iron bacteria posses an additional protection mechanism because of their iron or manganese incrustations. The mechanisms of hydrogenperoxide decomposition into water and oxygen are already well-documented (Broughton et al. 1947, Mani et al 1980). In addition, bacteriogenic iron hydroxides can differ in surface structure and reactivity to chemically precipitated iron hydroxides (Hallberg & Ferris 2004).

These factors could positively impact other biofilm bacteria closely associated to the iron precipitating species. A combination of extracellular catalases (Naclerio 1995) and interlaced iron hydroxides could pose an effective shield against H_2O_2 treatments and protect deeper layers of the biofilm from oxidative stress (Stewart 2000).

To asses the properties of iron hydroxides in response to hydrogen peroxide treatments, several experiments have been conducted during WELLMA-DNA.

6.1 Agar growth inhibition test
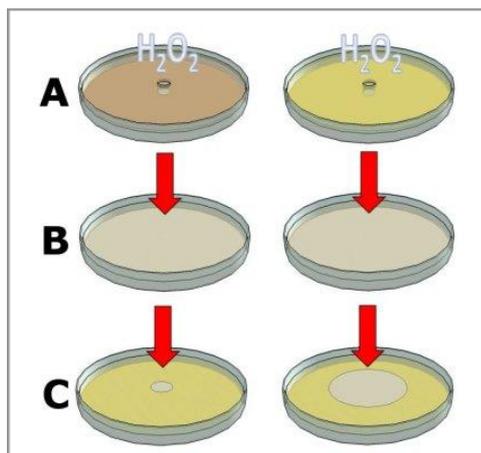
Fig.12: Growth inhibition test design

Over the course of a seminar paper (Kutun 2010), *Escherichia coli* bacteria have been cultivated on media containing chemically prepared iron hydroxides and a second batch of media containing no such additive. Subsequently, holes were stamped into the agar plates and H_2O_2 solution was applied into the holes. After one day of incubation, a metal stamp was used to transfer the bacteria onto a new solid medium. The resulting zones of growth inhibition were much larger for the bacteria originating from iron-free solid media. This indicates a protective property of the iron hydroxides, not only for the iron bacteria that precipitate them, but also for other bacteria in the biofilm.

Additional experiments conducted during the same seminar paper, supported these findings and indicated that an increase of concentration of H_2O_2 does not linearly increase the antimicrobial effect on ochre-encrusted biofilms. This may be related to the presence of potent catalase enzymes, which are able to decompose H_2O_2 very rapidly into water and oxygen. Preceding treatments with iron-solubilizing EDTA had a positive effect on removing encrusted biofilm structures with hydrogen peroxide. In addition to the removal of iron hydroxides, the observed reduction may be attributed to EDTA though, destabilizing the biofilm matrix and permeabilizing bacterial cell walls making them prone to subsequent antimicrobial treatments (Gray and Wilkinson 1965).

6.2 Decomposition of H_2O_2

During a diploma thesis (Papke 2011) supervised by the author, further experiments concerning the effect of hydrogen peroxide on groundwater biofilms have been conducted.

In order to monitor the decomposition of H_2O_2 in reaction to different substances, a test chamber was constructed containing an inert oxygen sensor spot (PreSens GmbH). Results showed that H_2O_2 was readily decomposed into oxygen and water by a pure culture of iron bacteria.

Subsequent experiments with chemically inactivated bacteria showed a reduced oxygen production and the same culture, heat-inactivated by autoclaving, showed only a fraction of oxygen production when treated with H_2O_2. This again indicates the presence of heat-sensitive catalase enzymes catalyzing the reaction. Also for pure chemically precipitated iron hydroxide, oxygen development could be observed, which corresponds to the results of S. Kutun (Kutun 2010) and may explain the protective properties of iron hydroxide.

6.3 Mechanism of biofilm removal

During other research projects, the main effect of hydrogen peroxide against biofilm structures has been assumed to be related to the shear forces connected to the oxygen production during catalytic decomposition (Schulte 2003). In the course of WELLMA-DNA this could be further explored. During a biofilm reactor trial (Papke 2010, Fig. 13), cell counts of exposed carrier materials treated with H_2O_2 indicated that especially long filamentous cells were detached from the surface while the bottom layers remained intact.
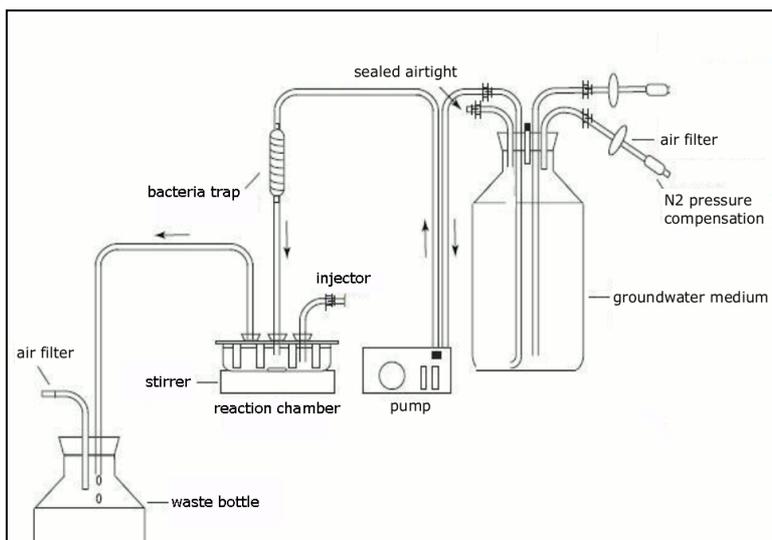
Fig.13: Biofilm reactor (after Röder 2006)

6.4 Adaptation

Results of subsequent population analysis were of peculiar interest, because a distinct population shift was observed, which in conjunction with microscopic analysis points towards a development of more H_2O_2-resistant populations. Even though these results were only observed under laboratory conditions, a similar mechanism seems likely in wells that undergo regular treatments. Microbial resistance to a host of different agents is already well described (Meyer and Cookson 2010, Flemming et al. 2011) and population shifts due to disinfection regimes are a common occurrence (Röder et al. 2010). Whether the resulting biofilm populations produce different types of incrustations has still to be determined, but could explain corresponding observations made by staff members of BWB during pump maintenance.

