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Microbial analyses of groundwater, bentonite and surfaces – post-test analysis of packages 4 (A05) and 5 (A06) from the MiniCan experiment

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Abstract

The MiniCan experiment consisted of five miniature canisters (300 mm long × 150 mm diameter) inserted in near horizontal boreholes with different amounts of bentonite clay and with 1 mm defects deliberately made on various positions of the copper canisters. The experiment was installed in late 2006 with the purpose to study corrosion of copper canister and cast iron insert by a possible leakage in the weld of the canister. During the project, water samples from inside of support cages for the low density bentonite, used in four of the packages, and of groundwater in the five boreholes have been taken. The water has been analysed regarding microbiology, including DNA analyses, chemical composition and dissolved gases. Package 3 (A04) was retrieved in 2011 and the corrosion was examined and the results were summarised in several reports and scientific papers.

The packages 4 (A05) and 5 (A06) were retrieved in 2015. Package 4 (A05) had fully compacted bentonite with dry density of 1 600 kg m^{-3} and one hole at the top and one at the bottom of the canister. Package 5 (A06) had no bentonite and two holes opposite each other at the top of the canister. Samples for microbiological and chemical analyses were taken from both groundwater and water from inside the support cages before the retrieval. In addition, samples for microbiological analyses were taken from surfaces of the two packages and bentonite from 4 (A05). After the microbiology sampling, a sampling campaign for corrosion analyses started. The corrosion results are compiled in a separate report.

The microbiological and chemical composition inside the packages differed significantly from the groundwater in MiniCan. The most pronounced differences were in the numbers of sulphate-reducing bacteria (SRB) and in the amount of ferrous iron. The number of SRB was below or close to the detection limit of the method in the groundwater samples and in the support cage of package 1 (A02). In package 2 (A03), the number of SRB was close to 8×10^3 mL^{-1} and in package 5 (A06) 1.1×10^3 mL^{-1}. The amount of ferrous iron in the support cages was 85, 77 and 24 mg L^{-1}, respectively in the packages 1 (A02), 2 (A03) and 5 (A06) to be compared to 0.1 mg L^{-1} in the groundwater in all boreholes in MiniCan. The number of SRB in package 1 (A02) had decreased from 2.3×10^3 mL^{-1} in 2011 to below detection in 2015 but had the highest amount of ferrous iron of the packages in 2015.

From surface and bentonite samples of package 4 (A05), with 1600 kg m^{-3} dry density bentonite, viable bacteria, both CHAB and SRB, could be detected but in low numbers compared to results from both packages 3 (A04) in 2011 and 5 (A06) in 2015. The number of SRB was around 20 cm^{-2} on the iron surface of the sandwich specimen and up to 100 g^{-1} of bentonite. The measured mean wet density value of 10 bentonite samples from package 4 (A05) was 1 930 kg m^{-3} and not 2 000 kg m^{-3} which is the stipulated wet density in a KBS-3 repository.

Since there was no bentonite in package 5 (A06), all samples were taken from surfaces of the support cage, canister and various specimens for corrosion studies placed on a nylon support rack. There were up to 5×10^5 SRB cm^{-2} on the canister and corrosion study items. The highest numbers were found on the iron surface of an iron-copper sandwich specimen and on the copper canister surface. DNA analyses confirmed that SRB together with sulphur-reducing bacteria were the dominating groups of bacteria on the surfaces. The surfaces were covered with thick precipitates of iron sulphide and the corrosion studies showed severe corrosion damages of most iron specimens in package 5 (A06). The results from package 5 (A06) were very similar to the results from the earlier retrieved package 3 (A04). The SRB data from surfaces in package 5 (A06) and from the support cage waters together with the high amounts of ferrous iron in the support cage waters confirm the presence of so called electric microbial corrosion, EMIC, where SRB use electrons directly from metallic iron in the sulphate-reduction giving a ratio of 1 to 3 of FeS and ferrous iron as products. The same ratio was found in package 3 (A04) in 2011 and since the amount of ferrous iron is at the same level also in packages 1 (A02) and 2 (A03), EMIC is most likely ongoing or have been ongoing, in these packages as well.

Contents

1 Introduction

1.1 The MiniCan project

The MiniCan project is located at the depth of 420 m in the Äspö Hard Rock Laboratory (HRL) research tunnel and the aim of the project was to study corrosion of the cast iron inserts if a leak was introduced in the outer copper-canister weld. The experimental part of MiniCan started in 2007 and consisted of five different packages (Table 1-1), denoted package A02–A06. Three of the MiniCan test copper canisters, A02–A04, were surrounded by bentonite clay in a support steel cage. The bentonite in these packages had a dry density of 1 300 kg m^{-3}, which is lower than what is planned to be used in a future repository. In A05, the bentonite was in direct contact with the copper canister with a dry density of 1 600 kg m^{-3} which is fully compacted according to the KBS-3 concept. Package A06 had no bentonite. In all the MiniCan copper canisters, holes with a diameter of 1 mm were introduced to allow Äspö groundwater to come in contact with the interior cast iron inserts. This was done to mimic an accidental leakage in a KBS-3 type repository for spent nuclear fuel. A full description of the design of the project is found in Smart and Rance (2009).

The progress of corrosion inside the support cages was followed by chemical and microbiological analyses sampled in May, August and September 2007, in October 2008 and in December 2010 (Lydmark and Hallbeck 2011). In 2011, the A04 package was retrieved and the results from the analyses of water, bentonite and surfaces of the equipment were presented in Hallbeck et al. (2011) and Smart et al. (2012, 2014).

This report summarises the microbiological, DNA, gas and chemical results from the retrieval of packages A05 and A06. In addition, water chemistry in groundwater and in the packages are presented and discussed.

Table 1-1. The design of the different MiniCan packages installed at the Äspö Hard Rock Laboratory in 2007.

Package	Sampling point	Filling of supporting cage	Introduced hole in the copper canister
A02	KA3386 A02	Bentonite pellet*	Hole (1 mm in diameter) top of copper canister
A03	KA3386 A03	Bentonite pellet*	Hole (1 mm in diameter) located at the bottom of the test canister
A04, retrieved in 2011	KA3386 A04	Bentonite pellet*	Holes (1 mm in diameter) located both at the bottom and top of the test canister
A05, retrieved in 2015	KA3386 A05	Bentonite blocks**	Hole (1 mm in diameter) located at the top of the test canister
A06, retrieved in 2015	KA3386 A06	None	Two holes (1 mm in diameter) located at the top of the test canister

* Bentonite pellet = highly permeble, low density bentonite (1 300 kg m^{-3}).

** Bentonite blocks = fully compacted bentonite (1 600 kg m^{-3}).

1.2 Hydrogen gas metabolism and anaerobic microbial corrosion

As stated above, the experimental part of the MiniCan project started in 2007 and aimed to examine how the corrosion of the cast iron inserts develops inside defect copper canisters. In a future spent nuclear fuel (SNF) repository, corrosion of the cast iron inserts will, in a worst-case scenario, expose the spent nuclear fuel to groundwater and by that release radionuclides into the surroundings. Another potential risk with anaerobic corrosion is that hydrogen gas can be produced from cathodic reduction of protons at the iron surface (King and Miller 1971). Hydrogen gas, H_2, is unwanted in the KBS-3 disposal for two reasons. The first is that an increase in gas volume will build up the pressure inside the barrier system. The second is that development of H_2 is closely linked to activity of sulphate-reducing bacteria (SRB) (Hallbeck 2014). H_2 is used as an energy source by many of the microbes in the deep granitic subsurface (Pedersen 1999, 2012), for example SRB, acetogens and methanogens.

SRB use SO_4^{2-} as electron acceptor and produce sulphide in their anaerobic metabolism according to Equation 1.

$$SO_4^{2-} + 8^{e-} + 8H^+ \rightarrow HS^- + 3H^+ + 4OH^- \quad \text{(Equation 1)}$$

As energy and electron donor, some SRB use organic compounds such as lactate or acetate and are called heterotrophic SRB. Heterotrophic SRB use the organic compounds also as carbon source.

The reaction for sulphate-reduction with acetate is found in Equation 2.

$$CH_3COO^- + SO_4^{2-} + 3H^+ \rightarrow CO_2 + H_2S + 2H_2O \quad \text{(Equation 2)}$$

Many SRB can also use H_2 as energy and electron donor.

$$4H_2(g) + SO_4^{2-} \rightarrow HS^- + 3H_2O + OH^- \quad \text{(Equation 3)}$$

H_2 is an excellent energy and electron source for SRB since there is no need for enzymatic degradation chain as the electrons are transported across the cell membrane via one dehydrogenase. H_2 has also high energy value.

In H_2 metabolism both CO_2 and acetate can be used as carbon source (Dinh et al. 2004). As an example is the assimilation of acetate and CO_2 by *Desulfovibrio aespoeensis* presented in Equation 4. *D. aespoeensis* is a sulphate-reducing bacterium isolated from groundwater in the Äspö HRL (Motamedi and Pedersen 1998). In the reaction, CO_2 is reduced by H_2 together with acetate, activated with the co-enzyme CoA, producing pyruvate to be used in cell synthesis:

$$CH_3COO\text{-}CoA + CO_2 + H_2 \rightarrow CH_3COCOO^- + H_2O + CoA \quad \text{(Equation 4)}$$

There are two types of iron corrosion processes caused by SRB, chemical microbially influenced corrosion, CMIC, and electrically microbially influenced corrosion, EMIC (Enning and Garrelfs 2014). In CMIC, heterotrophic SRB using organic molecules, here with acetate as example, as energy and electron donor, produce sulphide in their metabolism according to Equation 1 and 2.

The produced sulphide react with iron and hydrogen gas is produced together with iron sulphide, see Equation 5.

$$H_2S + Fe^0 \rightarrow H_2 + FeS(s) \quad \text{(Equation 5)}$$

The CMIC process, with sulphate-reduction and iron oxidation, is described with Equation 6:

$$CH_3COO^- + SO_4^{2-} + Fe^0 + 3H^+ \rightarrow CO_2 + FeS(s) + H_2 + 2H_2O \quad \text{(Equation 6)}$$

The hydrogen gas production from the formation of iron sulphide in CMIC, can be further utilized by SRB or other bacteria in their metabolism, see Equation 3, but the process depend on the organic molecules as energy and carbon source.

Cathodically produced H_2 from anaerobic corrosion of iron is believed to be directly scavenged by SRB if they carry the enzyme hydrogenase (Caffrey et al. 2007, Cord-Ruwisch and Widdel 1986). At neutral pH, this reaction is considered to be a rather slow process compared to CMIC and EMIC (Enning and Garrels 2014). The process will be a combination of Equation 6 and 3 but theoretically also 5.

$$Fe^0 + 2H^+ \rightarrow Fe^{2+} + H_2(g) \quad \text{(Equation 7)}$$

In EMIC, SRB withdraw the electrons directly from the metallic iron and use them in the sulphate-reduction metabolism and there is consequently no hydrogen gas formation step in this process. The SRB using EMIC are either autotrophic using CO_2 as carbon source or they use acetate as carbon source (Enning et al. 2012, Enning and Garrelfs 2014).

$$4Fe^0 + SO_4^{2-} + 3HCO_3^- + 5H^+ \rightarrow FeS(s) + 3FeCO_3(s) + 4H_2O \quad \text{(Equation 8)}$$

In this reaction, four iron atoms are oxidised giving one FeS in the reaction with the produced sulfide and three Fe^{2+} that will react depending on the chemical composition of the water. In Equation 8, this is illustrated with the formation of $FeCO_3$.

Autotrophic acetogens (AA) is a group of microorganisms that also can use H_2 as energy and electron source. The carbon source for AA is CO_2 and they produce acetate in their metabolism according to the reaction formula:

$$4H_2 + 2HCO_3^- + H^+ \rightarrow CH_3COO^- + 4H_2O \quad \text{(Equation 9)}$$

The produce acetate could be used by SRB or other anaerobic microorganisms.

1.3 Investigations of microorganisms, dissolved gas and chemical composition in MiniCan

Sampling for analyses of microorganisms, dissolved gas and chemistry in the groundwater and in the water from inside the support cages in the MiniCan packages A02–A06 have previously been done in May, August and September 2007, in October 2008, December 2010. Because of the compacted bentonite in A05, it has never been possible to sample water from the support cage of this package. The third package, A04 was retrieved in August 2011, and during the experimental period, samples for microbiology, chemistry and dissolved gas were taken and analysed (Eriksson 2007, Hallbeck et al. 2011, Lydmark and Hallbeck 2011). Table 1-2 shows information on sample names, sampling points, dates for sampling of dissolved gas, microbiology and water chemistry. Table 1-3 lists the performed analyses.

Retrievals of the copper canisters in the packages A05 and A06 were done during September–October, 2015 with an interval of 26 days. Before the retrieval, water was sampled from inside the support cages of A02, A03 and A06 but not from A05 as explained above. Groundwater from the boreholes in the packages A02, A03, A05 and A06 were sampled concurrently. The gas analyses comprised H_2, CO, Ar, CO_2, CH_4, C_2H_6, C_2H_4, C_2H_2, C_3H_8, C_3H_6, C_3H_4, He, O_2, and N_2. Sampling and analysis of microorganisms comprised total number of cells (TNC), the amount of adenosine tri-phosphate (ATP), culturable heterotrophic aerobic bacteria (CHAB), quantitative most probable number (MPN) of SRB and autotrophic acetogens (AA). DNA from surfaces and bentonite were sampled and analysed but due to the rapid development of new methods in the discipline, the methods for analyses have varied over the period of the MiniCan package. After the retrieval of A05, groundwater from the boreholes in A05 and A06 were filtered to collect cells for DNA extraction.

Table 1-2. Analyses performed in May, August and September 2007, in October 2008 and in December 2010 in the groundwater (G) and in water from inside the support cage (C) surrounding the canisters in the MiniCan packages. A04C was sampled 2011-08-22 and an additional groundwater chemistry sample was taken 2014-06-03.

Sample name	Sampling point	Sampling date dissolved gas	Sampling date microbes	Sampling date water chemistry
A02C	KA3386 A02Canister	2007-09-28, 2008-10-15, 2010-12-08	2007-08-21 2008-10-15 2010-12-08	2007-05-22 2008-10-15 2010-12-08 2014-06-03
A03C	KA3386 A03Canister	2007-09-28 2008-10-15 2010-12-08	2007-08-21 2008-10-15 2010-12-08	2007-05-22 2008-10-15 2010-12-08 2014-06-03
A03G	KA3386 A03Ground-water	2007-09-28 2008-10-15 2010-12-08	2007-08-21 2008-10-15 2010-12-08	2007-05-22 2008-10-15 2010-12-08
A04C	KA3386 A04Canister	2007-09-28 2008-10-15 2010-12-08 2011-08-22	2007-08-21 2008-10-15 2010-12-08 2011-08-22	2007-05-22 2008-10-15 2010-12-08 2011-08-22 2014-06-03
A05C	KA3386 A05canister	–[a]	–	–
A06C	KA3386 A06Canister	2007-09-28 2008-10-15 2010-12-08	2007-08-21 2008-10-15 2010-12-08	2007-05-22 2008-10-15 2010-12-08 2014-06-03

[a] Water could not be extracted from package A05C.

Table 1-3. Parameters analysed in the groundwater and in water from inside the support cages surrounding the canisters in the MiniCan packages.

Gas analyses		Microbial analyses		Chemical analyses	
Sampling vessel	**Analyses**	**Sampling vessel**	**Analyses**	**Sampling vessel**	**Analyses**
PVB sampler	H_2, CO_2, CO, CH_4, $C_{2-3}H_{2-8}$, O_2, He, Ar, N_2	10–100-mL anaerobic tube or bottle	TNC, ATP, CHAB, MPN SRB and AA	Plastic and glass bottles according to the requirements of the instructions for the methods.	SO_4^{2-}, HCO_3^- Fe^{2+}, S^{2-}, Cl^- pH, acetate

2 Material and Methods

2.1 Sampling procedures

2.1.1 Groundwater and water inside the support cages

Sampling for gas and microbiological analyses was done 2015-08-20. Microbiological samples were taken directly from the connection of the packages A02, A03 and A06 support cages with sterile tubing and attached valve. An anaerobic sampling vessel was attached via a sterile needle. An additional needle prevented dangerous pressure build-up during the filling. The same procedure was used to sample ground water in all packages.

The first 50 mL of water sampled from the MiniCan packages flushed the sample equipment and were discarded. The volumes used were, for acetate, 10 mL, MPN SRB, MPN AA, ATP and CHAB, 50 mL, TNC, 10 mL, dissolved gas, 250 mL and isotopes, 100 mL, respectively. The laboratory at Äspö HRL did the groundwater sampling for groundwater chemistry 2015-08-24. In Table 2-1 and Table 2-2, the samples taken from the packages A05 and A06 are compiled.

2.1.2 The retrieved packages A05 and A06

A06

A container filled with groundwater was placed in front of the package to be retrieved. Before the package was opened, the container water was infused with N_2-gas. The package was removed from the borehole, into the container and further into the transport cylinder, which was directly transported to the anaerobic box in one of the buildings in the Äspö HRL. The transport cylinder was placed in an anaerobic glove box overnight until anaerobic condition was reached.

The package A06 was retrieved 2015-09-30. Immediately after the support cage was transferred from the transport cylinder into the box, Figure 2-1, biofilms were sampled with sterile swabs from a 6 × 6 cm area from the outside of the support cage, Figure 2-2. The swabs were directly placed in an anaerobic tube with 10 mL medium. Biofilms were sampled from the nylon support rack and the iron- and copper sandwich test specimen, see Figure 2-3 and Figure 2-4. Surface samples were collected from inside the support cage, Figure 2-5, before the copper canister was removed and samples were taken, see Figure 2-6 and Figure 2-7. The sample locations and sample types are summarised in Table 2-1 and a more detailed description of the different specimens inside the support cage can be found in Smart and Rance (2009).

