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Microbial analyses of groundwater and surfaces during the retrieval of experiment 3, A04, in MINICAN

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1 Introduction

1.1 The MINICAN experiment

The MINICAN project is located at the depth of 450 m in the Äspö Hard Rock Laboratory (HRL) research tunnel. The aim of the project was to study corrosion of the cast iron inserts if a hole is introduced in the outer copper-canister. The experimental part of MINICAN started in 2007 and consists of five different experiment canisters (Table 1-1), denoted experiment A02–A06. Four of the MINICAN test copper canisters are surrounded by bentonite in a support steel cage, of which the bentonite in experiment A05 is fully compacted according to the KBS-3 approach (dry density 1,600 kg m^{-3}) and experiments A02–A04 are compacted with bentonite to a lower density than will be used (dry density 1,300 kg m^{-3}). Experiment A06 has no bentonite. In all the MINICAN copper canisters, holes with a diameter of 1 mm have been drilled to allow Äspö groundwater to come in contact with the interior cast iron inserts. This is done to mimic real accidental leakage during the KBS-3 type of long-time spent nuclear fuel storage. The project has been described in 1068871– Project Plan MINICAN, in AP TD F77.3-05-001, AP TD F77.3–08-44 and in AP TD F77.3.

Table 1-1. The MINICAN experiments installed at the Äspö Hard Rock Laboratory.

Experiment name	Sampling point	Filling of the cage around the test canister	Introduced hole in the copper canister to the cast iron
A02	KA3386 A02	Highly permeable, low density bentonite (1,300 kg m^{-3})	Hole (1 mm in diameter) located at the top of the test canister
A03	KA3386 A03	Highly permeable, low density bentonite (1,300 kg m^{-3})	Hole (1 mm in diameter) located at the bottom of the test canister
A04	KA3386 A04	Highly permeable, low density bentonite (1,300 kg m^{-3}	Holes (1 mm in diameter) located both at the bottom and top of the test canister
A05	KA3386 A05	Fully compacted bentonite (1,600 kg m^{-3}) from blocks	Hole (1 mm in diameter) located at the top of the test canister
A06	KA3386 A06	Only groundwater in contact with the test canister	Two holes (1 mm in diameter) located at the top of the test canister

1.2 Microbial corrosion and hydrogen gas metabolism

As stated above, the experimental part of the MINICAN project started in 2007 and aimed at, during a five year period, examine how the corrosion of the cast iron insert develops inside the perforated copper canisters. In the real waste repository, corrosion of the cast iron will in a worst-case scenario expose the spent nuclear fuel to groundwater and release radionuclides into the surroundings. Another potential risk with corrosion is that hydrogen gas can be produced because when iron comes in contact with anaerobic water, cathodic hydrogen is formed at the iron surface (King and Miller 1971). Hydrogen gas is unwanted in the KBS-3 storage for two reasons. The first is that an increase in gas volume will build up the pressure inside the system. The second is that development of hydrogen gas is closely linked to activity of sulphate-reducing bacteria (SRB). Hydrogen gas is used as an energy source for many of the microbes in the deep granitic subsurface (Pedersen 1999), in particular the SRB.

There are both autotrophic SRB, organisms that fix carbon dioxide into organic molecules, and heterotrophic SRB, organisms that use organic molecules for synthesis of cell material. The oxidation of hydrogen by SRB acts concurrently with reduction of sulphate to hydrogen sulphide. Cathodic hydrogen is believed to be directly scavenged by SRB if they carry the enzyme hydrogenase (Cord-Ruwisch and Widdel 1986, Caffrey et al. 2007). In addition, there are indications that SRB can use metallic iron directly in their metabolism as their electron donor (Dinh et al. 2004).

The microbial sulphate reduction process in anaerobic environment:

The hydrogen oxidation:

$H_2(g) \rightarrow 2H^+ + 2e^-$ (Eq 1-1)

The sulphate reduction:

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

With the total reaction:

$4H_2(g) + SO_4^{2-} \rightarrow HS^- + 3H_2O + OH^-$ (Eq 1-3)

(Dinh et al. 2004)

The heterotrophic SRB oxidise the organic compounds such as lactate or acetate with sulphate and produce sulphide, which indirect can corrode the iron chemically and form FeS.

The **indirect** reaction net formula is given as Equation 1-4:

$2[CH_2O \text{ (organic carbon)}] + 1⅓Fe + 1⅓SO_4^{2-} + ⅔H^+ \rightarrow 2HCO_3^- + 1⅓FeS + 1⅓H_2O$ (Eq 1-4)

(Dinh et al. 2004)

1.3 The microbial and chemical analyses

Analyses of microbial presence and activity, chemistry and dissolved gas in groundwater from the cages around the copper canisters were performed in May, August and September 2007, in October 2008 and December 2010. Before the retrieval of canister A04, one last sampling was made in August 2011. Sampling and analysis of gases comprised hydrogen, carbon monoxide, argon, carbon dioxide, methane, ethane, ethylene, propane, propene, propyne, helium, oxygen and nitrogen**.** Sampling and analysis of microorganisms comprised total number of cells (TNC), quantitative most probable number (MPN) of SRB and autotrophic acetogens (AA), culturable heterotrophic aerobic bacteria (CHAB) and analysis of adenosine triphosphate (ATP) measurement were performed.

The third experiment, canister A04 was retrieved in August 2011. Prior to this, samples for microbiology and chemistry were taken and analysed, as were done in September 2007, October 2008 and in December 2010 (Eriksson 2008, Lydmark and Hallbeck 2011) but this sampling reflected the composition of the microbial population and composition of gas and chemistry of the water in the support cage of the canister at the time of removal. This specific experiment followed up earlier studies and investigated how microbes were involved in the corrosion processes inside the MINICAN experiments.

Directly after retrieval of the A04 canister, biofilm formation was studied on the surface of the support cage. Samples for TNC, CHAB and SRB (cultivation and DNA technique) were taken from the surface of the steel support cage using the same sampling method and analyses as in the retrieval of the canisters Dh6 and Dh5, in the Prototype project. The A04 canister was held under water in a container during the sampling. Samples from the container water were also taken as comparison to the biofilms. The sampling and analysis procedures were described in the activity plan AP TD F77P3-11-052 using methods that have been described in (Pedersen, 2005) and use of pressure vessels as described in AP TD F63.1-07-020.

This report presents data from the analyses of water and gas in the water inside the A04 stainless steel cage, before the retrieval of the A04 canister, in comparison with data from previous analyses. It also presents the microbial data from biofilm samples taken from the surface of the support cage and surfaces of the miniature copper canister together with the presence of SRB, CHAB and iron-reducing bacteria (IRB) in the bentonite from A04.

2 Material and Methods

2.1 Sampling occasions and analyses performed before the removal

Sampling of water for gas-, microbe- and chemical analyses from the MINICAN experiments were performed in 2007, 2008 and in 2010. The results for these analyses were described by Eriksson (2008) and Lydmark and Hallbeck (2011). Before the retrieval of canister A04, groundwater was sampled and analysed in the same way. Table 2-1 shows information on experiment names, sampling points, dates for sampling of dissolved gas, microbiology and water chemistry. Table 2-2 lists the performed analyses.

Table 2-1. 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 cage (C) surrounding the canisters in the MINICAN experiments. A04-C was sampled 2011-08-22.

Canister name	Samples	Sampling date dissolved gas	Sampling date microbes	Sampling date water chemistry
A02C	KA3386 A02 Canister	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
A03C	KA3386 A03 Canister	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
A03G	KA3386 A03Groundwater	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 A04 Canister	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
A05-C	KA3386 A05-canister	–[a]	–	–
A06C	KA3386 A06 Canister	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

[a] Water could not be extracted from canister A05-C.

Table 2-2. Parameters analysed in the groundwater and in water from inside the cage surrounding the canisters in the MINICAN experiments.

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	According to SKB standard	SO_4^{2-}, HCO_3^- Fe^{2+}, S^{2-}, Cl^- pH, acetate

Before the retrieval of the A04 cage and canister, samples of the nitrogen infused water in the container, used as protection of the package against exposure to air, were taken for analyses of the microbial composition. Samples from the biofilm on the support steel cage were taken immediately when it was removed from the borehole. The microbiological analyses were TNC, CHAB and MPN for SRB and DNA samples for the microbial diversity.