6.5 Conclusion

Even though the antimicrobial efficiency of the H_2O_2 treatments seems to be greatly impaired by the presence of catalase enzymes in conjunction with metal hydroxides in the biofilm matrix, the effect of the shear force (due to oxygen production) on young biofilms was observable during lab experiments. Long filamentous bacteria seem to be especially vulnerable to this destabilization, while bacteria growing more adhesive to the surface (e.g. *Kineosporia sp.*), may be more resilient to detachment. This corresponds to recent studies, which have shown that bubble-induced detachment is mostly determined by the number of bubbles in contact with the cells (Barkley et al. 2004). So, the free surface area, which is determined by the morphology of the predominant species of the affected biofilm, may play an important role in the efficiency of the current treatment.

To utilize these mechanisms and vastly increase the effectiveness of the H_2O_2 treatment, a pre-treatment could be useful.

6.6 Pre-treatment: Bio-activation

In order to assess the potential for biological well regeneration, several trials with natural and artificial biofilms have been conducted. The experiments included oxygen limiting conditions as well as variations in substrates and flow velocities. Biofilms have been analyzed visually, microscopically and genetically.

Fig.14: Modified robbins device

In order to assess the reactions of natural ochreous deposits, sample slides, which had been exposed in a modified robbins device, fed with well water, were subsequently inserted into a biofilm reactor in the lab (Fig.13). During the experiment, oxygen supply was limited and nutrient supply was high.

Additional in-situ trials with the modified robbins devices at the wells TEGost-01-/86V and TEGost-02-/86V (Fig. 14) were conducted using ethanol as electron donor. Cloning, sequencing and subsequent phylogenetic analysis of the biofilm populations before and after the treatment have been performed.
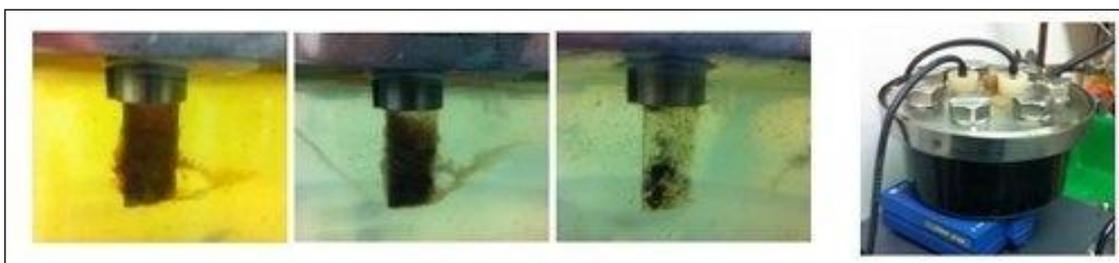
Fig.15: Phases of biofilm change

6.6.1 Results of pre-treatment

The biofilms exposed in the lab reactor (Fig.13) showed a gradual reduction in thickness and stability. Biofilms coating the exposed slides and reactor walls cleared from a brownish yellow coloration up to the point, where dark precipitations coated the whole reactor (Fig. 15).

Phylogenetic analysis of the samples before the ethanol treatment shows a great diversity of clones related to manganese and/or iron precipitating bacteria like *Gallionella sp.*, *Leptothrix sp., Caulobacter sp., Brevundimonas sp., or Sphingomonas sp.* in both wells. After treatmen,t the diversity was greatly reduced and the population shifted towards iron reducing bacteria like *Geothrix sp.* and *Geobacter sp.* Slides recovered from the robbins devices after the ethanol treatment showed a reduced thickness and black precipitates (Hannemann 2011).

6.6.2 Discussion

Results clearly indicate that a major population shift in the ochreous deposits is possible by altering the nutrient supply. Ethanol served as a model electron donor to gain further insight into the potential effects of this method and to speed up the oxygen consumption of the population. After the available electron acceptors oxygen and nitrate have been depleted, the population shifted towards iron-reducing species. This probably destabilized the ochreous deposits and filamentous organisms detached more easily. After the iron had been dissolved, most likely sulfate reduction started, resulting in the formation of hydrogen sulfide and subsequently iron sulfide. This reaction is undesirable though and the resulting population could promote metal corrosion, if left unattended (chapter 6). This makes the right timing for this method a major issue and a reliable tool for monitoring the population changes would be beneficial.