Table 2-1. Compiled information of the samples taken from the retrieved A06 package in the MiniCan project sampled 2015-09-30.

Sample name	Sample site	Number of samples	Sample type	Tool
A06OC	Outside support cage, Figures 2-1 and 2-2.	2	Biofilm	Sterile swab
A06IC	Inside support cage, Figure 2-5	2	Biofilm	Sterile swab
A06Cu	Copper canister, Figure 2-7	3	Biofilm	Sterile swab
A06Nyl	Nylon support rack, Figures 2-3 and 2-4	2	Biofilm	Sterile swab
A06SFe	Sandwich specimen iron, Figure 2-3	2	Biofilm	Sterile swab
A06SCu	Sandwich specimen copper, Figure 2-3	2	Biofilm	Sterile swab

Figure 2-1. *Transfer of the support cage of the MiniCan package A06, from the transport cylinder inside an anaerobic glovebox.*

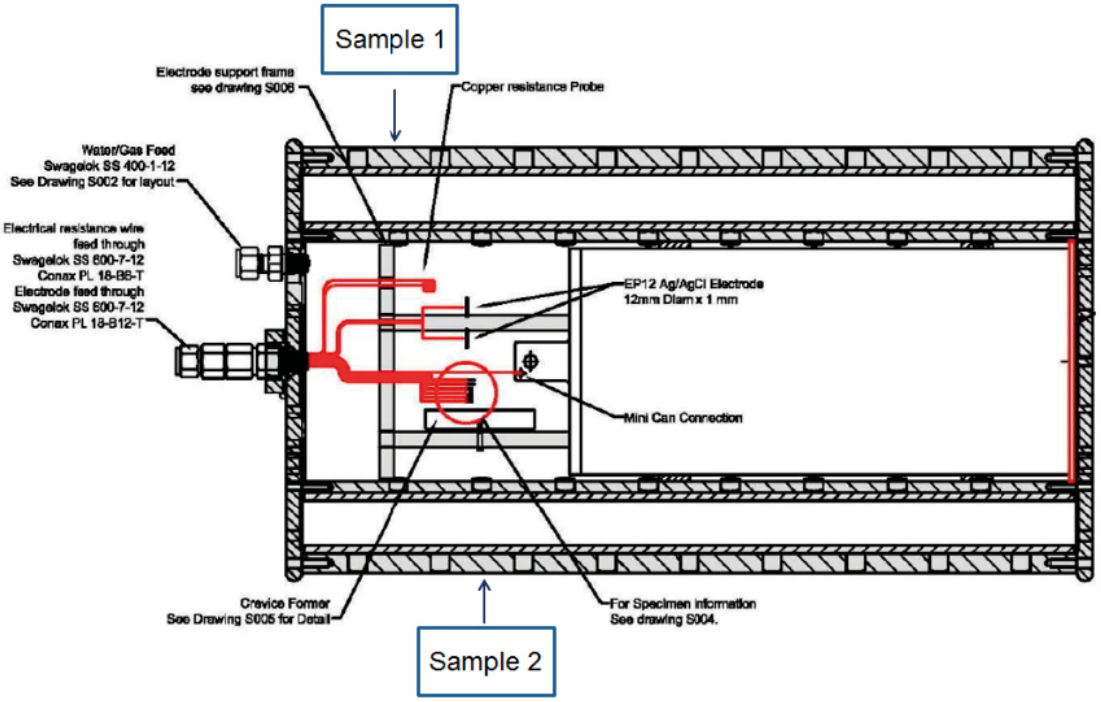

Figure 2-2. *The positions of the two samples taken from the outside of the support cage of package A06 in MiniCan.*

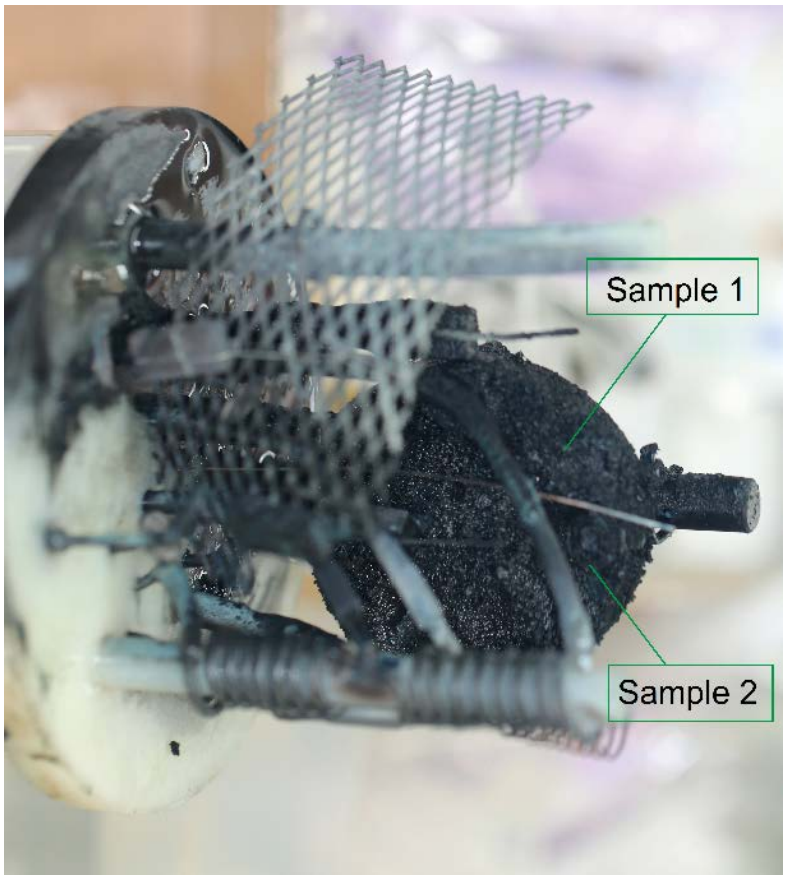

Figure 2-3. *The nylon support rack with electrodes and test specimens from the MiniCan package A06. The text squares show where the samples were taken.*

Figure 2-4. *The nylon support rack and test specimens from the MiniCan package A06.*

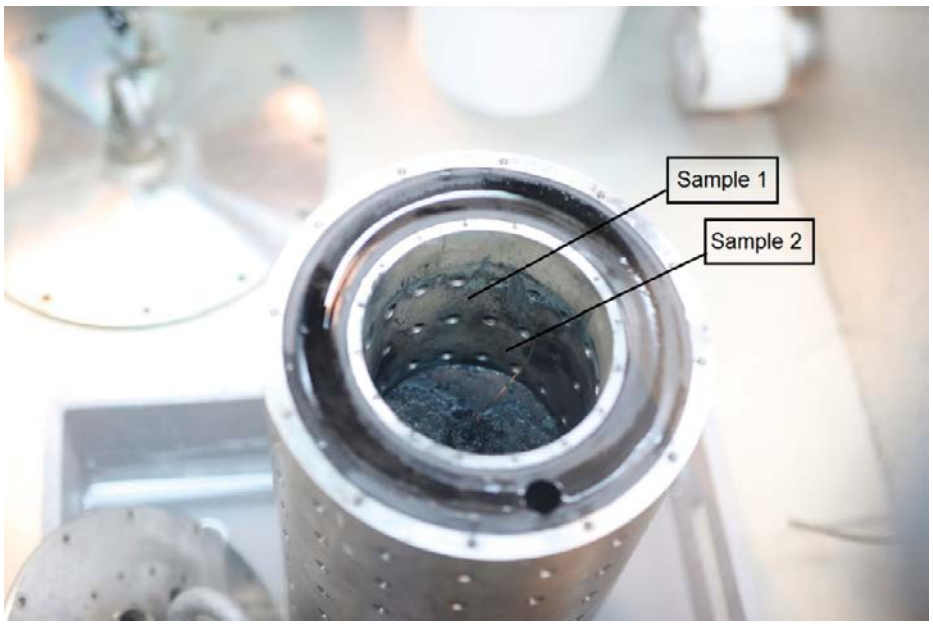

Figure 2-5. *The positions of the two samples from the inside of the support cage in the MiniCan package A06. The text squares show where the two samples were taken.*

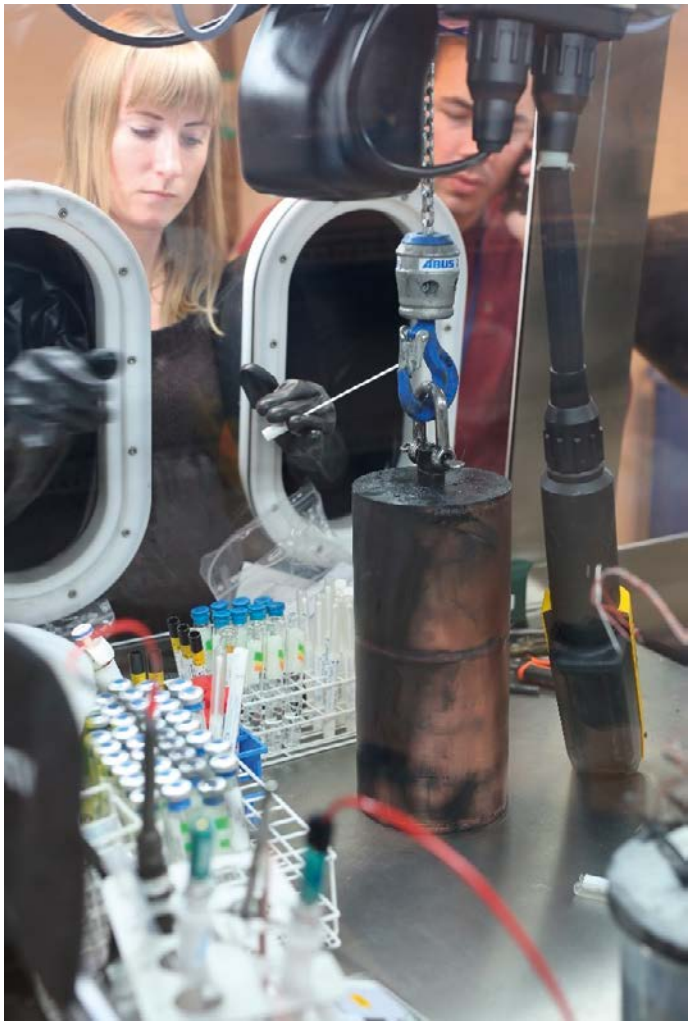

Figure 2-6. *Sampling from the A06 copper canister from the MiniCan project, inside the glove box.*

Figure 2-7. *Sampling for microbiological analysis from the copper canister surface of the MiniCan package A06. The text squares show where the three canister samples were taken.*

A05

The package A05 was retrieved 2015-10-28. The surface outside the support cage of A05 was sampled from a 6 × 6 cm area with sterile swabs and placed in anaerobic tubes, see Figure 2-2. The support cage of A05 was totally filled with bentonite clay and therefore it had to be cut into pieces to release the canister as illustrated in Figures 2-8, 2-9 and 2-10. There was no nylon support rack in this package. Bentonite samples were collected from around the iron and copper sandwich specimens, close to the copper canister, from the inside of the support cage and from the surrounding bentonite, see Figures 2-13 and 2-14. Biofilm samples from the iron part of the sandwich specimen were sampled as shown in Figures 2-11 and 2-12. The sample locations and samples types are summarised in Table 2-2.

Table 2-2. Compiled information of the samples taken from the retrieved A05 equipment in the MiniCan project sampled 2015-10-28.

Sample name	Sample site	Number of samples	Sample type	Tool
A05OC	Outside support cage, Figure 2-1	2	Biofilm	Sterile swab
A05IC	Inside support cage	2	Bentonite	Sterile scalpel
A05Cu	Copper canister	3	Bentonite	Sterile scalpel
A05SFe	Sandwich specimen iron, Figure 2-14	4	Biofilm and bentonite	Sterile swab and sterile scalpel
A05SCu	Sandwich specimen copper, Figure 2-14	2	Bentonite	Sterile scalpel
A05B	Bentonite	3	Bentonite	Sterile scalpel

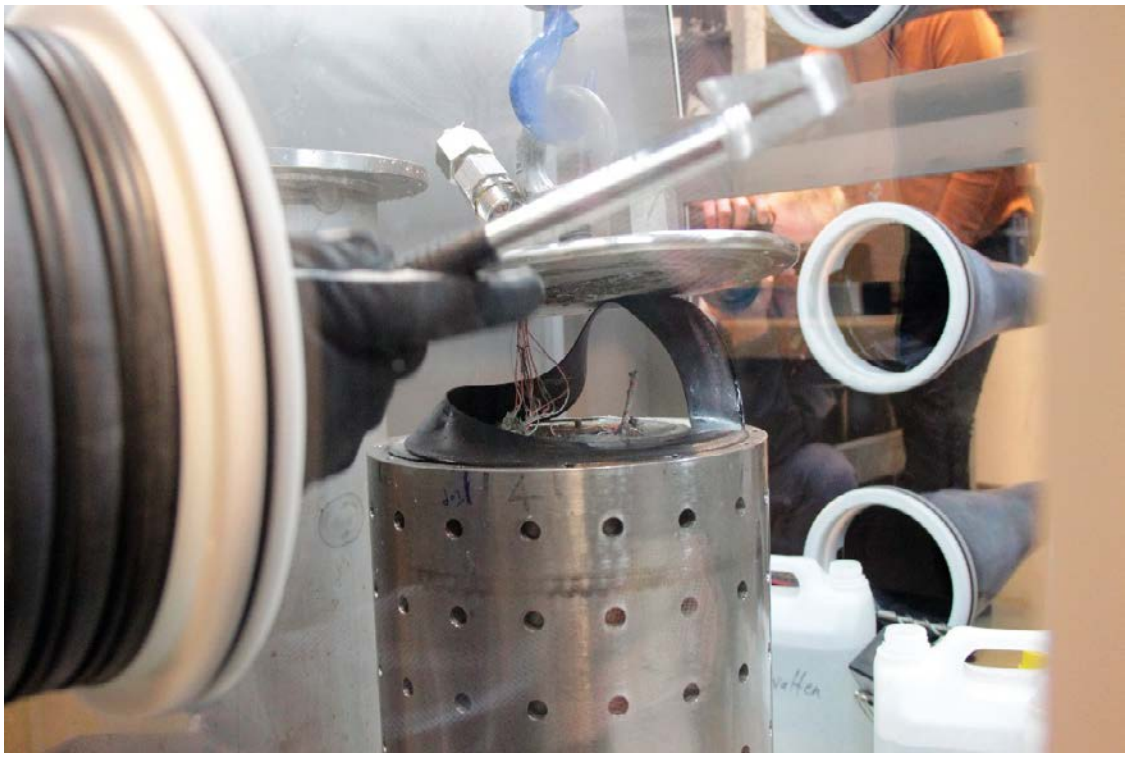

Figure 2-8. *Opening of the support cage of the retrieved A05 package in MiniCan.*

Figure 2-9. *The top of the A05 support cage from MiniCan with fully compacted bentonite.*

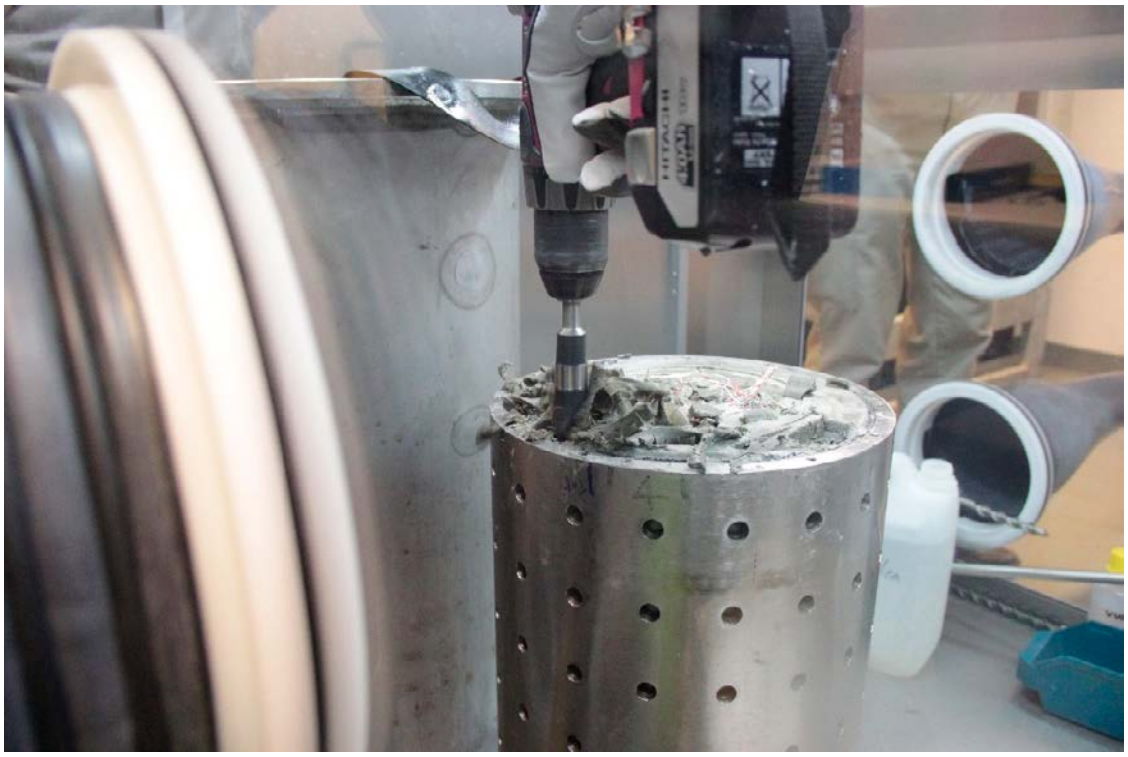

Figure 2-10. *Removal of bentonite from the A05 package in MiniCan to reach the test speciemens and copper canister inside the support cage.*

