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Microbial oxygen reduction during the REX field experiment

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Keywords: bacteria, groundwater, kinetic, methane, methanotrophs, microorganisms

Abstract

This report describes microbiological characterisation and quantitative tests of microbial oxygen reduction before (March -April 1998) and during the Redox EXperiment in detailed scale (REX) (July 1998-August 1999) in comparison with anaerobic groundwater sampled simultaneously from the inner section of KA2862A. The groundwater contained abundant and diverse microbial populations, which included both strict anaerobic ($2.5x10^2$-$7.7x10^2$ cells ml^{-1}), facultative aerobic ($3.3x10^2$-$8.5\ x10^4$ cells ml^{-1}), and microaerophilic organisms ($1.5x10^2$-$8.0\ x10^4$ cells ml^{-1}). Total counts of free-living microbes developed from $2.5x10^3$ up to $7.7x10^5$ cells ml^{-1} with oxidation of the REX groundwater. Oxygenation induced adaptive succession of the microbial population from anaerobic to microaerophilic and aerobic. Methanogens, microaerophilic heterotrophic, sulfate-and iron-reducing bacteria were replaced with methanotrophs, hydrogen-oxidising and aerobic heterotrophic bacteria after oxygen pulses in the REX chamber groundwater. The representation of a certain microbial group partly depended the on redox potential in the chamber. Methanotrophs, hydrogen-oxidising and heterotrophic organisms constituted up to 12%, 52% and 75% of total microbial count in the REX groundwater, respectively. Acetate and formate were actively used in the presence in oxygen. Addition of methane and hydrogen at *in situ* concentrations stimulated microbial oxygen reduction in REX groundwater up to two or three times. Methane oxidation rate in REX groundwater exceeded that in the anaerobic KA2862A groundwater by 3-4 orders of magnitude. Reducing capacity of microbial reactions was modelled and calculated in accordance with available electron donors, microbial activities and viable competent organisms. Oxygen was reduced (0.46-1.77 $\mu M\ day^{-1}$), carbon dioxide was produced (0.47-1.50 $\mu M\ day^{-1}$) and methane oxidised (0.49-3.33 $\mu M\ day^{-1}$) by unattached microbes in the REX groundwater *in vitro*. Oxygen uptake rate *in situ* exceeded the rates measured *in vitro* batch experiments by 2 orders of magnitude. The microbial Oxygen Reduction Coefficient (MORC) was a reproducible parameter and ranged from $1.17x10^{-9}$ to $2.53x10^{-9}$ μM of oxygen per cell per day in the REX groundwater. Microscopic and culturing results indicated the presence of biofilms on the surface of the drill core and the gold cap. The total number of attached and unattached cells inside the REX chamber in PEEK tubing, on the granite core and gold cap surfaces was $6.81x10^{10}$ cells. Viable counts in biofilms on tubes constituted 91% of whole microbial population. The drill core and the gold cap biofilms contained 8 % while the groundwater microorganisms constituted only 1% of the total microbiota. The viable numbers of attached cells in the REX chamber exceeded the numbers of unattached cells. Antimicrobial agents used under *in situ* conditions reduced partly the oxygen consumption. Culturing results showed that the microbial activity was not inhibited completely. Inhibition of viable microbial cells was 54% in the REX groundwater and 92% on REX tubes as determined by a most probable numbering method. Residual oxygen reduction observed *in situ* took place due to the microbial reduction of oxygen both in the groundwater and in the biofilms. Empirical model and results of the inhibiting experiment *in situ* suggest that attached and unattached organisms could be responsible for some or most of the oxygen uptake observed in the REX chamber.

KEYWORDS: microorganisms, groundwater, biofilms, molecular oxygen reduction, methane, hydrogen, oxidation, adaptive succession of population

Summary and conclusions

- The REX groundwater contained electron and carbon sources available to be oxidised with oxygen by the microbial groups present in the groundwater.
- Oxygenation stimulated a quantitative development of the microbial population in the groundwater. Total unattached microbial counts in the REX chamber increased in comparison with KA2862A groundwater.
- The percentage of unattached cultivable organisms increased with oxygenation of the groundwater in the REX chamber.
- Biofilms were formed on the granitic surface of the REX core and on the REX PEEK tube system. The numbers of cells attached as biofilms inside the *in situ* REX system exceeded the numbers of free-living cells.
- The groundwater contained abundant and diverse unattached microbial populations, which included both strict anaerobic, facultative aerobic and microaerophilic organisms. Oxygenation induced adaptive succession of the microbial population from anaerobic to microaerophilic and aerobic. Methanogens, microaerophilic heterotrophic, sulphate- and iron-reducing bacteria were replaced with methanotrophs, hydrogen-oxidising and aerobic heterotrophic bacteria during oxygen pulses in the REX chamber groundwater.
- Oxygen was reduced, carbon dioxide was produced and methane was oxidised *in vitro*. Oxygen reduction rates by the unattached microbiota *in vitro* were reproducible over different samplings.
- The pH was buffered by microorganisms in the course of oxygen reduction in the *in vitro* batch experiments.
- Application of the primary and secondary models demonstrated that the attached and unattached microorganisms could be responsible for the oxygen uptake observed under *in situ* conditions.

These findings are in excellent agreement with the current knowledge in microbiology.

Table of content

1 Introduction

Oxygen is an important groundwater constituent that determines both geochemical and microbiological events in an aquifer. Reducing processes in an aquifer involve a complex interaction of biotic and abiotic components. Aerobic respiration is fundamentally important for the functioning of an aquifer in transport and bioremediation processes. The oxygen-reducing capacity of microorganisms affects an aquifer both as an ecosystem and as a hydrological unit. However, there have been few studies of oxygen reduction in groundwater. The presence of attached and unattached microorganisms actively reducing oxygen may influence to what extent copper canisters of nuclear waste deposited in the future will corrode and leach radionuclides from the repository via groundwater. Therefore, the study of the occurrence and activity of microorganisms in the vicinity of nuclear waste repositories is very important.

The results of our previous studies showed that groundwater and granite surfaces contain abundant and diverse microbial populations (Kotelnikova, Pedersen, 1998b). The populations include not only anaerobic organisms (Kotelnikova, et al, 1998; Kotelnikova, Pedersen, 1998b), as could be expected in the reduced environment, but also facultative aerobic and microaerophilic organisms. Different microbially catalysed reactions may, theoretically, contribute to microbial biomass production and oxygen reduction in accordance with available nutrients (Kotelnikova, Pedersen, 1999).

In our studies on microbial oxygen reduction during the REX field experiment, our goal was to answer the following questions:

- Are there microorganisms in attached and unattached states in the REX chamber?
- Have the microorganisms enough biologically available energy sources to increase in number and to reduce oxygen?
- What portion of the directly counted cells is viable and actively oxygen reducing?
- What kind of microbial processes are responsible for biological oxygen reduction in the REX groundwater?
- Which part of oxygen reduction in the REX chamber is mediated by microorganisms, including both attached and unattached populations?

The borehole KA2861A was drilled for the REX field experiment near the borehole KA2862A during fall 1996. A double-packer system was installed in KA2861A, but the borehole appeared to have very low conductivity. The water from the inner section of the borehole KA2862A (7.38–15.98 m), neighbouring the REX packer system, served as stock water for the REX experiment. To answer the question about contribution of microorganisms to the oxygen reduction in REX *in situ* experiments the appearance of different aerobic and anaerobic microbial groups was studied repeatedly. Microbial processes and diversity at the REX site were studied in three steps, before, during and after the REX field experiment. In the first step, groundwater from KA2861A and KA2862A were sampled (March–April 1998). In the second step, the groundwater was sampled directly from the REX experimental chamber and from the inner section of KA2862A (July 1998–March 1999). In the third step, we sampled PEEK tubes and groundwater from REX and KA2862A before and after an inhibiting run and sampled

the REX drill core surface (May–August 1999). The first set of samples (980311) was designed as characterisation of the REX site (section 5.1), the second sampling set (980707–190599) was designed as a microbial control of REX *in situ* experiments (sections 5.2). Effect of crushed KA2861A core granite, uranin, methane and hydrogen on microbial oxygen reduction was studied (section 5.1.4, 5.1.5 and 5.4). Incubation conditions *in vitro* were as close as possible to those of the granite surroundings. The data are compared to the Replica experiment microbial data (section 5.3). Effect of microbial oxygen reduction on pH was studied (section 5.5). The third set of sampling was designed as attached microorganisms in the REX system (section 5.6) and inhibition of microbial reduction of oxygen in the REX chamber (section 5.7). The characterisation of the microbial effects and microbial O_2-reduction coefficient (MORC) were used for modelling oxygen reduction of the *in situ* REX experiment (section 5.8).

We collected data on the presence and amounts of unattached and attached microorganisms in the REX chamber, their viability, and the impact of the different microbial groups on the total microbial oxygen reduction. The microbial activities are probably distributed between unattached and attached populations, so-called biofilms. The contribution of the unattached and attached microbial oxygen reduction in the total reduction process during the REX field experiment is discussed (section 6.0).

2 Subjects of research

- Groundwater KA2861A and KA2862A at REX site before the experiment.
- REX chamber groundwater sampled during REX *in situ* experiment.
- Groundwater from the borehole KA2862A before it was used for oxygen pulse injections during the experiment.
- REX PEEK tubes, drill core and gold cap after the experiment.

3 Objectives addressed during the test

1. Determination of the total numbers of unattached and attached microorganisms.
2. Study of activity and viability of unattached and attached microorganisms.
3. Analysis of the physiological structure of unattached and attached microbial populations.
4. Study of microbial reduction of oxygen, carbon dioxide production and pH control *in vitro.*
5. Determination of the cell specific microbial oxygen reduction coefficient (MORC) in groundwater and in pure cultures.
6. Modelling of microbial oxygen reduction under *in situ* conditions.

4 Description of experimental procedures

4.1 Sampling

Samples were taken from the REX chamber (980707, 980722, 981029, 990128, 990312, 990519, and 990608), the inner section of KA2862A (7.38–15.98 m; 980311, 980707, 990128, 990312, 990519 and 990608), the outer section of KA2862A (6.82–6.92 m; 980311), the REX borehole KA2861A, the inner section of KA2861A (8.5–9.8 m) (980311) and from the outer section of KA2861A (0–8.5 m; 980311). The water was collected into empty sterile bottles, inoculated in liquid media for most probable counting and inoculated on solidified medium 10–15 min later. Biofilms on PEEK tubes used in the REX experiment in the field were sampled for 990510 before the inhibition experiment and 990608 after the addition of inhibitors. Biofilms on the drill core and gold cap were sampled for 990811. The sampling and analyses performed during the characterisation of the REX site and microbial control of the REX *in situ* experiment are described in Table 4-1.

4.2 Total microbial oxygen reduction

4.2.1 Microcosm experiments

The experiment was repeated five times with the REX groundwater and twice with KA2862A groundwater. The groundwater from each sample was placed in 25 ml-serum bottles in 10 ml portions. Four repetitions contained only groundwater without any additions. Uranin was added into four other repetitions in each groundwater sample to a final concentration 0.05 mg/l. Four repetitions of groundwater were added with 1 µM CH_4 and four repetitions with 1 µM H_2 or crushed granite (3 g of sterile crushed core minerals from KA2861A per vial).

Controls for all variants with additions (uranin, granite, methane or hydrogen) were processed with the inhibitors. Killed controls were added with (0.2 ml) formaldehyde (2% final concentration), (0.2 ml) sodium aside (1%), 400 mg/l streptomycin, 0.58 mg/l bacitracin.

All bottles were closed with butyl rubber stoppers and aluminium rings and contained air in the headspace. The initial concentration of oxygen in the headspace of the closed bottles filled with air was 20 %. The sample bottles were cultivated laying horizontally, in the dark, without shaking. The incubation temperature was 16°C. The gases (CO_2, O_2, CH_4) in the headspace of the experimental vessels were analysed with a gas chromatography (GC) system twice: once the next day after sampling and then again 14-15 days. The GC tests were followed by pH measurements. Concentrations of gases dissolved in the groundwater were calculated using the Henry low for chemically inert gases.

Table 4-1 *List of analyses performed under the characterisation phase and microbial control of in situ REX experiment.*

Task definition/description	KA2861A, inner section (8.5-9.8 m)	KA2861A, outer section (0-8.5 m)	KA2862A, inner section (7.38-15.98 m)	KA2862A, outer section (6.82-6.92 m)	REX chamber groundwater
Total number of microorganisms	980311	980311	980311, 980707, 990128, 990312, 990519, 990608	980311	980707, 980722, 981029, 990128, 990312
Microbial oxygen reduction	980311	980311	980311, 990128, 990312	980311	980707, 980722, 981029, 990128, 990312
Carbon dioxide production	980311	980311	980311, 990128, 990312	980311	980707, 980722, 981029, 990128, 990312
Effect of mineral phase from KA2861A granite on growth		980311	980311	980311	
Effect of uranin on O_2 reduction		980311	980311	980311	
Effect of methane and hydrogen on O_2 reduction	980311	980311	980311, 990128, 990312, 990519, 990608	980311	980707, 980722, 981029, 990128, 990312
Viable count of aerobic heterotrophs	980311	980311	980311, 980707, 990128, 990312, 990519, 990608	980311	980707, 980722, 981029, 990128, 990312, 990510,990519, 990608
Viable count of microaerophilic heterotrophs	980311	980311	980311, 980707, 990128, 990312, 990519, 990608	980311	980707, 980722, 981029, 990128, 990312, 990510, 990519, 990608
Viable count of methanotrophs	980311	980311	980311, 980707,990128, 990312, 990519, 990608	980311	980707, 980722, 981029, 990128, 990312, 990510, 990519, 990608
Viable count of hydrogen-oxidising bacteria	980311	980311	980311, 980707, 990128, 990312, 990519, 990608	980311	980707, 980722, 981029, 990128, 990312, 990510, 990519, 990608
Methane and hydrogen oxidising activities					980707
Anaerobic activities (methanogenesis, acetogenesis, methane-oxidation)					980707
Heterotrophic activity					980707
Viable count of methanogens			980707, 990128, 990312, 990519, 990608		980707, 980722, 981029, 990128, 990312, 990510, 990519, 990608
Viable count of sulphate-reducing bacteria			980707, 990128, 990312, 990519, 990608		980707, 980722, 981029, 990128, 990312, 990510, 990519, 990608
Viable count of iron-reducing bacteria			980707, 990128, 990312, 990519, 990608		980707, 980722, 981029, 990128, 990312, 990510, 990519, 990608
Cells attached on the inner surface of PEEK, drill core, gold cap					990510, 990608, 990811

4.2.2 Calculations of oxygen uptake in the microcosm experiments *in vitro*

All points were calculated as a mean of two independent replicates. Oxygen uptake and carbon dioxide released due to biogenic processes were calculated as the rates in non-inhibited samples minus the rates in the killed controls. The difference between them was evaluated with t-distribution statistical test. To calculate the percent of oxygen reduction at the expense of methane oxidation the molar ratios of gases consumed (CH_4, O_2) and produced (CO_2) were estimated considering that one mole of consumed methane demands 2 moles of oxygen. The rates of methane oxidation were calculated as the difference between the methane concentrations in the different analysing times and divided by time. At atmospheric pressure the maximum possible rate (or first-order kinetic) of methane oxidation was observed (Kotelnikova, Pedersen, 1998a). Carbon dioxide accounted for by oxygen reduction was calculated as 1:1 percentage for oxygen for carbon dioxide rates in non-inhibited samples minus the rates in the killed controls. The kinetic of microbial reduction of oxygen during 14 days was fitted to linear equation. Oxygen uptake in the microcosm experiments was calculated using following equation:

$$V = \frac{[O_2]_1 - [O_2]_2}{dT}$$

Methods used for gas chromatography, chemical analyses, determination of the total numbers of microorganisms in the groundwater with acridine-orange direct counting (AODC), and calculation of most probable viable microbial numbers (MPN) of aerobic and microaerophilic heterotrophs, methane-oxidising, hydrogen-oxidising (HOB), iron-reducing (IRB), sulphate-reducing bacteria (SRB) and methanogenic archaea were described earlier in SKB TR 99-17 (Kotelnikova, Pedersen, 1999). The method used for viable cell counts on the surfaces is described in section 5.6.

4.2.3 Microbial oxygen reduction coefficient (MORC)

The MORC was calculated as $A_{cell} = \frac{[O_2]_2 - [O_2]_1}{dT \times N_g}$. N_g is total acridine orange cell count in groundwater, O_2 is oxygen concentration registered in the gas space of the microcosms with the groundwater in time 1 and 2. We studied the reproducibility of the MORC and compared MORCs under different conditions. MORC may be expressed as nanomolar O_2 per day per cell.

5 Results

5.1 Microbial characterisation of groundwater at the REX site KA2861A and KA2862A (March - April 1998)

5.1.1 Gas content of the REX groundwater

The chemical and physical conditions determine the availability of microbial energy sources for oxygen reduction. The groundwater in KA2862A and KA2861A boreholes is of native deepest origin and represents the ancient in Äspö groundwater type. Gas analysis of the REX site groundwater showed presence of gases typical for Äspö groundwater but the gas composition was different. Content and analysis of gas dissolved in REX site groundwaters are described in Table 8-1. Hydrogen content was higher than in other groundwaters tested. Ratio helium: nitrogen in the gas exceeds the ratio in other Äspö groundwater. KA2861A groundwater contained higher amount of gas than KA2862A, including methane (10 times) and hydrogen (100 times), whereas KA2862A comprised more carbon dioxide (15 times).

Total acridine-orange count showed $1.3x10^5$ - $7.5x10^5$ cells ml^{-1} of groundwater. The lowest microbial number ($2.4x10^3$ cells ml^{-1}) has been observed in the inner section of KA2862A borehole water. MPN, plate count and an oxygen reduction microcosm test gave negative results for this groundwater (Table 8-2). The highest number of microbes was observed in the outer section of KA2861A (0-8.5 m).