7 Probe Design: Indicator System

In order to develop a molecular indicator system for microbiological well clogging, several steps had to be combined (Fig. 16).
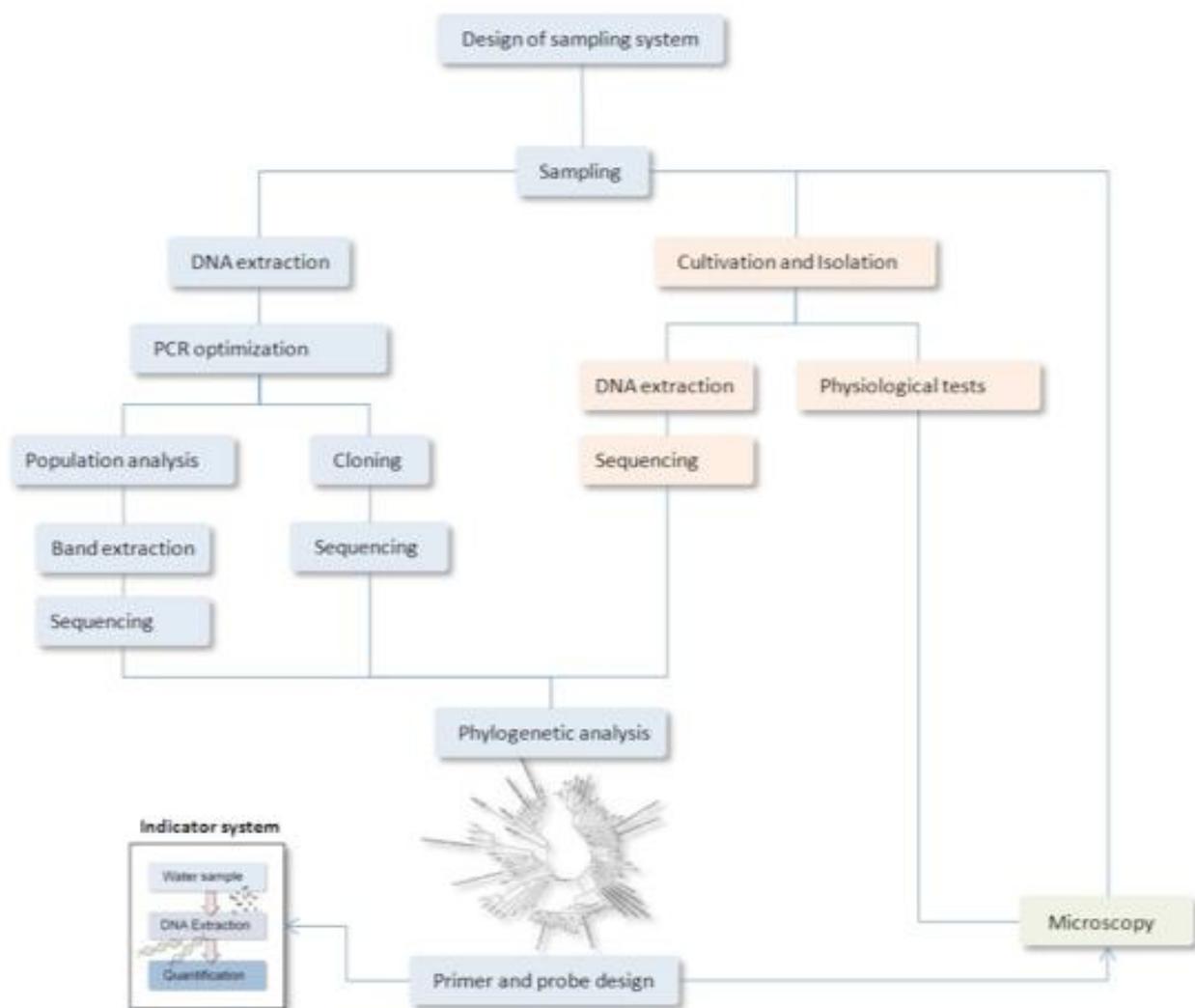
Fig.16: Development of indicator system

First, a sampling system had to be created (chapter 2.1) and applied to a multitude of wells in order to gain DNA samples and living bacteria for pure culture isolation, microscopic analysis and physiological tests. The data of all these experiments were combined in order to find bacteria that would constitute suitable candidates for a DNA-based test system.

Phylogenetic relationships, morphological and physiological features as well as the abundance in the analyzed wells led to a selection process of ochre related bacteria.
With the help of literature research and the „probeBase" (Loy et al. 2003) online tool it was checked, if suitable primers for the chosen group of bacteria already exist. Subsequently, with the help of the probe design tool Arb (Ludwig et al. 2004) and the phylogenetic analysis software Mega (Tamura et al. 2007, Tamura et al. 2011), suitable primers were calculated and checked using the „probeCheck" (Loy et al. 2008) as well as „probeMatch" (Kim et al. 2009) online tools. The result of this process is illustrated in the phylogenetic tree below (Fig.17).

The primer sets have been established in a diploma thesis (Täuber S. 2010) supervised by the author. The resulting primer sets can now serve as starting point for further refinement. Successive tests with wells showing different degrees of ochre formation followed by adjustment of the primers have to be conducted over longer periods of time, which could not be performed during WELLMA-DNA due to time constraints, but will be continued in ongoing projects.
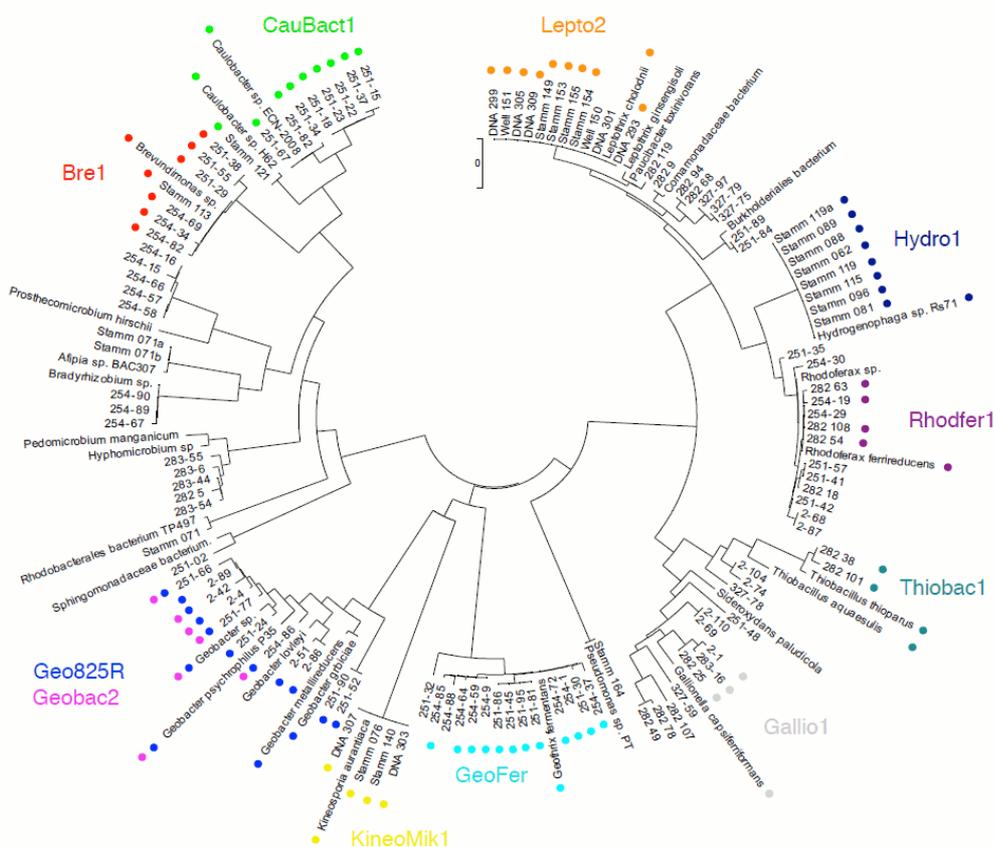
Fig.17: Result of primer design