Figure 2-11. *The bottom of the A05 support cage in MiniCan.*

Figure 2-12. *Removal of bentonite at the bottom of the A05 support cage from MiniCan to expose the copper canister.*

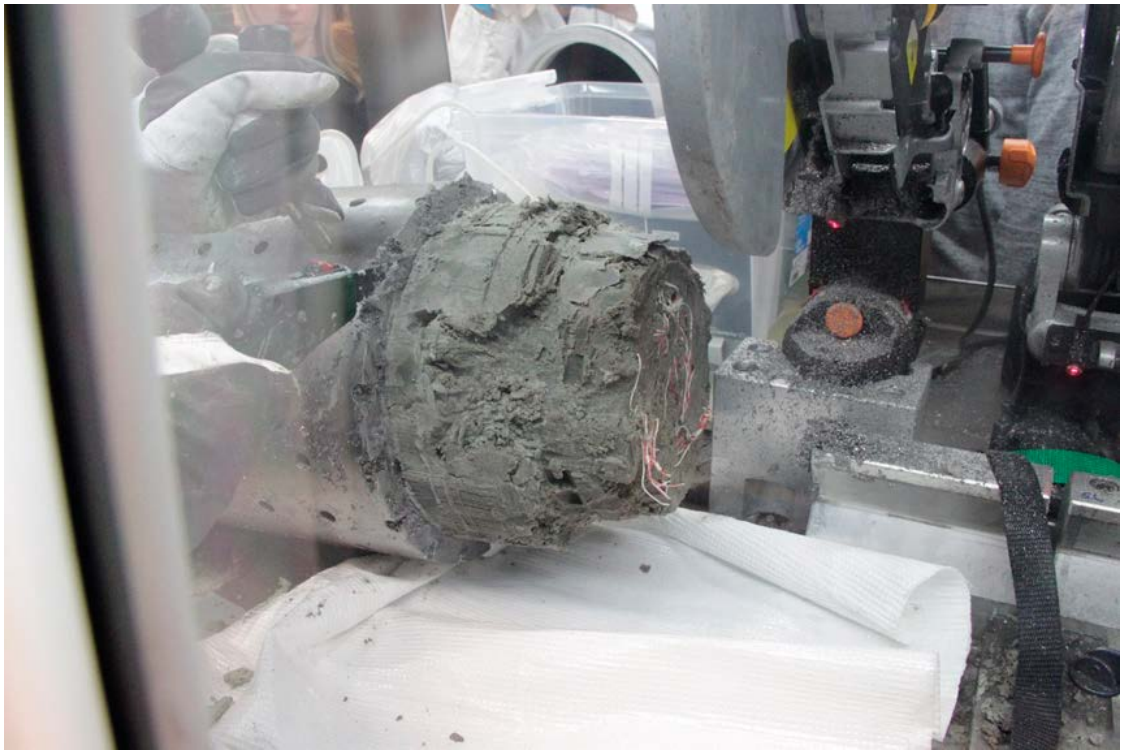

Figure 2-13. *The test specimens from the A05 package in MiniCan, surrounded by bentonite.*

Figure 2-14. *The sandwich specimen in compacted bentonite from the A05 package in MiniCan.*

2.1.3 Sampling for DNA analysis

Surfaces

In parallel to the microbial sampling on surfaces and bentonite from the retrieved A05 and A06 packages, samples were collected for DNA analysis using special DNA sampling swabs (Copan FloqSwab™, art.no: 4479438; Thermo Fisher Scientific). The swabs were rubbed gently over the moist surface to collect cells for subsequent DNA extraction. The swabs were stored in provided sample tubes in room temperature until analysis, according to the manufacturer's instructions.

Groundwater

Concurrently with the sampling from the A05 canister, groundwater from the boreholes in the A05 and A06 were sampled for DNA analyses by filtration. Groundwater was filtered using high pressure, stainless steel 47 mm filter holders (X4504700, Millipore AB, Solna, Sweden) prepared with 0.22 µm pore size water filters from the MO BIO Power Water kit filter units. The filter holders were equipped with pressure relief valves (Swagelok SS-RL3S6MM, SWAFAB, Sollentuna, Sweden) and a pressure gauge that enabled adjustment of a pressure drop over the filter between 200 and 400 kPa relative to the ambient aquifer pressure (Figure 2-15). Groundwater was filtered for 19 hours at a flow rate of 170–180 mL per minute.

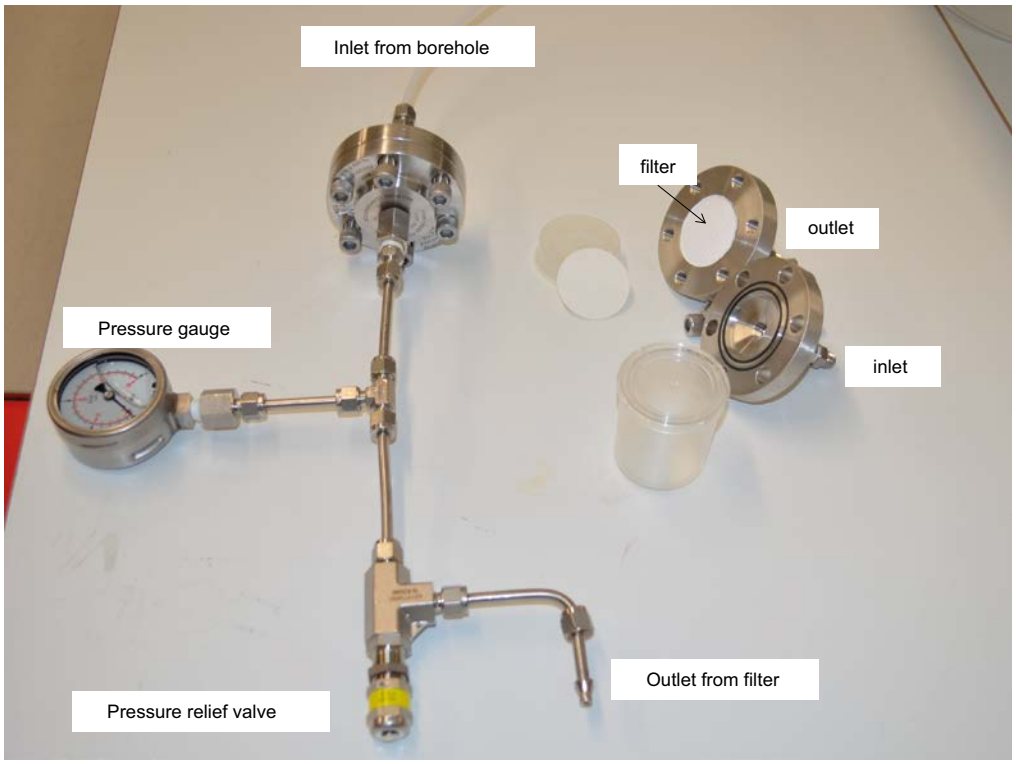

Figure 2-15. *Equipment used for groundwater filtration of the A05 and A06 packages.*

2.2 Analyses and data treatment

2.2.1 Total number of cells – TNC

The total number of cells (TNC) was determined using the acridine orange direct count method (Hobbie et al. 1977, Pedersen and Ekendahl 1990). From each sample, either water samples or dilution solution for surface samples, 1 mL was filtered (-20 kPa) onto a black polycarbonate filter (0.22 µm-pore-size), 13 mm in diameter (GTBP011300, Millipore, Solna, Sweden), thereafter stained with 0.2 mL acridine orange (AO) solution for seven minutes. The AO solution was prepared by dissolving 10 mg of AO in 1 L of a 6.6 mM sodium potassium phosphate buffer, pH 6.7. The filters were mounted between microscope slides and cover slips using fluorescence-free immersion oil. The number of cells was counted under blue light (390–490 nm) with a band-pass filter for orange light (530 nm), in an epifluorescence microscope (Nikon DIPHOT 300; Tekno-Optik, Göteborg, Sweden). Minimum 600 cells, or a minimum of 30 microscopic fields (1 field = 0.01 mm^2), were counted on each filter. Three filters were prepared per each sample. The results were calculated as the mean value of the three filters.

2.2.2 Adenosine tri-phophate – ATP

Groundwater for ATP extraction and analysis was collected in triplicates from each sample. The ATP Biomass Kit HS for determining total ATP in living cells was used (no. 266-311; BioThema, Handen, Sweden). This ATP biomass method has been evaluated for use with Fennoscandian groundwater, including Olkiluoto groundwater, and the results were published (Eydal and Pedersen 2007).

A new 4.0 mL, 12-mm-diameter polypropylene tube (no. 68.752; Sarstedt, Landskrona, Sweden) was filled with 400 µL of the ATP kit reagent HS (BioThema, Handen, Sweden) and inserted into an FB12 tube luminometer (Sirius Berthold, Pforzheim, Germany). The quick measurement FB12/Sirius software, version 1.4 (Berthold Detection Systems, Pforzheim, Germany), was used to calculate light emission as relative light units per second (RLU s^{-1}). Light emission was measured for three 5-s intervals with a 5-s delay before each interval, and the average of three readings was registered as a single measurement. The background light emission (I_{bkg}) from the reagent HS and the tube was monitored and allowed to decrease to a value below 50 RLU s^{-1} prior to registering a measurement. ATP was extracted from 100-µL aliquots of sample, by mixing for 5 s with 100 µL of B/S extraction solution from the ATP kit in a separate 4.0-mL polypropylene tube. Immediately after mixing, 100 µL of the obtained ATP extract mixture was added to the reagent HS tube in the FB12 tube luminometer, and the sample light emission (I_{smp}) was measured. Subsequently, 10 µL of an internal ATP standard was added to the reactant tube, and the standard light emission (I_{std}) was measured. The concentration of the ATP standard was 10^{-7} M. Samples with ATP concentrations close to or higher than that of the ATP standard were diluted with B/S extraction solution to a concentration of approximately 1/10 that of the ATP standard.

The ATP concentration of the analysed samples was calculated as follows:

$$\text{amol ATP mL}^{-1} = (I_{smp} - I_{bkg}) / ((I_{smp+std} - I_{bkg}) - (I_{smp} - I_{bkg})) \times 10^6 / \text{sample volume}$$

where I, represents the light intensity measured as RLU s^{-1}, smp represents sample, bkg represents the background value of the reagent HS, and std represents the standard (referring to a 10^{-7} M ATP standard).

2.2.3 Cultivable heterotrophic aerobic bacteria – CHAB

Petri dishes containing agar with nutrients were prepared for determining the numbers of CHAB in groundwater samples. This agar contained 0.5 g L^{-1} of peptone (Merck), 0.5 g L^{-1} of yeast extract (Merck), 0.25 g L^{-1} of sodium acetate (Merck), 0.25 g L^{-1} of soluble starch (Merck), 0.1 g L^{-1} of K_2HPO_4, 0.2 g L^{-1} of $CaCl_2$ (Merck), 10 g L^{-1} of NaCl (Merck), 1 mL L^{-1} of trace element solution and 15 g L^{-1} of agar (Merck) (Pedersen and Ekendahl 1990). The medium was sterilized in 1-L batches by autoclaving at 121 °C for 20 min, cooled to approximately 50ºC in a water bath, and finally distributed in 15-mL portions in 9-cm diameter plastic Petri dishes. Ten-times dilution series of culture samples were made in sterile analytical grade water (AGW) with 1.0 g L^{-1} of NaCl and 0.1 g L^{-1} of K_2HPO_4. 0.1-mL portions of each dilution were spread with an EasySpiral automatic spiral plater (Interscience, Saint Nom, France) in triplicates. The plates were incubated for 7 days at 20 °C, after which the number of colony forming units (CFU) was counted.

2.2.4 Most probable number – MPN SRB and MPN AA

Media for the MPN SRB and MPN AA were composed based on previously measured chemical data from Äspö HRL. The composition of the SRB medium was in g L^{-1}: NaCl, 7.0; $CaCl_2 \times 2H_2O$, 1.0; KCl, 0.67; NH_4Cl, 1.0; KH_2PO_4, 0.15; $MgCl_2 \times 6H_2O$, 0.5; $MgSO_4 \times 7H_2O$, 3.0. The AA medium in g L^{-1}: NaCl, 7.0; $CaCl_2 \times 2H_2O$, 1.0; KCl, 0.67; NH_4Cl, 1.0; KH_2PO_4, 0.15; $MgCl_2 \times 6H_2O$, 0.5. The medium was autoclaved and cooled under 80/20 % N_2/CO_2 gas. To the cooled medium was added vitamin and trace element solutions according to Table 2-3 A–D and pH was adjusted to between 7.2 and 7.5 with 1 M HCl or 1 M NaOH. Nine mL of final medium was dispensed anaerobically into 27 mL anaerobic tubes (Bellco glass Inc) that were sealed with butyl rubber stoppers and crimped with aluminium seals. Before filling, the sterile culture tubes were flushed with 80/20 % N_2/CO_2 gas.

Table 2-3 A–D. Composition of anaerobic media used for MPN SRB and MPN AA. All solutions were anaerobic.

A) Final medium **Component (mL L^{-1})**	**SRB**	**AA**
Basal medium, see text	885	890
Trace elements (Table C)	10	10
Vitamins (Table D)	10	10
Thiamine stock (Table B)	1.0	1.0
Vitamin B_{12} stock (Table B)	1.0	1.0
Fe stock (Table B)	5.0	5.0
Resazurin (Table B)	2.0	2.0
Cysteine hydrochloride (Table B)	10	10
$NaHCO_3$ (Table B)	60	60
Yeast extract (Table B)	10	1.0
Lactate (Table B)	5.0	–
Sodium sulphide (0.2 M)	10	10

B) Stock solutions **Component**	**Amount**
$NaHCO_3$	84 g L^{-1}
Thiamine chloride dihydrochloride in a 25 mM sodium phosphate buffer, pH 3.4	0.100 g L^{-1}
Cyanocobalamin (B_{12})	0.050 g L^{-1}
Yeast extract	50 g L^{-1}
$Fe(NH_4)_2(SO_4)_2*6H_2O$, initially dissolved in 0.1 mL of concentrated HCl	2 g L^{-1}
Resazurin	0.5 g L^{-1}
Cysteine-HCl	50 g L^{-1}
Sodium lactate solution	50 %

C) Trace element solution **Component**	**Amount**
Double-distilled H_2O	1 000 mL
Nitrilotriacetic acid	1 500 mg
$Fe(NH_4)_2(SO_4)_2*6H_2O$	200 mg
Na_2SeO_3	200 mg
$CoCl_2*6H_2O$	100 mg
$MnCl_2*4H_2O$	100 mg
$Na_2MoO_4*2H_2O$	100 mg
$Na_2WO_4*2H_2O$	100 mg
$ZnSO_4*7H_2O$	100 mg
$AlCl_3$	40 mg
$NiCl_2*6H_2O$	25 mg
H_3BO_3	10 mg
$CuCl_2*2H_2O$	10 mg

D) Vitamin mixture **Component**	**Amount**
Sodium phosphate buffer 10 mM pH 7.1	1 000 mL
p-Aminobenzoic acid	10 mg
Nicotinic acid	10 mg
Calcium D(+) pantothenate	10 mg
Pyridoxine dihydrochloride	10 mg
Riboflavin	10 mg
D(+)-biotin	5 mg
Folic acid	5 mg
DL-6-8-thiotic acid	5 mg

2.2.5 Inoculations and analysis of anaerobic microorganisms

All samples were transported and stored at 4 °C. Inoculations for MPN SRB and MPN AA, were performed in the laboratory in Mölnlycke the day after retrieval. After inoculations, addition of H_2 to an overpressure of 2 bars, was done to the AA cultures and all MPN tubes were incubated in the dark at 20 °C for 8 weeks. After incubation, the MPN tubes were analysed by testing for metabolic products and substrate consumption. Growth of SRB were detected by measuring sulphide production using the $CuSO_4$ method according to Widdel and Bak (1992) on a UV visible spectrophotometer (Genesys10UV, VWR, Stockholm, Sweden). Growth of acetogens were detected by means of acetate production using an enzymatic UV method (Enzymatic Bioanalysis Kit no. 10 139 084 035; Boehringer Mannheim/RBiopharm, Food Diagnostics, Göteborg, Sweden) with a UV visible spectrophotometer (as for SRB) and H_2-consumption by pressure measurements. Product formation at a concentration three times or above that of the sterile control tubes was taken as positive. The MPN procedures resulted in protocols with tubes that scored positive or negative for growth. The results of the analyses were rated positive or negative compared with control levels. Three dilutions with five parallel tubes were used to calculate the MPN of each group, according to the calculations by Greenberg et al. (1992).

2.2.6 Data treatment and graphics

Results were recalculated from the sampling area (biofilm samples), weight (bentonite samples) and the dilution step when the material were added to the anaerobic tubes. Data treatment and graphics were done with STATISTICA software, version 13 (Statsoft, Tulsa, OK, USA).

2.2.7 Molecular biology

The DNA from filter samples was extracted with Power Water DNA isolation kit (cat. no. 14900-100), while surface and bentonite samples were extracted with the Power Soil or PowerMax Soil DNA isolation kit (cat. no. 12888-100 and 12988-10, respectively) from MO BIO Laboratories, Carlsbad, CA, USA, according to the manufacturer's specifications. Total extracted nucleotide concentrations were measured using the Nanodrop ND-1000 UV-vis spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA) and double stranded (ds) DNA concentrations were measured fluorometrically using the Stratagene MX3005p fluorometer with MXPro software (Agilent Technologies Inc., Santa Clara, CA, USA) and the Quant-it™ Picogreen reagent kit from Molecular Probes (cat. no. P7589; Invitrogen, San Diego, CA, USA), according to the manufacturer's specifications. The extracted DNA was stored at −20 °C and subsequently used for sequencing.