5.1.2 Total number of microorganisms

Table 5-1 *Microbial numbers found at the REX site in March 1998.*

	Borehole section			
	KA2861A	**KA2861A**	**KA2862A**	**KA2862A**
Microbial group analysed	**8.5-9.8 m**	**0-8.5 m**	**7.38-16.0 m**	**6.8-6.9 m**
Total microbial counts, cells ml^{-1}	3.44×10^5	7.46×10^5	2.4×10^3	1.32×10^5
Most probable counts, cells ml^{-1}				
Heterotrophic microaerophiles	4.2×10^4	5.5×10^4		7.7×10^4
Heterotrophic aerobes	9.3×10^3	3.8×10^4	0	3.33×10^2
Methane-oxidising bacteria				
Group I	7.7×10^4	8.0×10^4	0	4.2×10^1
Group II and X	4.2×10^4	4.2×10^4	5×10^0	5×10^0
Hydrogen-oxidising bacteria	2.8×10^4	7.7×10^4	0	2.5×10^2

Presence of oxygen dependent organisms in the REX site groundwater was shown before the REX experiment. The structure of microbial population in two studied boreholes was described earlier (SKB TR 99-17, p.25). Here we report detailed data in the Tables 8-3 - 8-7. Structure of the aerobic microbial population in the groundwater KA2861A, section 0-8.5 m before the REX experiment initiation, 980311 is shown in Figure 5-1.

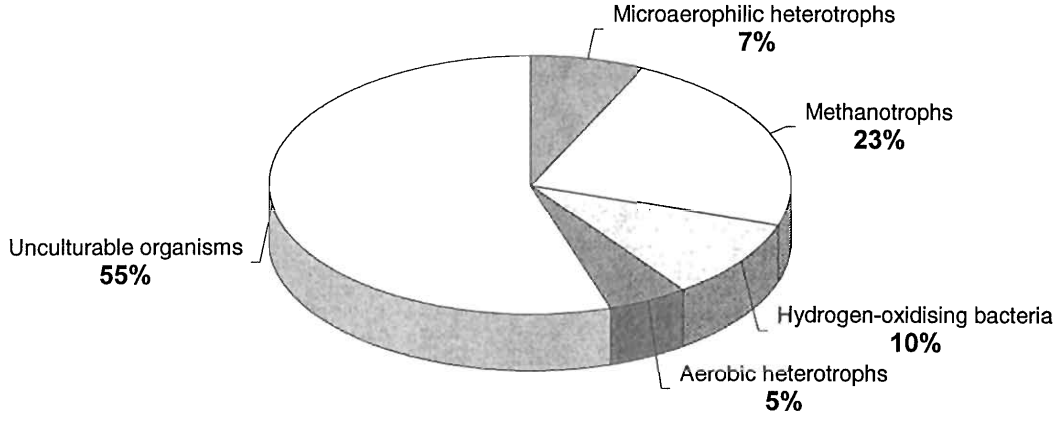


Figure 5-1 *Structure of the aerobic microbial population in the groundwater KA2861A, section 0-8.5 m before the REX experiment initiation, 980311. Anaerobic microorganisms were not analysed in these experiments.*

5.1.3 *In vitro* microbial oxygen reduction in groundwater at the site before REX experiment

Results of the microcosm experiments showed that before the REX field experiment microbial reduction of oxygen took place in the inner section of KA2861A, the outer section of KA2861A and the outer section of KA2862A groundwater. The oxygen reduction rates ranged between 1.26 and 2.78 µM per day (Table 5-2, and Table 8-8). Oxygen reduction in the inner section of KA2862A (0.34-0.71 µM per day) was observed only in variants added with 1 µM of methane or hydrogen. Oxygen reduction results are in good agreement with direct count and MPN observations (Table 5-1, Tables 8-3 -8-7). Oxygen competent microbial groups were active, they consumed organic matter, methane and hydrogen dissolved in the groundwater. The fact that oxygen reduction in groundwater with the lowest microbial number was not observed without addition of substrate shows the importance of microbial oxygen reduction.

Carbon dioxide production was observed simultaneously with oxygen uptake. Carbon dioxide production was greater in the outer section of KA2862A than in the outer section of KA2861A whereas oxygen uptake did not differ significantly. Aerobic oxygen reduction estimated from oxygen uptake corrected for chemical oxidation accounted for 50% of the total carbon dioxide production in the outer section of KA2862A. Relatively low carbon dioxide yield in the outer section of KA2861A supports the finding of a high hydrogen-oxidising bacteria count in this groundwater (Table 8-7).

Methane oxidation and methane-oxidising bacteria were observed in all groundwater tested (Table 8-5, 8-6 and 8-8). Methane oxidation accounted for up to 30% of the total oxygen uptake.

Table 5-2 *Microbial oxygen reduction at the REX site in March 1998.*

	Borehole section			
	KA2861A	**KA2861A**	**KA2862A**	**KA2862A**
Measurement	**8.5-9.8 m**	**0-8.5 m**	**7.38-16.0 m**	**6.8-6.9 m**
Total microbial reduction, nM O_2 day^{-1}	1200	1340	0	1620
O_2 reduction in presence of 5 w % mineral phase	n.d.	1390	0	2700
Carbon dioxide production, nM day^{-1}	3700	420	0	3230
Methane oxidation, nM day^{-1}	0.52	10-11	0.24	0.25
Hydrogen oxidation	+	+	+	+

5.1.4 Effect of uranin on microbial oxygen reduction

The effect of the widely used groundwater fluorescent tracer, uranin, was studied at the experimental concentration (0.05%), which is close to that used often in hydrological tests. Sterile stock solution of uranin (1%) was added to groundwater. Oxygen reduction and carbon dioxide production were followed during the 14-day experiment. The oxygen reduction rates in killed groundwater were subtracted from the samples added with uranin. The oxygen uptake, carbon dioxide production and pH were not affected by uranin in the KA2862A and the outer section of KA2861A. Nevertheless, the oxygen reduction was at the same level as in abiogenic control, whereas carbon dioxide production was inhibited and pH was decreased in the outer section of KA2862A (Table 8-8).

5.1.5 Effect of the mineral phase addition from the core granite on microbial oxygen reduction

Addition of a sterile mineral phase did not alter significantly the observed oxygen reduction rates in the groundwater tested (Table 5-2, Table 8-8). The observed differences in oxygen uptake with and without mineral phase were not statistically significant during the two-week test. The difference between oxygen reduction values in those samples was evaluated with t-distribution test at a significance level of P=0.95. We did not observe any oxygen reduction or carbon dioxide production in the presence of a mineral phase in the inner section of KA2862A, while it enhanced carbon dioxide production in the inner section of the KA2862A borehole. We performed short-time experiments where the effect of mineral phase probably was not clearly expressed.

5.2 Microbiological control during the REX field experiment (July 1998 - March 1999)

5.2.1 Total microbial number dynamic in the REX chamber in a course of oxygen injections

Total acridine-orange count showed $7.3x10^4$ - $2.5x10^5$ unattached cells per ml in anaerobic KA2862A groundwater and $2.4 x10^5$ - $7.7 x 10^5$ in aerated REX groundwater (Table 5-3, Tables 8-19, 8-20 and 8-21, Figure 5-2). The total microbial counts in the REX chamber were, as a rule, higher than in the stock anaerobic KA2862A groundwater. The observed differences in the total microbial counts were statistically significant and differed by 3.8-7.2 times. The difference between oxygen reduction values in those samples was evaluated with t-distribution test at a significance level of P=0.95.

Microbial counts in the groundwater of the REX chamber increased during the period of oxygen presence in the groundwater. Microorganisms using oxygen as an electron acceptor yield more energy than those using anaerobic respiration or fermentation. Further increase of microbial number in the groundwater may be limited by the electron donor availability.

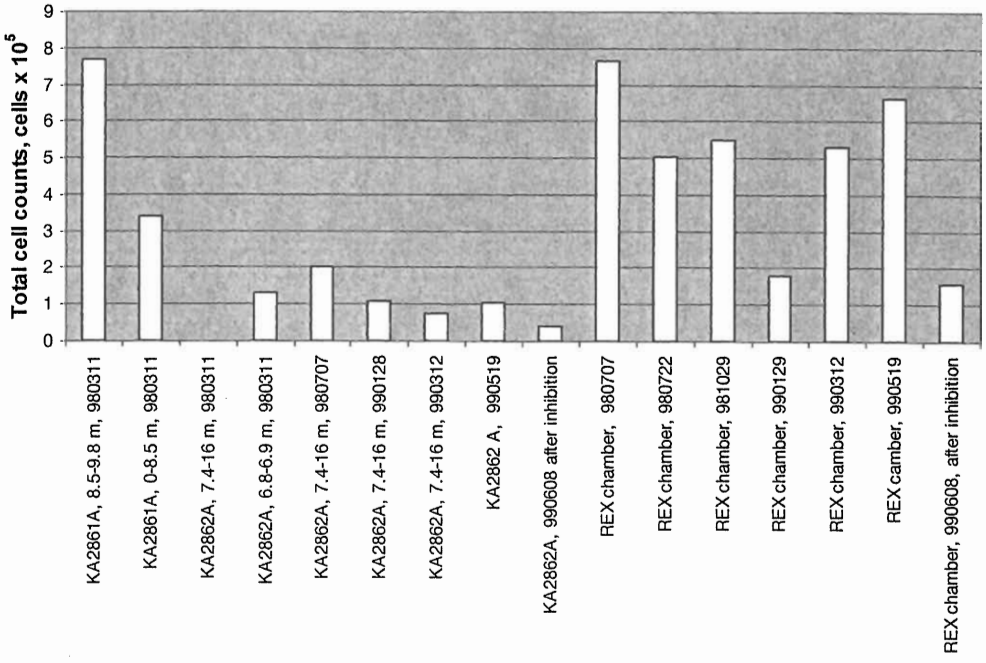


Figure 5-2 *Total microbial counts in the KA2862A groundwater and REX experiment chamber* in situ.

5.2.2 Structure of the microbial community in the REX site groundwater during the REX experiment

The structure of the microbial populations in the borehole KA2861A before the REX experiment (Figure 5-1) and in the borehole KA2862A (Figure 5-3), are compared to the structure of the microbial population in the REX chamber groundwater (Figures 5-4 and 5-5 and Table 8-10). We expressed the most probable number of different microbial groups as percentages related to the total count (Table 8-11). Comparison of the MPN counts in KA2862A groundwater (Table 5-3) with those in the REX chamber after oxygen pulses showed certain changes in the population structure (Table 8-10 and 8-11 and Figures 5-3, 5-4 and 5-5). For example, the fraction of anaerobic methanogens was 1.24–6.68% in KA2862A groundwater and only 0–1.63% in the REX groundwater. Sulphate-reducing bacteria constituted a significant part of microbial population in anaerobic KA2862A groundwater (14–55%), whereas their fraction in the REX chamber water did not exceed 0.98%. The heterotrophic microaerophilic component of the population was relatively constant: 1–58% in anaerobic water and 2–24% in oxidised REX water, whereas aerobic heterotrophs constituted 0–14% in the anaerobic water and 13–75% in the oxidised water. The fraction of hydrogen-oxidising bacteria increased from 0–12% in the anaerobic water to 13–52% in the oxidised water. Fraction of both hydrogen-oxidising and aerobic heterotrophs increased with the increase of redox potential in the REX chamber (Table 8-11). Counts of methanotrophic bacteria ranged from 0.3% in the anaerobic water to 10–12% in the aerobic waters. Maximal counts of microaerophilic heterotrophs and methane-oxidising bacteria were observed at redox potential in the REX chamber at +50 mV. These findings are in excellent agreement with the physiology of the microbial groups mentioned.

A wide variety of microorganisms, including fungi, facultatively anaerobic bacteria, and strict anaerobes are capable of reducing ferric iron. Some of these organisms are capable of completely oxidising organic compounds to carbon dioxide, whereas others are incomplete oxidisers. The use of common fermentative intermediates such as hydrogen, acetate, formate by dissimilative iron-reducing bacteria likely accounts for the majority of the iron-reduction. Iron-reducing bacteria are also known to catalyse the oxidation of aromatic compounds, including important intermediates of anaerobic decay. Some iron-reducers are capable of using a variety of electron acceptors, including Mn^{4+}, U^{6+} , NO_3^-, NO_2^-, SO_4^{2-}, and even oxygen.

The number of iron-reducing organisms increased from 1.2-2.3% in KA2862A to 0.2-55% in The REX chamber after low oxygen pulse in July 1998 (Figure 5-6). Chemical generation of ferrous iron as a result of auto-oxidation of ferric iron may explain, alternatively, the observed phenomena. It is probable that iron-reducing bacteria developed in the REX chamber because of the increased Fe (III) concentrations that followed the low oxygen pulse. After following oxygen pulses the numbers of iron-reducing bacteria did not exceed 2% of population in the REX groundwater. The viable counts of sulphate- and iron-reducing bacteria negatively correlated with increasing redox potential in the REX chamber (Table 8-11).

Our results demonstrated that the cultivable fraction of anaerobes dropped from at least 30% down to 9-10% while the fraction of cultivable facultative aerobic bacteria increased from 42 to 95% during the oxidative period.

Although hydrogen- and methane-oxidising organisms contributed to the oxygen reduction in the REX chamber, nevertheless, heterotrophic aerobes accounted for most of the oxygen reduction. Thus, the methane-oxidising, hydrogen-oxidising and heterotrophic aerobic bacteria were the dominating microbial groups in the REX groundwater during *in situ* experiment. Methane oxidation and methane-oxidising bacteria were observed in all groundwater tested (Table 5-1, 5-3, Table 8-10, 8-11).

It is necessary to mention that our analysis does not exclude possible overlap of physiological capacities of the microbial groups determined to be present. Whereas the ability to produce methane, reduce sulphate and oxidise methane is strictly determined for specific microbial groups, such abilities as oxidation of hydrogen, organic matter and respiration of iron or oxygen may be tied to organisms possessing ubiquitous metabolisms. Nevertheless, the data received are useful in the prediction of the dominating physiological types will be dominant with the appearance of oxygen. Comparison of the MPN in KA2862A groundwater and in the REX chamber after oxygen pulses showed that anaerobic and strict anaerobic organisms (sulphate reducing, methane producing and iron reducing) were sequentially replaced with microaerophilic (methanotrophs) and aerobic (hydrogen-oxidising bacteria) organisms (Figure 5-6). Heterotrophic microaerophils were replaced with aerobic ones. The number of organisms able to reduce oxygen increased. The observed behaviour of microbial populations was the first observation in deep subsurface groundwater under in situ conditions. This finding is of fundamental importance for understanding of the dynamic of microbial populations in a subterranean aquifer and provides the mechanistic explanation of microbial redox related processes. Theoretical model for the microbial adaptive succession was proposed (SKB TR 99-17, Kotelnikova, Pedersen, 1999)

Table 5-3 *Total and viable microbial numbers in the ground water KA2862A, sampled in a course of REX experiment directly from KA2862A borehole (cells ml^{-1}).*

	Sampling date		
Microbial counts	**980707***	**990128****	**990312*****
Total microbial counts (AODC), cell ml^{-1}	2.02×10^5	1.12×10^5	7.3×10^5
Heterotrophic aerobic microorganisms, cell ml^{-1}	2.4×10^4	5×10^3	0
Microaerophilic heterotrophic microorganisms, cell ml^{-1}	3×10^4	4.1×10^4	6×10^2
Microaerophilic methane-oxidising bacteria, group I, cell ml^{-1}	1.5×10^4	3×10^1	2×10^1
Aerobic hydrogen-oxidising bacteria, cell ml^{-1}	5×10^3	5×10^1	0
Anaerobic sulphate-reducing bacteria, cell ml^{-1}	2×10^3	2.5×10^4	3×10^4
Anaerobic iron-reducing bacteria, cell ml^{-1}	1×10^3	1×10^3	1×10^3
Anaerobic methane-producing archaea, cell ml^{-1}	5×10^1	4.0×10^4	0

* 28 days after oxygen injection, ** 14 days after oxygen injection, *** 12 days after oxygen injection.

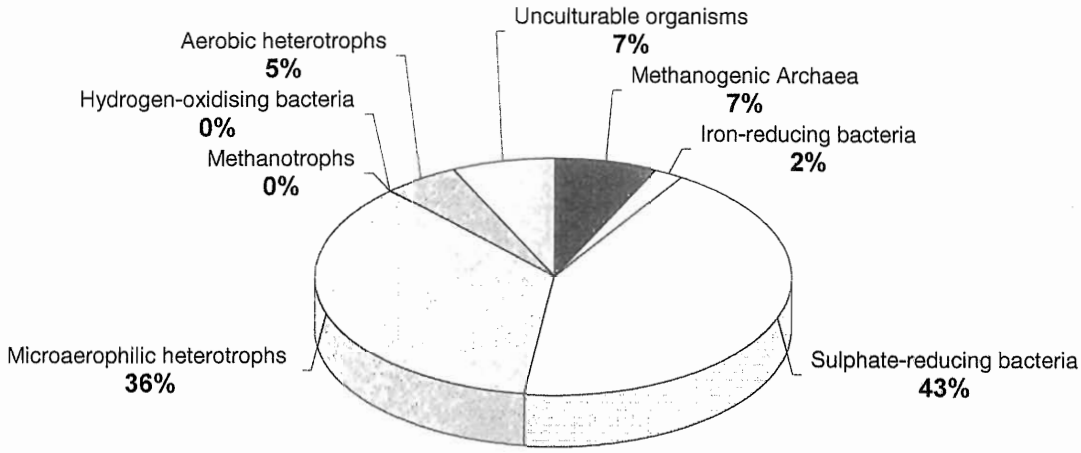


Figure 5-3 *Structure of the microbial population* in situ *found in the groundwater KA2862A, section 7.38-16 m, 990128.*

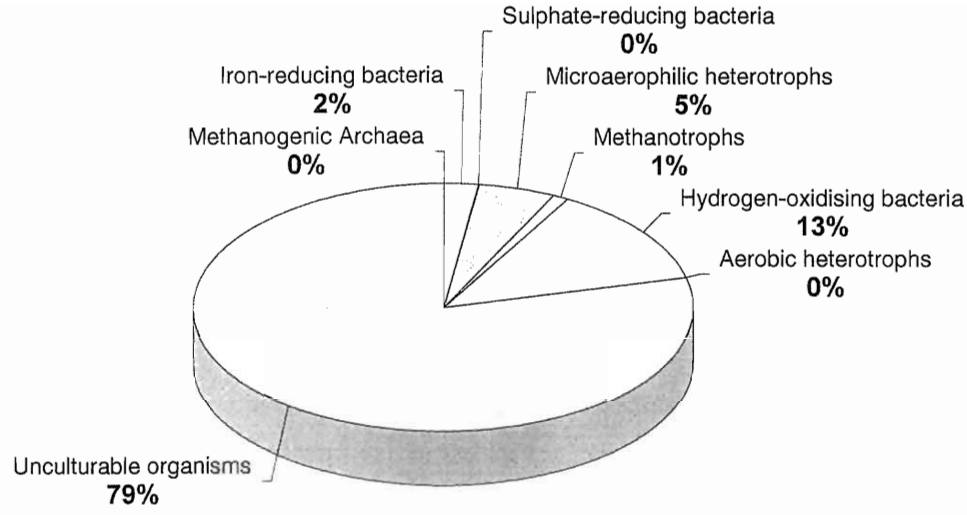


Figure 5-4 *Structure of the microbial population in the REX chamber, Redox -50 mV, 990312*