During further testing, not all of the primers turned out to be suitable for use in a molecular indicator system and after the selection, seven of the 11 initially tested primers remained as strong candidates for the detection of ochre formation associated bacteria.

Since the methodology and database for further adjustment and testing of additional primer sets is now established, it can be easily applied in the future. After a period of testing and calibration, recommended paths of utilization could include a qPCR kit for sensitive and quantitative detection of ochre forming bacteria or a micro array for broad and quick detection of many different indicator species.

The pPCR Assay

The exemplary generation of a qPCR protocol for the quantitative detection of the ochre associated bacteria, chosen in chapter 7 is presented in the following for Geobacter sp..

In order to establish a suitable qPCR protocol for the primers, we first need to generate a template standard for the respective primer set. The standard needs to contain a known copy of the target sequences that are intended to be detected in the environmental sample.

By utilizing the clone library that was created during WELLMA-DNA a multitude of suitable sequence fragments is now available. Plasmids of the clone „2-4" of the habitat TegHzk-13 contain the 16s rDNA sequence of Geobacter sp.. This can be retrieved and amplified by performing a PCR reaction with the primer set 63f, 1387r (Marchesi et al. 1998).

After the PCR product has been purified (e.g. with the Promega „Wizard® SV Gel and PCR Clean-Up System"), the product yield is quantified using a Thermo Fisher Scientific „NanoDrop 2000c" spectrophotometer. Subsequently the number of fragment copies in the PCR product can be calculated by counting the base pairs in the fragment sequence using a sequence analysis software (e.g. MEGA, Fig. 16) and the concentration of the standard.
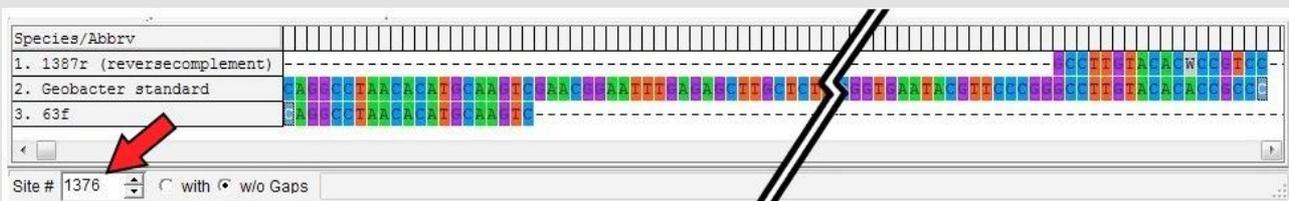
Fig.18: Representation of the DNA code in Mega

$$GC_{St} = c_{st} * 10^{-9} * N_a / M_{bp} * n_{bp}$$

GC_{st} = gene copies per µl
c_{st} = concentration of standard in ng / µl
M_{bp} = molar mass per base pair (660 g / mol)
n_{bp} = number of base pairs in the standard
N_a = avogadro constant $6.02214179 \times 10^{23}$ mol^{-1}

The resulting standard is then diluted tenfold each step, down to an appropriate template concentration, in order to obtain a standard curve of at least five steps (e.g. $10^5 - 10^1$ gene copies /µl). Each of the five standard dilutions is then used in a qPCR reaction, along with the environmental samples. In this study, we used the Corbett Rotor-Gene 6000 real-time DNA Cycler (LTF Labortechnik, Wasserburg) and the „mi-real-time EvaGreen® Master" mix (metabion International AG).

Protocol of the three step PCR:

Initial denaturation: 95 °C for 2 min
Start of cycle 40x
  Denaturation   95 °C for 15 sec
  Annealing   55 °C for 20 sec
  Elongation   72 °C for 30 sec
End of Cycle

The fluorescent dye contained in the reaction mix, intercalates with the DNA , and the PCR cycler can detect this by inducing a fluorescence. The resulting signal is directly proportionate to the template concentration at the respective PCR step. At the end of the PCR run, the Rotor-Gene application software calculates the detected gene copies for each sample and a report file can be generated.
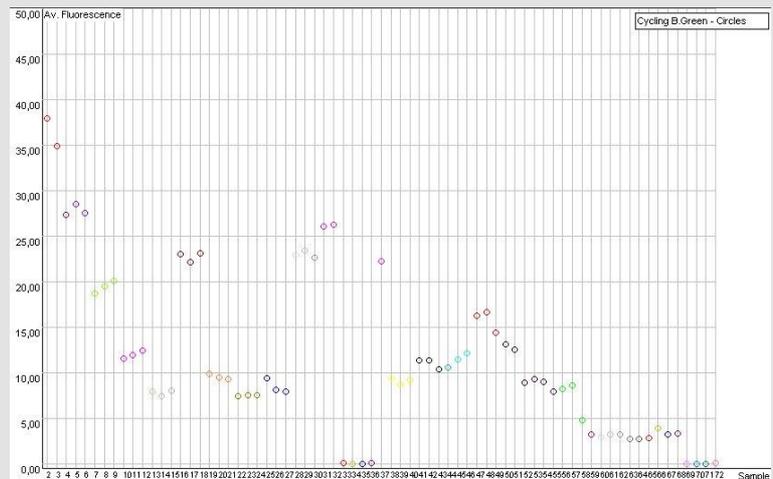
Fig.19: Result of qPCR

It would be expected, that samples containing a high number of gene copies of iron precipitating organisms, point to a high potential for ochre formation.