Amplicon library preparation was performed with primers designed for the hypervariable v6 region of the 16S Bacteria rRNA and modified to include indices and barcodes compatible with the Illumina NextSeq500 instrument. The 16S v6 target sequence forward primers were (967F) 5′-CTAACCGANGAACCTYACC- 3′, 5′- CNACGCGAAGAACCTTANC- 3, 5′-CAACGCGMARAACCTTACC - 3, 5′- ATACGCGARGAACCTTACC - 3 and reverse primer was (1064R) 5′- CGACRRCCATGCANCACCT-3 (Meyer et al. 2013). The amplification was carried out in triplicates with 33 µL reaction volume of 1.0 U Platinum Taq Hi-Fidelity Polymerase (Life technologies, Carlsbad CA), 1X Hi-Fidelity Buffer (Life technologies, Carlsbad CA), 200 µM dNTP PurePeak DNA Polymerase mix (Pierce Nucleic acid Technologies, Milwaukee, WI), 1.5 mM MgSO4, 0.2 µM of each primer and ~10 ng template DNA. Each primer pair had a no template control for each Multiplex IDentifier. Cycling conditions were; an initial 94 °C 3 min. denaturation, 30 cycles of 94 °C for 30 s, 60 °C for 60 s and 72 °C for 90 s followed by a final 10 min. extension at 72 °C. The triplicate PCR reactions were pooled and checked on 1 % agarose gel for amplicons that were of size 236 base pairs. Amplicons were sent on dry ice to the sequencing facility (tataabiocenter, Gothenburg, Sweden). Upon arrival amplicons were quality checked by capillary electrophoresis on a Fragment Analyzer, Advanced Analytical, and quantified using Picogreen ds DNA reagent (Life Technologies, Carlsbad, CA) and pooled in equimolar amounts for optimal cluster concentration. The Library pool was sequenced on a NextSeq500 Instrument from Illumina by a 150 base pairs paired-end run sequencing on high output mode with PhiX DNA at 25 % concentration, as the control DNA for the run.

Raw data files were provided in FASTAQ format and was delivered through the Illumina cloud-based sequence Hub Basespace. Envonautics bioinformatics consultants Ltd. performed the bioinformatics analysis using a phyton pipeline for 16S rRNA microbial amplicon sequencing data, version 2.0.0. The source code and further information on the workflow for the pipeline is found at: http://xapple.github.io/sifes/. To resolve the dataset, reads were de-multiplexed by identifying reads by their index and barcode. Paired-end recovers DNA sequence from both ends of the DNA template giving a complete overlap between the reads. The algorithm implemented in the Mothur software version 1.35.1 was used for requiring complete overlap of forward and reverse paired-end reads. Reads that did not assemble was rejected. A length cut-off rejected reads under 55 bases and over 140 bases. Further quality filtering to remove sequencing errors was done by searching for forward and reverse primer in reads. Sequences without a perfect match in either of the forward or reverse read were discarded together with reads with undetermined bases (N´s). A chimera check was applied using the UCHIME algorithm in deNovo and reference based mode (Edgar et al. 2011). All sources are available at http://drive5.com/uchime. The OTU clustering was performed with the UPARSE pipeline at 3 % dissimilarity distance threshold (Edgar 2013). Using the CREST classifier, the representative sequences for each OTU was search against the SILVA 123 16S Database set to 97 % threshold (Quast et al. 2013). Bar charts detailing the composition at different levels were constructed using the OTU table and taxonomic assignment.

Nucleotide sequences were submitted to the NCBI Sequence Read Archive (SRA) Repository with accession number SRP076858.

2.2.8 Gas analyses

Water samples from the PVB vessels were transferred to a vacuum container and any gas in the water was boiled off under vacuum, i.e. at water vapour pressure. The transfer time was approximately 20–30 min at 20 °C. The extracted gas volume was compressed and transferred to a 10-mL syringe (SGE Analytical Science, Melbourne, Victoria, Australia) and subsequently to a 6.6-mL glass vial. The sample vials were sealed with butyl rubber stoppers and aluminium crimps, evacuated and flushed twice with nitrogen (N_2), and left under high vacuum (1 Pa). A dehydrating gel was added to the vials to adsorb any traces of remaining water in the gas. The volumes of water sample and the extracted gas were measured and the volume of gas was calculated with the Ideal Law of Gas. Analysis was thereafter performed with gas chromatography. The gases were analysed with three different gas chromatographs equipped with four different detectors and three different carrier gases, He, Ar and N_2. By various configurations of these instruments all ranges of groundwater gases could be analysed. All chromatographs were calibrated with certified gas mixtures that mimic the gas composition of the analysed samples.

Helium (He), hydrogen (H_2), nitrogen (N_2), neon (Ne) oxygen (O_2) and methane (CH_4) >20 ppm are analysed on Varian CP-3800 gas chromatograph (Agilent Technologies Inc., CA, USA) with a 30 m high resolution capillary column (Bruker, SELECT PERMANENT GASES/CO_2 HR, CP7430) and He as carrier gas. The gases were detected with a thermal conductivity detector (TCD) at detector temperature 120 °C, the filament temperature of 220 °C and a column temperature of 45 °C. Methane (CH_4) and hydrocarbon gases (C1-C3) < 20 ppm were alternatively analysed on Varian CP-3800 gas chromatograph with a carboxen column (2 m ´ 1/8 in. diameter) and detected with the flame ionisation detector (FID) and N_2 as the carrier gas. A Bruker 450 gas chromatograph equipped with a CP7355 PoraBOND Q (50 m x 0.53 mm, ID), a CP7536 MOLSIEVE 5A PLOT (25 m x 0.32 mm, ID) and a Pulsed Discharge Helium Ionization Detector (PDHID) was employed for trace concentrations of hydrogen (H_2), oxygen (O_2), argon (Ar), carbon dioxide (CO_2) and hydrocarbon gases (C1-C3), (Bruker Daltonics Scandinavia AB, Vallgatan 5, SE-17067 Solna, Sweden). Hydrogen (H_2), argon (Ar) nitrogen (N_2), neon (Ne) oxygen (O_2) carbon monoxide (CO) and methane (CH_4) was also analysed on DANI Master GC using MXT-Molsieve 5A Plot 30 m × 0.53 mm × 50 µm and OPT 270M – MICRO thermal conductivity detector (TCD) system, with He, Ar or N_2 as carrier gases.

3 Results

3.1 Microbiology of groundwater and water inside the support cages in MiniCan

Data from the microbial analyses of groundwater and water inside the support cages in the MiniCan packages in 2015 are compiled in Table 3-1. Each microbiological parameter will be presented in the following sections.

3.1.1 ATP

The ATP data for groundwater (G) and water from inside the support cages (C) of the MINICAN packages are shown in Figure 3-1. The amount of ATP was between 1 000 and 3 000 amol mL^{-1} in all samples except in the groundwater sample from A03 (A03G) that had the highest ATP concentration, with 6 900 amol mL^{-1}, see also Table 3-1. The detection limit for the ATP analysis is < 1 000 amol mL^{-1} of sample. The lowest amount of ATP was found in the water from the inside support of the support cage in package A02, A02C.

3.1.2 TNC

The detection limit of the TNC method is around 1 000 cells mL^{-1}. The cage sample from the A03 had the significantly lowest number of TNC compared to the groundwater samples with detectable amounts of cells (Figure 3-2). A03 was only cage sample with TNC above the detection limit of the method, with 3 200 cells mL^{-1}. All groundwater samples had TNC above the detection limit except for the A06 groundwater sample (A06G). In this sample and for the samples from inside the support cages of A02 (A02C) and A06 (A06C), interfering materials disturbed the analysis. In order to override this problem, the samples were diluted but since the original number of cells in these samples were low, the increased detection limit of the method was not reached. The highest number of TNC was found in the groundwater sample from the A03, which correspond well with the ATP data (Figure 3-1). No significant difference in TNC was found between the groundwater samples from the A02, A03 and A05, Table 3-1. However, although the standard deviation overlaps, the tendency was shown from two separated methods, ATP and TNC, indicating that the A03G sample actually had the highest concentrations of ATP and TNC.

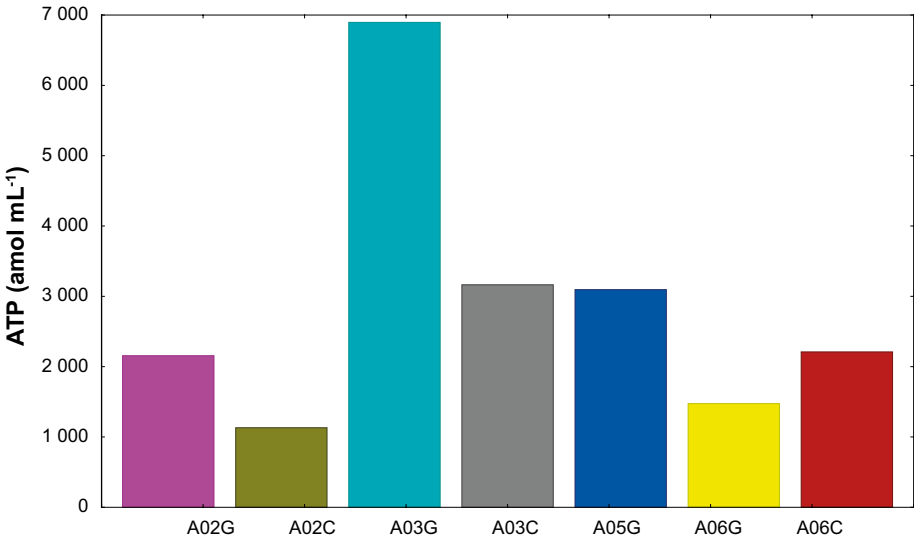

Figure 3-1. *The amount of adenosine triphosphate, ATP, in the groundwater (G) and water inside support cages (C) in the MiniCan packages in 2015.*

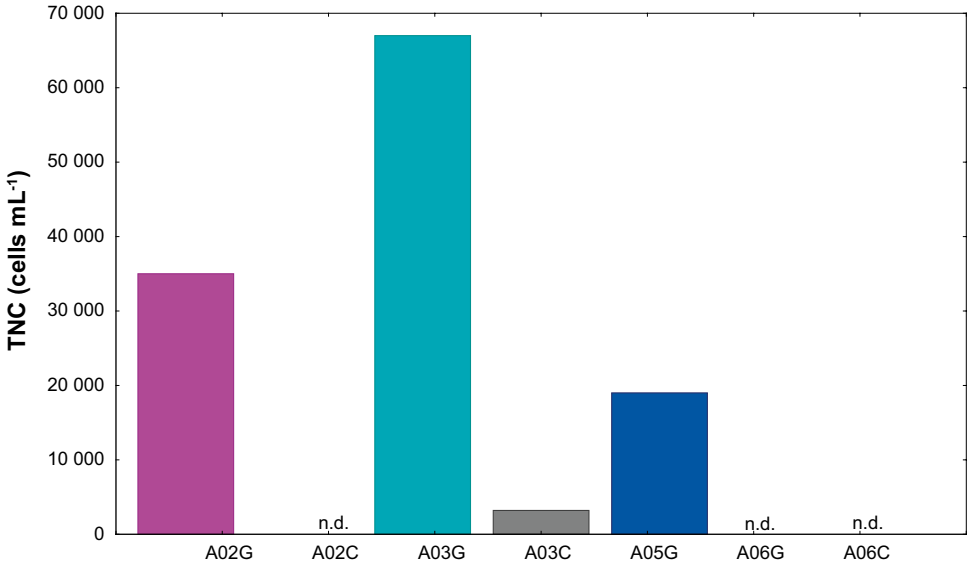

Figure 3-2. *The total number of cells, TNC, in the groundwater (G) and water inside support cages (C) in the MiniCan packages in 2015. Because of interfering materials no cells (marked as nd) were detected in the A02C, A06G and A06C samples.*

3.1.3 CHAB

Figure 3-3 shows CHAB data for groundwater and water from inside the support cages of the MiniCan packages. The significantly highest amounts of CHAB were found in A03 and the highest number was in the water of the support cage. Detectable CHAB concentrations were found in all groundwater samples except for A06 (A06G). Similarly, to the groundwater samples, all water samples from inside the support cages had detectable number of CHAB except for the A02 where the number of CHAB was below the detection limit, less than 7 CHAB mL^{-1}, of the method.

3.1.4 MPN AA

The groundwater sample from the A02 (A02G) was the only sample that had detectable number of AA; 0.2 AA mL^{-1} (see Table 3-1). The number of AA in groundwater samples from A03, A05 and A06, and water samples from inside the support cages of A02, A03 and A06 were all under detection (< 0.2 cells mL^{-1}) for the method.

3.1.5 MPN SRB

The significantly highest MPN of SRB was found in the water collected from inside the support cage of the A03 (Figure 3-4), with 8 000 cells mL^{-1}. This corresponded well with the CHAB data (Figure 3-3). The second highest MPN of SRB was observed in the cage sample from the A06, with 1 100 cells mL^{-1}. Low numbers of SRB were observed in the A03G and A05G samples (see Table 3-1). The detection limit of the method is < 0.2 SRB mL^{-1}.

To investigate if H_2 utilizing SRB were present in MiniCan, H_2 was added to the cultures after 8 weeks from inoculation and reanalysed after additional 8 weeks. Most of the samples had no or very small changes in SRB numbers compared to the first analysis except for the A06 cage sample (A06C) in which the SRB concentration increased from 1 100 to 1 400 cells mL^{-1}.

3.1.6 Acetate

The amount of acetate in the water samples ranged from approximately 1 to 18 mg L^{-1} (Table 3-1). Overall, higher amounts of acetate were found in the water samples from support cages compared to the corresponding groundwater samples, except for the A06 package in which the lowest acetate concentration in the support cages (A06C) was found. The highest acetate concentration was found in water from the support cage of A03 (A03C).

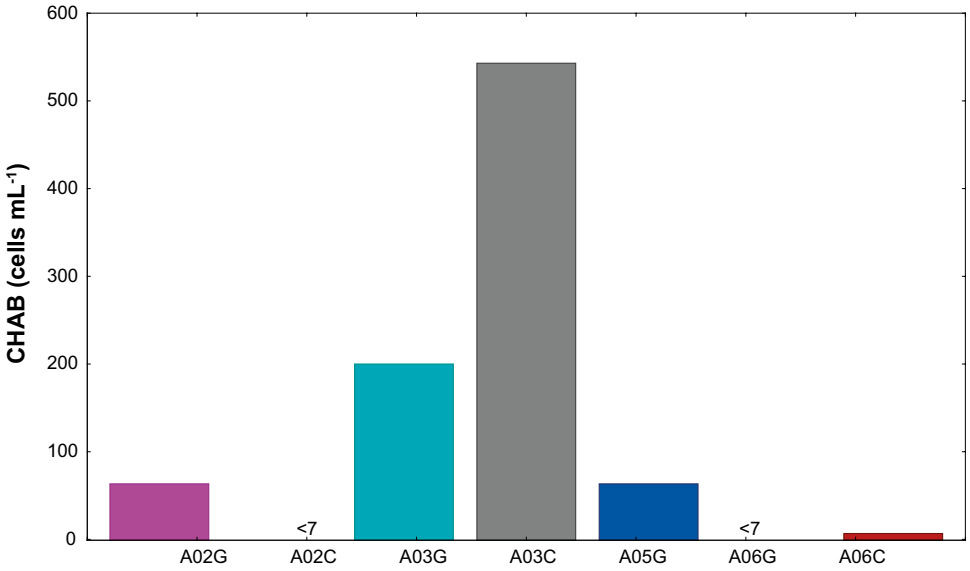

Figure 3-3. *The amount of cultivable heterotrophic aerobic bacteria, CHAB, in the groundwater (G) and water inside support cages (C) of the MiniCan packages in 2015.*

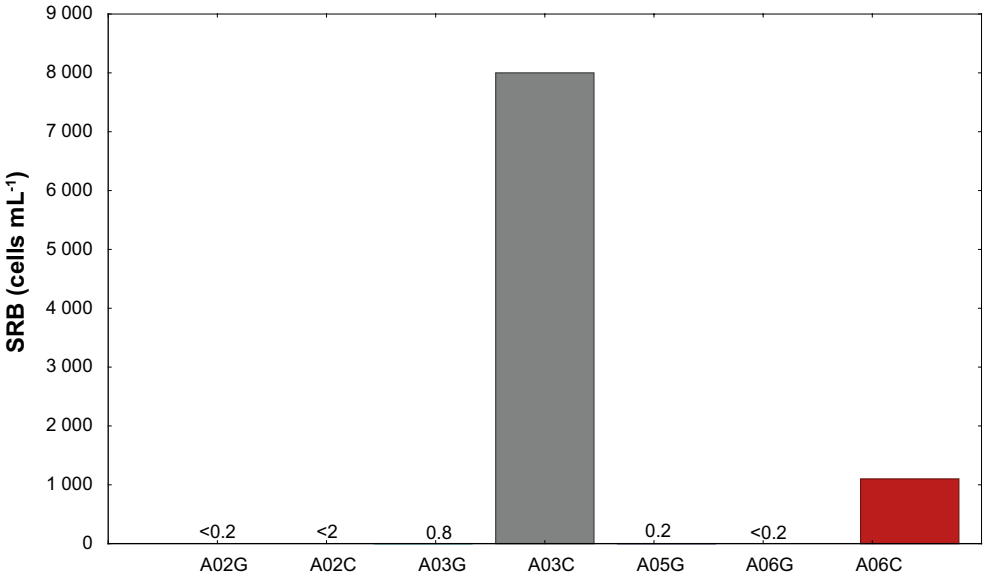

Figure 3-4. *The most probable number (MPN) of sulphate-reducing bacteria, SRB, in the groundwater (G) and water inside support cages (C) of the MiniCan packages in 2015. The number of SRB observed in the A03G and A05G were 0.8 and 0.2 cells mL^{-1}, respectively.*

3.2 Gas composition in groundwater and water inside support cages in MiniCan

The gas compositions from groundwater and water from inside the support cages of the MiniCan packages in 2015 are shown in Table 3-2. The uncertainty of the gas analyses is below 10 %.