2.2 Sampling procedures

2.2.1 The MINICAN experiment, sampling in the tunnel.

A sterile tube and junctions with a mounted valve, stopcock and a needle was attached to the connection of the A04 experiment in MINICAN. An anaerobic sampling vessel was attached to the sterile tube and the needle was penetrated through the septa of the vessel. During sampling, the stopcock

and the outflow from the SKB MINICAN connections were opened and the water filled the sampling vessels. An additional needle was penetrated through the septum of the sampling vessel shortly after the sampling begun, to eliminate dangerous pressure build-up.

In 2010 and 2011, the first 50 mL of water sampled from the MINICAN experiment flushed the sample equipment and were discarded. Acetate (10 mL), Fe^{2+} 10 mL, MPN SRB (10 mL), MPN AA (10 mL), TNC (30 mL), ATP (50 mL), CHAB (30 mL), dissolved gas (250 mL) and chemistry (2,500 mL), were used for respective analysis. Fe^{2+} and ATP were not analysed in 2011.

The container filled with groundwater was placed in front of the A04 experiment in MINICAN. Before the A04 experiment was opened, the container water was sampled with sterile 50 mL syringes and needles, see Figure 2-2 and Figure 2-3. The sampled water was added to the sampling vessels for microbiological analyses described in Table 2-2. The A04 experiment equipment was removed from the borehole into the container water. Samples from the A04 support cage were taken immediately with sterile 50 mL syringes and needles. The needles were placed on the surface and moved over a 3 × 3 cm area. The samples are called Cage 1, Cage 2 and Cage 3. For the positions of the samples, see Figure 2-1. The samples were transferred to the sampling vessels with syringes (Figure 2-4).

Measurements and inoculations of the samples from container water and support cage into growth media were made directly in the Äspö laboratory. The DNA samples were kept cool and brought to Microbial Analytic Sweden's laboratory in Mölnlycke for further treatment and analyses.

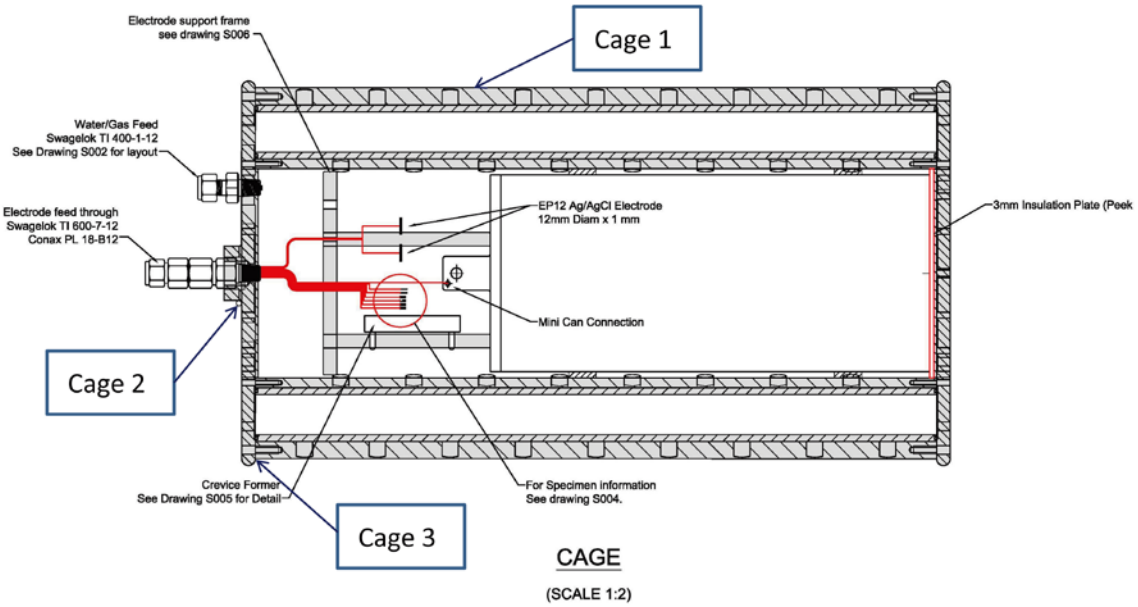

Figure 2-1. *The positions of the three samples from the support cage in experiment A04 of MINICAN.*

Figure 2-2. *Samples from container water and support cage surfaces were taken with sterile syringes and needles.*

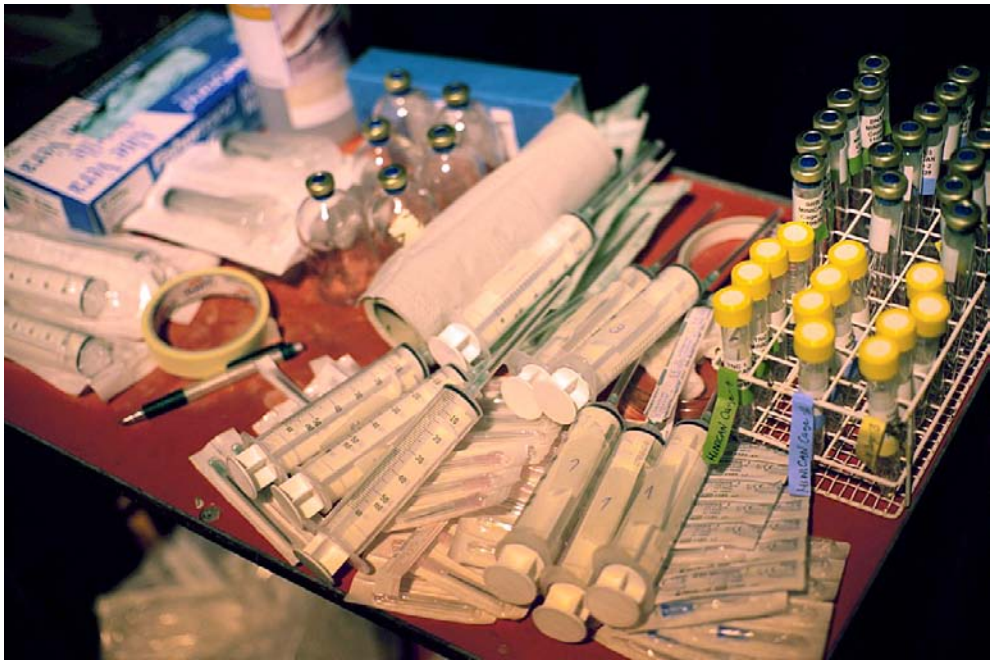

Figure 2-3. *Material for sampling during the removal of the A04 canister in the MINICAN experiment.*

Figure 2-4. *Sterile transfer of a water sample to an anaerobic, sterile test tube.*

2.2.2 MINICAN experiment, sampling from the copper canister.

The A04 canister was placed in a transport cylinder and shipped to the corrosion laboratory of SERCO in Culham, UK. At arrival, the transport cylinder was placed in an anaerobic glove box over night. The first samples were taken from bentonite when the support cage was opened. One gram of bentonite was added to each sample vessel with a sterile spoon inside the glove box. Immediately after the canister was lifted out of the support cage, samples from the copper surface were taken with sterile cotton swabs over an area of approximately 9 cm^2. The swabs were then placed in the sample vessels. Samples for TNC, CHAB and MPN for SRB were taken. Five swab samples for DNA, analyses were taken from each canister sample area, with DNA free, sterile cotton swabs and placed in sterile PBS solution. The inoculations were done immediately in the SERCO lab and the growth cultures were shipped to Microbial Analytics for incubation and analyses. DNA samples were kept cold and brought back by the personnel to the laboratory in Mölnlycke for treatments and analyses.

The bentonite showed red staining see Figure 2-6, Figure 2-7, Figure 2-8 and it was decided to make a MPN of IRB analysis of the bentonite in the laboratory in Mölnlycke, Sweden.