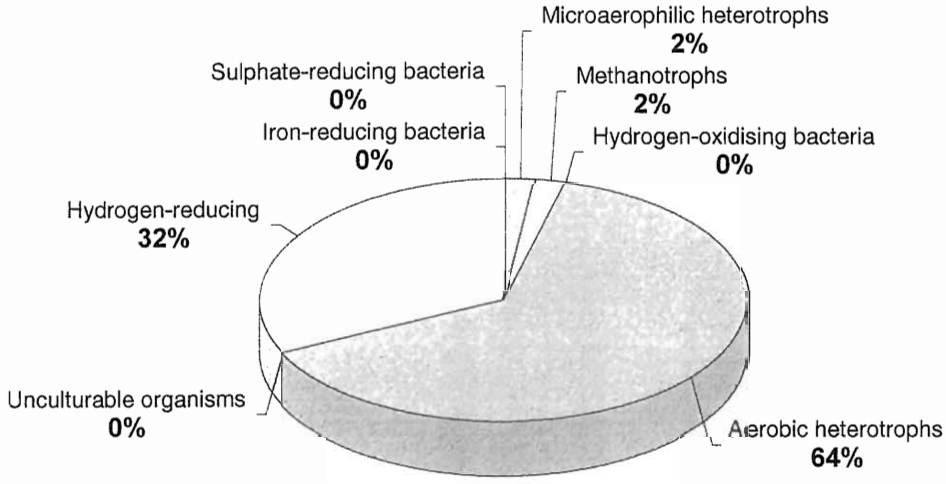


Figure 5-5 *Structure of the microbial population in the REX chamber, Redox +280 mV, 990312.*

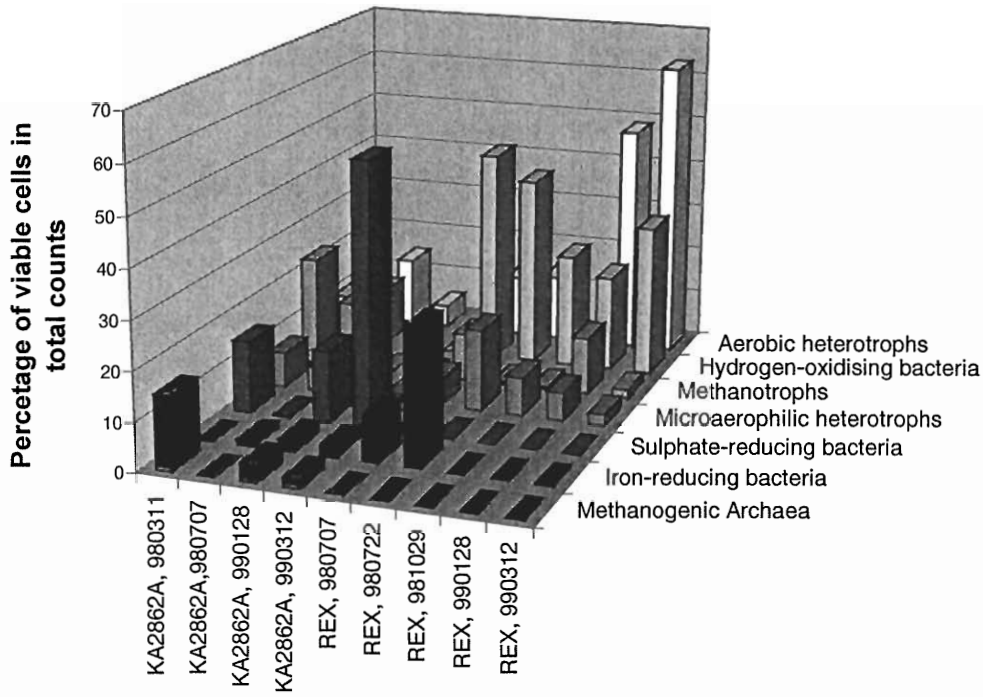


Figure 5-6 *Adaptive succession of the microbial community in KA2862A, 7.38-16 m, and in the REX chamber under different sampling occasions.*

5.3 Comparison of microbial numbers observed during the REX *in situ* and Replica experiments

Comparison of the data on microbial numbers showed that total microbial counts in the Replica experiment (Minutes of REX meeting, 991215, 990318) exceeded the AODC in the REX experiment by one order of magnitude (Table 8-9). This fact agrees with the difference of dissolved organic carbon concentrations between these two experiments. We have observed similar trends in the evolution of total microbial numbers both in the REX and Replica experiment. We observed an increase of the numbers with low oxygen pulses and a slight decrease with increasing concentrations of oxygen. The numbers had a tendency to increase as soon as the population adapted to the oxygen (Figure 5-2).

Heterotrophic aerobic counts were higher in REX in comparison with Replica groundwater. The numbers of iron-reducing bacteria were at the same level in these two experiments after first and third oxygen pulses but decreased with each new pulse. The same trend was observed in the REX and Replica experiments. We observed explosive growth of iron-reducing bacteria following low oxygen pulses in both experiments. The iron-reducing bacterial numbers are discussed in sections 5.2.2 and 6.1.

Hydrogen-oxidising bacteria were not studied during the Replica experiment. Methanotrophs were not found at same level in the Replica as in the REX. This discrepancy may be explained by lack of methane in the groundwater used in the Replica experiment. The ground water used for the REX experiment was impregnated with gas containing methane. In addition, it is known that methanotrophs may be inhibited by the presence of high-molecular organic carbon (Hanson, Hanson, 1996).

It is interesting that sulphate-reducing bacteria (SRB) being the main cultivable component in the anaerobic groundwater KA2862A did not disappear with oxygen pulses either in Replica or REX experiment. Their numbers dropped significantly after

oxygen injection. Although SRB are strictly anaerobic bacteria, they probably survived in the presence of oxygen in the anaerobic microenvironments. Some SRB are able to reduce iron and manganese (Cord-Ruwisch et. al, 1999). We did not observe development of SRB in the REX chamber because we sampled the groundwater a short time after the oxygen pulse when the redox potential was positive.

Comparison of microbial data received on biofilms in the Replica and REX experiments showed that viable microbial numbers are very close (Table 5-4). It is very difficult to learn if the cells are evenly distributed or not.

Despite observing different absolute numbers of microbial groups, we detected the same trends at the population level. Numbers of aerobic organisms increased, whereas the numbers of anaerobic organisms (sulphate-reducing, anaerobic heterotrophs, methanogens) decreased both in the Replica and REX experiments in a course of oxygen pulses. These data are in excellent agreement with the contemporary microbiology.

Table 5-4 *Comparison of viable microbial numbers on surfaces in Replica and REX experiments. The data for Replica experiment are taken from Trotignon et al. 1999.*

Experiment	Total viable microbial counts	Aerobic heterotrophic	Sulphate-reducing bacteria	Methane-oxidising group I	Iron-reducing bacteria
	Cells cm^{-2}				
Replica core I	3.88×10^8	6.00×10^4	1.30×10^4	<0.5	2.50×10^4
Replica core II	1.76×10^8	1.66×10^5	1.66×10^5	<0.5	1.66×10^4
Replica PETP cap	6.70×10^5	1.10×10^5	1.00×10^1	<0.5	2.40×10^1
REX drill core	3.54×10^8	1.10×10^4	1.20×10^3	2.31×10^2	1.70×10^4
REX gold cap	5.67×10^8	5.40×10^5	1.71×10^4	2.70×10^3	1.80×10^5
REX PEEK tube	1.36×10^5	1.66×10^4	1.10×10^5	4.00×10^3	2.70×10^2

Characterisation and comparison of microbial communities observed during the Replica and REX experiments showed certain trends in development and structure of the communities. Namely, after oxidation of the granite groundwater the following changes have occurred:

- The cultivable fraction of microorganisms increased.
- Anaerobic groups were replaced with aerobic and microaerophilic groups.
- With increase of oxygen concentrations microaerophilic heterotrophs were replaced with aerobic ones.
- Structure of microbial population in the REX chamber followed the changes of redox potential.
- Biofilms were built. They contained both aerobic and anaerobic organisms.
- Microbial counts were higher in biofilms formed on granitic surfaces than in biofilms formed on artificial plastic surfaces.

5.4 *In vitro* microbial oxygen reduction in the groundwater during the REX experiment

The microbial reduction of oxygen in vitro was studied during the REX experiment both in the REX groundwater and KA2862A anaerobic groundwater (Table 5-5, 5-6). The water from the experiment and from the anaerobic borehole was sampled under the same sampling occasions (Table 4-1) to see the difference in oxygen reduction rates or substrate specificity for the reduction. Although the groundwater in the REX chamber originated from the inner section of the KA2862A borehole, it has been in a contact with oxygen during and after the oxygen pulses, a principle difference, which suggests the differences in microflora and oxygen reduction activities.

Our results showed that microbial reduction of oxygen took place in the microcosms both with groundwater sampled from the REX chamber and from the KA2862A borehole. The oxygen reduction rates ranged between 0.46 and 1.77 µM per day (Table 5-2, 5-5, 5-6 and Table 8-13 and 8-14). Carbon dioxide was produced simultaneously with oxygen reduction. Production of carbon dioxide was not observed in the killed controls. Microbial oxygen reduction was estimated to provide 100% of the total carbon dioxide production in our microcosm experiments.

The oxygen reduction rates were close to those observed when the KA2862A water was first characterised (Table 5-2). The absolute rates of oxygen reduction and carbon dioxide production did not differ significantly ($p>0.01$) between REX chamber and KA2862A groundwater. Microbial reduction of oxygen was reproducible for the same groundwater under the same incubation conditions.

It is interesting, that the reduction level in KA2862A groundwater did not respond to the addition of methane or hydrogen, whereas the REX groundwater did (Tables 8-13 and 8-14). Oxidation of methane and hydrogen was more intensive in the REX groundwater than in KA2862A or KA2861A (Table 5-2, 5-5 and 5-6). The oxygen reduction in KA2862A groundwater could not be modulated by methane or hydrogen (Table 8-13) because counts of methane- and hydrogen oxidising bacteria were extremely low at those samples (Table 5-3).

It is remarkable that additions of methane or hydrogen in the gas phase stimulated oxygen reduction in the water containing higher numbers of respective physiological groups. Oxygen respiration results are in excellent agreement with direct and viable cell counts (Figure 5-9,Table 5-3, Table 8-10, 8-13 and 8-14). In a case where oxygen competent microbial groups were cultivable, they consumed organic matter, methane and hydrogen dissolved in the groundwater.

The number of organisms able to reduce oxygen may be used in evaluation of the microbial oxygen reduction coefficient (Section 4.2.3). We defined the **microbial oxygen reduction coefficient (MORC)** as oxygen reduction rate counted per cell in the groundwater. Our evaluations showed that the MORC reflects the average physiological state of cells and depends on the energy source currently available for microorganisms. Figures 5-7, 5-8, 5-9 and Table 8-15 demonstrates how much oxygen may be reduced per cell in the anaerobic groundwater and in the REX chamber groundwater under different sampling occasions. This parameter was reproducible during repetitive samplings. The MORC is close to that determined for pure cultures of methanotrophs isolated from Äspö (Table 8-16). The MORC may be evaluated in different types of groundwater and used for prediction of the microbial oxygen uptake potential based on total microbial counts or aerobic counts. In a case where the groundwater was not added with any electron donors the MORC ranged between 0.0009 and 0.0015 pM oxygen $cell^{-1}$ day^{-1} in the REX groundwater (Figure 5-7). Interestingly, those values ranged

between 0.001 and 0.012 pM oxygen $cell^{-1}$ day^{-1} in different sections of KA2861A and KA2862A boreholes (Figure 5-8). We did not perform any experiments with the additions of dissolved organic electron donors. It seems logical that MORCs in KA2862A and KA2861A groundwater (Figure 5-8, Table 8-8) are most representatives for the unattached population in the groundwater that is omitted in gaseous electron donors.

The main difference between REX and KA2862A and KA2861A groundwater is that REX groundwater has been oxygenated for a certain period of time whereas KA2862A and KA2861A have remained continuously anaerobic until the sampling. MORC value was lower in the REX groundwater in comparison with the groundwater originating from the anaerobic borehole. This fact may be explained by the presence of organic carbon available for microbial oxidation in the anaerobic groundwater. Probably this residual dissolved organic carbon was used during *in vitro* batch experiments. It is possible that the MORC values, which were revealed in our *in vitro* experiments with REX groundwater, could be underestimated. The biases could be caused by fact that (1) the groundwater was placed in the vials and the gas dynamic was followed without any additional electron donors; (2) dissolved organic carbon electron donors have been used before sampling; (3) the specific MORCs were only measured for cells in an unattached population.

Table 5-5 *Unattached microbial oxygen reduction activities* in vitro *found at the REX site.*

	Sampling date				
Measurement	**980707**	**980722**	**981029**	**990129**	**990312**
Total microbial reduction nM day^{-1}	725	720	460	0	1770
MORC, pmol oxygen $cell^{-1}day^{-1}$	0.001-0.0020	0.001-0.0014	0.001- 0.002	0	0.001-0.003
Carbon dioxide production nM day^{-1}	660	470	550	0	1500
Methane oxidation nM day^{-1}	2160	500	490	3330	1060
Hydrogen oxidation nM day^{-1}	1580	490	970	2440	470

Table 5-6 *Unattached microbial oxygen reduction activities* in vitro *found in KA2862A groundwater (section 7.38-16 m).*

	Sampling date		
Measurement	**980311**	**990129**	**990312**
Total microbial reduction nM day^{-1}	0	1230	60
MORC, pmol oxygen $cell^{-1}day^{-1}$	0	0.010	0.0008
Carbon dioxide production nM day^{-1}	0	150	2040
Methane oxidation nM day^{-1}	0	0	0
Hydrogen oxidation nM day^{-1}	0	0	0

Specific MORC for oxygen reduction ranged from 0.1 to 0.25pM oxygen $cell^{-1}$ day^{-1} in the REX chamber groundwater in the presence of methane. It ranged between 0.01 to 0.05pM oxygen $cell^{-1}$ day^{-1} in the presence of hydrogen (Figure 5-9). The potential for oxygen reduction in the REX groundwater with gaseous electron donors added was higher in comparison with those in the groundwater without any additions. Both oxygen reduction and carbon dioxide production could be stimulated by methane or hydrogen additions to the headspace in the REX groundwater (Table 8-14) and could not be stimulated in the anaerobic groundwater (Table 8-13). This finding agrees with progressive development of methane- and hydrogen oxidising microflora in the REX groundwater (Figure 5-6).

Comparison of the microbial oxygen reduction in the REX water and KA2862A groundwater showed the following facts:

- The microcosm measurements showed oxygen reduction by unattached microbes.
- Absolute reduction level did not differ significantly, whereas microbial oxygen reduction coefficient (MORC) was significantly higher in the KA2862A water.
- The specific MORC was a reproducible parameter for the groundwater during samplings and experiments.
- Oxygen uptake was not modulated by additions of methane or hydrogen in KA2862A groundwater, while it was in REX water.

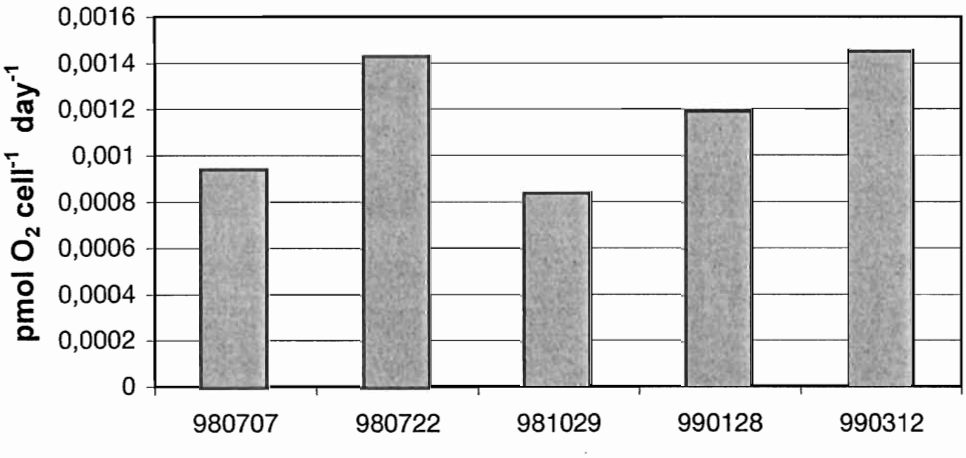


Figure 5-7 *Specific microbial oxygen reduction coefficients for the REX chamber groundwater without addition of any electron donors.*

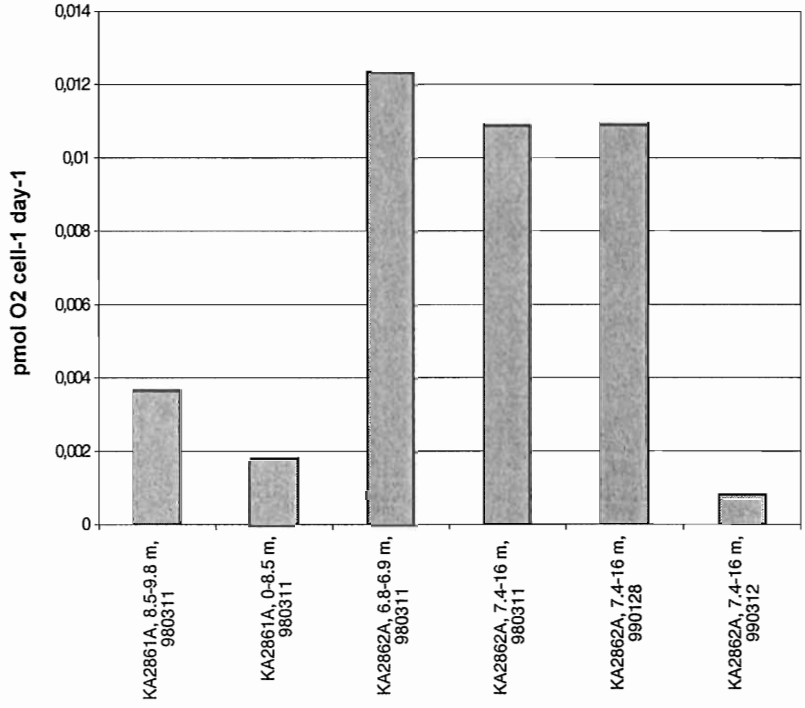


Figure 5-8 *Specific microbial oxygen reduction coefficients for KA2861A (REX) and KA2862A groundwater.*

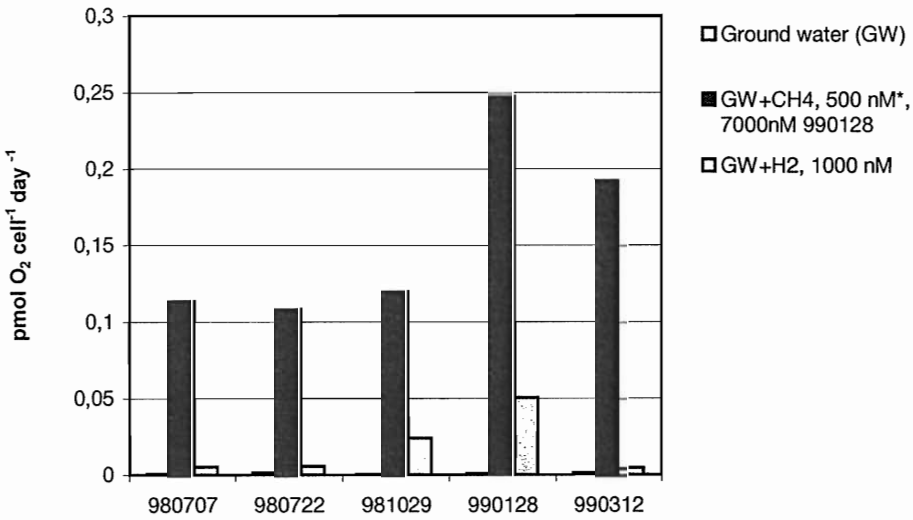


Figure 5-9 *Specific microbial oxygen reduction in the REX chamber groundwater after addition of methane or hydrogen in comparison with groundwater without any additions.*

5.5 Change of pH during *in vitro* oxygen reduction experiments

Our observations of pH changes during the batch experiments with groundwater showed decrease of pH values by 0.6–1.35 units during oxygen reduction (Table 8-12). Remarkably, the pH decrease in the inhibited controls was even higher than in non-inhibited samples (Table 8-12). Control experiments with sterile distilled water added

along with the inhibitors showed that the inhibitors did not contribute to acidification of the water. Microbes could not affect the pH dynamic in the inhibited samples. Therefore, we assumed that microorganisms hindered the pH decrease or buffered the groundwater samples. One more important aspect is that we did not register carbon dioxide production in the inhibited controls despite the pH decrease. This fact indicates that carbon dioxide in the non-inhibited samples was produced biologically.