By comparing the results of the gene copy number for several water samples with the actually observed ochre formation potential, the system can be calibrated and tested for real life applicability. A theoretical result sheet of a molecular detection campaign is illustrated in figure 20. It is important to note that the sampling and preparation procedures need to be followed strictly as stated in this report in order to ensure comparability of the results. Due to time constraints it was not possible to test and calibrate the system within the duration of this project.
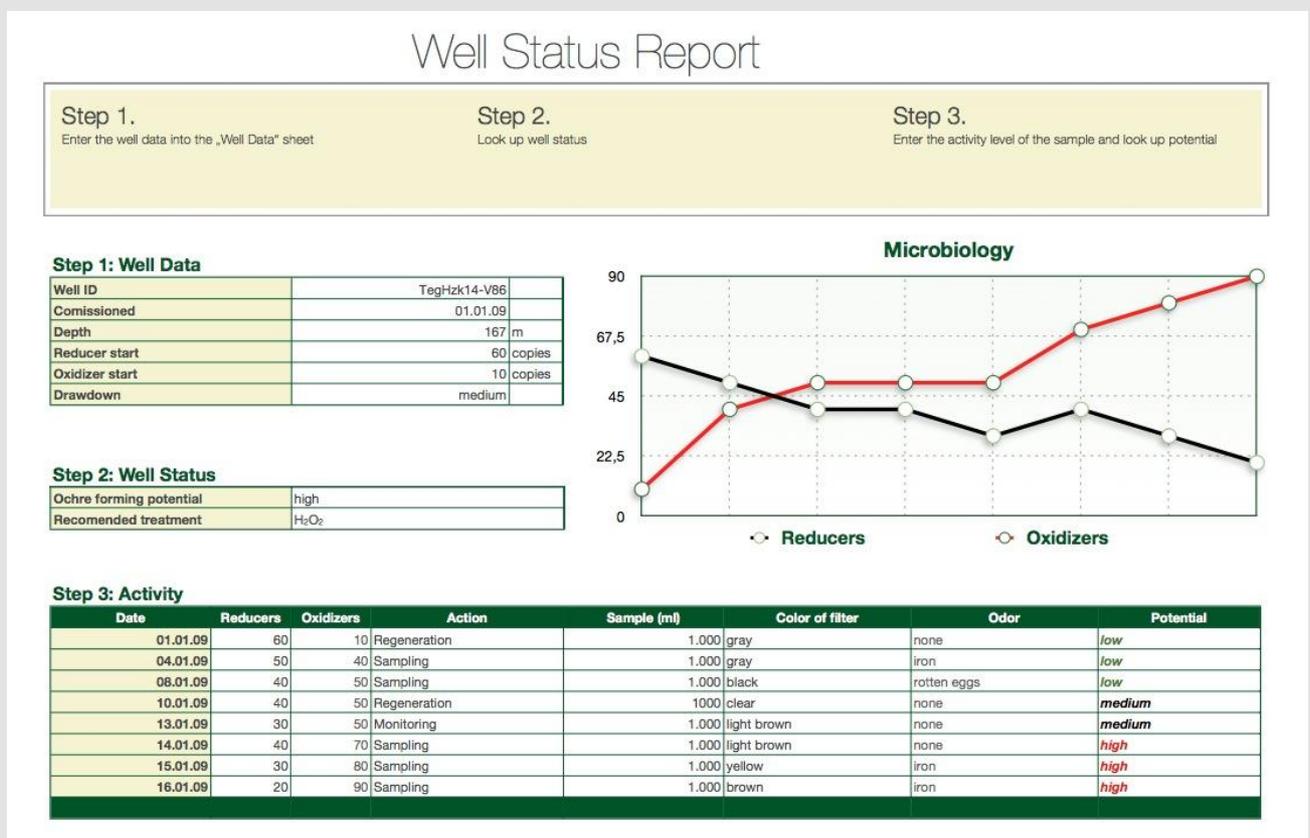
Fig.20: Possible result sheet design

8 Summary

During WELLMA-DNA, 13 diploma and bachelor theses along with several internships have been completed. A sampling system for biofilm samples as well as a sampling device for water samples have been designed and tested.

More than 400 DNA samples of different well sites have been collected and analyzed. Microbiological and molecular methods have been combined to gain a better understanding of the community composition of the ochre forming biofilms inside the wells. Molecular methods included PCR, DGGE, cloning and sequencing.

During the project, the bacterial populations of an unprecedented number of wells have been analyzed and several indicator bacteria for iron-related well clogging have been identified. Alongside iron-oxidizing bacteria, iron-reducing bacteria have been found in the wells and their potential for ochre-solubilization was confirmed. Alongside the molecular experiments, microbiological trials included the isolation of pure cultures, microscopic analysis and physiological tests. The morphology of the encountered iron bacteria could be classified into four different groups, which may have an impact on the rigidity of the biofilms on a macroscopic level. We were able to cultivate several of these indicator organisms, which could play an important role in the formation of ochreous deposits in the Berlin wells. During experiments utilizing microscopic flow cells, differences in growth rate and patterns of these ochre-forming bacteria have been observed.

For several of the identified indicator bacteria, primers have been calculated. These primers will allow for the first time to quantify the amount of indicator bacteria in a water sample and to derive operational pointers.

In addition, several experiments regarding the effect of hydrogen peroxide on ochre forming biofilms have been conducted and the effect of an additional electron donor (ethanol) on the communities has been tested. For future data acquisition and documentation, a guideline for classifying the degree of pump clogging has been developed.

9 Conclusions

The identification of several indicator organisms was successful and the development of a market-ready indicator system based on results of this work seems realistic and will be further pursued. The primers still need successive tests and adjustment, but the methodology is now established and first results of qPCR trials are very promising. In order for this molecular system to work, the sampling conditions have to be standardized according to the given protocols in this report. In addition, the wells should be in the same mode of operation for at least several hours before sampling to provide comparable results.