Figure 2-5. *The A04 cansister inside the support steel cage and bentonite. The photo shows sampling of bentonite with a sterile spoon.*

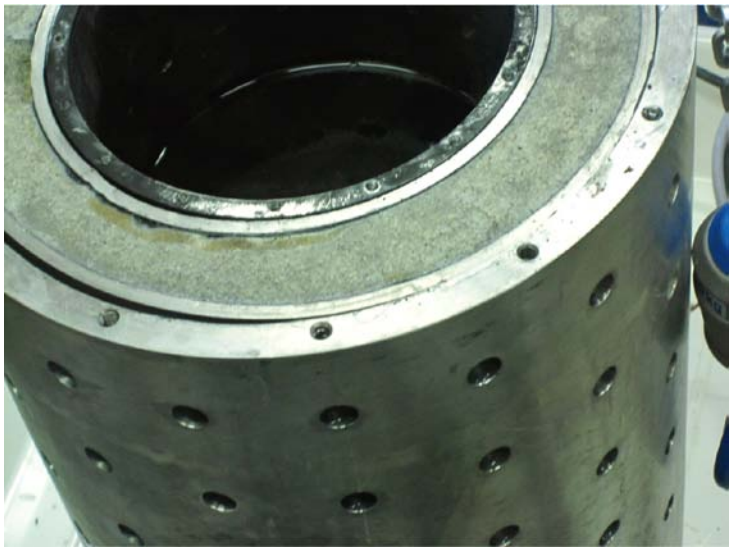

Figure 2-6. *The bentonite in A04 experiment before sampling.*

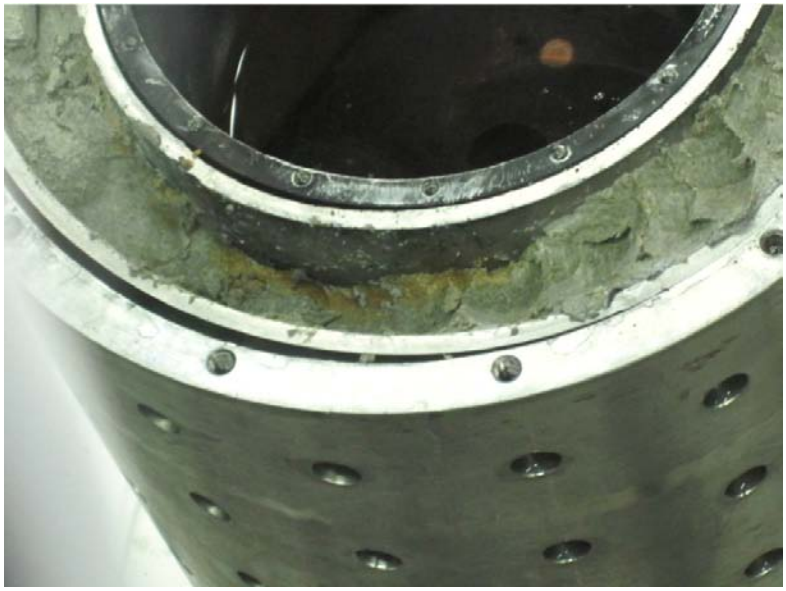

Figure 2-7. *The bentonite in A04 experiment after sampling.*

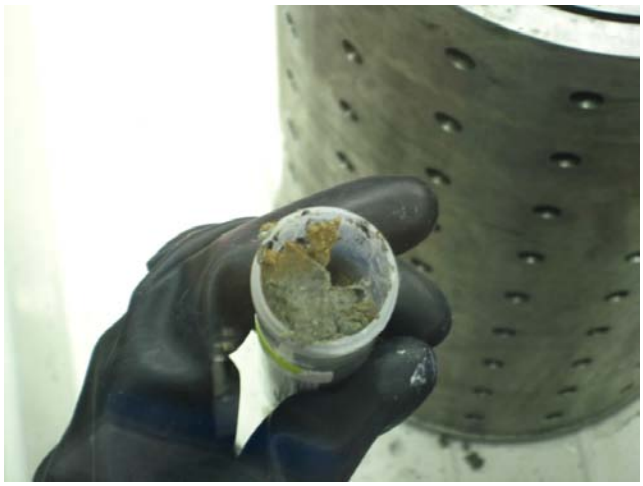

Figure 2-8. *The A04 bentonite in a sterile plastic test tube.*

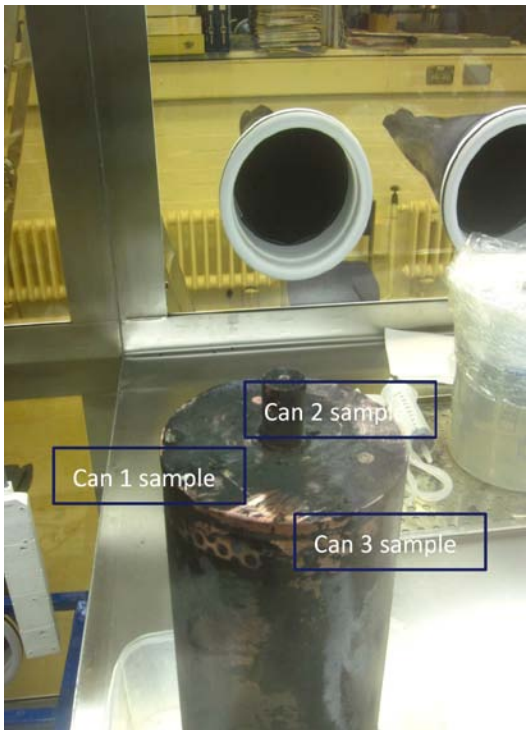

Figure 2-9. *The A04 copper canister inside the glove box. The text squares show where the three canister samples were taken.*

2.3 Analyses and data treatment

Gas analysis was performed as described previously by Pedersen et al. (2008b) and TNC, MPN SRB, MPN AA, and CHAB analyses were performed according to Hallbeck and Pedersen (2008).

Total DNA was extracted from the samples using the Power Soil or Power Water DNA extraction kit from MO BIO Laboratories according to the manufacturer's specifications. Nucleotide concentrations were measured using the nanodrop ND-1000 spectrophotometer (Thermo Scientific) and concentrations of double-stranded DNA were measured fluorometrically by using the MX3005P fluorometer with MXPro software (Stratagene) and the Quant-it™ Picogreen reagent kit from Molecular Probes. For the polymerase chain reaction (PCR), the DNA extract was added to a mixture of 2X iProof High fidelity mastermix (Bio-Rad Laboratories), 5 µM of each primer and ultra pure water (GIBCO). A first denaturation was performed at 98°C (30 s) and then a total of 30 cycles at 98°C (30 s), 60°C (30 s), and 72°C (40 s) followed by a final incubation at 72°C 5 min. The primers used were 27f and 1492r (Lane 1991) numbers corresponding to positions in E.coli Brosius (Brosius et al. 1978). The amplification products were purified with a QIAGEN QIAquick gel extraction kit following the manufacturer's specifications. The purified samples were cloned in to the pCR®2.1-TOPO vector and transformed into chemically competent TOP10 *Escherichia coli* cells with the TOPO TA cloning kit from Invitrogen following the manufacturer's instructions. From each DNA extraction, a total of 20 white clones containing the insert were randomly picked. Each colony was inoculated in 1 mL of Yeast-Tryptone Media (YT), Kanamycin (40 mg/mL) and incubated overnight at 37°C. The recombinant plasmids were extracted from the bacteria with the QIAGEN Miniprep kit following manufacturer's instructions. The sequencing was performed by using Eurofins MWG Operon Sequencing A la carte Service using the 907r sequencing primer (Ekendahl et al. 1994). The 16S rRNA gene clones were analysed using The BioEdit sequence alignment editor 7.1.3. The 16S rRNA reference gene E.coli Brosius with accession number J01695.2 was used as a sequence mask for the alignment of conserved regions within the 16S rRNA gene. The Sequences were screened for vector contamination using Vecscreen, a specialised Basic Local Alignment Search Tool (BLAST). The clones were compared to sequences available in the nucleotide database-BLAST. Sequence homology was analysed by using the nucleotide-nucleotide algorithm.

Data treatment and graphics were performed using STATISTICA software, version 10.0 (Statsoft, Tulsa, OK, USA).

3 Results

3.1 Microbial composition in experiment A04 in MINICAN

The microbial compositions inside the support cage of the MINICAN A04 experiment in 2007, 2008, 2010 and 2011 are shown in Table 3-1. Each microbiological parameter will be treated in the following sections.

