International
Progress Report

IPR-05-29

Äspö Hard Rock Laboratory

MICROBE

Analysis of microorganisms and gases in MICROBE groundwater over time during MINICAN drainage of the MICROBE water conducting zone

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November 2005

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Laboratory

Report no.
IPR-05-29
No.
F82K
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Date
November 2005
Checked by
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Date
December 2005
Approved
Anders Sjöland
Date
2006-01-25

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Keywords: ATP, Bacteria, Chloride, Microorganisms, Most probable number, Total number, Dissolved gas, KJ0050F01, KJ0052F01, KJ0052F03

Abstract

A field laboratory has been installed 450 m underground in the F-tunnel at the Äspö hard rock laboratory. The site selection of the MICROBE laboratory in the F-tunnel of Äspö has, until now, assured stable conditions. However, the situation has changed since the drilling of boreholes in January 2005 at the 450 m level for the "*in situ corrosion testing of miniature canisters*" MINICAN. This drilling caused a significant drainage of the formation from which MICROBE takes it groundwater. Until now (November 2005) more than 15 000 m^3 groundwater have been drained from KA3386A01. This is a very significant drainage. A completely new mixing situation has developed in the MICROBE formation during 2005. This report aims at the documentation of the transient in the microbial and chemical situation, including dissolved gas, caused by the so called MINICAN drainage. It will constitute a platform for follow up measurements once the situation has stabilised at new levels.

Significant increases in chloride concentrations indicate that deeper and saltier water is moving up from large depths towards the MICROBE formation. It remains to analyse the mixing history with M3 modelling. This should be done when the pressure and drainage conditions have been restored. Complete class 5 analyses must be performed to establish the new chemical conditions that have originated as a result of the MINICAN drainage. Deeper water is expected to carry more hydrogen and noble gases, and less CO_2. Although the gas data are a bit erratic, some individual gas analyses also suggest that deeper water is moving up towards the MICROBE boreholes. However, intensive mixing is expected with the measured drainage and this mixing may introduce variability over time. Variability of groundwater pressure due to opening and closing of boreholes, tide effects etc. may introduce wobbling effects over time that masks clear trends. The data on microbes has changed from being very reproducible to being very variable after the MINICAN drainage started. Microorganisms are very sensitive to changes in the environmental conditions. New analyses of microbes must be performed once the conditions have returned to those prevailing before the MINICAN drilling. However, it will be a totally new situation, because the introduction of new, deeper water to the MICROBE formation.

Sammanfattning

Ett underjordiskt fält-laboratorium har etablerats i F-tunneln i Äspölaboratoriet. Valet av plats gjordes för att garantera stabila förhållanden med avseende på grundvattenkemi och tryck. Dessa stabila förhållanden rubbades i januari 2005 när borrning av flera hål för MINICAN utfördes på 450 m nivån (MINICAN: *in situ corrosion testing of miniature canisters*). Den utförda borrningen skapade ett stort dränage av grundvatten från den vattenförande sektion MICROBE tar sitt grundvatten. Under perioden januari-november 2005 har mer än 15 000 m^3 grundvatten dränerats via ett borrhål med beteckningen KA3386A01. Detta är ett mycket betydande dränage. En helt ny situation av blandningsförhållanden har uppstått under 2005 i den formation MICROBE utnyttjar. Denna rapport syftar till att dokumentera vilka förändringar av de mikrobiella och kemiska (inklusive lösta gaser) förhållandena som MINICAN borrningen orsakat. Rapporten ska utgöra en platform för uppföljande mätningar när KA3386A01 har tätats och en nya, stabila förhållanden har inträtt.

En kraftig ökning av kloridkoncentrationerna har observerats vilket tyder på att djupare och saltare vatten rör sig upp från större djup mot den formation MICROBE tar sitt grundvatten. I ett senare skede ska situationen analyseras med M3 modellering. Den ska utföras när trycket återställts och dränaget stoppats. Nya klass 5 analyser måste genomföras för att fastställa den nya kemiska situation MINICAN dränaget orsakat. Grundvatten från stora djup under 450 m nivån kan förväntas innehålla mer vätgas och ädelgaser, jämfört med ett grundvatten från 450 m. Data på gasinnehållet tyder också på att djupare vatten rör sig upp mot MICROBE. Emellertid påverkar den kraftiga dräneringen blandningen av grundvatten från olika källor på ett variablet vis. Variabilitet i grundvattentryck orsakat av öppnande och stängade av borrhål, tidvatteneffekter, med mera kan skapa svängande effekter över tid vilket kan maskera tydliga trender i åtskilliga mätparametrar. Data på antal mikroorganismer av olika typer förändrades från mycket stabila och reproducerbara värden till att bli mycker variabla efter det att MINICAN dränaget började. Mikroorganismer är mycke känsliga för förändringar i miljön. Nya analyser av mikroorganismerna i de tre borrhål MICROBE utnyttjar behöver utföras när störningen från MINICAN eleminerats. Det kommer att vara en helt ny situtation, eftersom MINICAN dränaget fört upp en ny typ av djupare vatten med nya populationer av mikroorganismer.

Contents

1 Introduction

A field laboratory has been installed 450 m underground at the Äspö hard rock laboratory (Pedersen, 2005). This laboratory should serve many different purposes. Biocorrosion of copper, bioimmobilisation and biomobilisation of radionuclides and microbial redox control are projects presently being executed at the site. In addition, the laboratory should serve as a reference for the site investigations. Those projects are generally run over a long time and require stable groundwater conditions. The placement of the MICROBE laboratory in the F-tunnel of Äspö has, until now, assured such conditions. However, the drilling of boreholes in the search for a proper place for the "*Äspö Pillar Stability Experiment*" (APSE) during May-June 2002 changed this situation. The two boreholes KF0066A01 and KF0069A01 caused the salinity of KJ0050F01 and KJ0052F01 to rise more rapidly than before drilling. An accelerating up-coning effect was consequently observed. A third APSE borehole was drilled at NASA 3384, KA3386A01. This borehole intersects the formation from which the MICROBE boreholes get their water. Large diameter boreholes were drilled in January 2005 at the 450 m level for the "*in situ corrosion testing of miniature canisters*" MINICAN. This drilling intersected shallow fractures close to the tunnel wall that short-circuited KA3386A01 with the MINICAN boreholes. A significant drainage of the MICROBE formation started. Over less than a day, pressure drops of up to 300 KPa were registered in the MICROBE boreholes.

Until now (November 2005) more than 15 000 m^3 groundwater has been drained from KA3386A01. The outflow was 0.8 L/sec from January to the end of May, when the MINICAN boreholes were closed. After closure, the outflow stabilized at 0.5 L/sec. The inflow to the tunnel increased with about 30% from tunnel length 3179 and downwards. This is a very significant drainage. As there was a large pressure drop in the MICROBE boreholes that corresponds to the offset of this drainage, it can be concluded that the MICROBE groundwater formation has been under a strong drainage since January 2005. A completely new mixing situation has developed during 2005. The KA3386A01 borehole will be equipped with packers in December 2005 that will block the drainage. It is anticipated that the situation will stabilise on a new, more saline level. This report aims at the documentation of the transient in the microbial and chemical situation, including dissolved gas, caused by the so called MINICAN disturbance. It will constitute a platform for follow up measurement once the situation supposedly has stabilised at new levels.

2 Methods

2.1 Sampling and analysis of chemical components

Sampling for analysis of chloride concentration and conductivity were under taken from 050601 to 051026 at weekly intervals until 050921. Thereafter, sampling was done every 14 days. Analysis was performed by the chemistry laboratory at Äspö hard rock laboratory according to standard protocols.