5.6 Attached microorganisms in the REX system

5.6.1 The REX PEEK tube microflora

The REX PEEK tubes were collected 990510 before the inhibition and 990608 after the inhibition from the REX system. The pieces of the REX PEEK tube, 65 mm long, were collected. As a control, we analysed PEEK tubes that have not been used in the *in situ* REX experiment. After the sampling, both types of tubes were placed into sterile HOB medium and transported to the surface laboratory. The tubes were then brushed with sterile cotton pieces. The cotton pieces were rinsed in the 7.5 ml of sterile HOB medium. We analysed the viable cell counts on the inner surface of the PEEK tubes by diluting the cell suspension in a set of different media as described in SKB TR 99-17, section 6.7. The cell suspension was also diluted at 10 times the rate to 10^{-6} in the mineral HOB medium. Each dilution was plated on REX R2A medium and colony-forming units (CFU) were counted after 2 weeks of incubation at 16°C. The cells were calculated per square millimetre (Table 5-7). Sulphate-reducing and heterotrophic aerobic organisms were the most abundant cells attached per square millimetre on the PEEK tubes, which were used in the *in situ* REX experiment. We were able to culture aerobic hydrogen-oxidising, microaerophilic methane-oxidising and heterotrophic bacteria and anaerobic iron-reducing bacteria.

Then we did simple arithmetic calculations. The inner diameter of the PEEK tube was 3.15 mm. One square millimetre of the PEEK tube may be colonised by at least 1359 viable cells. If the length of the PEEK tube system used in the experiment was 45.94 meters, then the square of the total inner surface was:

$S = 2\pi \times R \times h = 2 \text{ x } 3.14 \text{ x } 1.575 \text{ x } 45.94 \text{ x } 10^3 = 4.54 \text{ x } 10^5 \text{ mm}^2$

Cell number = cells mm^{-2} tube square mm^2=1359 x 4.54 x 10^5= 6,18 x 10^{10} cells per 45.94 m

It means that the total count of cell in biofilms in the REX tubing system was around 6.18×10^{10} cells (Table 8-18). If the total volume of the groundwater in the REX system was around 1 L then the total counts of unattached cells observed experimentally in the REX were 7.65×10^8 cells/L. The viable cell counts on surfaces of the drill core and gold cap are 3,55 10^4 and 5.67 10^4 cells mm^{-2}, respectively. Our calculations showed that the viable counts in biofilms on tubes constituted 91% of whole microbial population. The drill core and the gold cap accounted for 8%, whereas the groundwater microorganisms constituted only 1% of the total microflora (Table 8-18). Thus, the viable microbial counts of the attached cells exceeded the unattached ones.

Table 5-7 Viable most probable cell counts of attached microorganisms in the REX chamber.

	Before inhibition	After inhibition			Inhibition of microbial numbers on the REX tubing
	PEEK tube	PEEK tube	Drill core	Gold cap	
Microbial group	**990510**	**990608**	**990811**	**990811**	**%**
Heterotrophic microaerophiles, cells mm^{-2}	22	1.5	2.17×10^{2}	1.85×10^{4}	95
Heterotrophic aerobes, cells mm^{-2}	166.00	74,2	1.06×10^{2}	5.40×10^{3}	55
Hydrogen-oxidising bacteria, cells mm^{-2}	15.36	3.27	3.51×10^{4}	3.51×10^{4}	79
Methane-oxidising bacteria, cells mm^{-2}	40	0	2.13	2.7×10^{1}	100
Group I					
Iron-reducing bacteria, cells mm^{-2}	2.74	26	1.71×10^{2}	1.85×10^{3}	0
Sulphate-reducing bacteria, cells mm^{-2}	1097	0	1.2×10^{1}	1.7×10^{1}	100
Methanogens, cells mm^{-2}	0	0	0	0	0
Total viable count, cells mm^{-2}	1359	105	3.55×10^{4}	5.67×10^{4}	92

Hydrophobic macromolecules, originating as excretion from living organisms or as lytic products from dead organisms, tend to partition at interfaces and, particularly, to absorb to solid surfaces to form conditioning film. These conditioning films alter the charge and free energy of the surface and act as concentrated energy substrates for microbes associated with the surface. In the groundwater flow system, there is a continuous input of nutrients that encourage rapid growth and reproduction of colonising bacteria and eventual build-up of biofilms. Bacteria are the primary organisms on surfaces, with small cells predominating. Successful colonisation of the surface depends on continued microbial adhesion, accompanied by growth of the adherent organisms, with a regular succession of bacterial types leading to the formation of a mature biofilm containing both aerobic and anaerobic bacteria, depending on the surfaces and environmental conditions encountered (Marshall, 1997).

5.6.2 Microbiological analyses of the drill core and gold cap after termination of the *in situ* REX experiment

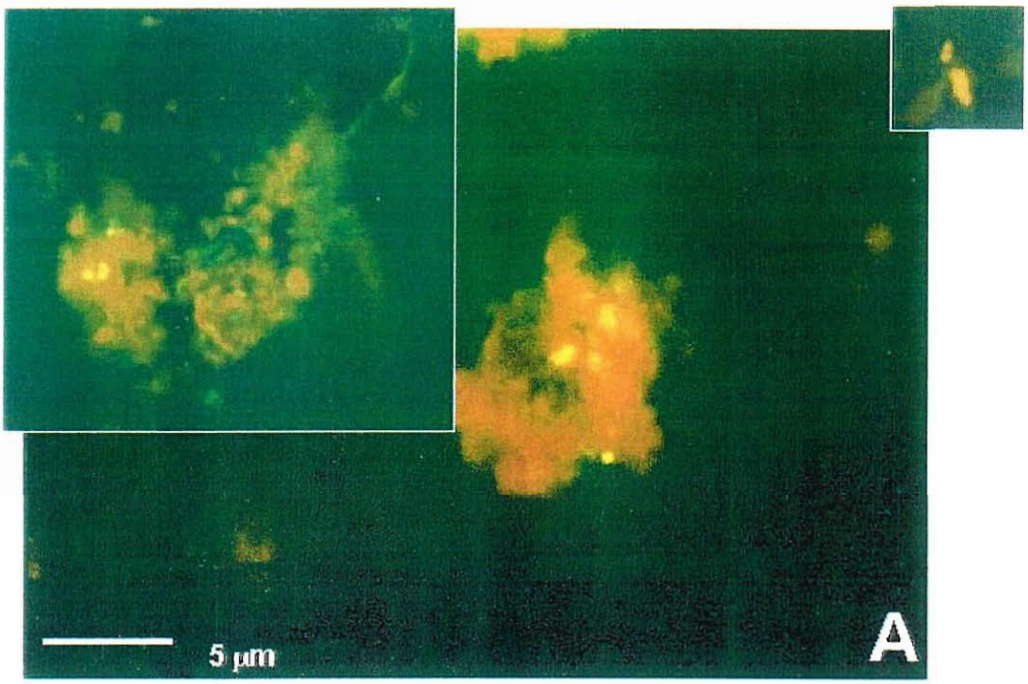
After completion of the *in situ* REX experiment, the drill core was taken out for examination. The drill core was sectioned at Äspö using a diamond saw. The surface was sampled with sterile cotton, which was rinsed in sterile mineral medium. The suspension was inoculated into serial dilutions. Viable and active cells were counted with MPN techniques (Table 5-7). Aerobic and microaerophilic heterotrophs, hydrogen-oxidising and iron-reducing bacteria were the most abundant cells attached on the drill core and gold cap surfaces. We were able to culture methane-oxidising and sulphate-reducing bacteria. We have found microbes at extremely high viable numbers on the REX drill core and the gold cap surfaces. This fact indicates that the microbial populations inhabited the surfaces as biofilms.

Several liquid samples were inhibited and transported to Gothenburg for the microscopic examination. The inhibited samples were filtered, stained with acridine orange and examined with a UV microscope. Biofilms were observed in 100% of the samples from the drill core (Figure 5-10A). Morphology of the cells observed in the biofilms was similar to the cell morphologies observed directly in the REX groundwater and cultures (Figure 5-10B).

Small pieces of the granite drill core were conserved with 2.0% glutaraldehyde, cooled and transported on ice to Gothenburg. The drill core from the *in situ* REX experiment was examined with confocal laser scanning microscopy (CLSM) in Lundberg Laboratory and scanning electron microscopy (SEM) at the Botanical Institute of Gothenburg.

CLSM showed that the surface of the drill-core granite was different from the control surface that was not in contact with the groundwater.

The most SEM-examined surface samples showed presence of microaggregates (Figure 5-11) and biofilm-like organic layers with embedded coccus-like bodies (Figure 5-12A). The control surfaces did not have such microaggregates (Figure 5-12B). The size of the microbodies was in the range of the bacterial cells observed in the REX groundwater cultures and directly in the REX groundwater. The size ranged between 300 and 650 nM for cocci and 300 × 3000 nM for rods. Analytical methods of high resolution (X-ray diffraction or plasma mass-spectroscopy) must be used to identify the chemical surface structure in the films to make definite conclusions about the nature of the surface microaggregates. However, our microscopic observations (UV, CLSM, SEM) indicated that the REX drill core and gold cap could be covered by biofilms.



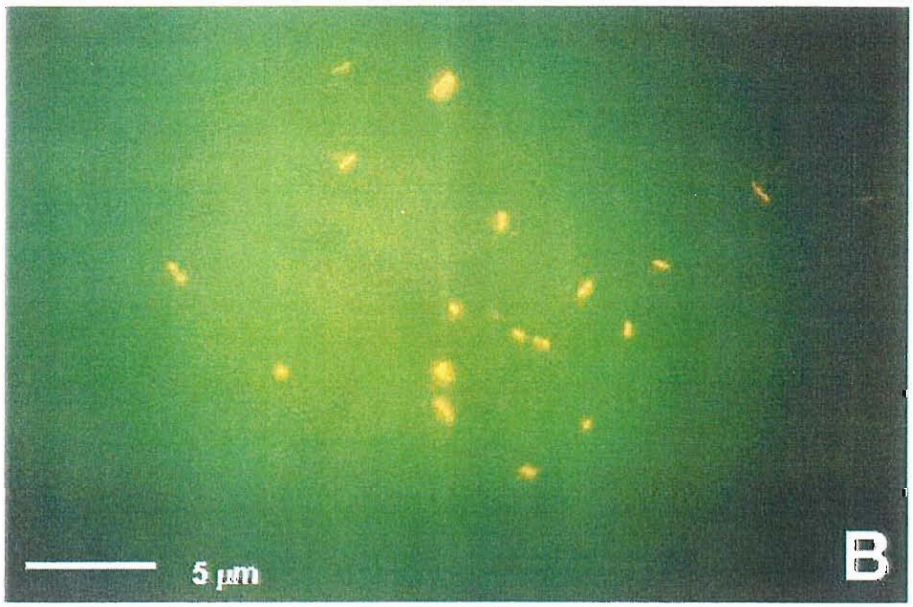


Figure 5-10 *(A) Acridine-orange stained biofilms from the REX drill core, sampled 990811; (B) Acridine-orange stained cells from the REX groundwater, 990519*

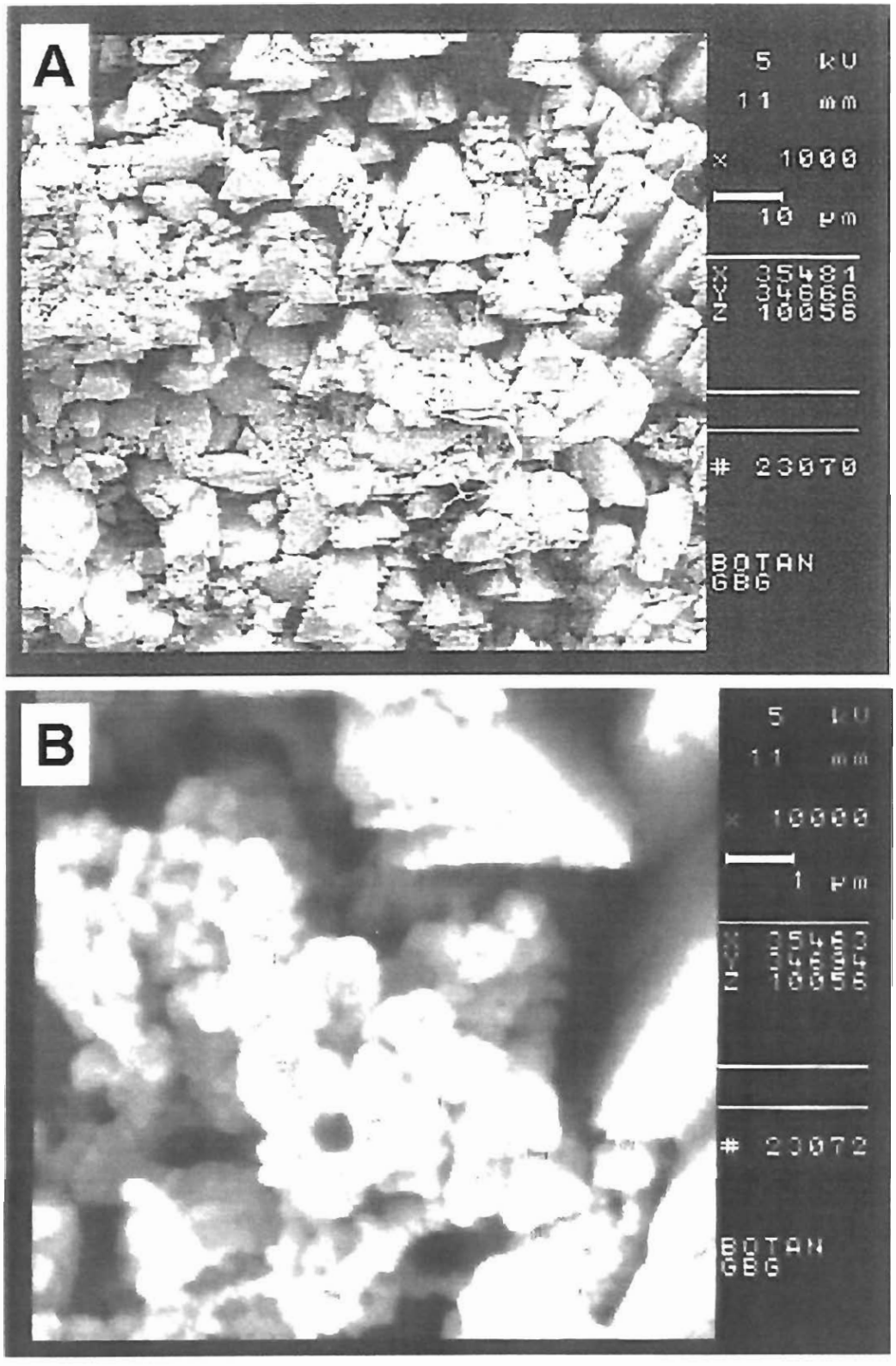


Figure 5-11 *(A, B) SEM image of the REX drill core surface after the in situ experiment.*