3.1.1 TNC

The TNC data for A04 from the four sampling occasions are found in Table 3-1. The TNC analyses of water from inside the cage of A04 in the MINICAN experiments were relatively stable during the years with numbers around 1×10^5 mL^{-1}, which is illustrated in Figure 3-1.

3.1.2 CHAB

The numbers of CHAB decreased from 2007 to 2008 (Table 3-1). In 2007, the number of CHAB inside the A04 MINICAN experiments was 530 cells mL^{-1} and in 2008 it was 20 cells mL^{-1}. The number of CHAB in 2010 was below detection but it increased to 667 cells mL^{-1} in 2011 as shown in Figure 3-2.

3.1.3 MPN AA

The numbers of AA in the A04 MINICAN experiment determined by MPN had decreased from 7,000 mL^{-1} in 2007 to below the detection limit in 2010 and 2011, see Figure 3-3.

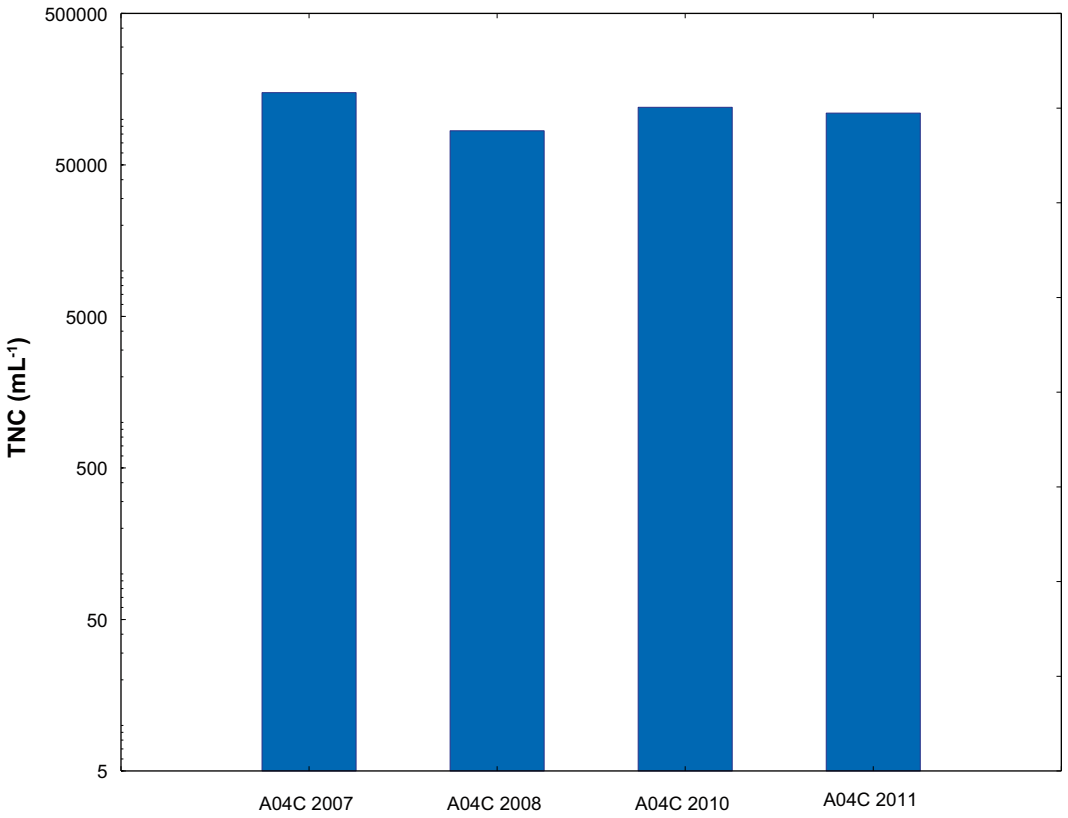

Figure 3-1. *Total number of cells, TNC, in samples from the A04 experiment cage in MINICAN from 2007, 2008, 2010 and 2011.*

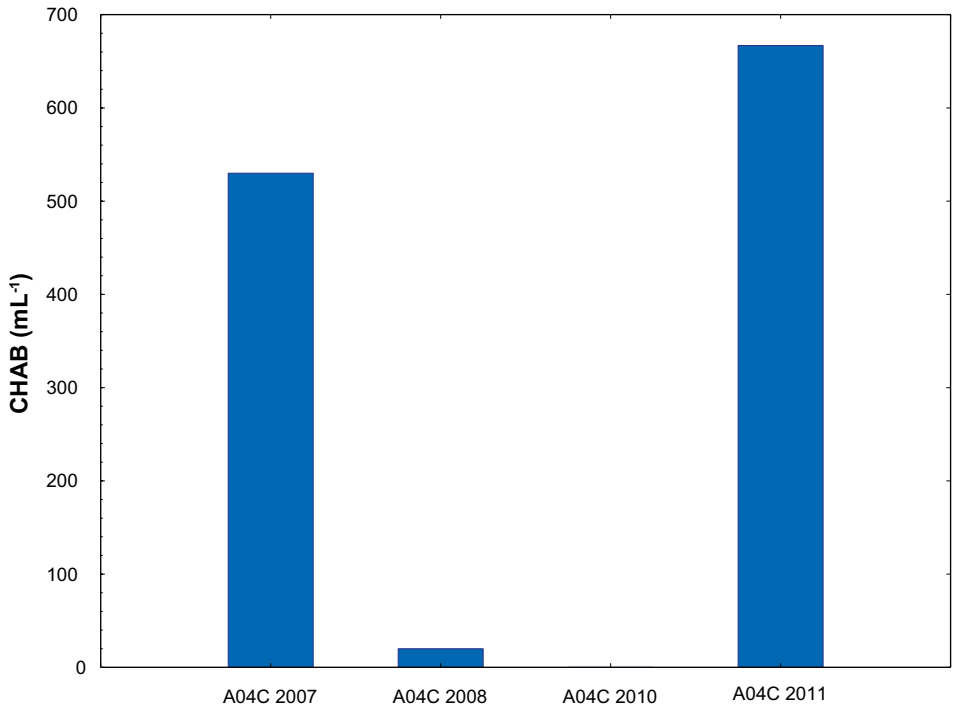

Figure 3-2. *The number culturable heterotrophic aerobic bacteria (CHAB), in samples from the experiment cage A04 in MINICAN from 2007, 2008, 2010 and 2011.*

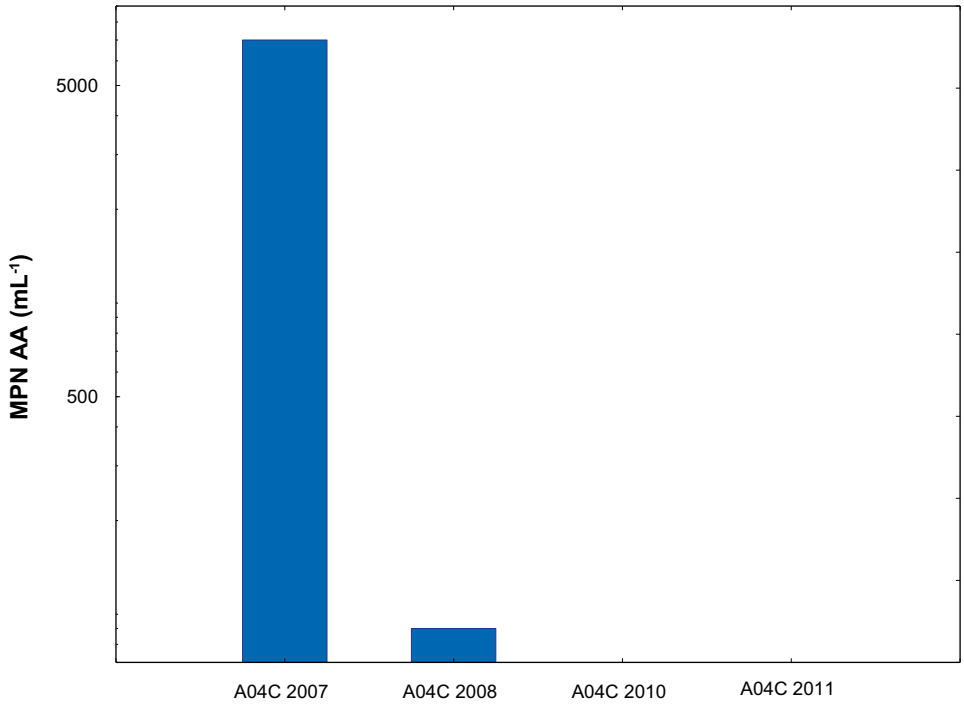

Figure 3-3. *The most probable number (MPN) of autotrophic acetogens, AA, in samples from the experiment cage A04 in MINICAN from 2007, 2008, 2010 and 2011.*