2.2 Sampling of gas

Gas was extracted using the MICROBE extractor as described elsewhere (See Fig 2-13, Pedersen, 2005). Approximately 90 ml groundwater was extracted in each extraction cycle. The volume of extracted gas was measured and transferred into a 6.6 ml vial with a butyl rubber stopper (Bellco glass Inc. (www.bellcoglass.com), product number 2048-117800) crimped with an aluminium seal (product number 2048-11020). The vials and tubes were evacuated and flushed with nitrogen twice and kept with high vacuum (<1 Pa) until use. Repeated extractions were made to obtain a total of about 10-11 ml gas. This gas volume was compressed in the 6.6 ml vial, thereby ensuring that air did not enter the Hamilton syringes during analysis (see below). Typically, two extractions were sufficient. The gas extracted 050524 was transferred to 27 ml anaerobic tubes and diluted with nitrogen.

2.3 Analysis of gas

2.3.1 Uncertainties

Volumes between 1 up to 250 µl were injected into the respective gas chromatograph using Hamilton syringes. The volume used is adjusted according to the range of the respective instrument and detector. Several injections are commonly needed to find the proper amount to inject for the respective gas. The uncertainty of the instruments and injections is very low, typically 0 up to 4 %. The used calibration gases have a maximum accepted mixing uncertainty of ± 2 %. The precision of the extractions is about ± 6 %. In total, the uncertainty is presently maximum ± 12.1 %. Small injection volumes (typically less than 10 µl) increase the uncertainty. This is sometimes the case for methane which can occur in very high concentrations.

2.3.2 Hydrogen < 20 ppm

Low concentrations of hydrogen (<20 ppm) is analysed on a KAPPA-5/E-002 analyser (http://www.traceanalytical.com) gas chromatograph equipped with a 156 × 1/16 inch stainless steel HayeSep in line with a 31 × 1/8 inch stainless steel Mole sieve 5A column which subsequently attach to a reductive gas detector (RGD). Nitrogen is used as carrier gas. The sample is injected into a 1000 µl injection loop. Dilution of the sample is commonly needed to reach the range of the instrument. This instrument has the most sensitive detector for hydrogen on the market. Detection limit on the instrument with a 1000 µl injection loop is 10^{-12} Litre (1 ppb).

2.3.3 Hydrogen > 20 ppm

High concentrations of hydrogen (>20 ppm) is analysed on a Star 3400CX Varian gas chromatograph (Varian AB, Sweden) using a thermal conductivity detector (TCD) with an oven temperature of 65°C, a detector temperature of 120°C, and a filament temperature of 250°C. The hydrogen gas is separated using a Porapak Q column (2m x 1/8 inch diameter) followed by a Molecular Sieve 5A column (6m x 1/8 inch) with Argon as carrier gas. Detection limit on the instrument with a 250 µl injection is 5×10^{-9} Litre (20 ppm).

2.3.4 Carbon monoxide

Carbon monoxide is analysed on a KAPPA-5/E-002 (http://www.traceanalytical.com) analyser gas chromatograph equipped with a 156 × 1/16 inch stainless steel HayeSep in line with a 31 × 1/8 inch stainless steel Mole sieve 5A column which subsequently attach to a reductive gas detector (RGD). Nitrogen is used as carrier gas. The sample is injected into a 1000 µl injection loop. Dilution of the sample is commonly needed to reach the range of the instrument. This instrument has the most sensitive detector for carbon monoxide on the market. The results are compared with the results obtained with the Varian 3400CX analyser and reported when they agree. Detection limit on the instrument with a 1000 µl injection loop is 10^{-12} Litre (1 ppb).

2.3.5 Helium

Helium is analysed on a Star 3400CX Varian gas chromatograph (Varian AB, Sweden) using a thermal conductivity detector (TCD) with an oven temperature of 65°C, a detector temperature of 120°C, and a filament temperature of 250°C. The helium gas is separated using a Porapak Q column (2m x 1/8 inch diameter) followed by a Molecular Sieve 5A column (6m x 1/8 inch) with argon as carrier gas. Detection limit on the instrument with a 250 µl injection is 5×10^{-9} Litre (20 ppm).

2.3.6 Nitrogen

Nitrogen is analysed on a Star 3400CX Varian gas chromatograph (Varian AB, Sweden) using a thermal conductivity detector (TCD) with an oven temperature of 65°C, a detector temperature of 120°C, and a filament temperature of 250°C. The nitrogen gas is separated using a Porapak Q column (2m x 1/8 inch diameter) followed by a Molecular Sieve 5A column (6m x 1/8 inch). Argon or helium can be used as carrier gas. The result obtained with argon is compared to the result obtained with helium and reported when they agree. Detection limit on the instrument with a 250 µl injection is 25×10^{-9} Litre (100 ppm).

2.3.7 Argon

Argon is analysed on a Star 3400CX Varian gas chromatograph (Varian AB, Sweden) using a thermal conductivity detector (TCD) with an oven temperature of 65°C, a detector temperature of 120°C, and a filament temperature of 250°C. The argon gas is separated using a Porapak Q column (2m x 1/8 inch diameter) followed by a Molecular Sieve 5A column (6m x 1/8 inch) with helium as carrier gas. Argon is very difficult to

separate from oxygen when present. The strategy is to analyse the total amount of oxygen and argon with this configuration. Then the result is reduced with the amount of oxygen analysed using argon as carrier gas. Detection limit on the instrument with a 250 µl injection is 25 x 10^{-9} Litre (100 ppm).

2.3.8 Carbon dioxide

Carbon dioxide is analysed on a Star 3400CX Varian gas chromatograph (Varian AB, Sweden) using a flame ionization detector (FID) with an oven temperature of 65°C and a detector temperature of 200°C. The carbon dioxide gas is separated using a Porapak Q column (2m x 1/8 inch diameter) and transformed to methane using a 10% Ni_2NO_3 "Methanizer" fed with hydrogen gas (9.375" x 1/8" diameter, temperature 370°C). Carbon dioxide is finally analysed as methane in on the FID with nitrogen as carrier gas. Detection limit on the instrument with a 250 µl injection is 0.1 x 10^{-9} Litre (0.4 ppm).

2.3.9 Methane

Methane is analysed on a Star 3400CX Varian gas chromatograph (Varian AB, Sweden) using a flame ionization detector (FID) with an oven temperature of 65°C and a detector temperature of 200°C. The methane gas is separated using a Porapak Q column (2m x 1/8 inch diameter) and analysed on the FID with nitrogen as carrier gas. Detection limit on the instrument with a 250 µl injection is 0.1 x 10^{-9} Litre (0.4 ppm).

2.3.10 Ethane, ethene+ethylene, propane and propene

Ethane, ethane+ethylene, propane and propene are analysed on a Star 3400CX Varian gas chromatograph (Varian AB, Sweden) using a flame ionization detector (FID) with an oven temperature of 65°C and a detector temperature of 200°C. The ethane, ethane, propane and propene gases are separated using a Porapak Q column (2m x 1/8 inch diameter) and analysed on the FID with nitrogen as carrier gas. Ethene and ethylene cannot be separated with the present configuration (Porapack Q). Detection limit on the instrument with a 250 µl injection is 0.1 x 10^{-9} Litre (0.4 ppm).

2.4 Sampling of microorganisms

A standard PVB sampler was pressurized with 200 KPa nitrogen in the lower compartment and subsequently attached to the respective borehole circulation day 1, inside the refrigerator (see Fig. 2-10 Pedersen, 2005). With 200 KPa nitrogen, the resulting sampling volume became approximately 180 ml. When installed, the sampler was filled and emptied three times, and then filled. After the last filling, circulation through the PVB was continued at about 30 ml/min over night. At morning day 2, the PVB was closed and detached. It was transported under cool conditions by car to the laboratory in Göteborg and sampling for analysis was generally started between 14.00 – 15.00 the same day. A set of 15 numbered anaerobic aluminium seal tubes (Bellco glass Inc. (www.bellcoglass.com), product number 2048-00150) with butyl rubber stoppers (product number 2048-117800) crimped with aluminium seals (product number 2048-11020) were filled with 10-12 ml groundwater sample each.