Even though there may be several yet unknown parameters, which can influence and alter the results, the presented method should deliver vastly superior results to alternative microbiological approaches like BART. The huge difference is that now a tool is at hand to quantify the indicator organisms and monitor their development (hence the ochre formation they induce) over a given time period.

Especially the ratio and changes of ochre-forming and ochre-solubilizing bacteria should generate very useful information about the state and development of the ochre formations.

The method renders it unnecessary to open the well head or pull the pump, since the detection of the bacteria is done with water samples. This allows for a more frequent and cost-effective monitoring and may serve as a decision aid for the cost-effective scheduling of regeneration procedures.

Results further indicate that H_2O_2 treatments are likely to target especially young filamentous organisms. This could be due to the steady generation of oxygen bubbles and the associated shear force. Incrusted bacteria probably benefit from the catalytic effect of iron hydroxide and catalase, which leads to the conclusion that older biofilms are better protected than young ones. Even though ethanol treatment proved to be a suitable method to revert the process of steady incrustation and switch the biofilm population to ochre-solubilizing species, it is unsuitable for drinking water systems and can only serve as a prove of concept. Nevertheless, a combined approach of biological iron reduction and subsequent H_2O_2 treatment would probably greatly increase the regeneration results. This is further pursued in the follow-up project ANTIOCKER.

10 Perspectives

In preparation of an EXIST start-up grant (BMWi funding), which is intended to start early next year, the results of this study will be reviewed and further investigations will be conducted with the goal to generate a fully functional indicator system, which can be applied in routine well monitoring.

Acknowledgement

Literature

Aklujkar M., N. D. Young, D. Holmes, M. Chavan, C. Risso, H. E. Kiss, C. S. Han, M. L. Land, and D. R. Lovley. 2010.
The genome of Geobacter bemidjiensis, exemplar for the subsurface clade of Geobacter species that predominate in Fe(III)-reducing subsurface environments.
BMC genomics 11:490.

Barkley S.,Johnson H., Eisenthal R., **Hubble J.** 2004
Bubble-induced detachment of affinity-adsorbed erythrocytes.
Biotechnology and applied biochemistry 40 (Pt 2) p. 145-9

Broughton D.B., Wentworth R.L. 1947
Mechanism of Decomposition of Hydrogen Peroxide Solutions with Manganese Dioxide. I
Journal of the American Chemical Society 69 (4) p. 741-744

Calcott P.H., MacLeod R.A. 1975
The survival of Escherichia coli from freeze-thaw damage: the relative importance of wall and membrane damage.
Canadian journal of microbiology 21 (12) p. 1960-8

Chan C. S., S. C. Fakra, D. C. Edwards, D. Emerson, and J. F. Banfield. 2009
Iron oxyhydroxide mineralization on microbial extracellular polysaccharides.
Geochimica et Cosmochimica Acta. Elsevier Ltd 73:3807-3818.

Clarke J.R. 2002
Molecular diagnosis of HIV.
Expert review of molecular diagnostics, 2 (3) p. 233-9

Conradi B. 2011
Investigation of mature biofilm populations in the distribution of drinking water with attention to bacteria of hygienic relevance
PhD thesis at the department of environmental microbiology of the TU Berlin

Corstjens P. L., de Vrind J. P., Westbroek P., de Vrind-de Jong E. W. 1992
Enzymatic iron oxidation by Leptothrix discophora: identification of an iron-oxidizing protein.
Applied and environmental microbiology 58 (2) p. 450-4

Cullimore D. R. 1999
Microbiology of well biofouling
CRC Press p. 456

De Lipthay J. R., Barkay T., Sørensen S. J. 2001
Enhanced degradation of phenoxyacetic acid in soil by horizontal transfer of the tfdA gene encoding a 2,4-dichlorophenoxyacetic acid dioxygenase.
FEMS microbiology ecology 35 (1) p. 75-84

Don R. H., P. T. Cox, B. J. Wainwright, K. Baker, and J. S. Mattick. 1991.
"Touchdown" PCR to circumvent spurious priming during gene amplification.
Nucleic acids research 19:4008.

Eberl H. J., Collinson S. 2009
A modeling and simulation study of siderophore mediated antagonism in dual-species biofilms.
Theoretical biology & medical modelling 6 (1) p. 30

Ehrlich. H. L. 2002.
Geomicrobiology 4th edition. Marcel Dekker, New York.

Elkins J.G., Hassett D.J., Stewart P.S., Schweizer H.P., McDermott T.R. 1999
Protective Role of Catalase in Pseudomonas aeruginosa Biofilm Resistance to Hydrogen Peroxide
Appl. Envir. Microbiol. 65 (10) p. 4594-4600

Emerson D., E. J. Fleming, and J. M. McBeth. 2010
Iron-oxidizing bacteria: an environmental and genomic perspective. Annual review of microbiology. Annual Reviews 64:561-83.

Emerson D., Moyer C. 1997
Isolation and characterization of novel iron-oxidizing bacteria that grow at circumneutral pH.
Appl. Environ. Microbiol. 63:4784–92.

Felföldi T., Tarnóczai T., Homonnay Z. G. 2010
Presence of potential bacterial pathogens in a municipal drinking water supply system.
Acta microbiologica et immunologica Hungarica 57 (3) p. 165-79

Flemming H-C., Wingender J., Szewzyk U. 2011
Biofilm Highlights (1st Edition)
Springer, ISBN-13: 978-3642199394

Glasauer S., Langley S., Beveridge T. J. 2001
Sorption of Fe (hydr)oxides to the surface of Shewanella putrefaciens: cell-bound fine-grained minerals are not always formed de novo.
Applied and environmental microbiology 67 (12) p. 5544-50

Gray W. ,Wilkinson S. G. 1965
The Effect of Ethylenediaminetetra-acetic Acid on the Cell Walls of Some Gram-Negative Bacteria
Microbiology 39 (3) p. 385-399

Hallberg R., Ferris F. G. 2004
Biomineralization by Gallionella
Geomicrobiology Journal 21 (5)