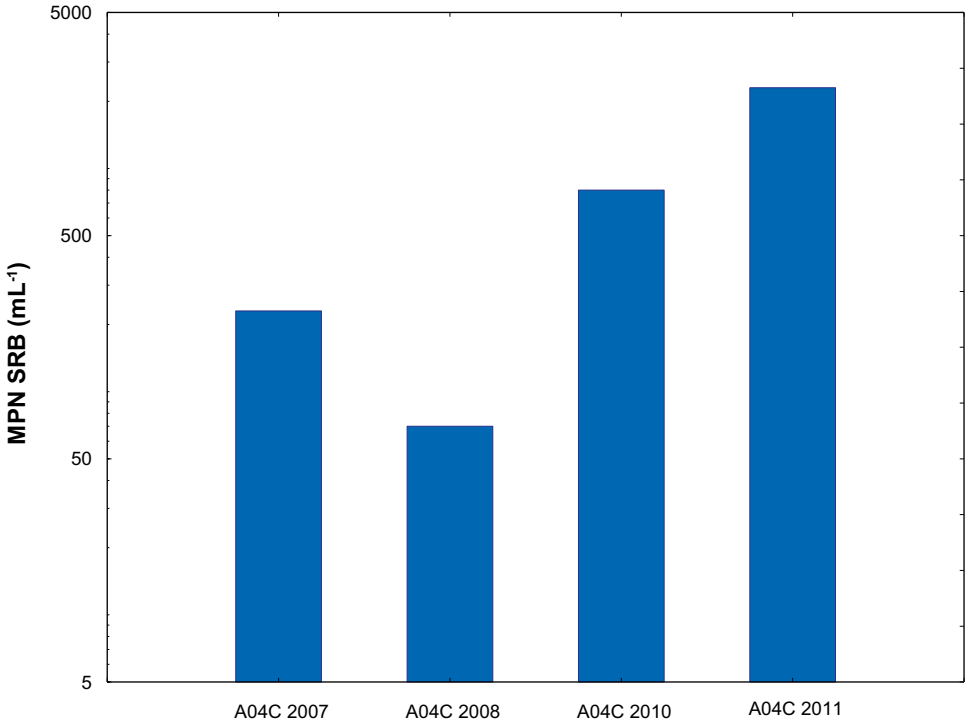

Figure 3-4. *The most probable number (MPN) of sulphate-reducing bacteria, SRB, in samples from the experiment cage A04 in MINICAN from 2007, 2008, 2010 and 2011.*

3.1.4 MPN SRB

The number of SRB in the A04 MINICAN experiment at the sampling in 2007 was 230 mL^{-1}. The number decreased from that in 2008 but in 2010 and 2011 the numbers have increased ten times to 800 mL^{-1} in 2010 and 2,300 mL^{-1} in 2011 (Figure 3-4).

Table 3-1. Microbial composition in samples from the A04 experiment in MINICAN 2007, 2008, 2010 and in 2011.

Year	ATP (amol mL^{-1})	TNC (±SD) (mL^{-1})	CHAB (±SD) (mL^{-1}) [a]	MPN SRB (lower and upper 95% confidence interval) (mL^{-1})	MPN AA (lower and upper 95% confidence interval (mL^{-1}) [c]	Acetate (mg L^{-1})
2007	15,500 ± 810	150,000 ± 61,000	530 ± 28	230 (90–860)	7,000 (3,000–21,000)	6.9
2008	27,400 ± 1,820	200,000 ± 22,000	bd	70 (30–210)	90 (30–290)	1.8
2010	6,800 ± 2,330	120,000 ± 7,800	bd	800 (300–2,500)	bd	1.8
2011	n.m	110,000± 3,500	667 ± 172	2,300 (900–8,600)	b.d.	3.4

3.2 Gas composition in MINICAN

The data for the complete gas composition inside the support cage of the A04 experiment in MINICAN from the samples from 2007, 2008, 2010 and 2011 are shown in Table 3-2.

3.2.1 Total gas volume

Figure 3-5 shows the total gas volumes dissolved in the groundwater inside the cage of the A04 MINICAN experiment. The volume was between 53 and 70 mL L^{-1} gas in this experiment.

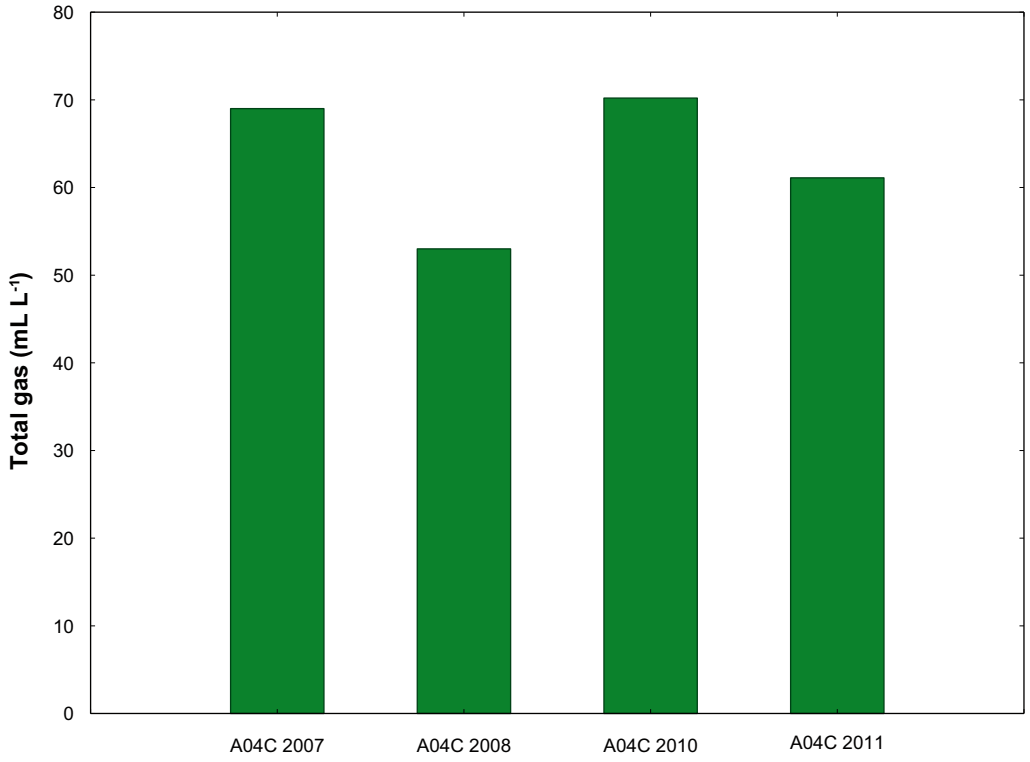

Figure 3-5. *The total amount of gas in groundwater from the cage of the A04 experiment in MINICAN from 2007, 2008 and 2010.*

3.2.2 The gas composition

The composition of the gas samples from 2007, 2008, 2010 and 2011 are compiled in Table 3-2. The largest volume of hydrogen was measured in 2010 with 17.2 µL L^{-1}. The volume of methane has decreased since the first sampling and analyse, from 245 µL L^{-1} to 192 µL L^{-1} in 2011. The same decreasing trend was found for carbon dioxide.

3.3 Water chemistry

In Table 3-4, data for some chemical parameters from the four sampling occasions are compiled. The sulphate concentration had decreased in A04, since the start of MINICAN, from 439 mg L^{-1} in 2007 to 271 mg L^{-1} in 2011. The decrease in sulphate concentration from December 2010 to August 2011 was 129 mg L^{-1}. During the same period, the ferrous iron concentration increased from 9.9 mg L^{-1} to 49.6 mg L^{-1}, pH decreased from 7.3 to 6.6, and the chloride concentration increased from 7,968 to 8,262 mg L^{-1}. The sulphide concentration had varied between 0.037 mg L^{-1} in 2007 to 0.045 mg L^{-1} in 2011.