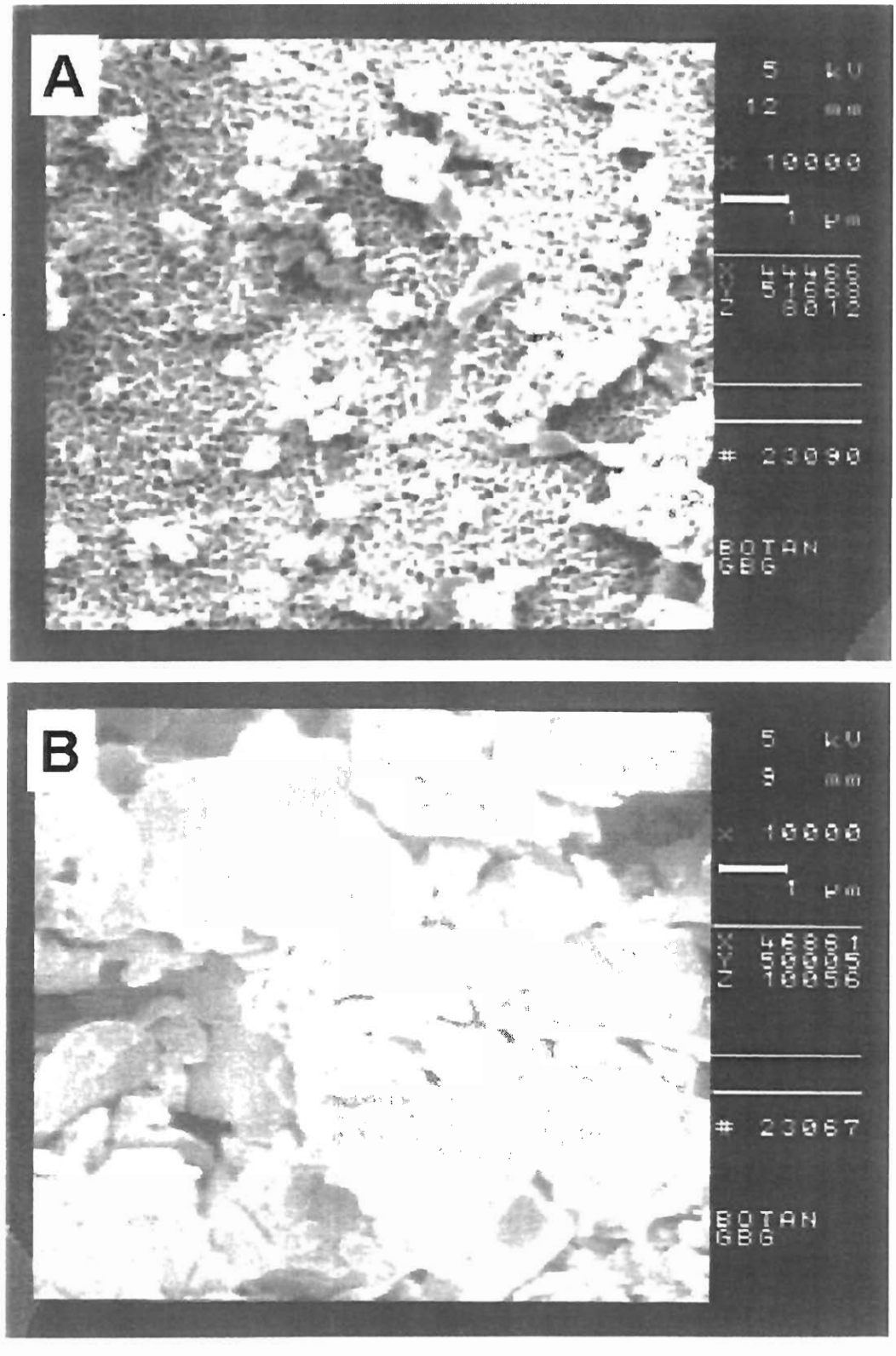


Figure 5-12 *(A) SEM image of biofilm on the REX drill core surface. (B). Non-altered surface.*

5.7 Inhibition of microbial reduction of oxygen in the REX chamber

The aim of the experiment was to inhibit microbial activities *in situ* by using strong and effective anti-microbial agents directly in the REX chamber. The list of the inhibitors and used concentrations are shown in Table 5-8. The total organic carbon concentration in REX groundwater does not exceed 0.8–1.4 mg/L. Thus, addition of the proposed amounts of the inhibitors could shift the organic carbon content to some extent. Therefore, we used the inhibiting agents minimising the organic carbon input. Provided that Eucaryotae are active in the groundwater, it would not be enough to use only an antibacterial agent. Metabolic inhibitors (for example sodium aside), affecting non-specifically both Eucaryotae (animal, yeast, fungi) and Procaryotae (bacteria), are advantageous because they impact a broad spectrum of organisms. Carbonyl cyanide 3-chlorophenylhydrozone (CCCP) inactivates cytochrom C and interacts with sodium proton pump and ATPase energy synthesis, and is not bacteria specific. We used the mixture of CCCP, Na_3N and bacitracin as main inhibitors, because they are effective at low concentrations and affected a broad spectrum of organisms. There was a possibility that the chosen inhibitors would not effective. For example, low sensitivity to the agent may be result of the non-permeability of microbial cell walls or biofilms. To be able to interpret the results of the experiment, we used a control of the cell viability both in the groundwater and on tubes before and after the experiment. The results are shown in Tables 5-7 and 5-9.

Table 5-8 *Inhibitors used during the* in situ *experiment, 990519-990608.*

Inhibitor	Functional characterisation	Concentration used
Sodium aside	Metabolic inhibitor of lipid peroxides, protein and replicate DNA synthesis both in Eucaryota and Prokaryote. Commonly used as preserving mean for biomaterials (500 µM)	6.2 mM
CCCP	Metabolic inhibitor of ionophor, cytochrom C and ATP-ase in Eucaryota, Archaea and Prokaryote	4.33 µM
Bacitracin	Antimicrobial agent, specific inhibitor of cell wall and protein synthesis both in Eubacteria and Archaea	0.2 mg/l

5.7.1 The inhibition of the unattached cells in the REX chamber and KA2862A under period 990510 –990811

As a control we tested microbial viability before and after the inhibiting experiment with MPN method. We have found active microorganisms both in the REX groundwater and on surfaces before and after the inhibition *in situ* experiment (Table 5-7 and 5-9). The MPN results showed that microorganisms were not completely inhibited in the groundwater and on surfaces. The active microbes contributed to the production of reducing compounds and direct oxygen reduction by cells. Total inhibition of viable microbial cells was 54% in the REX groundwater and 92% on REX tubes as measured just after the inhibition. The microflora could be totally inhibited or insensitive depending on the physiological group (Table 5-7 and 5-9). The inhibition percentages were close to the observed *in situ* experiment. Methane-oxidising, hydrogen-oxidising, sulphate-reducing and microaerophilic heterotrophs were the most sensitive microbial groups to the inhibitors used both in REX groundwater and on REX tubes. Aerobic heterotrophs were less sensitive to the inhibitors used (38–55%). Iron-reducing bacteria

were not inhibited. Their viable numbers increased after the inhibition. Their cell walls were probably not permeable for the inhibitors used. Iron-reducing bacteria replaced successively the sulphate reducers and heterotrophs.

Table 5-9 The viable cell counts of unattached microorganisms in the REX chamber

	Before inhibition		After inhibition		Inhibition in REX-KA2862A groundwater
	REX water	KA2862A	REX water	KA2862A	
Microbial group	**990519**	**990519**	**990608**	**990608**	**%**
Total microbial count, cells ml^{-1}	6.63 x 10^5	1.56 x 10^5	1.06 x 10^5	4.17 x 10^4	61-76
Most probable counts					
Heterotrophic microaerophiles, cells ml^{-1}	5.5 x 10^3	9.9 x 10^3	0	2.5 x 10^2	100-97
Heterotrophic aerobes, cells ml^{-1}	6.27 x 10^3	3.33 x 10^2	3.87 x 10^3	4.13 x 10^4	38-0
Hydrogen-oxidising bacteria, cells ml^{-1}	9.9 x 10^3	4.2 x 10^2	7.5 x 10^2	5.5 x 10^1	92-87
Methane-oxidising bacteria cells ml^{-1}	9.9 x 10^3	7.5 x 10^2	2.5 x 10^3	0	75-100
Group I					
Iron-reducing bacteria, cells ml^{-1}	7.5 x 10^1	1	7.5 x 10^3	4.6 x 10^1	0-0
Sulphate-reducing bacteria, cells ml^{-1}	0	2.5 x 10^2	0	2.5 x 10^2	0-0
Methanogens, cells ml^{-1}	0	0	0	0	0-0
Total viable count, cells ml^{-1}	3.16 x 10^4	1.11 x 10^4	1.46 x 10^4	5.55 x 10^2	54-95

After injection of the inhibitors into the REX groundwater, we were able to culture heterotrophic microorganisms attached on the PEEK tube both on solid and in liquid REX R2A medium (Table 5-7). The number of viable heterotrophs on the tube surfaces after inhibition was 55–92% lower than the numbers before the inhibition. It is interesting that methane-oxidising and sulphate-reducing bacteria were inhibited completely whereas the hydrogen-oxidising part of the population was inhibited only partly. Iron reducers were not inhibited at all. As a result of the inhibition the total viable counts observed on the REX PEEK tubes were decreased by 92%. These results showed that microorganisms were not completely inhibited in the biofilms.

We observed active microflora both on surface of the drill core and the gold cap two months after the inhibition. Thus, in accordance with the culturing results, the microbial activity was not inhibited completely. This incomplete inhibition means that the residual oxygen reduction observed *in situ* took place because of the microbial reduction of oxygen both in the groundwater and in the biofilms.

5.8 Modelling of oxygen reduction for the *in situ* experiment and microbial contribution

The curve fitting best used for the *in situ* data may be described mathematically as following:

$$f(t) = \sum Ae^{-k(t+\alpha)}$$

where A_n is linear coefficient, velocity coefficients are successive $K_1 > K_2 > K_3 > K_n$, α is shift of time (Figure 5-13).

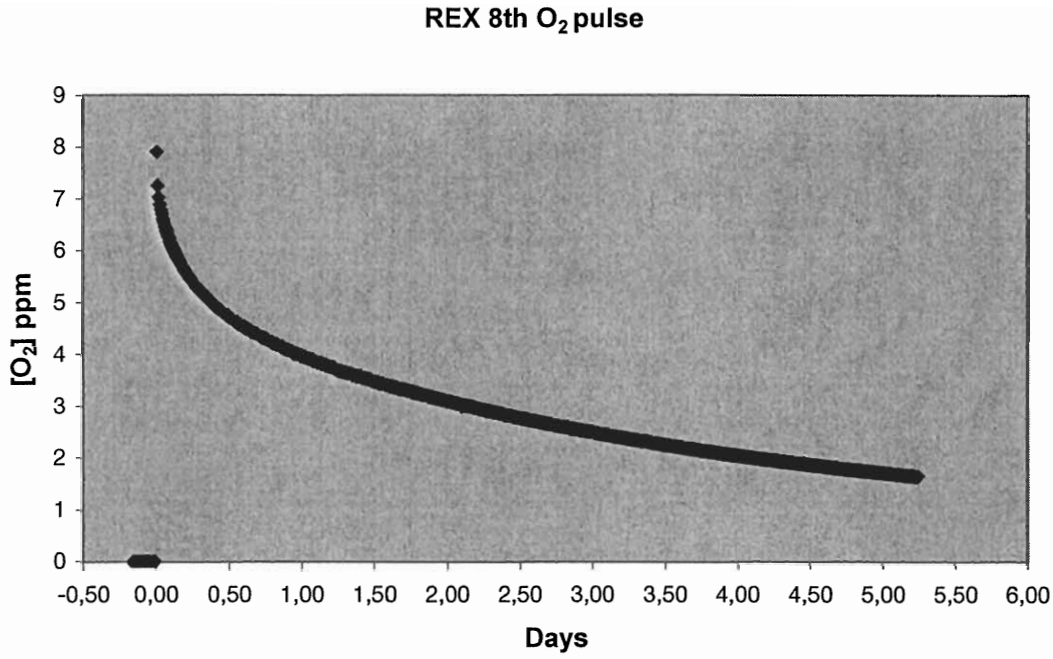


Figure 5-13 *Oxygen uptake in situ (8-th pulse) observed in REX field experiment (Puigdomenech et al. 1999)*

The process of oxygen uptake *in situ* may be described by a complex exponential function. The mathematical description does not give the mechanistic explanations; however, it shows that several processes contribute to the oxygen uptake. The velocity constants are regressing and time dependent. We integrated the curve and evaluated the linear rate constants on the different time intervals. The process follows at least three constants during the first three days: 130.9 μM per day at the beginning, 29.5 μM per day during the second day and 19.5 μM per day during the third day of the experiment. We assume the contribution of both microbial processes (particularly aerobic heterotrophs with low affinity to oxygen and high K_{mo} around 180–200 μM) and mineral reactions including auto-oxidation of ferrous iron, manganese (II), oxygen-mediated clay formation, bacterial-mediated smectite dissolution. The short time and fast reactions are typical for the mineral reactions. Successive contribution of different microbial groups makes the oxygen reduction robust (Figure 10 in TR 99-17, p.40). The experimental evidence for the microbial adaptive succession was found during the REX *in situ* experiment sampling (section 5.2.2). The second step is characterised by slower oxygen uptake (29.5 μM per day). The process is properly described by an exponential curve and fits to the Monod kinetic for biofilms (Figure 5-13 and 5-14). The rate constant is typical for the attached microflora (Table 8-15). Hydrogen-oxidising microflora contribute (probable K_{mo} are around 10–50 μM). Heterotrophic microaerophils are important (probable K_{mo} are around 5.0 μM). The detected microbial groups are probably active simultaneously, surviving in different microenvironments in the biofilms. This means that there is production of reduced species [Fe(II), Mn(II), S^{-2}] by anaerobic bacteria through the whole period of reduction. This fact is suggested by the presence of iron-reducing, sulphate-reducing and aerobic bacteria in the tested REX biofilms. The third step may be contributed by successive microbial oxygen reduction by unattached microflora with high affinity to oxygen (K_{mo} below 1.5 μM). The rate constant is close to the microbial one measured in the microcosm experiments with the groundwater (Table 8-15 , TR 99-17).

Iron reduction and microbial promotion of reduced species during this period (Figure 11, TR 99-17, p.41) may follow the microbial methane oxidation. The processes following the oxygen uptake lead to the evolution of negative redox potential and development of sulphate-reducing microflora (Replica report, 1999, draft).

Microbe-REX *in vitro* experiments produced data that could be described with the help of an exponential function (TR 99-17). The rate coefficients ranged between 0.31 and 4.5 μM per day in the microcosms. The rates were much below the rates observed *in situ*. There is an important difference in the *in vitro* and *in situ* systems. The laboratory microcosms contained only groundwater. The microbial population was stressed, depressurised, limited with natural substrates (including gases) and did not have natural adhesion surfaces. The laboratory microcosms did not contain any biofilms. However, comparison of the oxygen reduction kinetic observed in the *in situ* REX field experiment with the kinetic in Microbe-REX *in vitro* microcosms (page 28, 32, SKB TR 99-17) showed that the processes had similar natures. This similarity means that the process observed *in situ* may have a microbial nature. The overall oxygen reduction was calculated using following equation:

$$V = \frac{[\mathrm{O_2 initial}]_1 - [\mathrm{O_2}]_2}{dT}$$

and related to the initial oxygen concentrations (Figure 5-14). The data used for the calculations are shown in Table 8-17 These results pointed out that the higher the oxygen concentration, the higher the reduction rate measured under *in situ* conditions. K_{mo} is O_2 concentration when growth velocity is ½ of the maximum. Figure 5-14 shows that as K_{mo} could be approximated to 280 μM. An alternative curve was fitted to the empirical *in situ* data (Figure 5-14). The curve may be described by equation, which is shown in Figure 5-14. This equation may be eventually used for the calculation of time for oxygen reduction:

$$dT = \frac{[O_2 \mathrm{initial}]}{1.0763\,[\mathrm{O_2 initial}]0.6071}$$

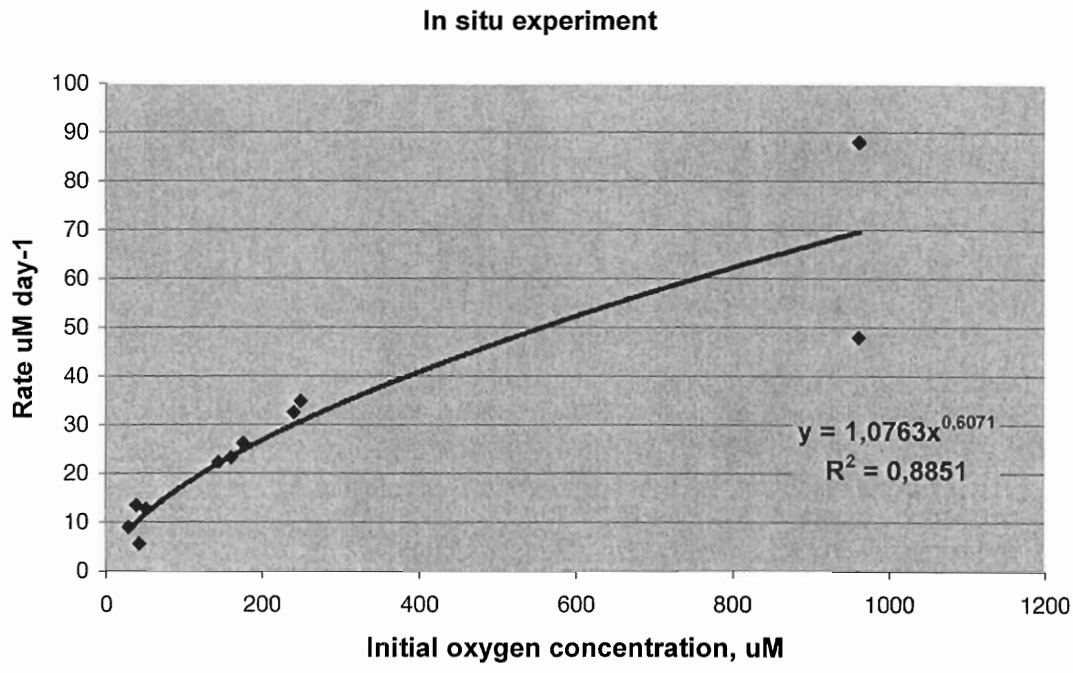


Figure 5-14 *Oxygen uptake under* in situ *conditions related to initial oxygen concentrations.*

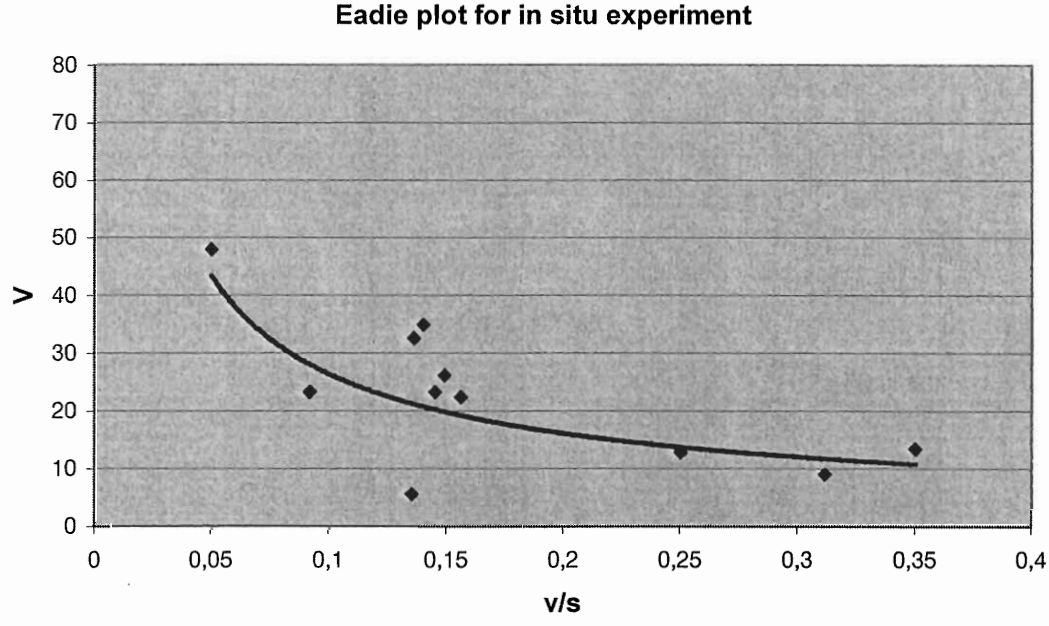


Figure 5-15 *Linear transformation (Eadier plot) of* in situ *data from REX field experiment (1998-1999). V is overall oxygen reduction per day (µM/day). V/S is ratio of V and initial oxygen concentration (day).*

Maximal rate was determined with the help of an Eadie plot (Figure 5-15) and is around 72 µM per day. Consideration of the rate constant of *in situ* experiment runs gave: V_{max} equal to 88 µM per day (Table 8-17). Thus, integration of the *in situ* data set indicates that attached and unattached microorganisms contribute the oxygen reduction and

evolution of low redox potential under *in situ* conditions during the tested period. The successive microbial contribution and the microbial promotion of the reduced species are particularly important.