2.5 Analysis of microorganims

2.5.1 Biomass – total counts

Total cell numbers were determined using the acridine orange direct count method (AODC) (Hobbie et al, 1977; Pedersen and Ekendahl, 1990). The three first filled tubes were used. From each tube 1 ml were filtered onto a black polycarbonate filters (0.22 µm) and stained with 0.2 mL acridine orange solution (10 µL/ml) for six minutes and then rinsed with double distilled water (DDW). On each filter, 30 sight field areas of 10^4 μm^2 were counted using a Zeiss epifluorescence microscope with a blue filter (530 nm). Two filters were prepared per tube. The results were calculated from an average of six filters from three tubes for each groundwater sample.

2.5.2 Biomass – ATP

Groundwater for ATP extraction analysis was collected in triplicate from one sample tube (2.4) and transferred into a sterile 15 mL Falcon centrifuge tube. Extraction and analysis of ATP concentrations were based on the methods by Lundin *et al*. (1986) and Lundin (2000). ATP was extracted from 100 µL aliquots of groundwater within an hour of collection using 100 µL BS-buffer (BioThema ATP Biomass Kit HS, Sweden). After extraction, 100 µL of the ATP extract was mixed with 400 µL of HS-buffer (BioThema ATP Biomass Kit HS, Sweden) in a FB12/Sirius luminometer (Berthold, Germany). The total volume of groundwater in the sample then became 50 µl. Prior to each sample measurement, light emission of the 400 µL HS-buffer without sample addition was allowed to diminish to less than 50 relative light units per second (rlu/s) in the luminometer. After addition of the ATP extracted sample, light emission was immediately determined using FB/Sirius PC Software quick measurement (Berthold, Germany). To account for attenuating effects from the sample on the enzymatic reactions (e.g. salinity, dissolved metals, sulphide), and to calibrate the luminometer readings, 10 µL of internal ATP-standard (0.1 µmole/L) was added to the extracted sample in the luminometer and light emission was measured again. All light emission measurements were performed three times. The equation used to calculate ATP-concentration in the sample was based on the average of three measurements as follows:

$$\text{pmol ATP / sample} = \frac{I_{smp} - I_{bkg}}{I_{(std + smp - bkg)} - I_{(smp - bkg)}} \qquad \text{(eq. 1)}$$

Where:

I: *light intensity measured as relative light units*
smp: *sample*
bkg: *background with the HS buffer*
std: *standard*

The amount of ATP per ml groundwater was calculated as:

amol/ml groundwater = pmol ATP/sample (from eq. 1) x SF x DF (eq. 2)

Where:

SF: *shift factor from pmol to amol = 10^6*
DF: *dilution factor to obtain amount of ATP per ml =1000/50*

2.5.3 Preparation of media for most probable numbers of cultivable anaerobic microorganisms

Media for the most probable number of organisms (MPN) in groundwater from MICROBE were composed based on previously measured chemical data from the site. This allowed artificial media to be composed that most closely resembled *in situ* groundwater chemistry for optimal microbial cultivation (Haveman and Pedersen, 2002). Media for the metabolic groups of nitrate reducing bacteria (NRB), iron reducing bacteria (IRB), manganese reducing bacteria (MRB), sulphate reducing bacteria (SRB), autotrophic acetogens (AA), heterotrophic acetogens (HA), autotrophic methanogens (AM) and heterotrophic methanogens (HM) were autoclaved and dispensed according to the various compositions outlined in Table 2-1 in anaerobically into 27 mL anaerobic aluminium seal tubes (Bellco glass Inc. (www.bellcoglass.com), product number 2048-00150) with butyl rubber stoppers (product number 2048-117800) crimped with aluminium seals (product number 2048-11020).

All culture tubes were flushed with 80/20% N_2/CO_2 gas and then filled with 9 mL of their respective media. For IRB, 1 mL of hydrous ferric oxide (HFO) was added to each culture tube. The HFO was prepared from $FeCl_3$. The final concentration of the iron solution was 0,44 M. For MRB, 2 mL of 135 mM MnO_2 solution (Lovley and Phillips, 1986) was added. The HM media also contained 20 mL/L of 100 g/L NaCOO, 3 mL/L 6470 mM trimethylamine, 4 mL/L methanol and 20 mL/L of a 20g/L solution of $NaCH_3COO$. The HA medium also contained 20 mL/L of 100 g/L NaCOO, 3 mL/L 6470 mM trimethylamine and 4 mL/L methanol. The final pH was adjusted to between 7 to 7.5 with 1 M HCl or 1 M NaOH.

2.5.4 Inoculations and analysis for anaerobic microorganisms

Inoculations for NRB, IRB, MRB, SRB, AA, HA, AM and HM were performed in the laboratory within six hours from sample collection for all boreholes. After inoculation, the headspace of only AA and AM was added with 80/20% H_2/CO_2 to an overpressure of 2 bars and all MPN tubes were incubated in the dark at 17 °C for 8 weeks. Analysis of the MPN tubes after incubation was performed by detecting metabolic products. The production of nitrate was determined using a HACH DR/2500, The chromotrophic acid method 10020 for water and waste water (0.2 – 30 mg/L NO_3^--N). The production of ferrous iron by IRB was determined using a HACH DR/2500 spectrophotometer (HACH company, Loveland Colorado, USA) and the 1,10 phenanthroline method (method no. 8146). Ferrous iron at concentrations twice that in the un-inoculated control tubes were taken as positive for IRB. The HACH method 8034 based on periodate oxidation was used in a similar way to determine Mn^{2+} concentrations in MPN tubes for MRB. Detection of SRB was assessed by measuring sulphide production using the $CuSO_4$ method according to Widdel & Bak (1992) on a UV visible spectrophotometer (Ultraspec 2000, Amersham Pharmacia Biotech). Methanogens were detected by the production of CH_4 in the culture tubes headspace by gas chromatography as previously described in 2.3.9. Acetogens were detected by acetic acid production using an enzymatic UV method (Enzymatic Bioanalysis kit, Boehringer Mannheim, Germany) using a UV visible spectrophotometer (as per SRB).

The MPN procedure results in a scheme with tubes that score positive or negative growth. The results from the analyses were graded positive or negative in comparison to a control. Three dilutions were used to calculate the most probable number of each respective group according to the calculations found in Greenberg *et al.* (1992).

Table 2-1. Composition of anaerobic media used for MPN cultivation of different metabolic groups of anaerobic microorganisms. All components were anoxic.

A) Ready medium	Metabolic group[a]				
Component (mL/L)	**NRB**	**IRB / MRB**	**SRB**	**AA & HA**	**AM & HM**
Basal medium (Table B)	925	940	960	860	890
Trace elements (Table C)	1.0	1.0	1.0	--	--
Trace elements (Table D)	--	--	--	10	10
Vitamins (Table E)	1.0	1.0	1.0	--	--
Vitamins (Table F)	--	--	--	10	10
Thiamine stock (Table G)	1.0	1.0	1.0	1.0	1.0
vitamin B_{12} stock (Table G)	1.0	1.0	1.0	1.0	1.0
Fe stock (Table G)	-	--	--	5.0	5.0
Resazurin (Table G)	-	--	1.0	2.0	2.0
cystein hydrochloride (Table G)	-	--	--	10	10
bromoethansulfonic (Table G)	-	--	--	30	--
Selenite-tungstate (Table G)	-	--	1.0	--	--
$NaHCO_3$ (Table G)	30	30	30	60	60
Yeast extract (Table G)	1.0	1.0	--	10	10
$NaCH_3COO$ (Table G)	25	25	--	--	--
Lactate (Table G)	5.0	--	5.0	--	--
KNO_3 (G)	10				
Sodium sulphide (0.2 M)			7.5	10	10

[a]NRB=nitrate reducing bacteria, IRB = iron reducing bacteria, MRB = manganese reducing bacteria, AA = autotrophic acetogens, HA = heterotrophic acetogens, AM = autotrophic methanogens, HM = heterotrophic methanogens