Table 3-2. Dissolved gas in samples from the A04 experiment cage in MINICAN collected in 2007, 2008, 2010 and 2011.*O_2 was below detection in all samples.

Experiment name	Gas/water (mL L^{-1})	H_2 (µL L^{-1})	CO (µL L^{-1})	CH_4 (µL L^{-1})	CO_2 (µL L^{-1})	C_2H_6 (µL L^{-1})	C_2H_{2-4} (µL L^{-1})	C_3H_8 (µL L^{-1})	C_3H_6 (µL L^{-1})	Ar (µL L^{-1})	He (µL L^{-1})	N_2 (µL L^{-1})
2007	69	0.10	0.72	245	1,850	0.12	0.02	<0.1	<0.1	842	4,920	61,100
2008	53	0.55	0.65	215	568	0.28	<0.1	0.05	<0.1	185	6,600	44,900
2010	69	17.2	0.25	167	465	0.1	<0.1	<0.1	<0.1	773	7,030	59,500
2011	61	1.18	0.82	192	445	0.26	<0.1	<0.1	<0.1	609	7,810	52,100

Table 3-3. Microbial composition in samples taken during the removal of A04 canister, container water and support cage surfaces, sampled 2011-08-22 in the Äspö tunnel.

Sample	TNC (±SD) (mL^{-1})	CHAB (±SD) (mL^{-1}) [a]	MPN SRB (lower and upper 95% confidence interval) (mL^{-1})	Acetate (mg L^{-1})
Container	2.90E+04 (±5.80E+03)	8.40E+03 (±2.00E+03)	230 (90–860)	3.4
Cage 1	5.00E+04 (±1.30E+04)	5.10E+03 (±1.10E+03)	800 (300–2,500)	n.m.*
Cage 2	5.00E+04 (±1.10E+04)	1.03E+04 (±1.15E+03)	230 (90–860)	n.m.
Cage 3	1.80E+04 (±2.80E+03	7.03E+03 ±(3.21E+02)	23 (9–86)	n.m.

*Not measured.

3.4 Microbial composition of the biofilm on the A04 support cage in MINICAN

Three samples were taken from the surface of the A04 support cage as soon as it was removed from the bore hole. Since the whole cage was submerged in water during the sampling, the same microbial analyses were done on the container water. In Table 3-3, the results from TNC, CHAB and MPN of SRB are shown from this sampling. The TNC data were about the same for all samples including the container water, with numbers between 2×10^4 and 5×10^4 mL^{-1}. The numbers of CHAB were also similar between the three sample sites and the container water. The highest was found in the sample from the Cage 2 place with 1.03×10^4 mL^{-1}. The difference in number of SRB between the sample sites and the container water was not significant and varied between 23 mL^{-1} at the cage 3 site and 800 at the cage 1 site. The number of SRB in the container water was 230 mL^{-1}. The acetate concentration in the water from the container was 3.4 mg L^{-1}.

3.5 Microbial composition of the bentonite in the A04 support cage of the in MINICAN

Table 3-5 includes the microbial data from the bentonite sample taken when the A04 support cage was opened. The number of CHAB, microorganisms that can grow with air and organic carbon as energy/electron donor, was 2.07×10^6 g^{-1} and the number of SRB was 9.0×10^4 g^{-1}. TNC was not possible to apply to bentonite. For the bentonite sample also MPN of iron-reducing bacteria (IRB) was performed and the result was 80 g^{-1}.

3.6 Microbial composition of the biofilm on the A04 copper canister in MINICAN

From Table 3-6 it can be seen that almost all cells at the sampled surfaces of the copper canister were SRB. The TNC data range from 5.0×10^3 to 1.0×10^4 cm^{-2} and the SRB data range from 1.9×10^3 to 2.6×10^4 cm^{-2}. The number of CHAB was highest at the Can 1 surface with 174 cm^{-2} and the lowest 1.9 cm^{-2}.

3.7 Microbial diversity of canister biofilm of the A04 experiment in MINICAN

Samples for DNA extraction were taken from container water, support cage surfaces, denoted Cage 1, Cage 2, Cage 3 and canister surfaces. The 16S rRNA gene from the extracted DNA was amplified with the polymerase chain reaction and cloned. Twenty clones from each sample were sequenced and compared to deposited 16S rRNA sequences in Genbank (NCBI 2011). The resulting identities and closest relative species are presented in Table 3-7. Clones that were closest related to species in the δ-Proteobacteria group are written in bold italic. Many SRB belong to this group. Many clones from the canister surface belonged to this group and the closest species found were often SRB.

One clone in each canister sample were closest related to *Desulfovibrio ferrophilus*, a SRB that can utilise metal iron or stainless steel as energy/electron donor in laboratory cultures (Dihn et al. 2004).

Table 3-4. Chemical composition of samples from the experiment A04 in MINICAN, sampled 2007, 2008, 2010 and 2011.

Year	SKB sample number	SO_4^{2-} (mg L^{-1})	HCO_3^- (mg L^{-1})	S^{2-} (mg L^{-1})	Fe^{2+} (mg L^{-1})	pH	Cl^- (mg L^{-1})
2007	14289	439	51	0.037	0.82	7.6	6,671
2008	14644	410	38	0.022	15.7	7.2	6,895
2010	20552	400	32	0.059	9.91	7.3	7,968
2011	20906	271	34	0.045	49.6	6.6	8,262

Table 3-5. Microbial composition in samples from the removal of A04 canister, the bentonite in the support cage, sampled 2011-09-02 in the SERCO lab, UK.

Sample	TNC (±SD) (g^{-1})	CHAB (±SD) (g^{-1})	MPN SRB (lower and upper 95% confidence interval) (g^{-1})	MPN IRB (lower and upper 95% confidence interval) (g^{-1})
A04 Bentonite	–	2.07E+06 (±5.03E+05)	9.00E+04 (3.00E+04–2.90E+05)	80 (30–250)

Table 3-6. Microbial composition of samples from the removal of A04 canister, the copper canister surfaces, sampled 2011-09-02 in the SERCO lab, UK.

Sample	TNC (±SD) (cm^{-2})	CHAB (±SD) (cm^{-2})	MPN SRB (lower and upper 95% confidence interval) (cm^{-2})
Can 1	1.04E+04 (±478)	174 (±44.9)	1.89E+04 (7.78E+03–5.00E+04)
Can 2	7.33E+03 (±1.67E+03)	1.86 (±1.28)	2.56E+04 (1.00E+04–9.56E+04)
Can 3	5.00E+03 (±9.89E+02)	1.48 (±1.28)	1.89E+03 (7.78E+02–5.33E+03)

Table 3-7. Microbial diversity in samples from the removal of A04 canister, container water, support cage surfaces and copper canister surfaces.