Our modelling approach was focused on a priori consideration of the presence of diverse microbial communities in the groundwater and on the rock surfaces. The first aspect of the approach includes knowledge of the potential for different microbially catalysed reactions (or demonstration of the presence of electron donors for microbial oxygen reduction and activity of specific microbial groups). The total oxygen reduction rate may be constituted by the sum of contributing rates of diverse microbial metabolisms. The rate may be used as a model for the sum of additives yielding total oxygen uptake rate in a case where the microbial populations are non-competing. The physiological structure of the mixed microbial population provides weight coefficients for each participating group (Kotelnikova & Pedersen, 1999).

Acetate and formate analysis in Äspö groundwater was performed using the UV-enzymatic method (Boehringer Mannheim) at 340 nm. The groundwater was collected in 20-ml portions and filtered using 0.2-µm pore filters to separate microbes. The data are averages of three repetitions in seven different Äspö groundwaters.

Data show that shallow Äspö groundwater contains 81–90% fulvic acids and 10–19% low-molecular organic acids, whereas deep groundwater contained 35–66% of fulvic acids and 34–64% of low-molecular organic acids (C. Petersson, unpublished data). Acetate and formate are low-molecular organic acids that are the main products of anaerobic degradation of organic carbon. In the presence of oxygen they are oxidised efficiently by microbes to carbon dioxide in KA2862A groundwater (SKB TR 99-17, p.34). The low-molecular organic acids may be more easily oxidised with oxygen in comparison with hardly degradable fulvic acids. We have found both acetate and formate at higher concentrations in the deep groundwater than in shallow ones. These observations indicate that deep Äspö groundwater contains higher contents of available organic electron donors for microbial oxygen reduction than shallow groundwater.

Oxygen-reducing capacity mediated by microorganisms may be expressed as:
$C = Y_1aC_1 + Y_2bC_2 + Y_3cC_3 + Y_nzC_n$

where Y_1 Y_2 Y_3 ...Y_n are stoichiometric (energy yielding) coefficients for an O_2 reduction reaction. The stoichiometric or energy-yielding coefficient shows how many moles of oxygen may be reduced at the expense of specific electron donors at 100% substrate-consuming efficiency (Table 5-10).

Here, *a*, *b*, *c*...*z* are weight coefficients for each physiological group of microorganisms participating in the reduction.

C_n is a O_2 reduction capacity of a particular group consuming a single energy source.

Table 5-10 *Approximated oxygen reducing capacity of the microbial reactions in the REX experiment.*

Microbial process	Energy giving reaction	Stoichometric coefficient	Energy donor concentration in groundwater μM	Percentage of responsible microbial group in population %	Oxygen reducing capacity, μM
Oxidation of formate	$2CH_2OO + O_2 \rightarrow 2CO_2 + 2H_2O$	0.5	5-52	5-75	1.25-19.5
Oxidation of DOC	$"C_6H_{12}O_6" + 6O_2 \rightarrow 6\ CO_2 + 6H_2O$	6	240-292	13-75	187-1314
Oxidation of acetate	$CH_3HCOO + O_2 \rightarrow 2CO_2 + 2H_2O$	1.0	3.8-34	5-75	0.19-25.5
Nitrification	$NO_2^- + 0.5O_2 \rightarrow NO_3^-$	0.5	n.d.	n.d.	n.d.
Nitrification	$NH_4 + O_2 \rightarrow NO_2 + H_2O + 2H$	1.0	13-18	n.d.	n.d.
Sulphur oxidation	$S^{2-} + O_2 + H_2O \rightarrow SO_4 + 2H^+$	1.0	0.3	n.d.	n.d.
	$S_2O_3^{2-} + 2O_2 + H_2O \rightarrow 2SO_4^- + 2H^+$	2.0	n.d.	n.d.	n.d.
Manganese oxidation, at pH 6.0	$2Mn^{2+} + 2H^+ + 0.5O_2 \rightarrow 2Mn^{3+}\ H_2O$	0.25	4.3-7.1	n.d.	n.d.
Iron oxidation at pH 6.5	$2Fe^{2+} + 2H^+ + 0.5O_2 \rightarrow 2Fe^{3+} + H_2O$	0.5	2.1-2.2	n.d.	n.d.
H_2 oxidation	$H_2 + 0.5O_2 \rightarrow H_2O$	0.5	0.98	28-52	0.14-0.26
CO –oxidation	$2CO + O_2 \rightarrow 2CO_2$	0.5	n.d.	n.d.	n.d.
Methane oxidation	$CH_4 + 2O_2 \rightarrow CO_2 + 2H_2O$	2.0	0.98	0.5-12	0.098-2.4

Using the proposed model, the reducing capacity mediated by microorganisms may be calculated for KA2862A groundwater. We observed that cultivable aerobic and microaerophilic heterotrophic organisms constituted between 4.6 and 76% of the whole population ($a = 0.76$), H_2-oxidising bacteria constituted 13–52% ($b = 0.52$), iron-reducing bacteria constituted 0.2–55% ($c = 0.55$) and CH_4 used 0.5–12% ($d = 0.12$) (Table 8-11). Stoichiometric coefficient for dissolved organic carbon is 60, for hydrogen-0.5, for methane – 2.0, and for dissolved ferrous iron - 0.25. Concentrations of the energy sources (S) measured during the REX experiment were dissolved organic carbon, 240–292 μM; H2, 0.98 μM; dissolved iron 11–36 μM; and CH4, 0.92 μM (Table 5-10). Efficiency coefficients may be approximated to 1.0 because we use a structural parameter based on cultivable cell counts. Thus, the buffering capacity mediated by known microorganisms in KA2862A groundwater may be calculated as:

$$C = 6 \cdot 0.76 \cdot 292 + 0.5 \cdot 0.52 \cdot 0.98 + 0.25 \cdot 0.55 \cdot 2.2 + 2 \cdot 0.12 \cdot 0.92 = 1332 \mu M$$

This equation means that the REX groundwater contains more than enough reduced compounds that may be oxidised by the microbial groups present in the groundwater. We did not study all of reactions listed in Table 5-10 because the predicted oxygen-reducing capacities, which may be mediated by nitrification, oxidation of carbon monoxide, sulphur species, iron and manganese, are expected to be low.

To be able to apply the model proposed earlier (Kotelnikova, Pedersen, 1999), we need to isolate pure cultures representing different physiological groups and study their kinetic characteristics. In the model the total oxygen respiration velocity was expressed by the sum of O_2 uptake rates of different contributing microbial groups at expense of the substrate available in the ground water. The O_2 reduction of separate physiological group is an additive in the summary respiration.

$$V_{total} = aV_{CH4} + bV_{H2} + cV_{DOC} + dV_{Fe2+} + eV_{Mn2+} + fV_S$$

$$v_S = V_{max} E \frac{[S]}{Km + [S]} \cdot \frac{[O_2]}{Km_0 + [O_2]}$$

where V_{max} is maximal uptake velocity of an energy source (S) by a competent microorganism present in ground water, E- efficiency coefficient, K is half-saturation constant of the organisms for S energy source, S_0 is initial energy source concentration in the ground water; O_2 is initial O_2 concentration in the ground water, K_o is half-saturation constant of O_2 reduction. The kinetic parameters $K_{m,}$, V_{max} and substrate efficiency coefficient E reflect biochemical capacities of individual physiological types.

Here we propose a model that is based on a consideration of the presence of unattached and attached microbial populations respiring oxygen in the REX chamber. This aspect of the model describes the total microbial reduction (R) assuming that two microbial populations contribute to the reduction, namely, unattached (R_g) and attached (R_b):

$$R = R_{groundwater} + R_{biofilm}$$

$$R_g = ([O_2]_2 - [O_2]_1)/dT$$

In other words, we assumed that microbes inhabiting groundwater (g) and biofilms on the PEEK tube, the REX drill core and gold cap surfaces (b) participated in oxygen reduction proportional to their cell densities (N_g and N_b), because the microbial population was adapted to the oxygen presence (section 4.2). In our model we assumed that all cells were equally active during time (dT). The MORC (A_{cell}) (Tables 8-15 -17) was used to reproduce microbial oxygen reduction by biofilms:

$$R = N_g A_{cell} + N_b A_{cell}$$

$$R = N_g ([O_2]_2 - [O_2]_1)/dT + N_b A_{cell}$$

The MORCs for unattached microorganisms in the groundwater were calculated as described in section 3.2.3. Microbial reduction (R) and cell density (N_g) in the groundwater were analysed empirically, and these values were used to calculate the specific MORCs (A_{cell}):

$$A_{cell} = ([O_2]_2 - [O_2]_1)/dT\ N_g$$

Acridine-orange direct counts were used as N_g values to count MORC in the REX groundwater. Experimentally elucidated microbial density in the biofilms (N_b) (Table 5-7) allowed us to model the microbial reduction (Table 8-15) as follows:

$$R_{biofilm} = N_b A_{cell}$$

The sum of the attached and unattached microbial oxygen reduction was compared with oxygen uptake rate calculated with *in situ* REX experiment results (Table 8-15 and 8-17). The rate was calculated using the same equation.

$$R_{in\ situ} = ([O_2]_2 - [O_2]_1)/dT$$

The use of the equation was based on the kinetic nature of oxygen uptake *in situ* (Figure 5-13).

Experimentally elucidated microbial density in the biofilms allowed us to build the primary model of the microbial reduction using the biofilms. The sum of the attached and unattached microbial oxygen reduction was compared with oxygen uptake rate calculated with *in situ* REX experiment results (Table 8-15 –8-17). The rates of *in vitro* microbial reduction were calculated using the same equation as for *in situ* oxygen reduction. Application of the primary model showed that the oxygen uptake coefficients calculated using the model fit with the empirical values (Table 8-15).

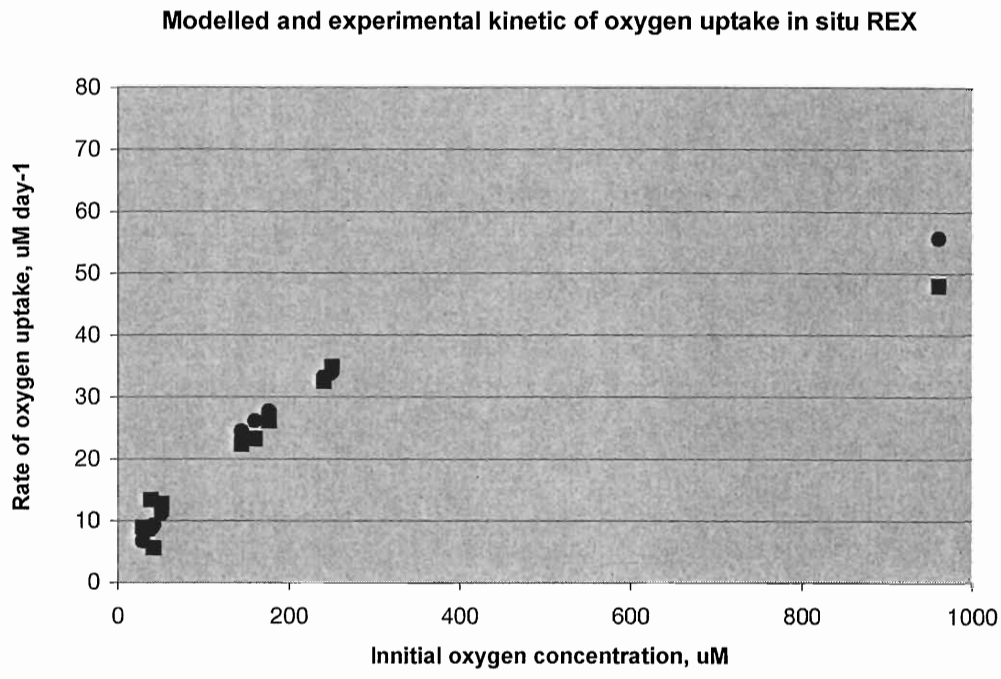


Figure 5-16 *Secondary model curve (square) and experimental data of the groundwater in the REX chamber (circle). The experimental data correlated to the Monod model with coefficient r=0.98.*

Important aspect of the model concerns the evaluation of the kinetic of the *in situ* oxygen reduction. The Michaeles-Menten kinetic was used to model oxygen reduction in the *in situ* experiment. This secondary model describing the effect of the initial oxygen concentrations on the oxygen reduction rate of the *in situ* experiment was empirically built using initial oxygen concentrations used *in situ*. Oxygen uptake rate calculations have been performed using the kinetic parameters V_{max}=72 µmol day^{-1} and K_m= 280 µM, which has been elucidated earlier (Figures 5-14, 5-15). The Michaeles-Menten–modelled curve was in accord with the *in situ* data (Figure 5-16). Over a whole range of O_2 concentrations tested *in situ*, the variation of (v) with respect to [O_2] follows the empirical Monod equation (Michaelis-Menten kinetic used for microbial growth):

$$v = V_{max} \frac{[O_2]}{Km_o + [O_2]}$$

where v is O_2 reduction velocity at a specific O_2 concentration, V_{max} is the maximum velocity when the organism is O_2 saturated and K_{mo} is O_2 concentration when growth velocity is ½ of the maximum.

Application of the primary and the secondary models demonstrated that the attached and unattached microorganisms could be responsible for the oxygen uptake observed under *in situ* conditions.

6 Discussion

The microbiological characterisation and quantitative tests of microbial oxygen respiration have been performed in two (the inner and the outer) sections of KA2861A and KA2862A groundwater before and during the field experiment (March 1998, January–August 1999). The REX chamber microflora was studied during the field experiment (July 1998–August 1999).

6.1 Structure and succession of microbial population in the groundwater

As a result of sampling and analysing the groundwater directly from the REX chamber it was shown that the groundwater contained an abundant and diverse microbial population. It included both strict anaerobic ($2.5 \times 10^2 - 7.7 \times 10^2$ cells ml^{-1}), facultative aerobic ($3.3 \times 10^2 - 8.5 \times 10^4$ cells ml^{-1}), and microaerophilic organisms ($1.5 \times 10^2 - 8.0 \times 10^4$ cells ml^{-1}).

Strictly anaerobic methanogens, sulphate-reducing bacteria and microaerophilic heterotrophic bacteria constituted a significant part of the cultivable microbial population in the anoxic KA2862A groundwater. Viable counts of anaerobic microorganisms such as methanogens, sulphate-reducing bacteria, decreased with oxygen pulses. Aerobic organisms comprised a high fraction of the population in the REX chamber. Aerobic methanotrophs, hydrogen-oxidising bacteria and heterotrophic bacteria constituted up to 22, 52 and 58% of total microbial counts, respectively. Our finding was that anaerobic and microaerophilic microbial groups were replaced with aerobic ones as oxygen concentrations increased in the groundwater.

Oxidation of the granite environment induced adaptive succession of the microbial population from anaerobic to microaerophilic and aerobic. The percentage of cultivable organisms increased with oxygenation of the groundwater in the REX chamber. The population was altered qualitatively. As soon as oxygen appears in the groundwater, dissolved organic carbon- and hydrogen-oxidising bacteria reduce it by catalytic reactions. These microbial groups are aerobic. They tolerate high oxygen concentrations. Oxygen is a high toxic oxidiser. It reacts chemically with the cell components and produces superoxide radicals inactivating the enzymatic activity. Aerobic microorganisms possess more powerful defence mechanisms against superoxide radicals in comparison with microaerophilic and anaerobic ones. Methanotrophes are microaerophilic organisms. They are active at lower oxygen concentrations than dissolved organic carbon and hydrogen-oxidising bacteria. While anaerobic organisms are inhibited, microaerophilic organisms take advantage and consume nutrients by reducing oxygen at low concentrations.

The effects of the microbial process on the groundwater chemistry in the groundwater from KA2862A exhibits a nearly complete sequence of classic water chemistry changes commonly observed in groundwater systems (SKB TR 99-17). The groundwater KA2862A is an example of an electron acceptor–limited environment because the groundwater is situated in a low conducting zone lacking recharge water inflow, which could bear oxidised compounds. Groundwater KA2862A is oligotrophic and contains 0.8 mg/L organic carbon. However, the system may not be defined as electron-donor deficient because methane and hydrogen have been observed as the dissolved gases in the groundwater. Consequently, microbial metabolism is constrained by the availability

of electron acceptors there. Because of active anaerobic microbial metabolism (iron-reduction, sulphate reduction, homoacetogenesis and methanogenesis), the amounts of available electron donors in the system (such as organic carbon, acetate, formate, hydrogen and methane) are large relative to electron acceptors (oxygen, ferric iron, sulphate and carbon dioxide). The electron acceptor–limited nature of KA2862A explains much of the behaviour of the microbial processes in this system when oxygen is introduced.