B) Basal medium	Metabolic group			
Component (g)	**NRB, IRB & MRB**	**SRB**	**AA & HA**	**AM & HM**
Double distilled H_2O	1000	1000	1000	1000
NaCl	15	15	15	15
$CaCl_2x2H_2O$	1.0	0.1	0.28	0.28
KCl	0.1	0.5	0.67	0.67
NH_4Cl	1.5	0.25	1.0	1.0
KH_2PO_4	0.6	--	--	--
K_2HPO_4	--	0.2	0.17	0.15
$MgCl_2x6H_2O$	0.1	1.2	5.5	5.5
$MgSO_4x7H_2O$	0.1	--	6.9	6.9
$MnCl_2x4H_2O$	0.005	--	--	--
$Na_2MoO_4x2H_2O$	0.001	--	--	--
Na_2SO_4	--	4.0	--	--

C) Trace element solution	
Component	**Amount**
Double distilled H_2O	1000 mL
Nitrilotriacetic acid	1500 mg
$Fe(NH_4)_2(SO_4)_2x6H_2O$	200 mg
Na_2SeO_3	200 mg
$CoCl_2x6H_2O$	100 mg
$MnCl_2x4H_2O$	100 mg
$Na_2MoO_4x2H_2O$	100 mg
$Na_2WO_4x2H_2O$	100 mg
$ZnSO_4x7H_2O$	100 mg
$AlCl_3$	40 mg
$NiCl_2x6H_2O$	25 mg
H_3BO_3	10 mg
$CuCl_2x2H_2O$	10 mg

D) Non-chelated trace element	
Component	**Amount**
Double distilled H_2O	987 mL
HCl (25%=7,7M)	12,5 mL
$FeSO_4x7H_2O$	2,1 g
H_3BO_3	30 mg
$MnCl_2x4H_2O$	100 mg
$CoCl_2x6H_2O$	190 mg
$NiCl_2x6H_2O$	24 mg
$CuCl_2x2H_2O$	2 mg
$ZnSO_4x7H_2O$	144 mg
$Na_2MoO_4x2H_2O$	36 mg

E) Vitamin mixture for NRB, IRB, MRB & SRB	
Component	**Amount**
Sodium phosphate buffer 10mM pH 7.1	100 mL
4-Aminobenzoic acid	4 mg
D(+)-biotin	1 mg
Nicotinic acid	10 mg
Pyridoxine dihydrochloride	15 mg
Calcium D(+)-pantothenate	5 mg

F) Vitamin mixture for AA, HA, AM & HM	
Component	**Amount**
Sodium phosphate buffer 10mM pH 7.1	100 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

G) Stock solutions	
Component	**Amount**
$NaHCO_3$	84 g/L
Thiamine chloride dihydrochloride in a 25 mM sodium phosphate buffer, pH 3.4	100 mg/L
Cyanocobalamine (B_{12})	50 mg/L
KNO_3	1000 mg/L
$NaCH_3COO$	100g/L
Yeast extract	50 mg/L
$Fe(NH_4)_2(SO_4)_2x6H_2O$,initially dissolved in 0.1 mL concentrated HCl	20 g/L
Resazurin	500 mg/L
Cysteine-HCl	500 mg/L
Bromoethansulfonic (BESA)	35 g/L
Sodium lactate solution	50%

3 Results and evaluation

3.1 Results

3.1.1 Chemistry

The concentrations of chloride showed significant increases for all three boreholes (Figure 3-1). KJ0052F03 showed the largest increase from just below 7000 mg/L up to 8500 mg/L. The smallest increase was connected to KJ0052F01 that increased about 500 mg/L. KJ0050F01 increased about 1000 mg/L. The conductivity data showed similar increases (Fig 3-2).

The long term trends of chloride concentrations in the boreholes are shown in Fig 3-3. There has been a slow increase, in particular for KJ0050F01 and KJ0052F01 since drilling of the boreholes. The data are sparse and it is not possible to evaluate if this increase was continuous or has more of a momentary character. There is a possibility that drilling of the APSE boreholes KF0066A01 and KF0069A01 early in 2003 may have short-circuited with the MICROBE boreholes. The groundwater of those boreholes has a deep chemical signature similar to that analysed in KAS03 during the pre-investigation stage. The chloride concentrations measured in august 2003 in KF0066A01 and KF0069A01 were 11652 and 10809 mg/L respectively.

The MINICAN boreholes were drilled in January 2005. The short term survey of chloride started in June 2005. Figure 3-3 shows that the MINICAN disturbance is very strong for KJ0052F03 and also large for KJ0050F01. KJ0052F01 increased initially, but seems to level out at about 9500 mg/L.