Hannemann C. 2011
Untersuchung eisenoxidierender und -reduzierender Biofilme in Trinkwasserbrunnen
Bachelor thesis at the department of environmental microbiology of the TU Berlin

Harrison A. P. 1955
Survival of bacteria upon repeated freezing and thawing.
Journal of bacteriology 70 (6) p. 711-5

Hubalek Z. 2003
Protectants used in the cryopreservation of microorganisms
Cryobiology 46 (3) p. 205-229

Jäger K. 2010
Isolierung und Charakterisierung von verockerungsrelevanten Mikroorganismen aus Trinkwasserbrunnen
Diploma thesis at the department of environmental microbiology of the TU Berlin

Kappler A., M. Benz, B. Schink, and A. Brune. 2004
Electron shuttling via humic acids in microbial iron(III) reduction in a freshwater sediment.
FEMS microbiology ecology 47:85-92.
Kim S.-J., S.-J. Park, Y.-S. Oh, S.-A. Lee, K.-S. Shin, D.-H. Roh, and S.-K. Rhee. 2011.
Shewanella arctica sp. nov., an iron-reducing bacterium isolated from Arctic marine sediment.
International journal of systematic and evolutionary microbiology.

Kim Y. J., N. Teletia, V. Ruotti, C. A. Maher, A. M. Chinnaiyan, R. Stewart, J. A. Thomson, and J. M. Patel. 2009
ProbeMatch: rapid alignment of oligonucleotides to genome allowing both gaps and mismatches.
Bioinformatics (Oxford, England) 25:1424-5.

Kinner N. E., Harvey R. W., Blakeslee K., Novarino G., Meeker L. D. 1998
Size-Selective Predation on Groundwater Bacteria by Nanoflagellates in an Organic-Contaminated Aquifer
Appl. Envir. Microbiol. 64 (2) p. 618-625

Krsek M. and Wellington E. M. H. 1999
Comparison of different methods for the isolation and purification of total community DNA from soil
Journal of Microbiological Methods 39 (1) p. 1-16

Lehmann S. 2009
Berechnung von FISH- Sonden für verblockungsrelevante Bakterien aus Trinkwasserbrunnen
Diplomarbeit im Studiengang Biotechnologie der Beuth Hochschule für Technik Berlin

Loy A., R. Arnold, P. Tischler, T. Rattei, M. Wagner, and M. Horn. 2008
probeCheck--a central resource for evaluating oligonucleotide probe coverage and specificity
Environmental microbiology 10:2894-8.

Loy A., M. Horn, and M. Wagner. 2003
probeBase: an online resource for rRNA-targeted oligonucleotide probes
Nucleic acids research 31:514-6.

Ludwig W., O. Strunk, R. Westram, L. Richter, H. Meier, Yadhukumar, A. Buchner, T. Lai, S. Steppi, G. Jobb, W. Förster, I. Brettske, S. Gerber, A. W. Ginhart, O. Gross, S. Grumann, S. Hermann, R. Jost, A. König, T. Liss, R. Lüssmann, M. May, B. Nonhoff, B. Reichel, R. Strehlow, A. Stamatakis, N. Stuckmann, A. Vilbig, M. Lenke, T. Ludwig, A. Bode, and K.-H. Schleifer. 2004.
ARB: a software environment for sequence data.
Nucleic acids research 32:1363-71.

Mah T-F. C. and O'Toole G. A. 2001
Mechanisms of biofilm resistance to antimicrobial agents
TRENDSinMicrobiology Vol.9No.1January2001

Mani B., Ravi Mohan Ch., Sitakara Rao V. 1980
Kinetics of decomposition of hydrogen peroxide on Fe(III)−Al(III) hydroxide-oxide systems
Reaction Kinetics and Catalysis Letters 13 (3) p. 277-284

Marchesi J. R., T. Sato, A. J. Weightman, T. A. Martin, J. C. Fry, S. J. Hiom, D. Dymock, and W. G. Wade. 1998
Design and evaluation of useful bacterium-specific PCR primers that amplify genes coding for bacterial 16S rRNA.
Applied and environmental microbiology 64:795-9.

Martin-Laurent F., Philippot L. , Hallet S., Chaussod R., Germon J. C., Soulas G., Catroux G. 2001
DNA extraction from soils: old bias for new microbial diversity analysis methods.
Applied and environmental microbiology 67 (5) p. 2354-9

Mend J., Teske A. 2005
Expanding frontiers in deep subsurface microbiology
Palaeogeography, Palaeoclimatology, Palaeoecology 219 (1-2) p. 131-155

Meyer B.,Cookson B. 2010
Does microbial resistance or adaptation to biocides create a hazard in infection prevention and control?
The Journal of hospital infection 76 (3) p. 200-5

Mikkelsen H., Sivaneson M., Filloux A. 2011
Key two-component regulatory systems that control biofilm formation in Pseudomonas aeruginosa.
Environmental microbiology 13 (7) p. 1666-81

Muyzer, G., De Waal, E.C., and Uitterlinden, A.G. 1993
Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA.
Appl. Environ. Microbiol. 59: 695-700

Naclerio G., Baccigalupi L., Caruso C., De Felice M., Ricca E. 1995
Bacillus subtilis Vegetative Catalase Is an Extracellular Enzyme.
Applied and environmental microbiology 61 (12) p. 4471-3

Nevin, K. P. ; Lovley, D. R. 2002
Mechanisms for Accessing Insoluble Fe(III) Oxide during Dissimilatory Fe(III) Reduction by Geothrix fermentans.
Appl. Environ. Microbiol. 68 , Nr. 5, S. 2294–2299

Oliver J. D. 2005
The viable but nonculturable state in bacteria.
Journal of microbiology (Seoul, Korea) 43 Spec No p. 93-100

Pedersen K. 2010
The deep biosphere
GFF 132 (1) p. 93-94

Picardal F. W., Z. Zaybak, A. Chakraborty, J. Schieber and U. Szewzyk 2011
Microaerophilic, Fe(II)-dependent growth and Fe(II) oxidation by a Dechlorospirillum species.
FEMS Microbiology Letters 319,51-57

Popiol M. 2009
Charakterisierung der Verockerung von Trinkwasserbrunnen
Maser thesis at the department of environmental microbiology of the TU Berlin

Quiagen GmbH 2011
mericon™ Pathogen Detection Handbook
For detection of pathogens in food or animal feed samples using real-time PCR

Reguera G., McCarthy K. D., Mehta T. , Nicoll J. S. , Tuominen M. T. , and Lovley D. R. 2005
Extracellular electron transfer via microbial nanowires.
Nature. Macmillian Magazines Ltd. 435:1098-101.