6.2 Microbial effects on pH

Remarkably, the decrease in pH values in the inhibited controls was even higher than in non-inhibited samples (Table 8-12). We did not observe any carbon dioxide production in the controls that indicates that carbon dioxide in the non-inhibited samples was produced biologically. Microorganisms probably buffered the groundwater by producing carbon dioxide. Increase of alkalinity and pH was observed in the REX experiment (Puigdomenech et al, 1999), whereas in the Replica (Trotignon et al, 1999) slight acidification was observed. The difference could be caused by periodically changing water in the REX and using the same water during the Replica. Replica water had higher organic content than REX water. The heterotrophic microbial processes promoted incomplete oxidation of the organic matter that leads to formation of organic acids and acidification in the Replica. Low pH in biofilms accelerated weathering of silica, manganese, and iron from the minerals. Microbial iron reduction may immobilise sorbed metals by incorporating them into a new mineral phase that is resistant to release by ion exchange (Cooper et al, 1999). In addition, it was shown that aerobic bacteria can dissolve iron oxides through exopolysaccharide formation (Hersman et al, 1999). Heterotrophs are potentially able to interact with minerals in a variety ways, including through exoproduct formation, e.g.. organic acids, chelating agents and exopolymers. Both aerobic and anaerobic heterotrophic bacteria can dissolve potassium-feldspar, releasing Al and Si from the mineral. Some colonising microorganisms produce a reactive microenvironment at the mineral surface altering mineral-water equilibria at that surface. Native subsurface bacteria preferentially colonise and weather silicates that contain apatite (Roger et al, 1999). All these facts help interpret the chemical dynamic in the REX and Replica experiments.

Our results on total microbial numbers at the REX site agreed with the data in the literature (Pedersen, Ekendahl, 1990; Stevens et al, 1993) and with our expectations. Although the presence of aerobic microflora in Äspö groundwater was demonstrated earlier (Pedersen, Ekendahl, 1990), the important question was if the organisms have enough biologically available energy sources to increase in number and to reduce oxygen.

6.3 Dissolved organic carbon in the groundwater and microorganisms

A major part of dissolved organic carbon in surface water is composed from humic acids whereas fulvic acid is one of the major components in subsurface groundwater (Pettersson et al, 1990). Fulvic acid content was below 10 mg per litre in Äspö ground water and ranged between 10 and 650 mg per litre in other studied granitic subsurfaces in Sweden (Pettersson et al, 1990). Substrates such as fulvic acids (aromatic hydrocarbons) are often non-degradable under anaerobic conditions. They are expected to be oxidised in the presence of an electron acceptor such as oxygen (Aragno, Schlegel, 1991; Busse, Auling, 1991; Hanson, Hanson, 1996). Simultaneously, humic substances are strong reducing agents and may contribute to the oxygen reduction to a small extent. To distinguish chemical reduction caused by reduced minerals and fulvic acids from

microbial reduction, we used biological inhibitors in the controls. Dissolved organic carbon content ranged between 240 and 292 μM. Theoretical microbial oxygen reduction capacity in the REX groundwater is around 1334 μM.

Increase of dissolved organic carbon content was observed both in the REX (Puigdomenech et al, 1999) and Replica (Trotignon et al, 1999) experiments. We have found that autotrophic hydrogen-oxidising bacteria were abundant in the REX system. Iron-reducing and sulphate-reducing bacteria inhabited the biofilms. Some iron-reducing bacteria may use hydrogen as the electron donor and grow autotrophically (Balashova, Zavarzin1979; Lovely et al 1997). Sulphate reducers are also autotrophic. Methanotrophs use methane as the sole source of carbon and energy to produce biomass and carbon dioxide. All of these microbial groups are good candidates for production of organic carbon.

Theoretically, there are enough electron donors dissolved in KA2862A groundwater to support microbial oxygen reduction (Kotelnikova, Pedersen, 1999). Composition of electron donors dissolved in the REX site groundwater was different from other Äspö groundwater; namely, hydrogen content (around 2 μM) was higher than in other groundwater tested. Methane content was lower (0.1–1.0 μM) in comparison with other groundwater.

6.4 Iron-reducing bacteria

Our unexpected finding was the number of bacteria using ferric iron as an electron acceptor increased from 2.5×10^2 up to 2.8×10^5 cells ml^{-1} with initial oxidation of the REX groundwater in July 1998. The same was observed during the Replica experiment (Trotignon et al, 1999). Iron-reducing microorganisms were abundant in the diorite-associated biofilms in the REX and Replica experiment. They replaced heterotrophic aerobes and sulphate-reducing bacteria after the inhibition of the REX experiment.

Most of the known iron-reducing bacteria are facultative anaerobes. They may survive and develop in the presence of oxygen. However, the environment must be oxygen-free to allow the microbial iron reduction. It is questionable if iron-reducing organisms may develop before the concentrations of the dissolved oxygen drops. The redox values decreased several days later after an oxygen pulse. As soon as redox potential dropped, anaerobic bacteria, namely, iron-reducing, developed and reduced ferrous iron to ferric iron using organic electron donors or hydrogen available in the groundwater. This process may be why we did not observe further increase in numbers of iron reducers during the later sampling occasions in October 1998 and January, February and March 1999. The redox potential in the REX chamber has never been below +50 mV during this period. The same is true for sulphate-reducing bacteria and methanogenic Archaea; oxygen and high redox potential hinder their development (Kotelnikova & Pedersen, 1999).

The core mineral consists of Äspö diorite, which includes chlorite, biotite and hematite. The chlorite has a high portion of ferric iron (50–80%). The diorite contains 6–7 wt % of iron in the form of Fe_2O_3, $FeCO_3$ and FeS_2 (Tullborg E-L., personal communication). As result of groundwater and rock interaction, the dissolution of chlorite, pyrite and manganese took place (Nordström, et al, 1990). Microbial formation of magnetite (Fe_3O_4) from FeOOH at alkaline pH was reported (Bell et al, 1987). Microbial weathering of iron-containing minerals is a well-known phenomena (Ehrlich, H-L, 1990). If complexing agents such as humic acids abound, the ferric iron may be mobilised from minerals in ore. Mobilised iron would be chemically oxidised at higher oxygen concentrations and pH. Significantly higher concentrations of Fe^{2+} in high

Redox containing O_2 was observed in the East Swamp groundwater around Underground Research Laboratory Site in South Eastern Manitoba in Canada (Gascoyne et al, 1997). A large increase in iron and manganese concentrations in the oxic groundwater was observed during the Replica experiment (Trotignon et al, 1999). The dissolution was observed at high redox potential (Redox = +230 mV).

The oxidation of the iron-containing minerals released into the liquid phase probably took place in the REX chamber because the redox potential ranged between Redox +100 and +300 mV after oxygen pulses. Ferrous iron, which is abundant there, probably was chemically oxidised to ferric iron, thereby providing renewable electron acceptor for iron reduction (SKB TR 99-17). Ferric iron is a typical electron acceptor for the subsurface microflora. Iron and manganese are important as intermediate oxidants and redox intermediates between oxygen and organic carbon electron donors, like for example, lactate, formate, acetate or hydrogen.

After depletion of ferric iron, sulphate resulting from abiogenic sulphide oxidation becomes a principal electron acceptor indicating initiation of sulphate reduction, as it was observed in the Replica long-term experiment (Trotignon et al, 1999).

If microbe-mediated hydrogen production takes place at the surface in the presence of reduced minerals under anoxic conditions, iron- or sulphate-reducing bacteria may release ferrous iron into the groundwater. The probability of such events in the mineral phase was not studied under close *in situ* conditions. In the case of microbially mediated ferrous iron containing mineral dissolution from the granitic core, the effect may be seen as alteration of oxygen content in the presence of crushed granite. In our experiments we observed an increase of oxygen reduction and carbon dioxide production in the groundwater from the outer section of the KA2862A borehole with the addition of 5% crushed granite from the REX core. To be conclusive, further studies need to be done.

6.5 Microbial activity in the groundwater

The next question was: what part of the cells counted with AODC is actively oxygen consuming? It is an important question, considering some of cells observed with AODC technique may be dormant or unable to reduce oxygen. Are uncultivable cells dead?

We showed that number of viable aerobic microorganisms, total microbial counts and cultivable fraction of population increased after contact with oxidised groundwater in the REX chamber. Observed microbial numbers were higher in the aerated REX than in the anaerobic KA2862A groundwater, which indicates that oxygen may stimulate development of microbial population *in situ*. Aerobic fraction constituted up to 99% of microbial population in the REX chamber in March 1999. The number of microbes increased after oxidation in the Replica, as well (Trotignon et al, 1999).

The activity of individual cells was studied earlier using a microautoradiography technique at Äspö HRL. It was shown that 98% of cells observed in the groundwater incorporated radiolabeled leucine (Pedersen, Ekendahl, 1990). Pyrite precipitate that was found on the mineral phase of the KA2861A (Eva-Lena Tullborg) indicates that sulphate-reducing bacteria were active *in situ*. The activity of the microbial population in subsurface groundwater may vary depending on the availability of electron donors for the microbial population. We have shown the presence of viable and active organisms reducing oxygen both with radioisotopic technique and by numbering viable organisms (SKB TR 99-17, Kotelnikova, Pedersen, 1999). The overall rates of microbial metabolism registered with the radioisotope technique in the REX groundwater were on

the order of 0.02–12.0 mM per litre per day, which is faster than in other subsurface environments (Phelps et al, 1989; Chapelle, Lovely, 1990; Murphy et al, 1992). The radioactive laboratory measurements tend to overestimate natural respiration rate by a factor of 1000 to 100,000. *In situ* microbial oxygen reduction was assessed in deep groundwater in Cape Cod, Massachusetts. The $^{18}O_2$ test indicated a respiration rate of 0.8 µM per day, whereas microcosm experiments with groundwater and sedimentary minerals gave 100-fold higher rates than tracer test rate estimates (Smith et al, 1999). Using the same tracer, $^{18}O_2$ gave similar results, 1.1 µM per day (Penarrieta, 1998). For example, the natural biogenic calcite formation may be extremely slow process. One model shows that 0.04 µg calcite should have formed in 1 g of rock over 47 years (Tobin et al, 1999).

Our direct measurements of microbial oxygen uptake, carbon dioxide production and methane oxidation in KA2862A and REX groundwater confirmed that cells were not dormant or inhibited with oxygen. In addition, we were able to culture up to 98% of microorganisms sampled from the REX chamber. We have shown that 20–99% of the viable cells could reduce oxygen in the REX experiment groundwater.

6.6 Microbial reduction of oxygen

Unattached oxygen-consuming microbial activity in Äspö groundwater was demonstrated during microbiological control of the *in situ* REX experiment. The incubations were kept at *in situ* temperature and in the native groundwater without any additions to simulate *in situ* conditions or stimulate organisms that are representative of the groundwater. Phsychrophilic and psychrotrophic organisms dominated aerobic oxygen reduction in the groundwater studied (Kotelnikova et al, 1998; Kotelnikova et al, 1999; Kaluznaya et al, 1999). The *in vitro* incubations of the groundwater were limited in time to minimise growth.

We have shown microbial oxygen uptake, carbon dioxide production and methane oxidation in the REX groundwater that confirmed previous observations (Kotelnikova, Pedersen, 1998a; Kotelnikova, Pedersen, 1999). The absence of carbon dioxide production and the low level of oxygen reduction in the killed controls indicated that abiotic oxygen reduction was not significant in the groundwater.

After sampling the 28 of January 1999, methane and hydrogen were added at concentrations exceeding *in situ* (7 µm) results, which further enhanced oxygen uptake (Table 8-14). The increase of oxygen uptake upon addition of methane or hydrogen concentrations in the *in vitro* experiments demonstrated that the microorganisms in our *in vitro* tests were electron-donor limited. However, the oxygen reduction rates counted per cell in the REX groundwater *in vitro* were close to those measured for pure cultures isolated from other Äspö sources at excess of methane. Microbial oxygen reduction coefficients in the groundwater were similar to in pure cultures (Table 8-15 and 8-16). This means that our *in vitro* evaluations of the specific microbial oxygen reduction may be regarded as close to *in situ* activities and may be used in modelling.

6.7 What kind of microorganisms was responsible for oxygen reduction during the REX experiment?

What kind of microbial processes were responsible for biological oxygen reduction from the REX groundwater? Different microbial catalysis impacted the oxygen reduction in accordance with available electron donors, microbial activities and viable competent organisms. Our most probable numbering and gas chromatography measurements demonstrated that only part of the oxygen reduction was due to

organotrophic oxygen reduction, whereas the rest were coupled to methane and hydrogen oxidation. Radioisotope experiment showed that methane, hydrogen, acetate and formate were actively used in the presence of oxygen (Kotelnikova, Pedersen, 1999). The microbial reoxidation of reduced inorganic compounds like CO, Mn^{2+}, Fe^{2+} and reduced sulphur species was not studied.

The unattached organisms reduced oxygen at reproducible rates after repetitive and periodical samplings. The MORC in the REX chamber groundwater without substrate additions were slightly lower than the rates in newly oxidised KA2862A groundwater collected from the anaerobic borehole. This fact suggests that aerobic heterotrophic bacteria in the REX groundwater progressively removed bioavailable organic carbon matter in the presence of oxygen. Methane, hydrogen and dissolved organic carbon are present in many subsurface environments (Aravena, Wassenaar ,1993).

6.8 Attached microflora

The microbial oxygen reduction rates were reproducible over different samplings. Experimental *in vitro* oxygen reduction rates by unattached organisms ranged between 0.47 and 1.78 µM per day. The oxygen reduction observed in the *in situ* experiment ranged from 12 to 35 µM O_2 per day. The difference in the levels of oxygen reduction observed in the *in vitro* and *in situ* experiments may indicate that: 1) *in vitro* conditions did not encourage development of microbial oxygen reduction activity or 2) non-identified mechanisms of oxygen reduction were involved in the *in situ* experiment.

The first alternative seems to be true because microorganisms were electron donor limited under *in vitro* conditions. The second alternative may include mineral reactions, diffusion of anaerobic groundwater into the REX chamber and ***reduction of oxygen by biofilms***. The chloride and calcium containing groundwater was pumped through the system and bore certain amounts of nutrients and electron donors. These factors are supposed to contribute to biofilm formations on surfaces of the PEEK tubes, drill core and gold cap in the REX chamber.

After sampling of the PEEK tube we showed that biofilms developed on inside surfaces of PEEK tubes that were used in the *in situ* REX experiment and on the REX drill core and gold cap. Our results are consistent with the data received in the other laboratory. Biofilm development has been observed on the mineral surface in the Replica pilot experiment (Minutes of REX meeting, 151299, TR 99).

According to previous observations in Äspö and data in the literature, the cell density of the biofilms may exceed those observed in the groundwater. Previous biofilm development experiments have indicated active attached microbial population of up to 2.2×10^9 cells cm^{-2} on surfaces exposed to flowing groundwater (0.2 mm s^{-1}) over eight weeks (Pedersen, Ekendahl, 1990). The viable numbers counted in the biofilms both in Replica and REX experiments are very similar and in agreement with the data in the literature.

Oxygen reduction efficiency of attached cells is expected to be higher than those of unattached cells, because the biofilms contain a denser cell population. The cell population is expected to be more adapted to aerobic conditions than the free-living cells because it has been exposed to oxygen periodically with each oxygen pulse. Thus, the oxygen uptake in biofilms may exceed the oxygen uptake in the groundwater. We do not have data to be able calculate the biofilm MORC. We used the MORC of unattached cells to reproduce oxygen respiration in biofilms.

6.9 Modelled reduction of oxygen by unattached and attached microorganisms

Thus, our qualitative evaluations showed that microbial reactions consuming oxygen take place in the oxygenated groundwater, but the most important question was: which part of oxygen reduction was mediated by microorganisms? To answer this question two approaches have been used. The first was modelling and calculating microbial oxygen reduction on the basis of the values retrieved experimentally on specific MORC, kinetics and cell densities of both attached and unattached populations. The second was experimental inhibition of microbial activity directly during the *in situ* experiment.

We modelled total microbial reduction assuming that two microbial populations contribute to the reduction, namely, unattached and attached. In other words, we assumed that microbes inhabiting groundwater and biofilms on tube and mineral core surfaces participated in oxygen reduction proportionally to their cell densities. In our model we assumed that all cells were evenly active. The specific microbial oxygen reduction coefficient was used to reproduce microbial oxygen reduction by biofilms. Because we have shown that up to 99% of cells in REX groundwater were viable, we used acridine-orange direct cell counts in our model. Microbial reduction and density were analysed empirically in the groundwater. These values were used to calculate the microbial oxygen-reduction coefficient. Experimentally elucidated microbial density in the biofilms allowed us to build the primary model of the microbial reduction by the biofilms. The sum of the attached and unattached microbial oxygen reduction was compared with the oxygen uptake rate calculated with *in situ* REX experiment results (Table 8-15 and 8-17). The microbial oxygen uptake *in vitro* was calculated using the same equation as for oxygen reduction *in situ*.

The Michaeles-Menten kinetic was used to model oxygen reduction in the *in situ* experiment. This secondary model describing the effect of the initial oxygen concentrations on the oxygen reduction rate of the *in situ* experiment was empirically built from the experimental data concerning Äspö groundwaters. This work pointed out that the higher the oxygen concentration, the higher the reduction rate measured under *in situ* conditions.

Application of the primary and secondary models showed that the oxygen uptake coefficients calculated using the model fit with the empirical values. The model calculations based on empirical data on microbial numbers and MORC gave the oxygen reduction values close to the rates measured under *in situ* conditions (Table 8-15 and 8-17). The Michaeles-Menten modelled curve was in agreement with the *in situ* data. These facts indicate *that attached and unattached microorganisms were responsible for the oxygen uptake observed under in situ conditions.* Kinetic of the oxygen reduction in the Replica experiment was also close to that of the microbial kinetic. It is remarkable that reduction time in the Replica experiment decreased with each new oxygen pulse (Minutes of REX meeting, 151299). This observation agrees with the biological nature of oxygen reduction and our finding of successive adaptation of the microbial population. This adaptation phenomenon is not expressed in the REX because of water replacements.