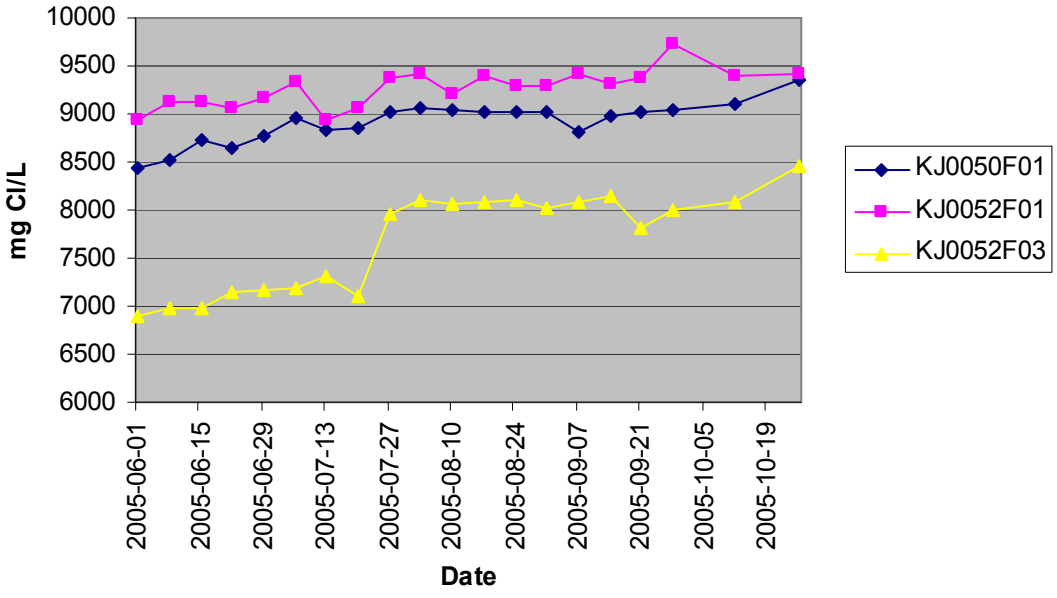

Figure 3-1. *Concentration of chloride over time in the groundwater.*

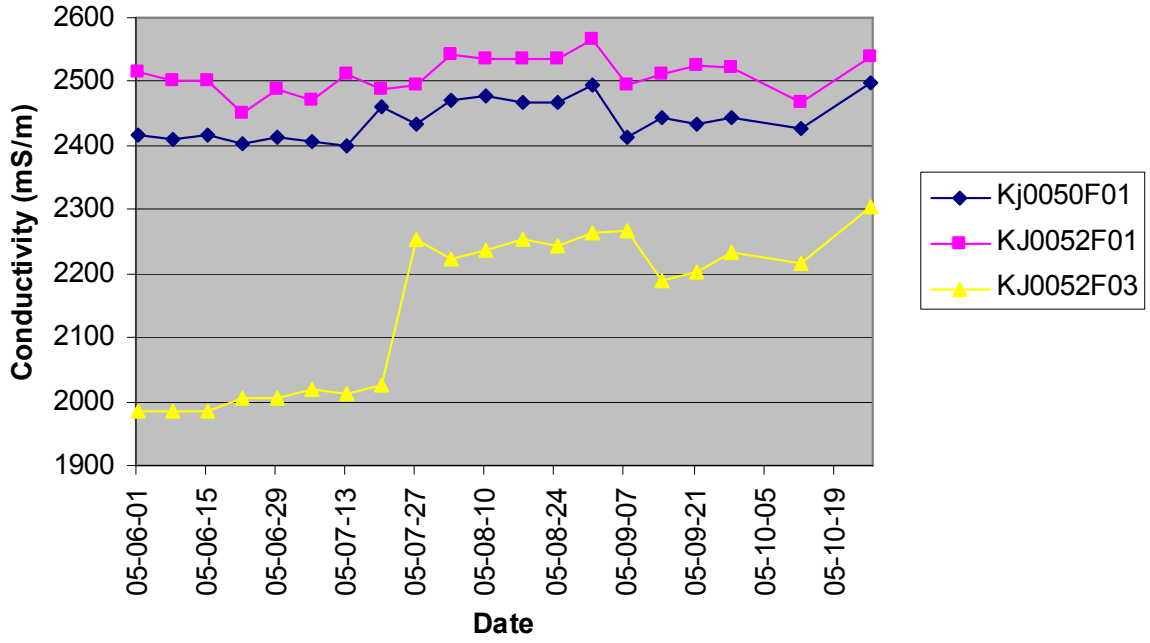

***Figure 3-2**. Conductivity over time of the groundwater.*

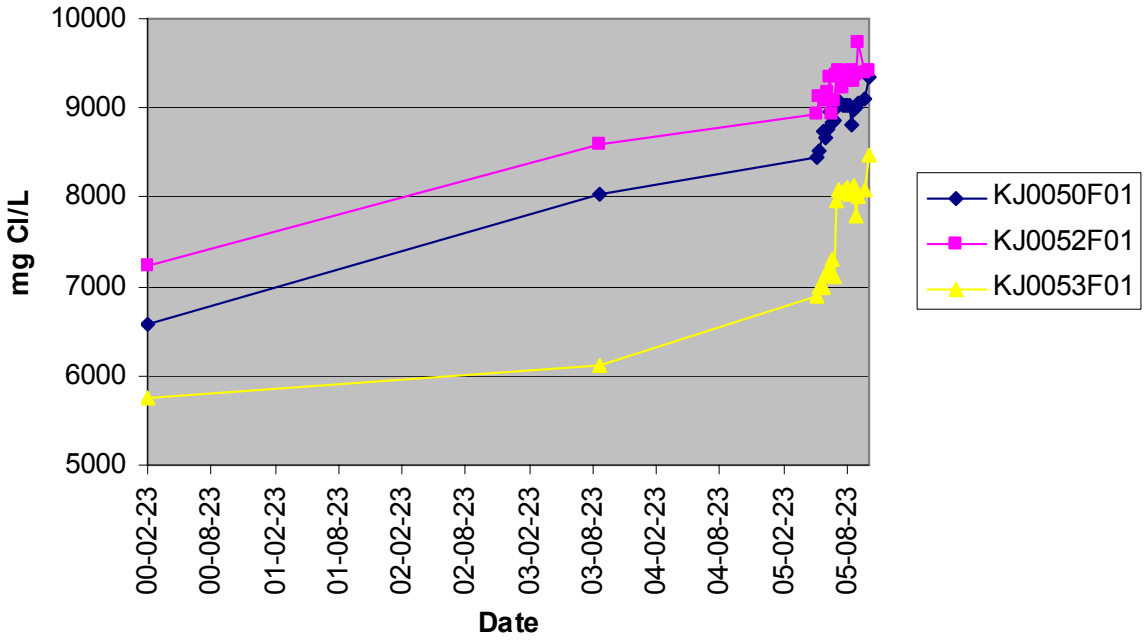

***Figure 3-3.** The long term increase of chloride in the groundwater.*

3.1.2 Gas

The volumes of dissolved gas show some changes over time (table 3-1). The respective components also show variations. In particular, hydrogen was increasing in KJ0052F01. Else, the variability shows no clear trends.

Table 3-1. Gas content in the groundwater.

Borehole	Sample date	volume	H_2	He	Ar	N_2	CO_2	CH_4	C_2H_{2-4}	C_2H_6
		mL/L	µL/L	mL/L	mL/L	mL/L	mL/L	mL/L	µL/L	µL/L
KJ0050F01	050524	44.9	44.4	6.46	0.808	37.2	0.044	0.330	0.127	0.037
KJ0050F01	050606	39.1	67.2	7.51	0.627	30.8	0.037	0.318	0.034	0.074
KJ0050F01	050830	39.0	19.1	8.13	0.618	33.1	0.019	0.283	0.040	0.090
KJ0052F01	050524	51.3	13.1	9.15	0.651	41.2	0.027	0.281	0.083	0.049
KJ0052F01	050606	41.2	46.0	7.38	0.876	32.8	0.017	0.272	0.010	0.060
KJ0052F01	050830	53.2	57.9	10.8	0.508	43.9	0.024	0.226	0.020	0.820
KJ0052F03	050524	42.7	1.14	7.02	0.368	35.0	0.030	0.316	0.093	0.055
KJ0052F03	050606	30.9	0,72	4.33	0.479	26.0	0.012	0.200	0.007	0.044

3.1.3 Microorganisms

The total numbers of microorganisms decreased in KJ0052F01, was almost indifferent in KJ0052F03 and varied without a clear trend in KJ0050F01 (Fig. 3-4). There was no clear trend for ATP in KJ0050F01 and KJ0052F01, while KJ0052F03 showed an increasing trend (Fig 3-5).

The MPN data was very reproducible for the two first measuring occasions (November and early February. Figs 3-6 to 3-11). The two last measurements were all variable. Consequently, after the MINICAN boreholes were drilled, the stability from the two first sampling times were not reproduced.

Some clear trends were observed. IRB and MRB increased in KJ0052F01. SRB decreased in all boreholes from several thousands per ml down to numbers at or below detection. This is consistent with the loss of a heavy smell of sulphide from the groundwater in all boreholes. Further, there was a clear decrease in the numbers of acetogens in KJ0050F01.