3.2.1 Total gas volume

The total volumes of dissolved gas in the groundwater and water from inside support cages in MiniCan in 2015 are found in Figure 3-5. The total volume of gas ranged from 65 to 97 mL L^{-1}. For all packages, higher dissolved gas volumes were extracted from the water inside the support cages compared to the groundwater samples. The highest and lowest gas volumes were respectively found in the A06 and A03, for both groundwater and samples from inside the support cages.

3.2.2 H_2

Figure 3-6 shows the H_2 data. The highest H_2 concentration, 155 µL L^{-1}, was observed in the water sample from inside the support cage of A03 (A03C). A02 was the only package with detectable amounts of H_2 in both the groundwater sample and water sample from inside the support cage. Measurable H_2 concentrations were found in samples from inside the support cages in all packages.

3.2.3 CH_4

The CH_4 concentration in groundwater and water inside support cages in the MiniCan package are found in Figure 3-7. The amount of CH_4 ranged from 103 to 254 µL L^{-1}. The water samples from inside the support cages had higher amounts of CH_4 than the groundwater samples. The A02 and A05 groundwater samples had the highest and lowest CH_4 concentration, respectively. Similarly, in the water samples from inside the support cages the highest amount of methane was observed in the A02 package and the lowest was found in A06.

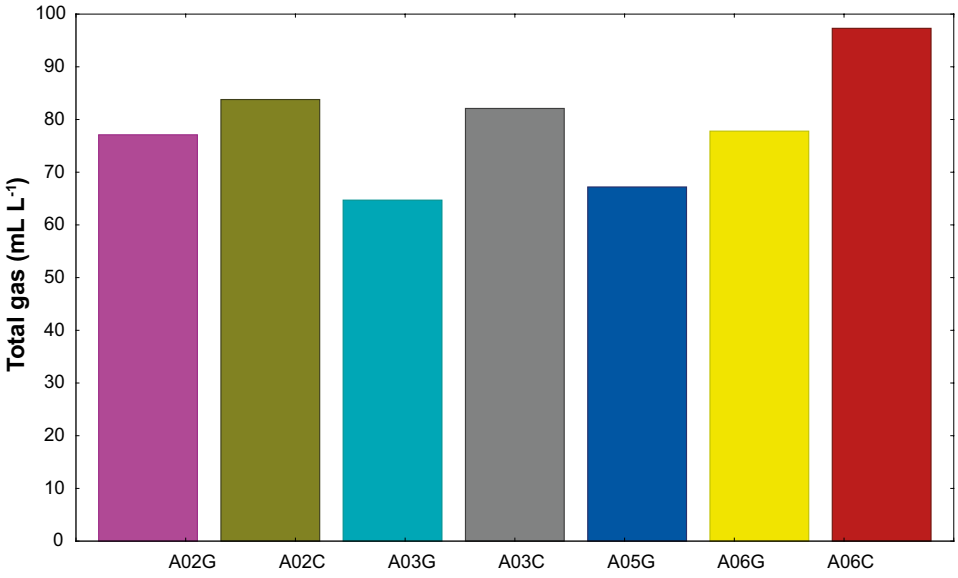

Figure 3-5. The total volumes of dissolved gas in groundwater (G) and water inside support cages (C) in MiniCan packages in 2015.

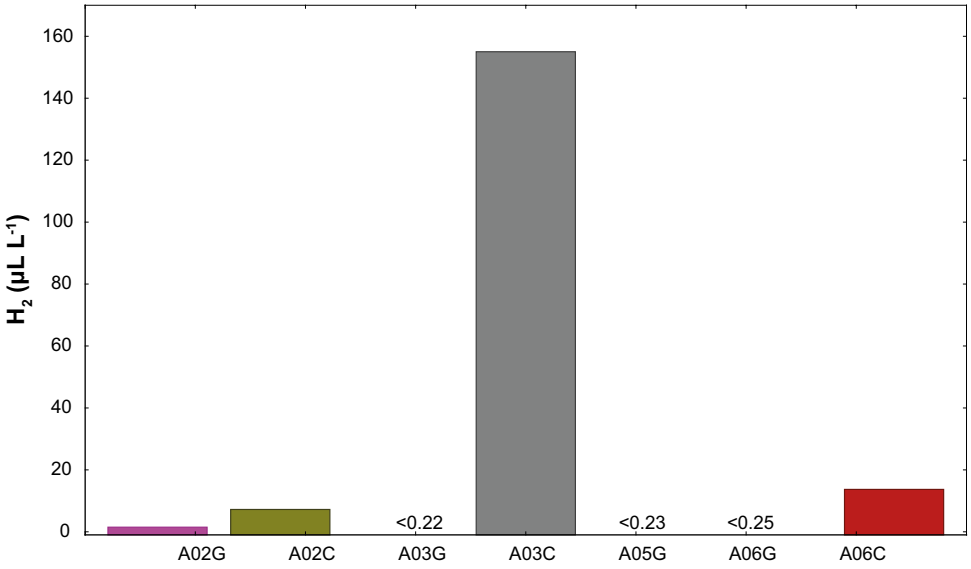

Figure 3-6. The amount of hydrogen, H_2, in the groundwater (G) and water inside support cages (C) in MiniCan packages in 2015.

3.2.4 CO_2

The CO_2 data are presented in Figure 3-8. Overall, the highest and lowest amounts of CO_2 were observed in A02. Again, higher amounts of CO_2 were detected in the water from the support cages compared to the groundwater samples. The highest amount of CO_2 in groundwater was found in A06.

3.2.5 The gas composition

The gas data are compiled in Table 3-2. The most abundant gas in the MiniCan experiement was nitrogen. Nitrogen was detected in all packages, from 57 to 82 mL L^{-1}. The argon concentration was ranged from 626 to 837 µL L^{-1}. Helium was the second most abundant gas in the MiniCan, ranged from 6.9 to 14 mL L^{-1}. The gases carbon monoxide, ethane, ethene, ethyne, propane, propene and propyne were all under detection for the methods.

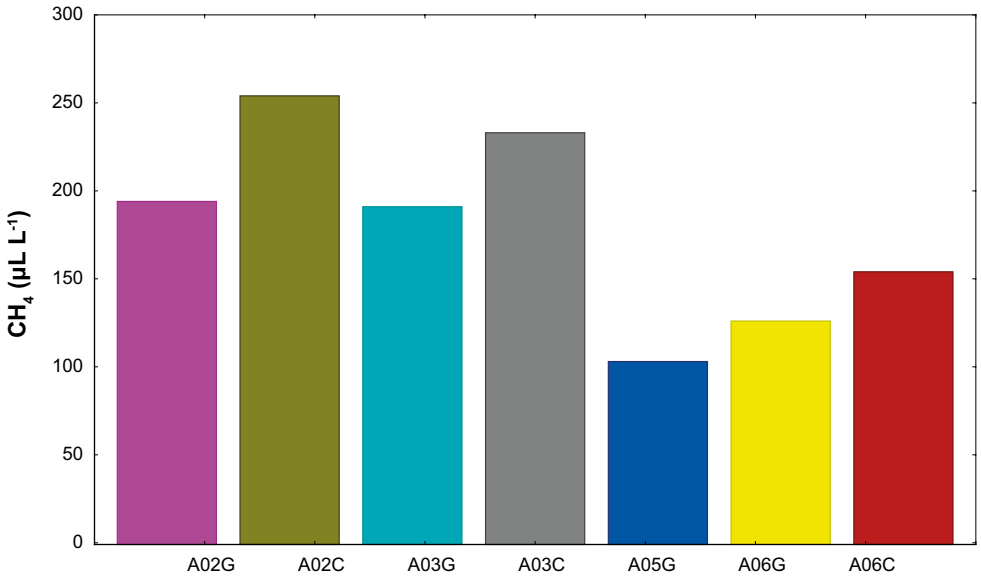

Figure 3-7. *The amount of methane, CH_4, in the groundwater (G) and water inside support cages (C) in MiniCan package in 2015.*

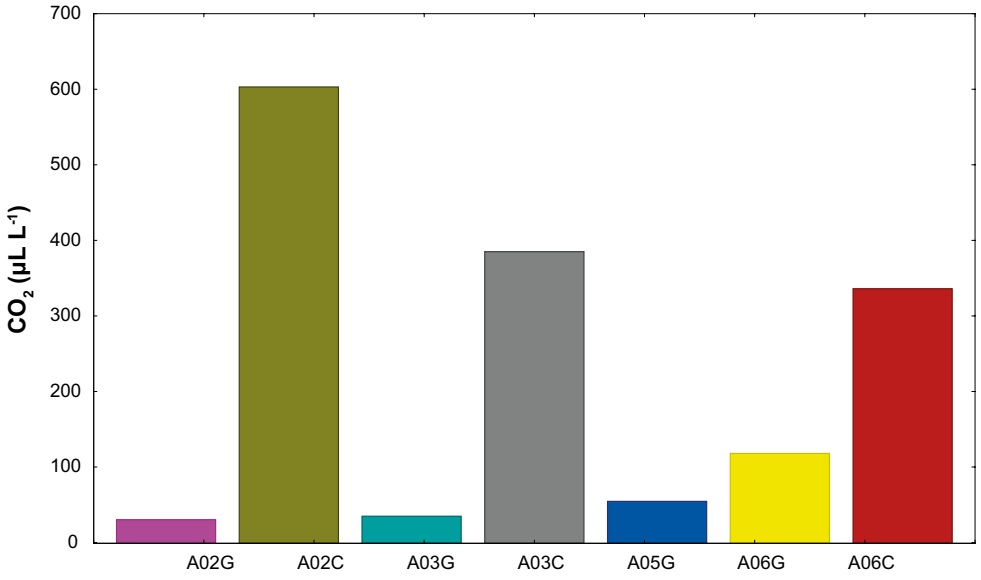

Figure 3-8. *The amount of carbon dioxide, CO_2, in groundwater (G) and water inside support cages (C) in MiniCan packages in 2015.*

Table 3-1. Microbial composition in groundwater (G) and water inside support cages (C) in the MiniCan experiemtn in 2015.

Sample name	ATP (amol mL^{-1})	±SD ATP (amol mL^{-1})	TNC (cells mL^{-1})	±SD TNC (cells mL^{-1})	CHAB (cells mL^{-1})	±SD CHAB (cells mL^{-1})	MPN SRB (cells mL^{-1})	95 % confidence interval SRB (cells mL^{-1})	MPN AA (cells mL^{-1})	95 % confidence interval SRB (cells mL^{-1})	Acetate (mg L^{-1})
A02G	2200	420	35000	28000	60	10	<0.2	–	0.2	0.1–1	2
A02C	1200	330	n.d.*	n.d.	<7	–	<2	–	<2	–	3
A03G	6900	810	67000	23000	200	30	0.8	0.3–2.4	<0.2	–	2
A03C	3200	120	3200	1300	540	60	8000	3000–25000	<2	–	18
A05G	3100	100	19000	820	60	6	0.2	0.1–1.1	<0.2	–	13
A06G	1500	530	n.d.	n.d.	<7	–	<0.2	–	<0.2	–	13
A06C	2200	930	n.d.	n.d.	7	10	1100	400–3000	<2	–	1

* n.d: no data because of interfering materials.

Table 3-2. Gas composition, corrected for air contamination, in groundwater(G) and water inside support cages (C) in the MiniCan experiment in 2015.

Sample name	Gas/water (mL L^{-1})	H_2 (µL L^{-1})	CH_4 (µL L^{-1})	CO_2 (µL L^{-1})	CO (µL L^{-1})	Ar (µL L^{-1})	He (µL L^{-1})	N_2 (µL L^{-1})	C_2H_6 (µL L^{-1})	C_2H_4 (µL L^{-1})	C_2H_2 (µL L^{-1})	C_3H_8 (µL L^{-1})	C_3H_6 (µL L^{-1})	C_3H_4 (µL L^{-1})
A02G	77.1	1.49	194	30.3	<0.84	670	9670	66500	<0.08	<0.17	<0.17	<0.08	<0.17	<0.34
A02C	83.8	7.22	254	603	<1.94	653	9710	72500	<0.19	<0.38	<0.38	<0.19	<0.38	<0.72
A03G	64.7	<0.22	191	34.9	<0.75	641	6920	56900	<0.07	<0.14	<0.14	<0.07	<0.14	<0.28
A03C	82.1	155	233	385	<0.88	733	11100	69500	<0.09	<0.18	<0.18	<0.09	<0.18	<0.36
A05G	67.2	<0.23	103	54.5	<0.77	626	8570	57800	<0.08	<0.16	<0.16	<0.08	<0.16	<0.32
A06G	77.8	<0.25	126	118	<0.85	746	9920	66900	<0.08	<0.16	<0.16	<0.08	<0.16	<0.32
A06C	97.3	13.7	154	336	<1.05	837	13700	82200	<0.10	<0.20	<0.20	<0.10	<0.20	<0.40

3.3 Chemical composition of groundwater and water in support cages in MiniCan

A selection of water chemistry data is shown in Table 3-3. Overall, high concentrations of ferrous iron, sulphide and chloride were found in the water from the support cages, compared to the groundwater samples; a somewhat higher conductivity was as well observed in the cage samples. The groundwater samples had higher pH and carbonate alkalinity than the cage samples. In addition, the sulphate concentrations were higher in the groundwater samples compared to the cage samples, except for A06 in which the sample from inside the support cage had higher sulphate than the corresponding groundwater sample. It should be noted that the volume inside the support cage of A06 was around 26 litres compared the volume inside the support cages of A02 and A03 which have a volume of around 2.6 litres.

Table 3-3. Chemical composition in groundwater(G) and water inside support cages (C) of the MiniCan experiment in 2015.

Sample name	SO_4^{2-} (mg L^{-1})	Fe^{2+} (mg L^{-1})	pH (field)	HCO_3 (mg L^{-1})	S^{2-} (mg L^{-1})	Conductivity (mS m^{-1})	Cl^- (mg L^{-1})
A02G	524	0.1	7.8	11	< 0.019	2394	9014
A02C	142	85	7.3	6.4	0.02	2454	9174
A03G	515	0.1	7.7	10	< 0.019	2433	8998
A03C	407	77	7.6	5.9	0.025	2450	9001
A05G	496	0.2	7.6	19	< 0.019	2520	9225
A06G	527	0.1	7.7	13	< 0.019	2719	10140
A06C	620	24	7.5	2.6	0.031	3203	12190

Measurement of the sulphur isotope S-34 in sulphate was done in water from inside the support cages of A02 and A03 and the corresponding groundwater for the packages. The data show that the SO_4^{2-} was heavier in the cage water, $\delta^{34}S$ of 16.88 and 17.57, respectively, compared to values in the groundwater with $\delta^{34}S$ of 14.95 and 15.78, for the respective package. The data support that sulphate reduction has been ongoing in the support cages since microbial sulphate-reducing activity enrich for SO_4^{2-} with the heavier sulphur isotope.

3.4 Microbiology of biofilm and bentonite from the retrieval of package A05 in MiniCan

Table 3-4 and Table 3-5 show the microbiology data of biofilm and bentonite samples from the retrieved package A05 in MiniCan, 2015. Each microbiological parameter will be presented in the following sections. Biofilms were sampled from two surface positions on the iron sandwich specimen, (A05SFe_1) and (A05SFe_2), and outside the support cage, (A05OC_1) and (A05OC_2). Bentonite samples were collected at two positions inside the support cage, (A05IC_top) and (A05IC_middle), in connection with the iron of the sandwich specimen, (A05SFe_3) and (A05SFe_4), and at the copper of the sandwich specimen, (A05SCu_1) and (A05SCu_2). Bentonite was also collected from three positions in contact with the copper canister, (A05Cu_top), (A05Cu_middle) and (A05Cu_bottom), and of the surrounding bentonite, (A05B_middle) and (A05B_bottom), see information in Table 2-2.

3.4.1 TNC

The two samples from outside the support cage, (A05OC_1) and (A05OC_2) were the only samples from the A05 retrieval had number of cells above the detection limit of the method, see Table 3-4. Because of interfering materials and possibly even absence of cells, no cells could be detected in other biofilm or bentonite samples from the A05 retrieval. The samples had to be diluted and thus the number of cells became below detection limit for the method.

3.4.2 CHAB

CHAB were detected in one of the samples from the surrounding bentonite, (A05B_bottom), and in one of the bentonite samples from inside the support cage, (A05IC_middle), but because the variation in data, the differences between them may not be significant (Table 3-4). Of the surface samples, low amounts of CHAB were found in the samples from outside the support cage of the A05 package, (A05OC_1) and (A05OC_2). The difference between the samples is not significant due to the large variation between the duplicate samples (Table 3-4). In other biofilm and bentonite samples, the number of CHAB were below detection limit for the method.

3.4.3 MPN SRB

There were viable SRB in the two surface samples from the iron surface of the sandwich specimen and in 6 of the 12 bentonite samples taken from the retrieved A05 package (see Tables 3-4 and 3-5). On the iron surface, there were around 20 SRB cm^{-2} and in the bentonite samples there were from 27 to 107 SRB g^{-1} wet weight. The difference between the bentonite samples is not significant within the 95 % confidence interval. Viable SRB were found in bentonite from the inside surface of the support cage, in one of the samples from the canister surface, on both the iron and copper surfaces of the sandwich specimen and in bentonite in the middle of the compacted bentonite barrier.

3.4.4 MPN AA

No AA were detected on the surfaces or in the bentonite samples from the retrieval of package A05 (see Tables 3-4 and 3-5).

Table 3-4. Microbial composition on surfaces from retrieval of package A05 in MiniCan. Surfaces i.e. Outside support cage, OC; Sandwich specimen Iron, SFe.