Roeder R.S., Lenz J.,Tarne P.,Gebel J.,Exner M.,Szewzyk U. 2010
Long-term effects of disinfectants on the community composition of drinking water biofilms.
International journal of hygiene and environmental health 213 (3) p. 183-9

Rönsch S. 2011
Etablierung einer Oligonukleotid-Sonde zur Detektion eisenablagernder Bakterien aus Trinkwasserbrunnen
Diplomarbeit am Fachgebiet Umweltmikrobiologie der TU Berlin

Roden E., D. Sobolev, B. Glazer, and G. Luther. 2004.
Potential for Microscale Bacterial Fe Redox Cycling at the Aerobic-Anaerobic Interface. Geomicrobiology Journal. Taylor & Francis 21:379-391.

Rauch R. 2010
Einfluss von Wasserstoffperoxid auf Biofilmpopulationen in Trinkwasserbrunnen
Diploma thesis at the department of environmental microbiology of the TU Berlin

Schenker I., F. Filser, T. Aste, and L. Gauckler. 2008.
Microstructures and mechanical properties of dense particle gels: Microstructural characterisation.
Journal of the European Ceramic Society 28:1443-1449.

Schmidt B. 2008
Physiologie und Phylogenie eisenoxidierender Bakterienisolate auf schadstoffhaltigen Medien
Diplomarbeit, FG Umweltmikrobiologie, TU Berlin

Schulte S. 2003
Wirksamkeit von Wasserstoffperoxid gegenüber Biofilmen
Dissertation: Universität Duisburg-Essen, Fakultät für Naturwissenschaften, Institut für Chemie

Siqueira J.F. and Rôças I.N. 2005
Exploiting Molecular Methods to Explore Endodontic Infections: Part 1
Current Molecular Technologies for Microbiological Diagnosis
Journal of Endodontics, Vol. 31, Issue 6, Pages 411-423

Stach J. E.M., Bathe S., Clapp J. P., Burns R. G. 2001
PCR-SSCP comparison of 16S rDNA sequence diversity in soil DNA obtained using different isolation and purification methods.
FEMS Microbiology Ecology 36 (2-3)   p. 139-151

Stams A. J. M., Plugge C. M. 2009
Electron transfer in syntrophic communities of anaerobic bacteria and archaea.
Nature reviews. Microbiology 7 (8) p. 568-77

Stenson A. C. 2009
Fourier transform ion cyclotron resonance mass spectral characterization of metal-humic binding.
Rapid communications in mass spectrometry : RCM 23 (4) p. 465-76

Stewart P. S. , Roe F., Rayner J.,Elkins J.G., Lewandowski Z.,Ochsner U.A.,Hassett D.J. 2000
Effect of catalase on hydrogen peroxide penetration into Pseudomonas aeruginosa biofilms.
Applied and environmental microbiology 66 (2) p. 836-8

Stotzky G. , Goos R. D. und Timonin M. I. 1962
Microbial changes occurring in soil as a result of storage
Plant and Soil, Volume 16, Number 1, 1-18

Straub K., Benz M., Schink B., Widdel F. 1996
Anaerobic, Nitrate-Dependent Microbial Oxidation of Ferrous Iron
Appl. Envir. Microbiol. 62 (4) p. 1458-1460

Straub K., M. Hanzlik, and B. E. Buchholz-Cleven. 1998.
The use of biologically produced ferrihydrite for the isolation of novel iron-reducing bacteria.
Systematic and applied microbiology 21:442-9.

Straub K., Benz M., Schink. B. 2001.
Iron metabolism in anoxic environments at near neutral pH.
FEMS microbiology ecology 34:181-186.

Sunda W. G., Kieber D. J. 1994
Oxidation of humic substances by manganese oxides yields low-molecular-weight organic substrates
Nature 367 (6458) p. 62-64

Sutherland I.W. 2001
Biofilm exopolysaccharides: a strong and sticky framework
Microbiology 147 (1) p. 3-9

Szewzyk, U. and Schink, B. 1988
Surface Colonisation by and Life Cycle of Pelo- bacter acidigallici Studied in a Continuous-flow Microchamber,
Journal of General Microbiology, Vol. 134, p. 183-190

Tamura K., J. Dudley, M. Nei, and S. Kumar. 2007
MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0.
Molecular biology and evolution 24:1596-9.

Tamura K., D. Peterson, N. Peterson, G. Stecher, M. Nei, and S. Kumar. 2011
MEGA5: Molecular Evolutionary Genetics Analysis using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods.
Molecular biology and evolution, doi: 10.1093/molbev/msr121

Wackett L. P., Ellis L. B. 1999
Predicting biodegradation.
Environmental microbiology 1 (2) p. 119-24

Waters C. M., Bassler B. L. (2005)
Quorum sensing: cell-to-cell communication in bacteria.
Annual review of cell and developmental biology 21 p. 319-46

Wendlandt, E. (2001)
Mikrodurchfluss und Kulturküvette, Patentschrift Feb. 2001, Deutsches Patent- und Markenamt,
Patentnummer: DE 19738626C2

Winston V., Thompson TL. (1979)
Isolation and characterization of a bacteriophage specific for Sphaerotilus natans which contain an unusual
base in its deoxyribonucleic acid.
Appl Environ Microbiol. May;37(5):1025-30.