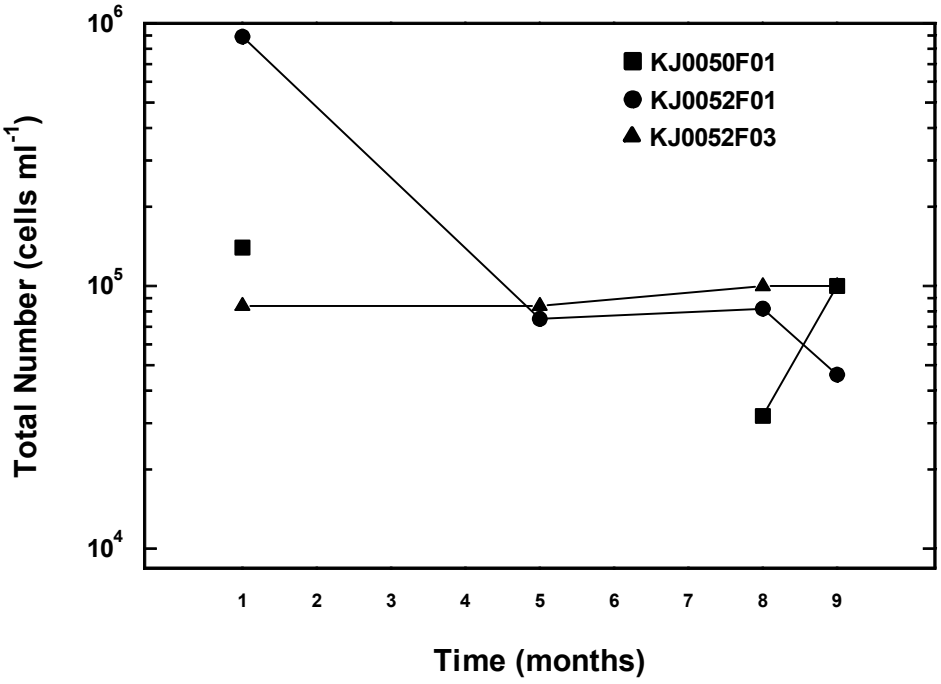

Figure 3-4. *Total numbers of microorganisms in the groundwater.*

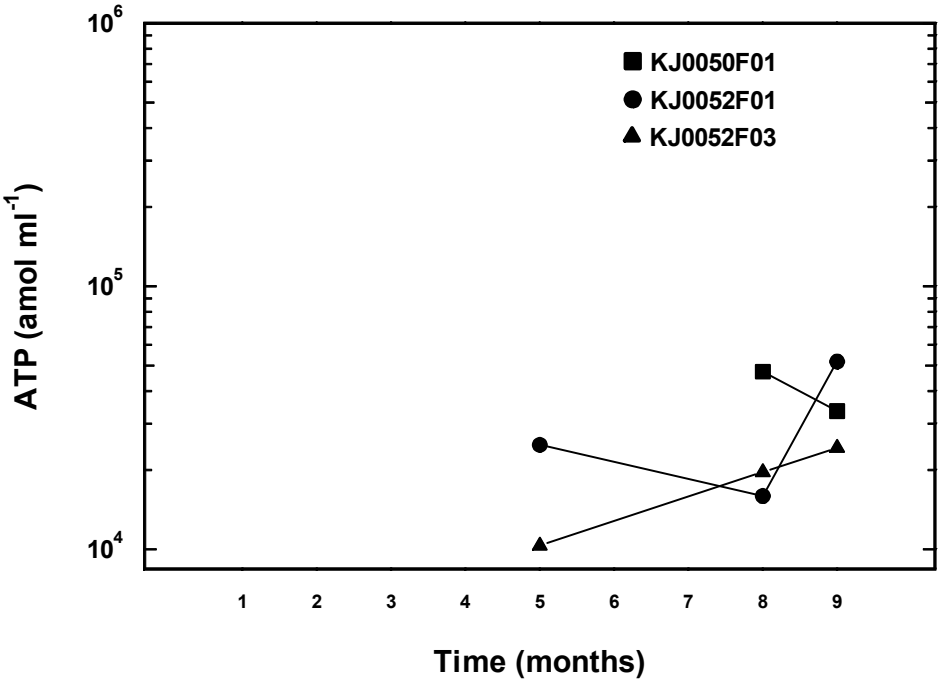

Figure 3-5. *Amounts of ATP in the groundwater.*

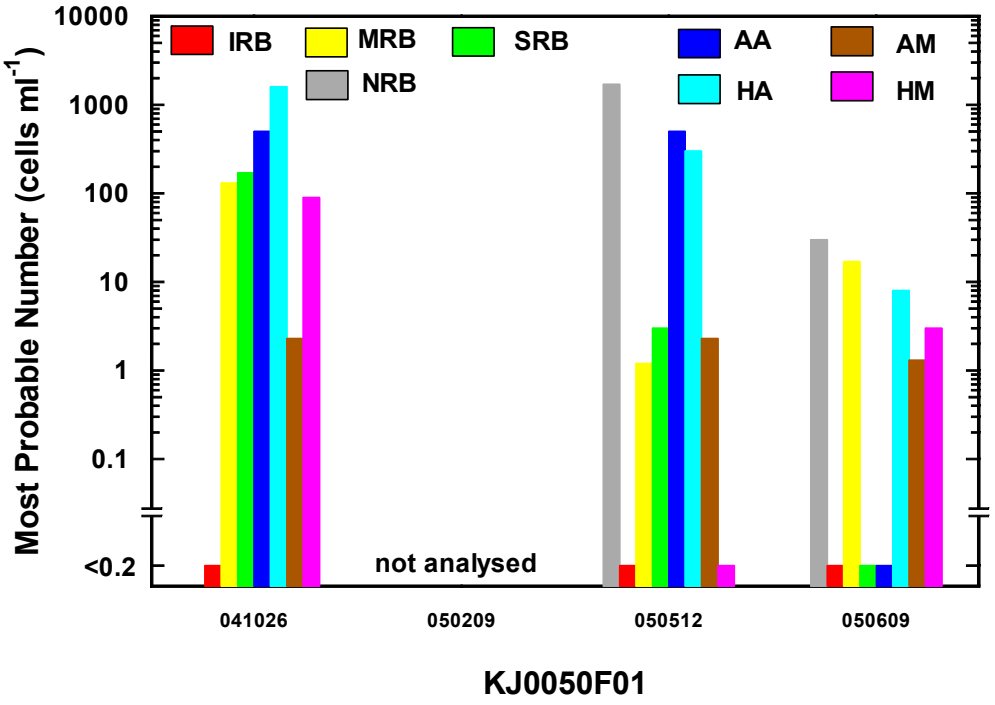

Figure 3-6. *Most probable number of physiological groups in KJ0050F01.*

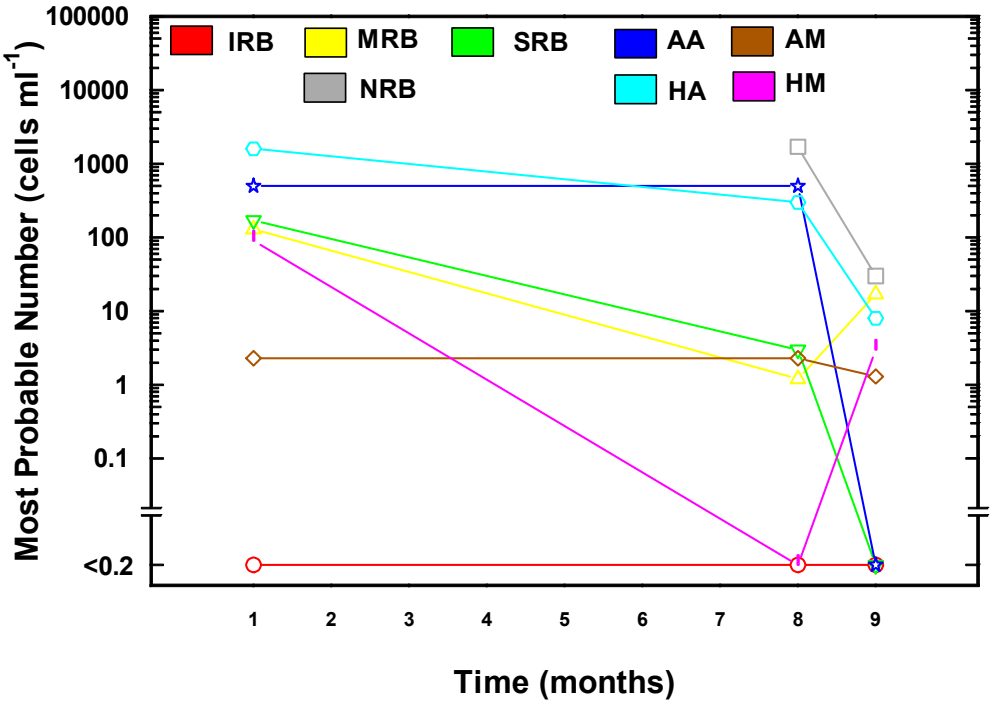

Figure 3-7. *Most probable number of physiological groups in KJ0050F01.*

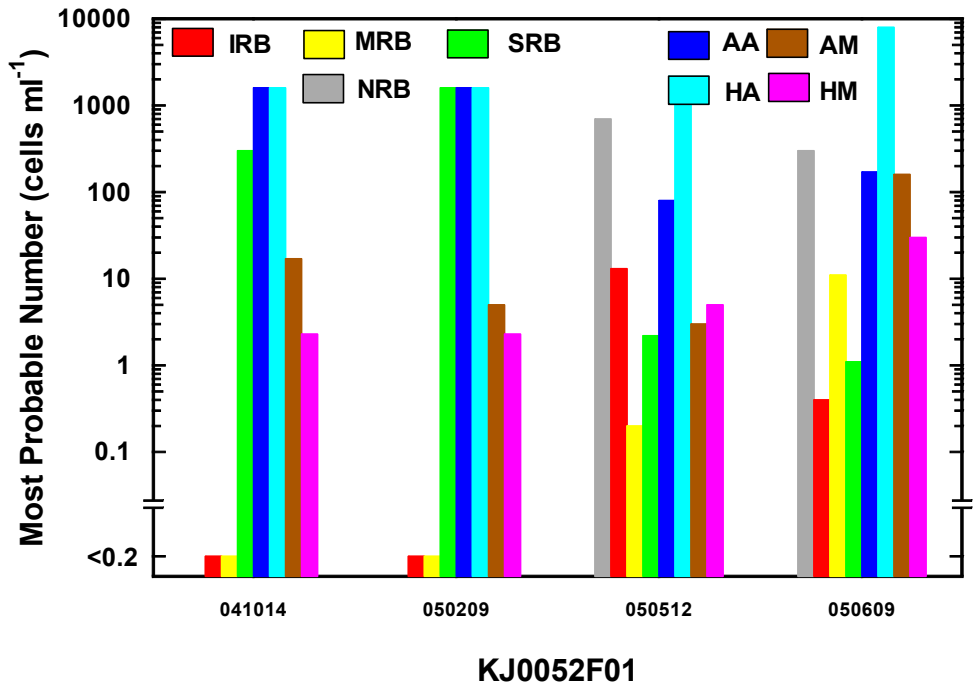

Figure 3-8. *Most probable number of physiological groups in KJ0052F01.*

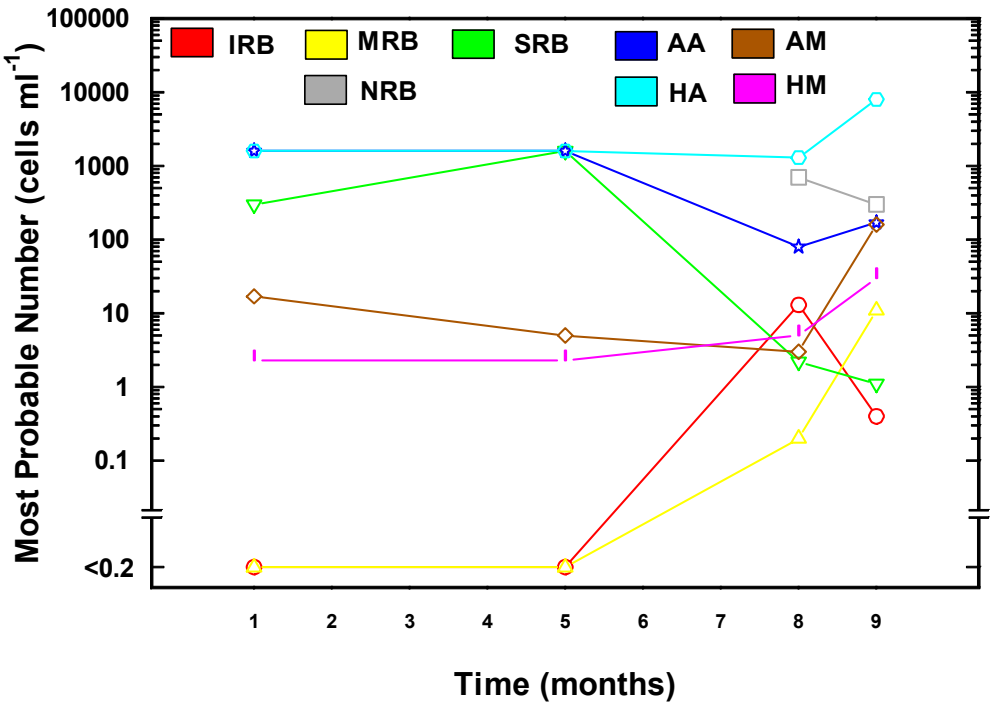