Sample name	TNC (cells cm^{-2})	±SD (cells cm^{-2})	CHAB (cells cm^{-2})	±SD (cells cm^{-2})	MPN SRB (cells cm^{-2})	95 % confidence interval (cells cm^{-2})	MPN AA (cells cm^{-2})	95 % confidence interval (cells cm^{-2})
A05OC_1	1330	280	210	80	0.056	0.028–0.3	<2	–
A05OC_2	560	60	230	10	<2	–	<2	–
A05SFe_1	n.d.*	–**	<70	–	28	8–84	<2	–
A05SFe_2	n.d.	–	<70	–	16	4–68	<2	–

* n.d: no data because of interfering materials.
** not applicable.

Table 3-5.Microbial composition in bentonite samples from retrieval of package A05 in MiniCan. Bentonite samples i.e. Inside support cage, IC; Copper canister, Cu; Sandwich specimen Iron, SFe; Sandwich specimen Copper, SCu; Bentonite, B

Sample name	TNC (cells g^{-1})	±SD (cells g^{-1})	CHAB (cells g^{-1})	±SD (cells g^{-1})	MPN SRB (cells g^{-1})	95 % confidence interval (cells g^{-1})	MPN AA (cells g^{-1})	95 % confidence interval (cells g^{-1})
A05IC_middle	n.d.*	–**	890	770	<2	–	<2	–
A05IC_top	n.d.	–	<70	–	27	13–147	<2	–
A05Cu_top	n.d.	–	<70	–	<2	–	<2	–
A05Cu_middle	n.d.	–	<70	–	<2	–	<2	–
A05Cu_bottom	n.d.	–	<70	–	27	13–147	<2	–
A05SFe_3	n.d.	–	<70	–	107	40–320	<2	–
A05SFe_4	n.d.	–	<70	–	<2	–	<2	–
A05SCu_1	n.d.	–	<70	–	53	13–200	<2	–
A05SCu_2	n.d.	–	<70	–	27	13–133	<2	–
A05B_top	n.d.	–	<70	–	<2	–	<2	–
A05B_middle	n.d.	–	<70	–	27	13–147	<2	–
A05B_bottom	n.d.	–	1330	0	<2	–	<2	–

* n.d: no data because of interfering materials.
** not applicable.

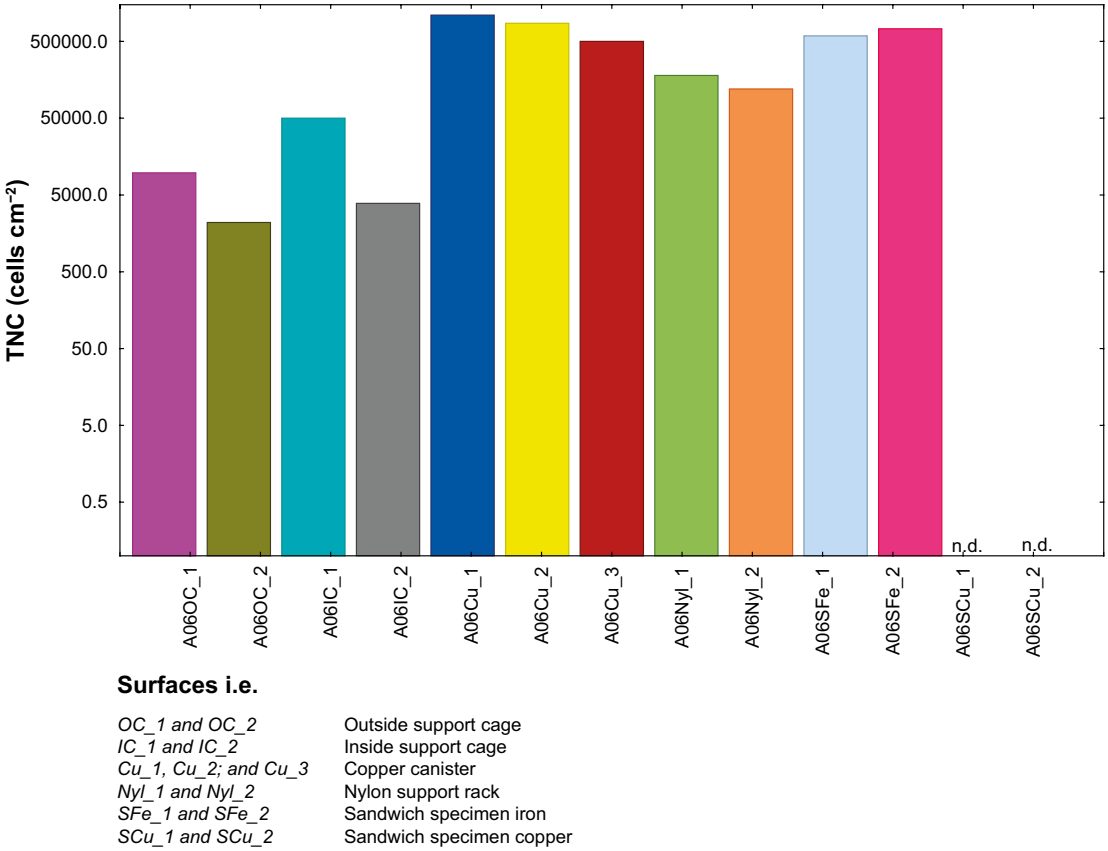

Figure 3-9. *The total number of cells, TNC, of surfaces sampled during the retrieval of package A06 in MiniCan in 2015. nd = no data. Note the logarithmic scale on the Y-axis.*

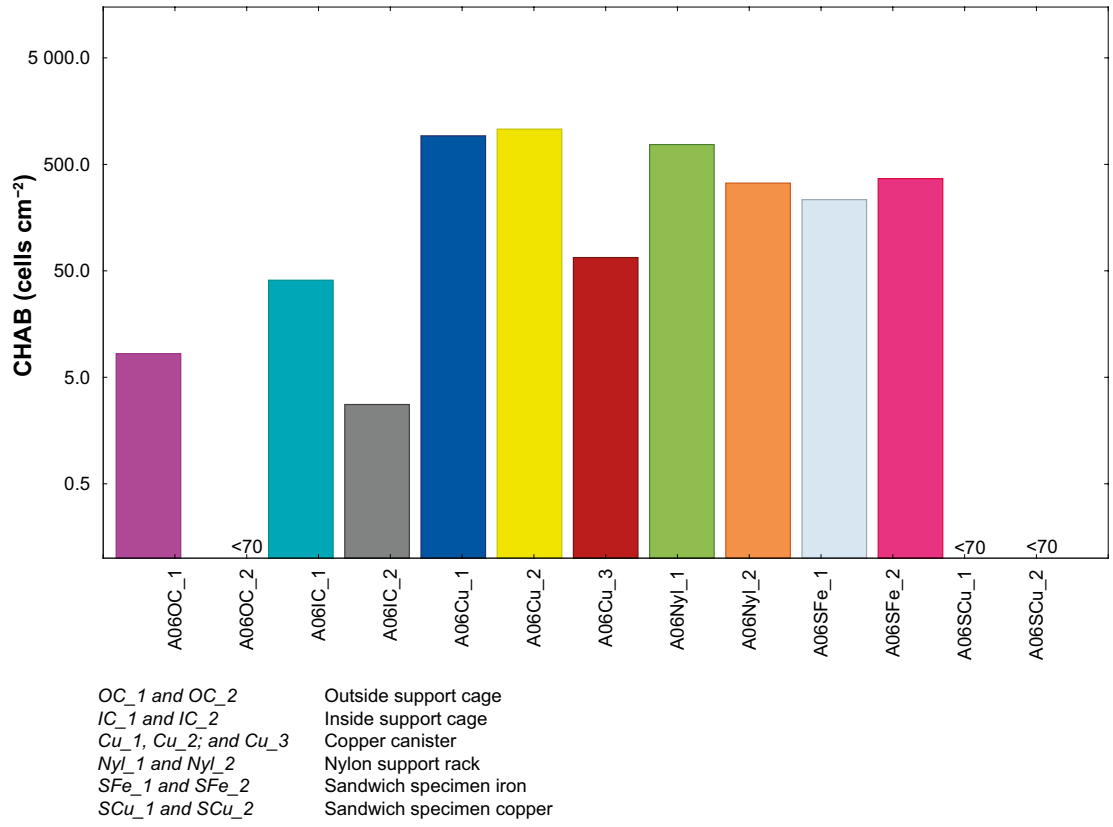

Figure 3-10. *The amount of cultivable heterotrophic aerobic bacteria, CHAB, of surfaces sampled during the retrieval of package A06 in MiniCan in 2015. Note the logarithmic scale on the Y-axis.*

3.5 Microbiology of biofilm from the retrieval of package A06 in MiniCan

The microbial composition data of biofilms from the retrieved package A06 in MiniCan in 2015 are shown in Table 3-6. Each microbiological parameter is presented in the following sections. Biofilms were sampled from two surface positions each from the outside of the support cage, (A06OC_1) and (A06OC_2), the inside of the support cage, (A06IC_1) and (A06IC_2), the nylon support rack, (A06Nyl_1) and (A06Nyl_2), iron sandwich specimen, (A06SFe_1) and (A06SFe_2) and copper sandwich specimen, (A06SCu_1) and (A06SCu_2). Surface samples were also collected from three positions on the copper canister, (A06Cu_1), (A06Cu_2) and (A06Cu_3) see information in Table 2-1.

3.5.1 TNC

TNC data from surfaces sampled from A06 are found in Figure 3-9 and in Table 3-6. During the sampling of the canister, the surface dried out and became white, which may be the explanation for the variation in TNC numbers between the first and third samples. The highest number of TNC was observed in the second sample position of the copper canister, but because of the large variation in data the differences are not significant. The significantly lowest TNC number was found in the second sample from outside the support cage, (A06OC_2), with 2 190 cells cm^{-2}. Because of interfering materials i.e. minerals and iron precipitates, no cells could be detected in the copper sandwich specimen samples; the samples were diluted and by that, the number of cells became to be below the detection limit for the method.

3.5.2 CHAB

Figure 3-10 shows the CHAB data from biofilms sampled A06. The highest amount of CHAB was found in the second sampled position from the copper canister, but in respect to standard deviation overlaps, the differences between the samples may not be significant (Table 3-6). The significant lowest amount of CHAB was found in the second sample from inside of the support cage (A06IC_2), with 3 cells cm^{-2}. Higher CHAB concentrations were found in the first sampled positions, except for the iron sandwich specimen where the second sample had higher amounts of CHAB than the first. This was also observed between the first and second sampled positions from the copper canister. The second sampled position from outside the support cage and samples from the copper sandwich specimen had CHAB concentrations below detection limit for the method.

3.5.3 MPN AA

Figure 3-11 shows the AA data from surfaces sampled from the retrieved package A06. High MPN of AA were found on the iron of the sandwich specimen samples (A06SFe_1 and A06SFe_2), but because of the variation in data, the differences may not be significant (Table 3-6). Low amounts of AA were found in samples from inside the support cage, in two of the copper canister samples, and in one of the samples from the nylon support rack and copper sandwich specimen. The number of AA from outside the support cage and in one of the samples from the copper canister, nylon support rack and copper sandwich specimen were all under detection for the method.

3.5.4 MPN SRB

The highest number of SRB in A06 (Figure 3-12) was found on one of the surface samples of the iron sandwich specimen (A06SFe_1), with 500 000 cells cm^{-2}. The significant lowest number of SRB was observed in the second sample from inside of the support cage (A06IC_2), with 0.1 cells cm^{-2}. No SRB were detected on the outside of the support cage or in one of the samples from the nylon support rack (Table 3-6), they were under the detection limit for the method.

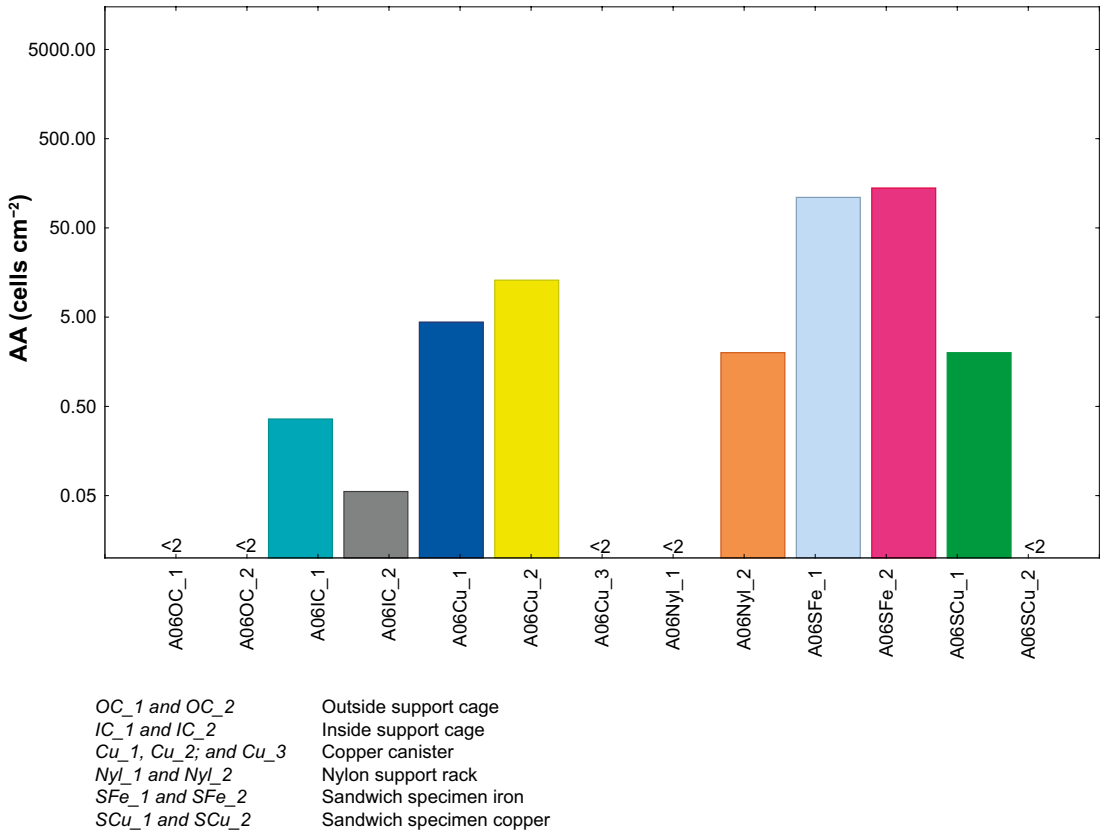

Figure 3-11. *The most probable number (MPN) of autotrophic acetogens, AA, of surfaces sampled during the retrieval of package A06 in MiniCan in 2015. Note the logarithmic scale on the Y-axis.*

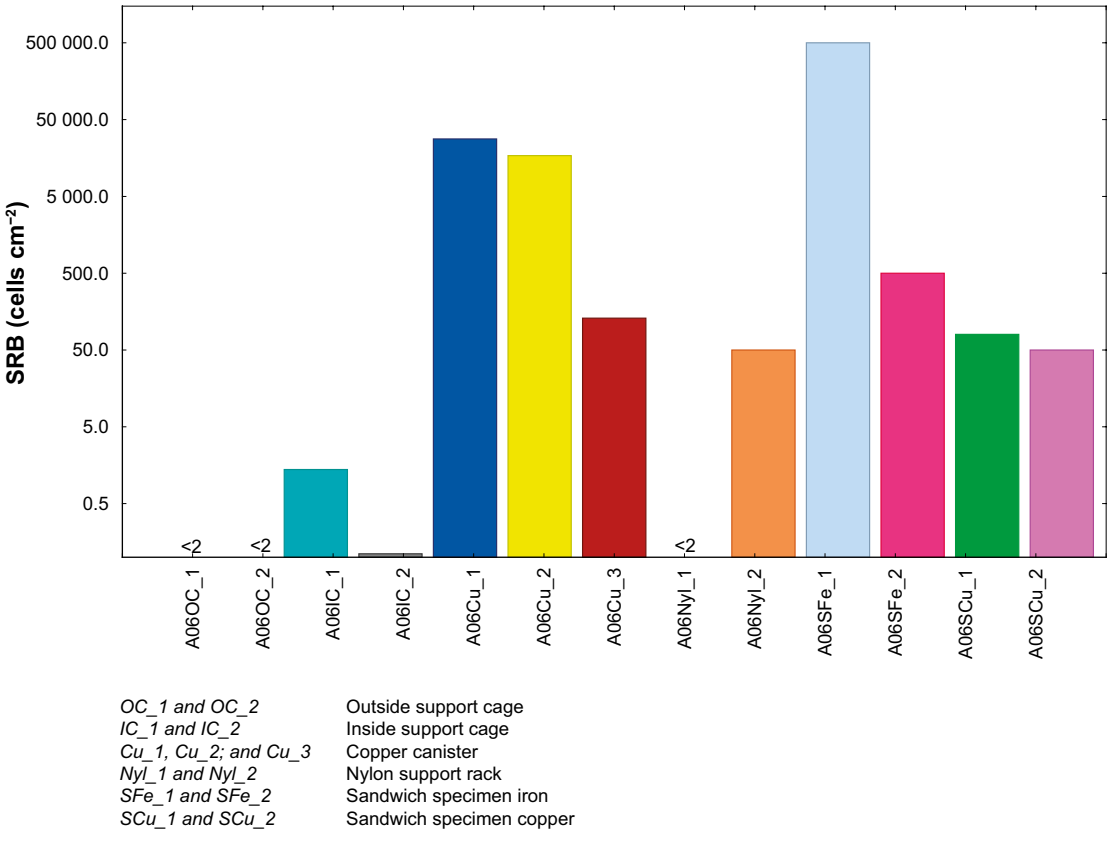

Figure 3-12. *The most probable number (MPN) of sulphate-reducing bacteria, SRB, of surfaces sampled during the retrieval of package A06 in MiniCan in 2015. Note the logarithmic scale on the Y-axis.*

Table 3-6.Microbial composition of surfaces from retrieval of package A06 in MiniCan. Surfaces i.e. Outside support cage, OC; Inside support cage, IC; Copper canister, Cu; Nylon support rack, Nyl; Sandwich specimen iron, SFe; Sandwich specimen copper, SCu.