Sample and clone number	Identity closest match GenBank (%)	Accession number Genbank	Species	Group	Properties of closest relatives
Cage 1–12	99	DQ833393	*Brevundimonas* sp. BVF-1	Alphaproteobacteria	Stalked bacterium
Cage 1–7	99	NR_042819	*Dechloromonas hortensis* strain MA-1	Betaproteobacteria	
Cage 2–1	99	GU584164	*Sulfitobacter* sp. 204Z-5	Alphaproteobacteria	
Cage 2–10	***87***	***CP000252***	***Syntrophus aciditrophicus***	***Deltaproteobacteria***	***Acetogen***
Cage 2–12	99	AJ244706	Brevundimonas mediterranea	Alphaproteobacteria	Stalked bacterium
Cage 2–13	99	DQ833393	*Brevundimonas* sp. BVF-1	Alphaproteobacteria	Stalked bacterium
Cage 2–17	92	NR_042090	*Dechloromonas denitrificans* strain : ED1	Betaproteobacteria	
Cage 2–2	***84***	***NR_025406***	***Desulfococcus biacutus strain DSM 5651***	***Deltaproteobacteria***	***SRB***
Cage 2–4	98	FJ527418	*Rheinheimera* sp. JSM 083085	Gammaproteobacteria	
Cage 3–1	99	EU000234	*Flavobacterium gelidilacus* strain KOPRI_22147	Bacteroidetes	
Cage 3–12	99	HQ588831	*Sphingobium* sp. BZ13	Alphaproteobacteria	
Cage 3–13	99	NR_029023	*Hydrogenophaga atypica* strain BSB 41.8	Betaproteobacteria	
Cage 3–14	99	NR_042819	*Dechloromonas hortensis strain* MA-1	Betaproteobacteria	
Cage 3–16	86	AB478415	Ignavibacterium album	Chlorobi	
Cage 3–17	87	FJ502233	*Methylopila* sp. JZL-4	Alphaproteobacteria	Methyl oxidising bacterium
Cage 3–18	***86***	***EF428583***	***Beggiatoa sp. 'Chiprana'***	***Deltaproteobacteria***	***Sulphide oxidising bacterium***
Cage 3–19	***81***	***CP001629.1***	***Desulfomicrobium baculatum DSM 4028,***	***Deltaproteobacteria***	***SRB***
Cage 3–20	96	CP001312	*Rhodobacter capsulatus* SB 1003	Alphaproteobacteria	
Cage 3–7	98	GQ221766	*Prosthecomicrobium* sp. ATCC 27825	Alphaproteobacteria	Stalked bacterium
Cage 3–8	***98***	***CP000089***	***Dechloromonas aromatica RCB***	***Deltaproteobacteria***	
Container 9	99	EF540480	*Rhizobium* sp. 8_4V	Alphaproteobacteria	Nitrogen fixing bacterium
Container 1	99	NR_042819	*Dechloromonas hortensis* strain MA-1	Betaproteobacteria	
Container 10	99	NR_042819	*Dechloromonas hortensis* strain MA-1	Betaproteobacteria	

Sample and clone number	Identity closest match GenBank (%)	Accession number Genbank	Species	Group	Properties of closest relatives
Container 12	99	EF093132	*Brevundimonas* sp. VTT E-052914	Alphaproteobacteria	Stalked bacterium
Container 13	90	HM569768	T*halassolituus* sp. IMCC1883	Gammaproteobacteria	
Container 15	***90***	***EF092443***	***Bacteriovorax sp. NF3***	***Deltaproteobacteria***	
Container 16	84	FN668941	Clostridium difficile	Clostridia	
Container 19	99	FR733676	Gemmobacter aquatilis	Alphaproteobacteria	
Container 2	98	AJ289884	*Thiobacillus* Q	Betaproteobacteria	Sulphide oxidising bacterium
Container 20	***80***	***AF030438***	***Desulfomicrobium baculatum DSM 4028***	***Deltaproteobacteria***	***SRB***
Container 5	92	FR872934	Clostridium sp. strain AN-AS8	Clostridia	
Container 7	83	CP002048	*Syntrophothermus lipocalidus* DSM 12680, complete genome	Clostridia	Acetogen
Container 8	***87***	***CP002629***	***Desulfobacca acetoxidans DSM 11109***	***Deltaproteobacteria***	***SRB, acetate oxidising***
Can 1–1	99	NR_029319	*Pseudomonas anguilliseptica* strain S 1	Gammaproteobacteria	
Can 1–12	99	AF439803	*Pseudomonas anguilliseptica* strain BI	Gammaproteobacteria	
Can 1–14	86	AB478415	Ignavibacterium album		
Can 1–16	***97***	***AY274449***	***Desulfovibrio ferrophilus***	***Deltaproteobacteria***	***SRB, metallic iron oxidiser***
Can 2–1	96	JN679850	*Pseudomonas fluorescens* strain 1573	Gammaproteobacteria	
Can 2–10	***99***	***NR_042143***	***Desulfobacula toluolica Tol2 strain DSM 7467***	***Deltaproteobacteria***	***SRB***
Can 2–11	***99***	***AY771932***	***Delta proteobacterium LacK10***	***Deltaproteobacteria***	***SRB***
Can 2–13	***92***	***NR_026423***	***Desulfocapsa sulfexigens strain SB164P1***	***Deltaproteobacteria***	***SRB***
Can 2–15	***98***	***AY274449***	***Desulfovibrio ferrophilus***	***Deltaproteobacteria***	***SRB, metal iron oxidiser***
Can 2–16	99	AF237677	Pseudomonas stutzeri	Gammaproteobacteria	
Can 2–17	87	AB541983	Prolixibacter bellariivorans	Gammaproteobacteria	
Can 2–19	90	NR_040971	*Leptolinea tardivitalis* strain YMTK-2	Gammaproteobacteria	
Can 2–2	99	U65012	*Pseudomonas stutzeri*	Gammaproteobacteria	Groundewater bacterium, nitrate reducing
Can 2–20	99	FN995247	*Pseudomonas stutzeri*	Gammaproteobacteria	Groundwater bacterium, nitrate reducing

Sample and clone number	Identity closest match GenBank (%)	Accession number Genbank	Species	Group	Properties of closest relatives
Can 2–4	***90***	***NR025407***	***Desulfomicrobium norvegicum strain DSM 1741***	***Deltaproteobacteria***	***SRB***
Can 2–5	***99***	***CP001629.1***	***Desulfomicrobium baculatum DSM 4028***	***Deltaproteobacteria***	***SRB***
Can 2–7	***99***	***AY771932***	***Delta proteobacterium LacK10***	***Deltaproteobacteria***	***SRB***
Can 2–8	***99***	***AY771932***	***Delta proteobacterium LacK10***	***Deltaproteobacteria***	***SRB***
Can 2–9	90	NR_040971	*Leptolinea tardivitalis* strain YMTK-2		
Can 3–1	94	NR_024886	*Fusibacter paucivorans* strain SEBR 4211	Clostridia	
Can 3–10	89	NR_040971	*Leptolinea tardivitalis* strain YMTK-2		
Can 3–12	***100***	***NR_029307***	***Desulfovibrio aespoeensis Aspo-2 16S ribosomal RNA***	***Deltaproteobacteria***	***SRB from Äspö groundwater***
Can 3–13	96	FR872932	*Clostridium* sp. AN-AS6C	Clostridia	
Can 3–14	98	NR_042321	Hoeflea alexandrii strain : AM1V30	Alphaproteobacteria	
Can 3–15	89	DQ833401	*Sphaerochaeta* sp		
Can 3–17	***98***	***AY274449***	***Desulfovibrio ferrophilus***	***Deltaproteobacteria***	***SRB, iron metal oxidiser***
Can 3–19	98	NR_026326	*Acetobacterium malicum* strain DSM 4132	Clostridia	
Can 3–20	89	DQ833401	*Sphaerochaeta* sp. RCcp2		
Can 3–3	***99***	***NR_042143***	***Desulfobacula toluolica Tol2 strain DSM 7467***	***Deltaproteobacteria***	***SRB***
Can 3–4	***99***	***NR_042143***	***Desulfobacula toluolica Tol2 strain DSM 7467***	***Deltaproteobacteria***	***SRB***
Can 3–5	***99***	***CP001629.1***	***Desulfomicrobium baculatum DSM 4028***	***Deltaproteobacteria***	***SRB***
Can 3–8	90	AB623230	*Bacteroidetes* bacterium 4F6B	Bacteroides	
Can 3–9	***96***	***HE600854***	***Desulfovibrionaceae bacterium PR7_B02***	***Deltaproteobacteria***	***SRB***

4 Discussion

4.1 The microbiology, gas content and chemistry of experiment A04 in MINICAN

4.1.1 Microbiology of A04, numbers and physiological groups

Samples for microbiological analyses have been taken from the water inside the support cage of A04 at four occasions in 2007, 2008, 2010 and the last in 2011, the day before the removal of the A04 canister. The TNC has not differed significantly during the years of the experiment and lies at the approximately value of 1×10^5 mL^{-1}, see Figure 3-1. This value corresponds well with the mean number of cells in Fennoscandian Shield groundwater systems (Hallbeck and Pedersen 2012, Hallbeck and Pedersen 2008, Kotelnikova and Pedersen 1998, Pedersen et al. 2008).