Figure 3-9. *Most probable number of physiological groups in KJ0052F01.*

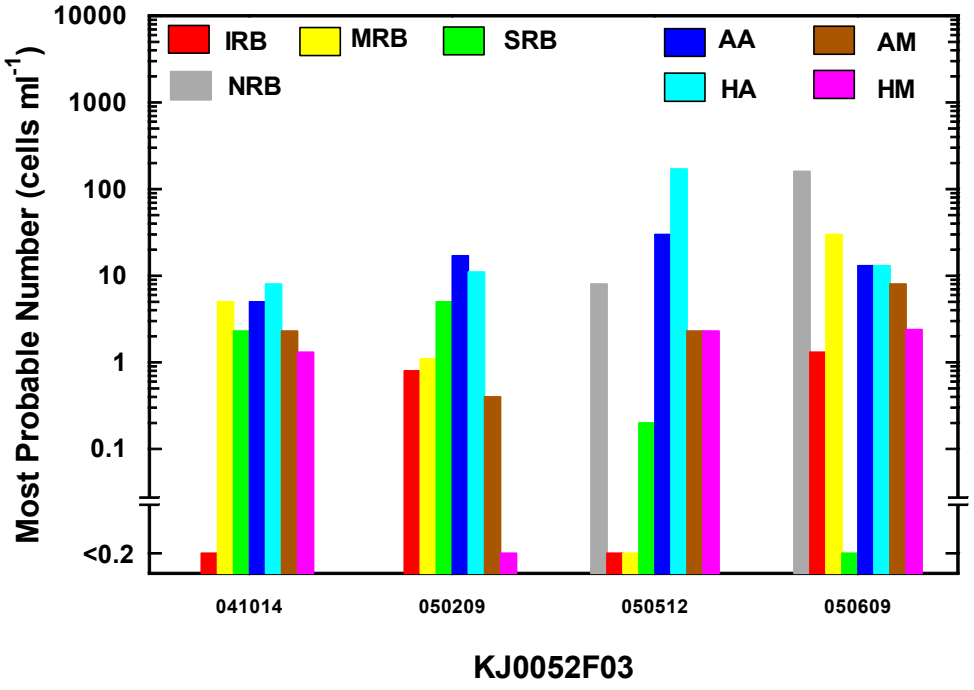

Figure 3-10. *Most probable number of physiological groups in KJ0052F03.*

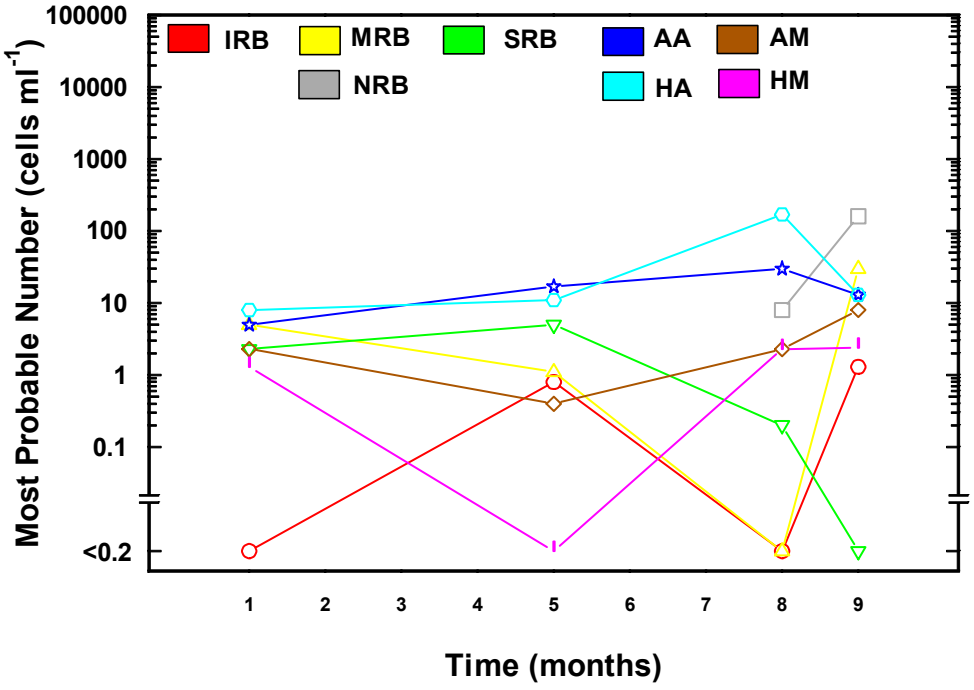

Figure 3-11. *Most probable number of physiological groups in KJ0052F03.*

3.2 Evaluation of the results

In general, the results confirm that the MINICAN drainage has generated a very large disturbance on the MICROBE site. Most previously collected data on chemistry, gas and microbes is no longer valid. A complete new characterisation must be performed when the drainage and pressure conditions have been restored.