Sample name	TNC (cells cm^{-2})	±SD TNC (cells cm^{-2})	CHAB (cells cm^{-2})	±SD CHAB (cells cm^{-2})	MPN SRB (cells cm^{-2})	95 % confidence interval SRB (cells cm^{-2})	MPN AA (cells cm^{-2})	95 % confidence interval AA (cells cm^{-2})
A06OC_1	9720	3330	8	3	< 2	–	< 2	–
A06OC_2	2190	720	< 70	–	< 2	–	< 2	–
A06IC_1	50000	11000	40	2	1.4	0.6–4	0.4	0.1–1.1
A06IC_2	3890	830	3	3	0.1	0.03–0.5	0.06	0.03–0.3
A06Cu_1	110000	18000	930	230	28000	12000–72000	4.4	1.8–11
A06Cu_2	860000	390000	1070	115	17000	7000–48000	13	5–38
A06Cu_3	500000	140000	70	60	130	50–390	< 2	–
A06Nyl_1	180000	1500	770	350	< 2	–	< 2	–
A06Nyl_2	120000	73000	330	150	50	20–170	2	1–11
A06SFe_1	590000	87000	230	150	500000	200000–2000000	110	40–300
A06SFe_2	730000	160000	370	150	500	200–1700	140	60–360
A06SCu_1	< 10000	–*	< 70	–	80	30–250	2	1–11
A06SCu_2	< 10000	–	< 70	–	50	20–170	< 2	–

* not applicable.

3.6 DNA analyses of groundwater, biofilm and bentonite from the retrieval of packages A05 and A06 in MiniCan

3.6.1 Groundwater in A05 and A06

Microorganisms in groundwater in A05 and A06 were collected by filtration for 19 hours at a flow rate of 170–180 mL per minute, giving a filtrated volume of approximately 200 L on each filter. The DNA extraction generated 7 and 18 ng μL^{-1} eluted DNA from respective groundwater sample.

A05

The closest related species, genera or family, deposited in the SILVA data base (Quast et al. 2013), to the sample sequences for the borehole in package A05 is compiled in Table 3-7.

It can be seen that around 18 % (marked with yellow) of the total reads, which in this sample were 1 316 669, had the closest match with sulphide-producing bacteria, either sulphate-reducers or sulphur-reducers and almost 67 % of the reads grouped in unclassified or unclassified Bacteria. A closer check with the group Bacteria_unclassified revealed that many matches but with similarities lower than the threshold set in the sequence analysis, were related to sequences from microbial analyses of deep groundwater in Finland (Pedersen et al. 2015).

Table 3-7. Sequence data from the groundwater in package A05 in MiniCan.

Closest match in SILVA database	No of reads	Percentage of total reads (%)
Bacteria_unclassified	598 990	46.1
unknown_unclassified	137 377	10.6
Desulfatiglans	89 984	6.9
S15A_unclassified	67 720	5.2
Atribacteria_Unclassfiied	60 557	4.7
Desulfobacteriaceae_unclassified	58 040	4.5
SEEP-SRB1	53 147	4.1
SM1H02-unclassified	37 412	2.9
Sphaerochaeta	26 463	2.0
Desulfobacula	24 007	1.8
Plantomycetes_unclassified	23 556	1.8
Eucarya_unclassified	22 098	1.7
Coxiella	21 881	1.7
Bacillaceae_unclassified	17 557	1.4
Anaerolineaceae_unclassified	17 307	1.3
Syntrophobacterales_unclassified	16 891	1.3
Oleispira	13 299	1.0
OPB4110966	10 966	0.8
Deltaproteobacteria_unclassified	10 680	0.8
Proteobacteria_unclassified	8 737	0.7

A06

In Table 3-8, the results from the comparison of the reads from the 16S rDNA sequencing of the sample from groundwater in A06 are compiled. In this sample 90 % of the reads were closest related to an unclassified *Desulfuromanadaceae*. This family consists sulphur-reducing bacteria that produce sulphide. In the table, sulphide producing families or genera is marked in yellow. The sulphide producers are either sulphate- or sulphur-reducing. The sulphide-producers comprise 94 % of all reads, which were 2 624 867 in this sample. The family *Desulfobacteraceae*, including the genuses *Desulfobacula* and *Desulfatiglans*, are examples of sulphate-reducing bacteria present in the groundwater of A06. The clone named SEEP-SRB1 is also a sulphate-reducing bacterium suggested as a partner in anaerobic methane-oxidation. *Hydrogenophaga* is a H_2-oxidising bacterium that could interact with produced H_2 from anaerobic corrosion.

Table 3-8. Sequence data from the groundwater in the package A06 in MiniCan.

Closest match in SILVA database	No of reads	Percentage of total reads (%)
Desulfuromonadaceae_unclassified	2265562	89.5
Bacteria_unclassified	59574	2.4
Desulfuromonadales_unclassified	55029	2.2
IheB3-7_unclassified	47751	1.9
Bacteriodetes_unclassifed	39642	1.6
Sunxiuqinia	31622	1.2
Desulfobacteraceae_unclassified	19575	0.8
Desulfobacula	14386	0.6
Firmicutes_unclassified	11571	0.5
SEEP-SRB1	11019	0.4
Mobilitalea	10021	0.4
Unknown_unclassified	9361	0.4
Desulfatiglans	8986	0.4
Desulfurivibrio	8430	0.3
Gammaproteobacteria	7558	0.3
Atribacteria_unclassified	6043	0.2
Hydrogenophaga	5734	0.2
Coriobacteriaceae_unclassified	4916	0.2
Anaerolineaceae	4895	0.2
Proteobacteria_unclassified	3192	0.1

3.6.2 Biofilms and bentonite in A05 and A06

Sufficient amounts of DNA for sequencing were achieved from three surfaces in A06, one from the canister surface and two from the inside surface of the support cage. None of the bentonite samples from A05 had enough DNA for the following sequence analysis.

A06

The sequence data from the A06Cu1 sample is compiled in Table 3-9. In total 88 % of the reads, 1 119 845, were affiliated with sulphide-producing bacteria. The dominant group, 58 %, was the genera *Desulomicrobium*, which is a sulphate-reducing group of microorganisms. There was also one acetogenic group, *Acetobacterium*, with 0.1 % of the reads.

In Tables 3-10 and 3-11, the sequence data of the two samples taken from the inside surface of the support cage in A06 are presented. In the samples, 59 % of the population in A06ic_1 and 87 % of the bacterial population in A06ic_2, belonged to sulphide-producing bacteria. Also here bacteria belonging to the genus *Desulfomicrobium* were large parts of the population, 22 % in A06ic_1 and 36 % in A06ic_2. Some sequences could not be resolved at genus level but clustered at family level to families belonging to sulphide-producers, either as sulphate-reducers or as sulphur-reducers.

Table 3-9 Sequence data from the canister surface in the package A06 in MiniCan.

Closest match in SILVA database	No of reads	Percentage of total reads (%)
Desulfomicrobium	612712	58.5
Deltaproteobacteria_unclassified	261438	24.9
Bacteria_unclassified	134701	12.9
Desulfobacteraceae_unclassified	29881	2.9
Sphaerocheata	21605	2.1
Desulfobacula	14656	1.4
Proteobacteria_unclassified	12727	1.2
IheB3-7_unclassified	9224	0.9
Desulfarculus	4718	0.5
Firmicutes_unclassified	3635	0.3
Anaerolineaceae_unclassified	2519	0.2
Acholeplasma	2477	0.2
Dethiosulfatibacter	2440	0.2
Desulfovibrio	1561	0.1
Coriobacteriaceae_unclassified	1528	0.1
Desulfovibrio	1270	0.1
Alteromonadaceae_unclassified	1144	0.1
Gammaproteobacteria_unclassified	825	0.1
Acetobacterium	784	0.1

Table 3-10. Sequence data from the inside surface of the support cage, A06ic_1, in the package A06 in MiniCan.

Closest match in SILVA database	No of reads	Percentage of total reads (%)
Bacteria_unclassified	444485	29.4
Deltaproteobacteria_unclassified	394116	26.1
Desulfomicrobium	337958	22.4
Desulfarculus	90577	6.0
Sphaerochaeta	33806	2.2
Desulfobacteriaceae_unclassified	32047	2.1
Proteobacteria_unclassified	26232	1.7
Staphylococcus	26018	1.7
Propionibacterium	24266	1.6
Firmicutes_unclassified	19951	1.3
Desulfobacula	19856	1.3
Desulfuromonadaceae_unclassified	11988	0.8
Acholeplasma	10578	0.7
Gammaproteobacteria_unclassified	10088	0.7
Shewanella	8615	0.6
Oleispira	7995	0.5
IheB3-7_unclassified	7458	0.5
Hydrogenophaga	7049	0.5
Streptococcus	6758	0.4
Alteromonadaceae_unclassified	6356	0.4

Table 3-11. Sequence data from the inside surface of the support cage, A06ic_2, in the package A06 in MiniCan.

Closest match in SILVA database	No of reads	Percentage of total reads (%)
Desulfomicrobium	372 803	36.1
Coriobacteriaceae	302 890	29.3
Desulfobacula	249 323	24.1
Deltaproteobacteria_unclassified	163 694	15.9
Bacteria_unclassified	80 943	7.8
Desulfuromonadaceae_unclassified	75 200	7.3
Firmicutes_unclassified	27 610	2.7
IheB3-7	22 922	2.2
Desulfobacteraceae_unclassified	16 986	1.6
Proteobacteria_unclassified	16 175	1.6
Desulfarculus	10 231	1.0
Sphaerochaeta	7 004	0.7
Staphylococcus	5 489	0.5
Propionibacterium	4 918	0.5
Demequina	4 309	0.4
Acholeplasma	4 262	0.4
Desulfuromonadales_unclassified	2 752	0.3
Bacteriodetes_unclassified	2 752	0.3
Desulfovibrio	1 572	0.2
Streptococcus	1 500	0.1

4 Discussion

The MiniCan experiment included five packages with miniature copper canisters as described in Section 1.1. The package A04 was retrieved in 2011 and the microbiological results were summarized in Hallbeck et al. (2011). The main findings in that investigation were that at some surfaces, i.e. the copper canister, almost the total microbial population consisted of SRB. The molecular characterization showed that some SRB belonged to a species, *Desulfovibrio ferrophilus* (Dinh et al. 2004) that has been shown to use electrons directly from metallic iron and stainless steel in their metabolism and sulphide production without the intermediate step of H_2-formation, the so-called EMIC, see Section 1.2. The analyse of the weight loss coupon showed that there was no metallic iron left. The A04 package had bentonite with a dry density of 1 300 kg m^{-3} in the support cage. In 2015 the packages A05, with bentonite at 1 600 kg m^{-3} dry density and package A06, without bentonite, were retrieved.

4.1 Microbial, chemical and gas composition from borehole groundwater and water from the support cages in MiniCan

Before the work to retrieve the canisters started, water samples were taken from groundwater in the boreholes and from the support cages of the remaining packages A02, A03, A05, and A06. The microbial composition, water chemistry and gas composition were analysed. The results from each of the four packages will be discussed below and compared to results from earlier investigations, performed in 2007, 2008 and 2010 (Lydmark and Hallbeck 2011).

4.1.1 Borehole groundwater

Groundwater samples were taken in the boreholes of A02, A03, A05 and A06. The chemistry data from the groundwater show that the water composition in A02, A03 and A05 were similar but the groundwater in the A06 package differed from the other three with higher amounts of SO_4^{2-} and Cl^- and also has higher conductivity. This difference was found also in the water inside A06 which had higher SO_4^{2-}, Cl^- and conductivity both in this sampling campaign and in the earlier analyses (Lydmark and Hallbeck 2011). There was no measurable sulphide in any of the groundwater samples and ferrous iron was low, 0.1–0.2 mg L^{-1}.

The gas composition of the groundwater samples from the four packages were similar. H_2 was found in the groundwater of A02. In the earlier analyses on groundwater from A06, there have been measurable amounts of H_2 in all samples from 2007, 2008 and 2010. During the five years from the last analyses there have been activities in the Äspö HRL that have influenced the groundwater and that can explain the difference in H_2 concentrations in these analyses.

The groundwater in A03 had the highest TNC and ATP and measurable amounts of SRB. SRB was also detected in groundwater from A05 but not in any other groundwater sample.

4.1.2 Package A02

The microbiological analyses from the A02 package showed values below detection for all microbiological culturing methods, CHAB, MPN SRB and MPN AA, and values below 1 000 cells in the TNC analyses and ATP value that corresponds to cell numbers around 1 000 mL^{-1}, 1 200 amol mL^{-1}. Bacterial cells have been measured to have an average of 0.4 amol ATP per cell in Scandinavian groundwater from 100 m down to 1 000 m depth depending on depth and available energy (Eydal and Pedersen 2007). In earlier analyses of the water inside the support cages in MiniCan, A02 had the highest amounts of SRB compared to the other packages. In 2010 the MPN of SRB was 24 000 mL^{-1}. The chemistry inside the support cage of A02 had also changed considerably since 2010. The total amount of iron was 85 mg L^{-1} in 2015 compared to 56 mg L^{-1} in 2010 and 143 mg L^{-1} in 2014. The amount of SO_4^{2-} had decreased from 376 mg L^{-1} in 2010 to 142 mg L^{-1} in 2015. The trend of increasing amounts of iron and decreasing amounts of SO_4^{2-} was observed also

from the first analyses in 2007 to the ones done in 2014. It seems as the microbial population had changed in the support cage with no measurable SRB and few other bacteria as well. One explanation for this could be that the iron in electrodes and test specimens are now totally oxidised and by that no energy is available for the SRB inside the support cage. There were measurable amounts of H_2 in this support cage, see Figure 3-6, and the amounts of the other gases measured were in the same range as in the water from the other support cages.

4.1.3 Package A03

The TNC and ATP in the water from the A03 support cage were around 3 000 mL^{-1} and around 3 000 amol mL^{-1}, respectively, which gives 1 amol ATP $cell^{-1}$. These values indicate that the bacteria were active in this cage. This assumption is supported by the value for the MPN SRB of 8 000 mL^{-1}, which was the highest number of SRB measured in this sampling campaign. There were also measurable amounts of CHAB in the water, 540 mL^{-1}. The chemistry data from the A03 support cage shows ferrous iron of 77 mg mL^{-1} which is in the same range as in the A02 support cage. The amount of SO_4^{2-} was 407 mg L^{-1} which is somewhat lower than in 2010, 430 mg L^{-1}, but it seems as there has been exchange with groundwater in this package. The amount of H_2 was 155 µL L^{-1} in the support cage water and this was the highest measured value in the sampling campaign of 2015. The other measured gases were in the same range as in the support cages and in the groundwater samples in the other packages.

4.1.4 Package A05

This package had compacted bentonite with a dry density of 1 600 kg m^{-3}. It has never been possible to sample water from this support cage due to the bentonite. The design of this package was confirmed after the retrieval of the canister, see Figure 2-8 and 2-9. The entire void volume inside the support cage was filled with bentonite.

4.1.5 Package A06

There was no bentonite in the support cage of the A06 and therefore the water volume in this support cage was larger than the volumes in the A02, A03 and A04 support cages. There were CHAB and SRB present in the water in the A06 support cage. The number of CHAB was low with 7 mL^{-1} but the number of SRB was in the same range as in A03 with 1 100 mL^{-1}. It was difficult to count TNC in the sample from A06 but the ATP was 2 200 amol mL^{-1} which is in same range as in the other support cages. The amount of total iron was 24 mg L^{-1} which is less than half of the amount in A02 and A03. This could be explained by that the water volume was larger in A06 than in the other two which dilute the amount of iron. The amount of SO_4^{2-} in the water in A06 was the highest measured during this sampling occasion, 620 mg L^{-1}. The amount of SO_4^{2-} together with the amount of Cl^- in A06 has been higher than in the water in A02, A03, A04 from start of MiniCan. This indicate that the A06 package gets its water from another source than the other three packages. There were measurable amounts of H_2 in the water in A06.

4.1.6 SRB, H_2, SO_4^{2-} and Fe^{2+} in MiniCan

Data for SRB, H_2, SO_4^{2-} and Fe^{2+} from all analyses of canister water from 2007 to 2015 were compiled and the results are displayed in Figures 4-1 to 4-4. In 2014, only water for chemical analyses was sampled by the Äspö HRL laboratory personnel and there are no SRB or H_2 data available for this date.

SRB

Figures 4-1 to 4-4 show that the A02 package has changed in the microbial composition compared the other packages and in 2015, the number of SRB in the inside water was below detection. In 2011 on the other hand A02 had the highest number of SRB found in the MiniCan package, 24 000 mL^{-1}. The reason for this change might be that the iron specimens in this package are now totally corroded and no energy from the iron oxidation is available or that no or lesser amount of groundwater has entered after the last sampling of groundwater in 2014. The package A03 showed the highest number of SRB in 2015, about the same number as in 2010. The number of SRB in A06 was lower than in 2010.