The culturable heterotrophic bacteria (CHAB) can grow on organic compounds with oxygen as electron acceptor. These organisms can be facultative anaerobes and by that be able to survive also in anaerobic environments. The number of CHAB in the A04 experiment has differed through the years. In the sample taken in 2007, the number was 530 mL^{-1}, after that the number of CHAB decreased and in 2010, the number was below detection. Before the removal of A04, the number had increased to 667 mL^{-1}. Before the last sampling in 2011, some pumping of the borehole KA3386 was done to fill up the container used in the retrieval of the A04 canister. This could affect the number of CHAB if the water inside the support cage was changed during the pumping. There might have been a possible growth-stimulating effect of the bentonite. The MX-80 bentonite (used in the MINICAN experiments) contains about 0.20–0.25% organic carbon, which potentially can serve as a carbon and energy source for subsurface bacteria able to utilise organic carbon.

The autotrophic acetogens (AA) grow with hydrogen as energy source and electron donor and with carbon dioxide as carbon source and electron acceptor. These bacteria were high in numbers when analysed 2007 but decreased in 2008. In 2010 and 2011, the numbers of AA were at or below detection indicating that the AA had disappeared from the water phase in the A04 experiment in MINICAN. There was no obvious reason for this but one explanation could be competition for the hydrogen from an increasing population of SRB or that the AA population became attached to surfaces were the hydrogen production occurred during the metal corrosion. Some clones from the sequencing of the 16S RNA gene were similar to acetogens, see Table 3-7.

Sulphate-reducing bacteria (SRB) have been present in the MINICAN experiments from the start in 2007. The number of SRB in A04 had increased in 2010 compared to the previous samplings but the experiment had lower numbers than the other experiments in MINICAN at the occasion (Lydmark and Hallbeck 2011). In 2011, the number of SRB in A04 was 2,300 mL^{-1}, an increase of 35% in nine months. The percentage SRB of TNC for A04 in 2010 was 0.6% in A04C and was 2% in 2011. The SRB has certainly increased its part of the population the last year. One explanation for this could be an increased iron oxidation in the system. The ferrous iron concentration in A04 increased from 9.9 mg L^{-1} in 2010 to 49.6 mg L^{-1} in 2011. Ferrous iron concentrations may have increased due to corrosion of the cast iron insert or the support steel cage and this process generates hydrogen gas. Hydrogen support growth of SRB as discussed in the introduction. From the previous results from sampling in the MINICAN project, presented in Lydmark and Hallbeck (2011), it was concluded that the corrosion in A04 MINICAN cage was less than in the other experiments, A02, A03 and A05. The results from 2011 suggest that the corrosion has accelerated in A04 since the sampling in December, 2011. No comparison can be made with the other experiments in 2011, since no sampling was made in the other experiments of MINICAN.

The hydrogen concentration in A04 has been the lowest of the MINICAN experiments from 2007 to 2010 (Lydmark and Hallbeck 2011). The hydrogen concentration in 2011 was lower than in 2010, 1.18 µL L^{-1} compared to 17.1 µL L^{-1}. It can be complicated to draw conclusions from the concentration of hydrogen since it is one of the preferred energy sources for many SRB and other microorganisms. Production and consumption rates would be more accurate to measure but is of course much more challenging.

The sulphate concentration in A04 has decreased from 439 mg L^{-1} at the start in 2007 to 271 mg L^{-1} in 2011. The decrease from December 2010 to August 2011 was 129 mg L^{-1} compared to the decrease of 19 mg L^{-1} from 2007 to 2008 and 10 mg L^{-1} from 2008 to 2010. An increased sulphate reduction could be one explanation for the higher decrease the last year than between the others. Another possible explanation could be a change in the origin of the water in A04 of MINICAN. An indication for this could be that chloride concentration has increased over the years, from 6,671 mg L^{-1} in 2007 to 8,262 mg L^{-1} in 2011, see Table 3-4.

4.1.2 Microbiology of biofilms in experiment A04 in MINICAN

The results from the samples of the biofilm on the support steel cage of A04 showed that there was no thick biofilm on these surfaces when compared to the microbiological results from the container water. There was no significant difference in the number of TNC, CHAB and MPN of SRB between the samples Cage 1–3 and Container, see Table 3-3. The DNA sequence data also confirm this since the closest related species in these four samples were similar to results from other groundwater samples from Äspö.

On the other hand, the microbiology of the copper canister surfaces was more specific and several of the clones obtained, were most similar to different SRB about also to some acetogens, see Table 3-7. The black biofilm that can be seen on the canister surface, Figure 2-1, consisted mostly of SRB. The mean number of SRB for the three canister sample areas was 1.5×10^4 cm^{-1} ($\pm$SD 1×10^4) and the mean of TNC for the areas was 7.4×10^3 cm^{-2} ($\pm$SD 2.5×10^3). That TNC was lower than the MPN of SRB can be explained by difficulties to count stained cells in large amounts of metal sulphides in the sample preparations for TNC. The microbiology data from the canister surfaces show clearly that almost the whole population was SRB. The numbers of CHAB were low which was expected since the corrosion process produced high amounts of hydrogen and by that selected for SRB.

The difference in microbiology between the support cage and the copper canister is most likely explained by the exposure to flowing water to the support cage but the more or less stagnant water inside the cage that the copper canister encountered. The SRB prefer slowly flowing or stagnant water to high flow rates.

It has been suggested that some SRB can use the electrons directly from the oxidation of iron in metallic iron or stainless steel (Dinh et al. 2004). The authors isolated one strain of SRB with this feature and the organism was called *Desulfovibrio ferrophilus.* One clone from each of the canister surface DNA samples, were closest related to this species. Interestingly, the same clones were also even more closely related to one strain of SRB isolated from a MPN tube in a project on bacteriophages in Äspö groundwater. The isolate originated from groundwater in the core-drilled borehole KA3110 in the Äspö tunnel (Eydal et al. 2009). This suggests that there are SRB in the groundwater that can utilize electrons directly from metallic iron in their sulphide production without the step of hydrogen formation.

The data from the microbial analyses of bentonite clay from the A04 experiment showed that there were many CHAB, 2.07×10^6 g^{-1} and 9.0×10^4 g^{-1} SRB. The bentonite was red and very porous at some places which led to the decision to analyse for the presence of IRB. The MPN showed that there were 80 IRB g^{-1} of red stained bentonite. It could be microbial iron-reduction that had affected the clay. Another observation was that close to the red bentonite, there was also more of the black sulphide precipitate. The red staining of the bentonite continued deeper into the clay, as could be seen after the sampling were done (Figure 2-7).

Conclusions

The following conclusions can be drawn from the results of the microbial analyses of surfaces from the retrieved support steel cage, bentonite clay and copper canister together with samples of groundwater in the A04 experiment of MINICAN.

- There has been a succession in the microbial populations in the A04 MINICAN experiment from a more complex population with CHAB, AA and SRB in 2007 towards populations with high numbers of SRB and low or no CHAB and AA in 2011.

- The increase in the number of SRB, concentration of sulphide and ferrous iron together with decrease of sulphate were higher between 2010 and 2011 than between the previous measurements 2007, 2008 and 2010 suggesting accelerated iron corrosion in the experiment A04 in MINICAN.
- The biofilm on the support steel cage was thin and consisted of a variety of groundwater bacteria similar to the bacteria found in the groundwater used in the container for the A04 retrieval.
- The biofilm on the copper canister consisted of SRB to a very large extent. This was confirmed with both a culture-dependent technique and with a DNA technique.
- There is evidence from the DNA results that some of the SRB belonged to a species 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 hydrogen formation. A close relative to this type of SRB has been isolated from groundwater in the borehole KA3110 in the Äspö HRL.

Future research

The MINICAN experiment A04 has during the time MINICAN has been in operation, shown the lowest number of SRB and the lowest ferrous iron concentrations of the monitored experiments, A02–A06. Still, there was a large effect on the number of SRB on the canister and the effect of metal corrosion on the sulphide production. The MINICAN project is a unique project that can increase the knowledge and understanding of the effect of metal corrosion on growth of SRB and their sulphide production. It would be of great advantage to continue the monitoring programme to be able to further confirm the findings from the retrieval of A04 and by that support the understanding of the microbiology behind the sulphide production in technical systems used in groundwater environment.

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