3.2.1 Chemistry

The increases in chloride concentrations indicate that deeper and saltier water is moving up from large depths via KA3386A01 and out through the fractures around the MINICAN boreholes. The drainage was very large and calculations show that 15 000m^3 of groundwater have left the MICROBE formation. This groundwater must of course be replaced. As the chloride concentrations are rising, it suggests that deeper groundwater is replacing the drained water. Part of the water that replace the drained water can originate from more shallow aquifers. It remains to analyse the mixing history with M3 modelling. This should be done when the pressure and drainage conditions have been restored. Complete class 5 analyses must be performed to establish the new chemical conditions that have originated as a result of the MINICAN drainage.

3.2.2 Gas analysis

Deeper water is expected to carry more hydrogen and noble gases, and less CO_2. Although the gas data are a bit erratic, some individual analyses suggest that deeper water is moving up towards the MICROBE boreholes. However, intensive mixing is expected with the measured drainage and this mixing may introduce variability over time. Variability of groundwater pressure due to opening and closing of boreholes, tide effects etc. may introduce wobbling effects over time that masks clear trends.

3.2.3 Microbiology

The data on microbes has changed from reproducible to very variable over time. This is a predicable effect from the increased groundwater flow and mixing due to the MINICAN drainage. Microorganisms are very sensitive to changes in the environmental conditions. They respond quickly with growth or death when new conditions evolve. New mixing conditions and increased flow may trigger biofilm development. Erratic detachment from such biofilms will introduce strong variability in samples that is difficult to overcome. Each set of microbial analyses are very time and cost consuming which hinder repeated measurements. In a system with variable mixing conditions as discussed for the gas, microbiology data will vary significantly over time, just as found in this investigation.

New analyses of microbes must be performed once the conditions have returned to those prevailing before the MINICAN. However, it will be a totally new situation, because the mixing of new, deeper water into the MICROBE formation has introduced a completely new situation at the MICROBE laboratory.

4 Conclusions

KJ0052F03 showed the largest overall disturbance of the three boreholes. It also harbours the lowest numbers of cultivable microorganisms. This borehole is presently too disturbed to be used for research.

KJ0050F01 showed moderate overall disturbance. Provided SRB will return, it can be chosen for experiments with biocorrosion of copper and possibly migration (MICOMIG) experiments.

KJ0052F01 showed the lowest overall disturbance. This borehole is best suited for the planned redox experiments (MICORED) and it may also be used for migration (MICOMIG) experiments.

5 References

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6 Appendix

Table 5-1. Chemistry data for chloride and conductivity

Date	Chloride concentration (mg Cl/L)			Conductivity (mS/m)		
	KJ0050F01	KJ0052F01	KJ0052F03	KJ0050F01	KJ0052F01	KJ0052F03
2000-02-23	6580	7230	5750	1800	1930	1610
2003-09-15	8040	8590	6110	2140	2231	1700
2005-06-01	8442	8933	6896	2416	2515	1986
2005-06-08	8519	9132	6980	2411	2503	1984
2005-06-15	8735	-	-	2416	-	-
2005-06-22	8655	9069	7147	2402	2451	2006
2005-06-29	8772	9177	7176	2412	2488	2006
2005-07-06	8956	9344	7181	2408	2471	2019
2005-07-13	8824	8936	7303	2398	2511	2011
2005-07-20	8853	9067	7114	2460	2487	2027
2005-07-27	9015	9368	7962	2432	2493	2253
2005-08-03	9069	9423	8094	2470	2541	2222
2005-08-10	9032	9215	8052	2478	2536	2238
2005-08-17	9031	9392	8085	2466	2537	2255
2005-08-24	9022	9299	8100	2467	2535	2242
2005-08-31	9019	9301	8024	2496	2565	2264
2005-09-07	8819	9411	8090	2412	2493	2266
2005-09-14	8980	9305	8137	2445	2511	2189
2005-09-21	9017	9377	7803	2433	2525	2203
2005-09-28	9049	9730	8002	2443	2521	2234
2005-10-12	9100	9391	8083	2428	2469	2216
2005-10-26	9356	9423	8464	2498	2538	2303

Table 5-2. Total number of cells and concentration of ATP

Borehole (sample date)	Total counts (cells ml^{-1})			ATP (amol ml^{-1})		
	AODC	Standard deviation	Number of observations	ATP	Standard deviation	Number of observations
KJ0050F01 (2004-10-26)	140000	42000	6	-	-	-
KJ0050F01 (2005-02-09)	-	-	-	-	-	-
KJ0050F01 (2005-05-12)	32000	17000	6	47348	1409	3
KJ0050F01 (2004-06-09)	100000	9500	6	33573	9836	3
KJ0052F01 (2004-10-14)	890000	170000	6	-	-	-
KJ0052F01 (2005-02-09)	75000	29000	6	24959	18247	3
KJ0052F01 (2005-05-12)	82000	36000	6	15954	4416	3
KJ0052F01 (2004-06-09)	46000	22000	6	51751	13175	3
KJ0052F03 (2004-10-14)	84000	27000	6	-	-	-
KJ0052F03 (2005-02-09)	84000	20000	6	10340	580	3
KJ0052F03 (2005-05-12)	100000	28000	6	19666	2656	3
KJ0052F03 (2004-06-09)	110000	18000	6	24299	3405	3

Table 5-3. Most probable number (MPN) of metabolic groups of microorganisms in KJ0050F01

Metabolic groups	Cells ml^{-1}			
	2004-10-26	**2005-02-09**	**2005-05-12**	**2005-06-06**
Nitrate reducing bacteria	-	-	1700	30
Iron reducing bacteria	<0.2	-	<0.2	<0.2
Manganese reducing bacteria	130	-	1.2	17
Sulphate reducing bacteria	170	-	3.0	<0.2
Autotrophic acetogens	500	-	500	<0.2
Heterotrophic acetogens	1600	-	300	8.0
Autotrophic methanogens	2.3	-	2.3	1.3
Heterotrophic methanogens	90	-	<0.2	3.0

Table 5-4. Most probable number (MPN) of metabolic groups of microorganisms in KJ0052F01

Metabolic groups	Cells ml^{-1}			
	2004-10-26	**2005-02-09**	**2005-05-12**	**2005-06-06**
Nitrate reducing bacteria	-	-	700	300
Iron reducing bacteria	<0.2	<0.2	13	0.4
Manganese reducing bacteria	<0.2	<0.2	0.2	11
Sulphate reducing bacteria	300	1600	2.2	1.1
Autotrophic acetogens	1600	1600	80	170
Heterotrophic acetogens	1600	1600	1300	8000
Autotrophic methanogens	17	5.0	3.0	160
Heterotrophic methanogens	2.3	2.3	5.0	30

Table 5-5. Most probable number (MPN) of metabolic groups of microorganisms in KJ0052F03

Metabolic groups	Cells ml^{-1}			
	2004-10-26	**2005-02-09**	**2005-05-12**	**2005-06-06**
Nitrate reducing bacteria	-	-	8.0	160
Iron reducing bacteria	<0.2	0.8	<0.2	1.3
Manganese reducing bacteria	5.0	1.1	<0.2	30
Sulphate reducing bacteria	2.3	5.0	0.2	<0.2
Autotrophic acetogens	5.0	17	30	13
Heterotrophic acetogens	8.0	11	170	13
Autotrophic methanogens	2.3	0.4	2.3	8.0
Heterotrophic methanogens	1.3	<0.2	2.3	2.4