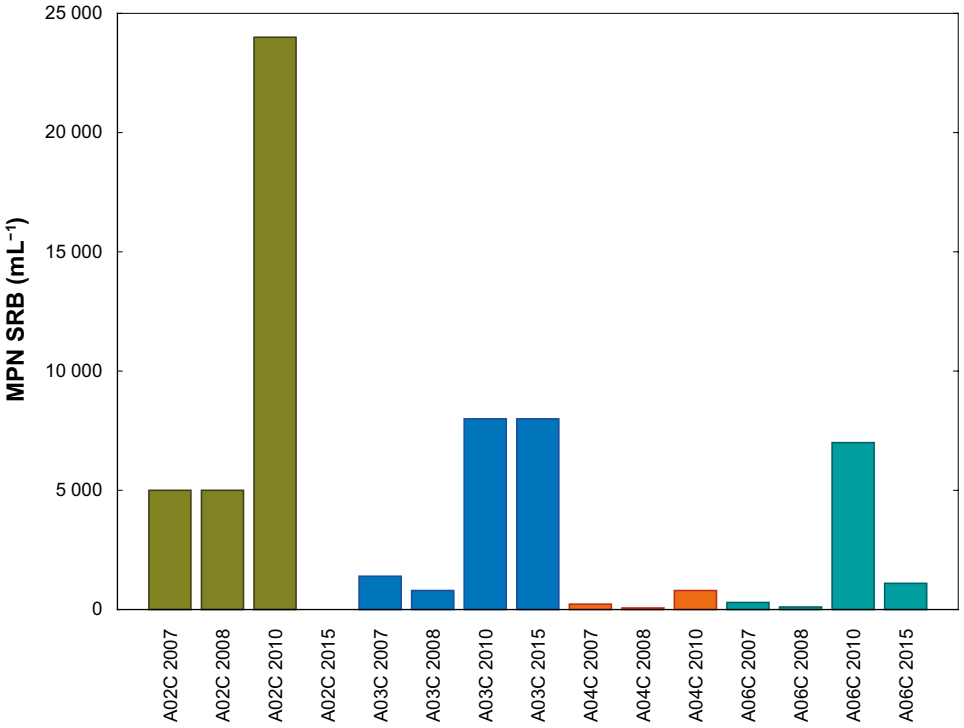

Figure 4-1. *The most probable number of sulphate-reducing bacteria in water from the inside the support cages in MiniCan from 2007 to 2015. The packaget A04 was retrieved in 2011.*

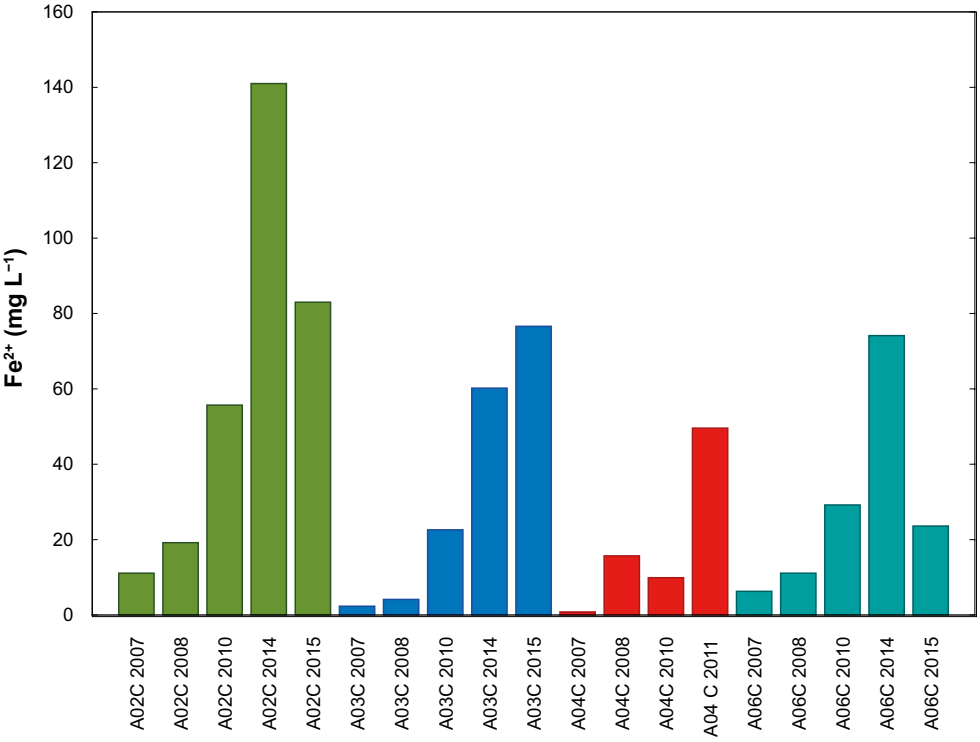

Figure 4-2. *The amount of ferrous iron in water from the inside of the support cages in MiniCan from 2007 to 2015. The package A04 was retrieved in 2011.*

Fe^{2+}

The amount of ferrous iron in the water inside the support cage in A03 has increased continuously since the experiment started in 2007. In the cage water in A02 and A06 the amount of ferrous iron was lower 2015 than in 2014. From Figure 4-2 it can be seen that the highest amount of ferrous iron was measured in A02 in 2014 with 143 mg L^{-1}. The time between the analyses in 2014 and 2015 was one year only and can be the reason for the lower amounts of ferrous iron measured in 2015 in A02 and A06 compared to the four years between analyses in 2010 and 2014. After sampling, groundwater replaces the sampled volume and dilutes the chemical components inside the support cage and after one year the corrosion process has produced less Fe^{2+} than after four.

H_2

The amount of H_2 in the water inside the four support cages are shown in Figure 4-3. The amount of H_2 vary between the packages and in time and there is no special trend. The highest amount found 2015 was in A03.

SO_4^{2-}

The amount of SO_4^{2-} was lower in the A02 already in 2014, compared the others, see Figure 4-4. The explanation for this is not obvious. The water inside the support cage was replaced by groundwater after sampling and since the amount of SO_4^{2-} in the A02 groundwater was the same as in ground-water in boreholes in A03 and A05, the amount of SO_4^{2-} in A02 would be the same as in these two packages. The SRB activity must therefore have been high in A02 from 2010 and the years after lowering the amount of SO_4^{2-}. The amount of SO_4^{2-} in A06 have been around 100 mg L^{-1} higher than in the three other packages.

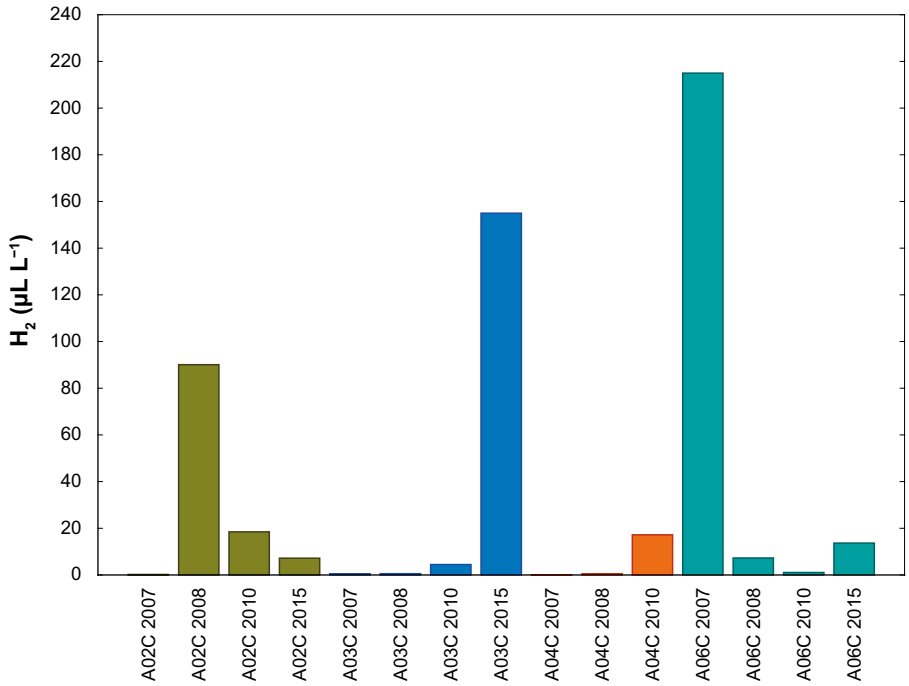

Figure 4-3. *The amount H_2 in water from the inside of the support cages in MiniCan from 2007 to 2015. The package A04 was retrieved in 2011.*

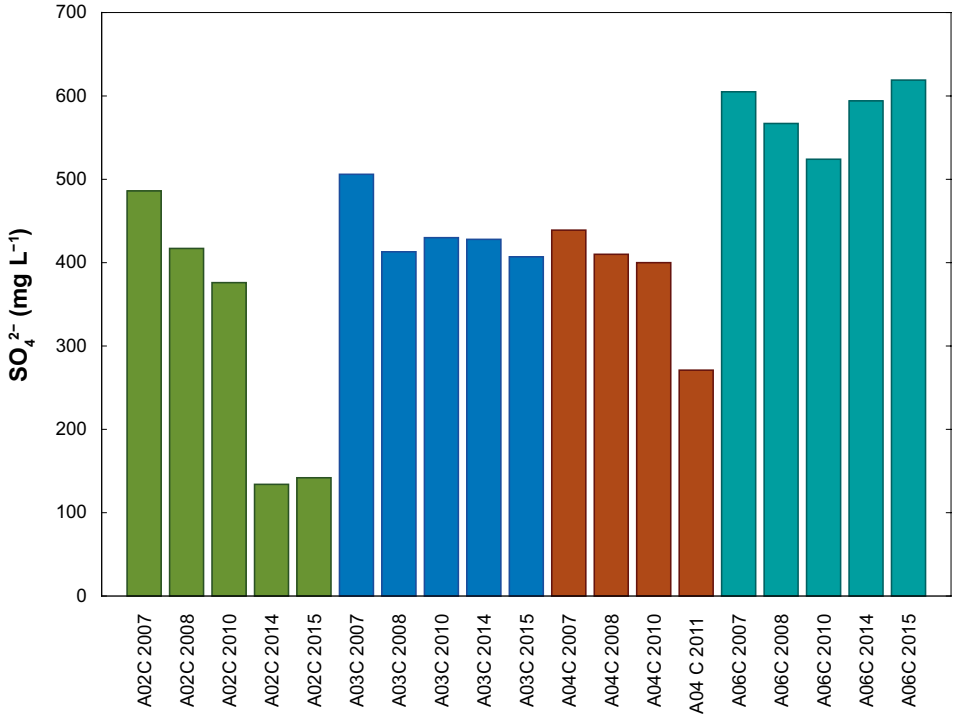

Figure 4-4. *The amount of SO_4^{2-} in water from the inside of the support cages in MiniCan from 2007 to 2015. The packageA04 was retrieved in 2011.*

4.2 Microbial composition of biofilm and bentonite from the retrieval of packages A05 and A06 in MiniCan

4.2.1 A05

The support cage in the A05, retrieved in October 2015, was completely filled with compacted bentonite. It was therefore decided that also the bentonite in close contact with the surfaces was to be sampled. There were viable SRB found in three of four swab samples and in 6 of the 12 bentonite samples, see Table 3-4. The places with viable SRB were spread among the different sample sites of A05; one of the samples from the inside of the support cage, bentonite in contact with the bottom of the canister, bentonite in contact with the iron of the sandwich specimen, both of the samples of bentonite in contact with the copper of the sandwich specimen and a bentonite sample from the middle of the bentonite surrounding the canister. Viable CHAB were found in the bentonite in contact with the inside of the support cage and in bentonite from the bottom of the package. No MPN cultures of AA showed growth. The wet density of the bentonite from A05 was measured in 10 samples and the mean wet density was 1 930 (SD 0.02) kg m^{-3}. The lowest measured value was 1 902 and the highest 1 959 kg m^{-3}. In bentonite from the A04 Package there was 2×10^6 CHAB per gram of wet bentonite with dry density of 1 300 kg m^{-3} (Hallbeck et al. 2011). The wet density was not measured in bentonite from A04.

Data from earlier studies have shown similar results of survival of SRB in compacted bentonite. Laboratory studies have shown numbers around 200 SRB g^{-1} wet weight bentonite with a dry density of 1 600 kg m^{-3} (Bengtsson et al. 2016). In that study the bentonite was inoculated with a pure culture of SRB. On the other hand, one bentonite sample from the retrieved A04 package had 9×10^4 SRB g^{-1} of wet 1 300 kg m^{-3} bentonite which is about 10 000 times more than in the samples from A05 bentonite (Hallbeck et al. 2011) and correspond with the values from the laboratory study where MPN for SRB gave 10 to 100 times higher number of surviving SRB in bentonite with a dry weight density of 1 300 kg m^{-3} than in the samples with 1 600 kg m^{-3} (Bengtsson et al. 2016). The DNA content in samples taken from A05 was too low for DNA sequence analyses.

4.2.2 A06

The support cage of the A06 was retrieved in September 2015 and had no bentonite inside and therefore all surface samples were taken with swabs. The amount of SRB on the surfaces in A06 was unevenly distributed. The highest numbers of SRB were found in one sample from the copper canister surface with 2.8×10^4 cm^{-2} and 5×10^5 cm^{-2} in one sample from the iron surface of the sandwich specimen. In these two areas the SRB were one tenth of the total population on the canister and almost the total population on the iron surface. On the other hand, samples from other areas of the same specimen had lower numbers of SRB. There are two possible reasons for this. One is that there is an uneven distribution of sulphide precipitates and by that also uneven distribution of SRB. The other reason could be that the surfaces dried out very quickly and that would also affect the recovery from the surfaces giving lower numbers of SRB in the MPN analyses.

Growth of AA was found on several surfaces, see Figure 3-11 and Table 3-6, in opposite to package A05 where no growth of AA was observed. Growth of AA was especially observed at the iron surface of the sandwich specimen. The reason for this could be that the AA used H_2 from the anaerobic corrosion of the iron. Growth of CHAB was observed on most of the surfaces in A06. The lowest values of CHAB were found on the stainless steel of the support cage and the copper surface of the sandwich specimen. These findings correspond to results from a study on sulphide production in borehole sections in Äspö HRL (Drake et al. 2014) where borehole equipment was investigated and the number of microorganisms and SRB were analysed on the different metal parts of the packer system in two boreholes in Äspö HRL. It was then found that the bacteria were attached to the aluminium parts of the packer system and not to the stainless steel parts.

The results from A06 agree with results for MPN SRB from the retrieval of package A04 in 2011, which had similar values of SRB on the canister surface, from 2×10^3 to 2×10^4 cm^{-2} compared to one of the samples from the A06 canister with 3×10^4 cm^{-2}. No samples were taken from the sandwich specimen in 2011 but one of the samples from A06 had even higher number with 5×10^5 cm^{-2}. The high number of SRB on the iron surface of the sandwich specimen explains the appearance of this piece with thick layer of iron sulphide and corrosion damage underneath, see Figures 2-3 and 2-4 in Gordon et al. (2017). The presence of bentonite in A04 did not affect the possibility for SRB to colonize the surface of the copper canister.

The DNA analyses from samples taken after the retrieval of A06 showed that 88 % of all reads clustered with sulphide producing bacteria and 55 % of the total belonged to the genus *Desulfomicrobium*, a SRB that also was present on the surface of the A04 canister as revealed by the cloning results presented in Hallbeck et al. (2011). The sequencing gives the relative abundance of the sequences which is not the case in cloning. Cloning does not give a relative abundance even if the species that dominate in a population in theory should appear more often than rare species. Also on the surfaces of the support cage the populations were dominated by sulphide producing bacteria.

4.2.3 Microbially influeced corrosion in MiniCan

The results from the microbiological analyses together with the studies of the corrosion made by Gordon et al. (2017) confirm that SRB were responsible for the damage of the iron parts in A06. The results correspond with the findings from the examination of the package A04 in 2011 (Hallbeck et al. 2011, Smart et al. 2012, 2014). The increasing amount of ferrous iron in the water of A06 but also A02-A04 during the experimental period verify that one of the major corrosion processes in MiniCan has been EMIC, i.e. that SRB utilizing electrons directly from the metallic iron. For each produced FeS in this process there will be three additional Fe^{2+} released, see section 1.2 in the Introduction. This will not be the result if CMIC, the corrosion done by heterotrophic SRB utilizing organic energy and carbon source, would be dominating. In that process the FeS ratio should be one to one without an increase in ferrous iron concentration (Enning and Garrelfs 2014). The DNA data from the retrieval of package A04 support this by the affiliation of several clones to SRB known to have this ability (Hallbeck et al. 2011).

4.3 In summary

A05

- There were viable SRB and CHAB on surfaces and in bentonite in contact with the metal surfaces at several places in A05, the package with compacted bentonite with a mean wet density of 1 931 kg m^{-3}. There was measurable corrosion on the weight specimens of copper and iron and there were also copper sulphides and iron sulphides on the respective surfaces, indicating that there have been active SRB at some point during the MiniCan experiment.

A06

- There were SRB and CHAB in the same numbers on surfaces in package A06 without bentonite as in package A04, retrieved in 2011, with 1 300 kg m^{-3} dry weight bentonite.
- There has been corrosion of iron specimens in A06 as can be seen in the amount of ferrous iron in the water, the thick precipitates of FeS, and the corroded iron electrodes and iron part of the sandwich specimen.
- The high amounts of SRB on the surfaces, confirmed with quantitative culturing and DNA technique, and the thick precipitates of FeS, show that SRB were involved in the anaerobic iron corrosion.
- The ratio of 1:3 for FeS and Fe^{2+} confirm that EMIC is the dominating MIC process.
- There were viable autotrophic acetogens, AA, on the iron surface of the sandwich specimen in the A06 without bentonite possibly involved in the corrosion by the consumption of H_2 and production of acetate that serves as carbon source for certain SRB.

A02 and A03

- The number of SRB inside the support cage in A02 had since 2011 decreased to under detection and the amount of ferrous iron in the support cage water was 140 mg L^{-1} in 2014 but 80 mg L^{-1} in 2015.
- The number of SRB in water inside the support cage in A03 was at the same level as in 2011. The amount of ferrous iron had increased since the sampling in 2014, from around 60 mg L^{-1} to almost 80 mg L^{-1}. The ATP value of 1 amol $cell^{-1}$ shows that the bacteria in this package were active.

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