The inhibition experiment with the REX chamber provided additional evidence for the microbial contribution. The kinetic of the oxygen uptake was altered. The rate of the oxygen uptake in the REX chamber dropped (Table 8-17). The oxygen reduction was inhibited *in situ.* Total inhibition of viable microbial cells was 54% in the REX groundwater and 92% on REX tubes as determined with MPN method after the inhibition. We observed active microflora both on surface of the drill core and the gold

cap two months after the inhibition. Thus, in accordance with the culturing results, the microbial activity was not inhibited completely, which means that the *residual oxygen reduction observed in situ took place because of the microbial respiration of oxygen* both in the groundwater and in the biofilms.

The integration of the* in situ *data set indicates that attached and unattached microorganisms contribute to the oxygen reduction and evolution of low redox potential under* in situ *conditions during the tested period. The successive microbial contribution and the microbial promotion of the reduced species are particularly important.

7 References

Aragno M, Schlegel H.G. (1991) The mesophilic hydrogen-oxidising (Knallgas) bacteria. In: Ballow A, Truper HG, Dworkin M, Harder W, Schleifer K-H, editors. *The Prokaryotes.* New York: Springer-Verlag, 344-84.

Aravena R, Wassenaar L.I. (1993) Dissolved organic carbon and methane in a regional confined aquifer, southern Ontario, Canada: Carbon isotope evidence for associated subsurface sources. *Applied Geochemistry* 8:483-93.

Busse HG, Auling G. (1991) The genera Alcaligenes and "Achromobacter". In: Ballows A, Trupper HG, Dworkin M, Harder W, Schleifer K-H, editors. *The Prokaryotes.* New York: Springer-Verlad, 2544-55.

Balashova V V, Zavarzin G.A. (1979) Anaerobic reduction of ferric iron by hydrogen bacteria. *Mikrobiologiya* 48:773-778 (Engl. Transl. P. 635-639)

Chapelle, F.H., Lovley, D.R. (1990) Rates of microbial metabolism in deep coastal plain aquifers. *Appl. Environ. Microbiol.* 56, 1865-1874.

Cord-Ruwisch, R, Lovely D.R Schink B. (1999). Growth of *Geobacter sulfurreducens* with acetate in syntrophic cooperation with hydrogen-oxidizing anaerobic partners. *Appl. Environ. Microbiol.* 64:2232-2242.

D.C. Cooper, F.W. Picardal, J. Rivera. (1999) Trace metal immobilization via Microbial Reduction of Iron Oxide Minerals. 4-th International symposium on subsurface microbiology, Abstract book, Vail, Colorado. P.42.

Ehrlich H.L. (1990) Geomicrobiology. New York and Basel: Marcel Dekker.

Hanson R.S, Hanson T.E. (1996) Methanotrophic bacteria. *Microbiol Rev* 1996;60:439-71.

Hersman L.E, Forsythe J. H, Maurice P.A, Sposito G. (1999) Dissolution of Fe oxides by an aerobic bacterium. 4-th International symposium on subsurface microbiology, Abstract book, Vail, Colorado. P.46.

Hungate R.E. (1969) A roll tube method for the cultivation of strict anaerobes. In: Norris JL, and Ribbons DW, editors. *Methods in Microbiology.* New York: Acad. Press. Inc., 117-32.

M^{c}Eldowney, S, Valsami-Jones, E., Gaze, W. and M^{c}Lean, J. (1999) Dissolution of Potassium-Feldspar by Heterotrophic Bacteria. 4-th International symposium on subsurface microbiology, Abstract book, Vail, Colorado. P.46.

Kaluzhnaya, M.G., Khemelina, V.N., Kotelnikova, S., Holmquist, L., Pedersen, K. (1999) *Methylomonas scandinavica*, a new methanotrophic bacterium from deep granitic ground waterof Äspö Hard Rock laboratory in Sweden. *Syst Appl Microbiol.*, 22: 565-572.

Koch AL. (1994) Most probable numbers. In: Gerhardt P, Murray RGE, Wood WA, Krieg NR, editors. *Methods for General and Molecular bacteriology.* Washington: American society for microbiology, 257-60.

Kotelnikova S, Pedersen K. (1997) Evidence for methanogenic *Archaea* and homoacetogenic *Bacteria* in deep granitic rock aquifers. *FEMS Microbiol Rev* 20:327-39.

Kotelnikova SV, Macario AJA, Pedersen K. (1998) *Methanobacterium subterraneum*, a new alcalophilic, eurythermic and halotolerant methanogen isolated from deep granitic groundwater. *Int J Syst Bacteriol* 48:357-67.

Kotelnikova S, Pedersen K. (1998a) Microbial oxygen consumption in Äspö tunnel environments. In: Swedish Nuclear Power Fuel, editor. *Progress report HRL 98-11.* Stockholm: SKB, 1-98.

Kotelnikova SV, Pedersen K. (1998b) Distribution and activity of methanogens and homoacetogens in deep granitic aquiferes at Äspö Hard Rock Laboratory, Sweden. *FEMS Microbiol Ecol* 26:121-34.

Kotelnikova, S., Pedersen, K. (1999) Microbial oxygen reduction in the Äspö tunnel: the Microbe-REX project. SKB Technical report 99-17. Swedish Nuclear Waste Fuel Company (SKB), Stockholm. pp 1-87.

Kotelnikova S. Molleström C. and Pedersen K. (1999) Methane oxidation in deep granitic groundwaters, manuscript.

Lovely D.R, Philips E.J.P. (1986) Availability of ferric iron for microbial reduction in bottom sediments of the fresh water tidal Potomac River. *Appl Environ Microbiol* 52:751-7.

Lovely D.R, Phillips E.J. (1988) Novel mode of microbial energy metabolism: organic carbon oxidation coupled to dissimilatory reduction of iron or manganese. *Appl Environ Microbiol* 54:1472-80.

Lovley D.R, Coates J.D, Saffarini D, Lonergan D. J. (1997) Diversity of dissimilatory Fe(III)-reducing bacteria. In: Winkelman G, Carrano CJ, editors. *Iron and related transition metals in microbial metabolism.* Amsterdam, The Netharlands: Harwood academic publisher, 187-215.

Marshall K.C. (1997) Colonization, adhesion and biofilms. In Manual of environmental microbiology. ASM press, Washington:358-359.

Murphy EM, Schramke J.A, Fredrickson J.K, Bledsoe H.W, Francis A.J, Sklarew D. (1992) The influence of microbial activity and sedimentary organic carbon on the isotope geochemistry of the Middendorf aquifer. *Water Resour Res.* 28:723-40.

Murphy, E.M., Schramke, J.A., Fredrickson, J.K., Bledsoe, H.W., Francis, A.J., Sklarew, D.S., Linehan, J.C. (1992) The influence of microbial activity and sedimentary organic carbon on the isotope geochemistry of the Middendorf aquifer. *Water Resour Res.* 28, 723-740.

Niemelä S. (1983) NMKL Report. Uppsala: Nordic committee on food analysis.

Nordström, D.K. Puigdomenech, I. McNutt, R.H. (1990) Geochemical modelling of water/rock interactions at the Osamu Utsumi mine and Morro do Ferro analogue study sites, Pocos de Caldas, Brazil Technical report 1990 SKB, Stockholm.

Pedersen K. (1995) Microbiological investigations. In: Banwart S, editor. *The redox experiment in block scale.* Stockholm: SKB,134-67.

Pedersen, K., Ekendahl, S. (1990) Distribution of bacteria in deep granitic groundwaters of Southeasthern Sweden. *Microbiological Ecology* 20, 37-52.

Phelps, T.J., Raione, E.G., White, D.C., Fliermans, C.B. (1989) Microbial activities in deep subsurface environments. Geomicrobiology Journal.7, 79-91. PETTERSSON C, EPHRAIM J, ALLARD B, BOREN H. Characterization of humic substances from deep groundwaters in granitic bedrock in Sweden. In *SKB Technical Report* Edited by PETTERSSON C. 1990 90-29, p 40. Swedish Nuclear Fuel and Waste management Co, Stockholm.

Penarrieta C.T. (1998) Assesment of microbial oxygen consumption in a Cap Cod aquifer using a natural gradient tracer test and core incubations: Bouldert, Iniversity of Colorado, Department of Civil Engenering, unpublished, MS. Thesis 131p.

Pettersson C, Ephraim J, Allard B. Boren H. (1990) Characterization of humic substances from deep groundwaters in granitic bedrock in Sweden. In *SKB Technical Report* Edited by Pettersson C 90-29, p 40. Swedish Nuclear Fuel and Waste management Co, Stockholm.

Preuss A, Schauder R, Fuchs G, Stichler W. (1989) Carbon isotop fractionation by autotrophic bacteria with three different CO_2 fixation pathways. *Z Naturforsch [A]* 44c:397-402.

Puigdomenech I, Banwart S A, Bateman K, Griffault L,Gustafsson E, Hama K, Kotelnikova S, Lartiquee J-E, Michaud V, Milodovski A E, Morosini M, Pedersen K, Rives Perez J, Trogtignon L, Tullborg E-L, West J, and Yoshida H. (1998) Äspö HRL- Redox experiment in detailed scale (REX): first project status report SKB in press 111 p.

Rogers, J.R., Bennett, P.C., Hiebert, F.K., and Choi, W.J. (1999) Nutrient-Driven Colonization and Weathering of Silicates. . 4-th International symposium on subsurface microbiology, Abstract book, Vail, Colorado, p.46.

Smith, R L. Böhlke, J K. Revesz, K M. Yoshinari, T. Hatzinger, P B. Penarrieta, C T. Repert, D A. In situ assessment of the transport and microbial consumption of oxygen in ground water, Cape Cod, Massachusetts. In: Proceedings of the technical meeting, Charleston, South Carolina, March 8-12,1999 Morganwalp, D W. Buxton, H T. Eds. West Trenton, New Jersey, 1999, p. 317-322.

Tobin, K.J.1, Colwell, F.S., Onstott, T.C., Fredrickson, J., and Smith, R (1999) Modern calcite precipitation in a fractured aquifer waste plume: reconciling laboratory and natural microbial CO_2 mineralization. In: 4-th International symposium on subsurface microbiology, Abstract book, Vail, Colorado, p.47.

Trotignon, L, Michaud V, Lartigue J-E, Eisenlohr L, Ambrosi J-P.(1999) Participation in the REX project (Äspö HRL): presentation and interpretation of the results of the Replica experiment, ANDRA TR (draft) 54 p.

Smith R L, Bohlke J K, Revesz K M, Yoshinari T. Hatzinger P B, Penarietta C T, Perert D A(1999). In situ assessment of the transport and microbial consumption of oxygen in ground water, Cape Cod, Massachusetts. 4-th International symposium on subsurface microbiology, Abstract book, Vail, Colorado

Scnurer A, Houwen FP, Svensson BH. (1994) Mesophilic syntrophic acetate oxidation during methane formation by a triculture at high ammonium concentration. *Arch Microbiol* 162:70-4.

Stevens, T.O., McKinley, J.P., Fredrickson, J.K. (1993) Bacteria associated with Deep, Alkaline, Anaerobic Groundwaters in Southeast Washington. *Microb Ecol.* 25, 35-50.

Stumm W, Morgan JJ. (1981) Oxidation and reduction. In: Stumm M, editor. *Aquatic chemistry.* New York: A Wiley Interscience publication, 418-502.

Wolin EA, Wolin MJ, Wolf J. (1963) Formation of methane by bacterial extracts. *J Biol Chem* 238:2882-6.

Zehnder AJB, Huser B, Brock TD. (1979) Measuring radioactive methane with the liquid scintillation counter. *Appl Environ Microbiol* 37:897-899.

8 Appendix

Table 8-1 *Dissolved gas in the REX groundwater sampled 970411.*

		Borehole	
		KA2861A	KA2862A
N_2 (µM)	[RSD%]	1992 [2.23]	301 [1.55]
He (µM)	[RSD%]	173 [26]	0.74 [15]
H_2 (µM)	[RSD%]	1.96 [10]	0.014 [85]
CH_4 (µM)	[RSD%]	1.13 [27]	0.09 [72]
CO_2 (µM)	[RSD%]	0.89 [6]	14 [11]

Table 8-2 *Acridine Orange Direct counts (AODC) in the groundwaters that was sampled 980311.*

	Borehole			
	KA2861A		KA2862A	
Numbers of cells	**8.5-9.8 m**	**0-8.5 m**	**6.8-6.9 m**	**7.4-16.0 m**
Average (cells ml^{-1})	3.4×10^5	7.4×10^5	1.3×10^5	2.3×10^3
Minimum (cells ml^{-1})	4.0×10^5	6.2×10^5	1.0×10^5	9.7×10^2
Maximum (cells ml^{-1})	-	7.9×10^5	1.2×10^5	5.4×10^3
RSD, %	22.11	11.89	29.54	84.97

Table 8-3 MPN of heterotrophic microorganisms counted in modified liquid R2A medium, 980311.

	Borehole			
	KA2861A		KA2862A	
Numbers of cells	**8.5-9.8 m**	**0-8.5 m**	**6.8-6.9 m**	**7.4-16.0 m**
Average, (cells ml^{-1})	8.5×10^3	1.5×10^3	1.9×10^4	0
Minimum (cells ml^{-1})	3.3×10^3	5.5×10^2	6.7×10^3	0
Maximum (cells ml^{-1})	4.2×10^4	5.5×10^4	7.7×10^4	0
Percent of total number,%	12.20	7.37	58.68	0.00

Table 8-4 *Viable heterotrophic microorganisms counted on modified solid R2A medium, 980311.*

	Borehole			
	KA2861A		KA2862A	
Numbers of cells	**8.5-9.8 m**	**0-8.5 m**	**6.8-6.9 m**	**7.4-16.0 m**
Average, (cells ml^{-1})	6.7×10^3	2.1×10^4	1.7×10^2	0
Minimum, (cells ml^{-1})	3.3×10^3	1.2×10^4	1.3×10^2	0
Maximum, (cells ml^{-1})	9.3×10^3	3.8×10^4	3.3×10^2	0
Percent of total number,%	2.70	5.09	0.25	0.00

Table 8-5 *MPN of methane-oxidising bacteria (MOB) counted in NMS+Cu medium, 980311.*

	Borehole			
	KA2861A		KA2862A	
Numbers of cells	**8.5-9.8 m**	**0-8.5 m**	**6.8-6.9 m**	**7.4-16.0 m**
Average, (cells ml^{-1})	1.9×10^4	2.0×10^4	8.5×10^1	0
Minimum, (cells ml^{-1})	6.7×10^3	8.0×10^3	3.3×10^1	0
Maximum, (cells ml^{-1})	7.7×10^4	8.0×10^4	4.2×10^1	0
Percent of total number,%	22.36	23.24	0.0	0,00

Table 8-6 *MPN of methane-oxidising bacteria (MOB) counted in NMS-Cu medium, 980311.*

	Borehole			
	KA2861A		KA2862A	
Numbers of cells	**8.5-9.8 m**	**0-8.5 m**	**6.8-6.9 m**	**7.4-16.0 m**
Average, (cells ml^{-1})	8.5×10^3	8.5×10^3	1.0×10^0	1.5×10^0
Minimum, (cells ml^{-1})	3.3×10^3	3.3×10^3	2.0×10^{-1}	5.0×10^0
Maximum, (cells ml^{-1})	4.2×10^4	4.2×10^4	4.1×10^0	5.5×10^0
Percent of total number, %	12.20	12.20	0.001	0.002

Table 8-7 *MPN of hydrogen-oxidising bacteria (HOB) counted in HOB medium.*

	Borehole			
	KA2861A		KA2862A	
Numbers of cells	**8.5-9.8 m**	**0-8.5 m**	**6.8-6.9 m**	**7.4-16.0 m**
Average, (cells ml^{-1})	4.8×10^3	1.9×10^3	4.6×10^1	0
Minimum, (cells ml^{-1})	1.8×10^3	6.7×10^2	1.7×10^1	0
Maximum, (cells ml^{-1})	2.8×10^4	7.7×10^4	2.5×10^2	0
Percent of total number, %	8.13	10.32	0.19	0.00

Table 8-8 *Effect of uranin, CH_4, H_2 and the mineral phase (crushed granite from KA2861A core) on microbial oxygen reduction in the groundwater from the REX site sampled 11.03.98*

Treatment	Oxygen consumption μM day^{-1}	pm oxygen cell^{-1} day^{-1}	Carbon dioxide production μM day^{-1}	Methane oxidation nM day^{-1}	delta pH
KA2862A, section 6.8-6.9 m					
Groundwater (GW)	1.62	0.0120	3.23	0.25	0.95
GW + 0.05% uranin	0	0	2.13	0	1.87
GW+5% mineral phase	2.78	0.0212	4.92	0	0.56
RSD,%	5.6		21	12	1.5
KA2861A, section 8.5-9.8 m					
Groundwater (GW)	1.26	0.0036	3.7	0.52	0.37
RSD,%	32		0.8	2.2	1.5
KA2861A, section 0-8.5 m					
Groundwater (GW)	1.34	0.0018	0.42	10.79	0.34
GW + 0.05% uranin	1.39		0.51	10.79	0.4
GW+5% mineral phase	1.39		4.68	11.2	0.14
RSD,%		38	50	5	1.3

RSD indicates percent of relative standard error in the analytical procedure based on repeated measurements. The rates are presented as difference between rates in non-inhibited sample and killed control. The killed control contained 2% formaldehyde, 1% sodium aside, 1 mg/l mercury chloride, 400 mg/l streptomycin and 0.58 mg/l bacitracin

Table 8-9 *Composition of the microbial population in the Replica and* in situ *REX experiments.*

Microbial group	Replica experiment		REX chamber	
	1-st pulse	3-d pulse	980707	980722
Total number (cells ml^{-1})	2.7×10^6	4.6×10^6	7.7×10^5	5.0×10^5
Heterotrophic aerobic microbes (cells ml^{-1})	2.5×10^2	6.0×10^3	8.0×10^4	5.6×10^4
Heterotrophic microaerophiles (cells ml^{-1})	na*	na	8.5×10^3	8.5×10^4
Heterotrophic anaerobes (cells ml^{-1})	6×10^0	2.5×10^0	na	na
Methane-utilising bacteria (cells ml^{-1})	<0.5	<0.5	1.9×10^4	4.6×10^2
Hydrogen-utilising bacteria (cells ml^{-1})	na	na	3.0×10^4	8.8×10^4
Iron-reducing bacteria (cells ml^{-1})	2.5×10^4	6.0×10^4	2.8×10^4	4.8×10^4
Sulfate-reducing bacteria (cells ml^{-1})	2.5×10^1	<0.5	1.8×10^3	2.4×10^2
Methanogens (cells ml^{-1})	<0.5	<0.5	4.6×10^2	3.1×10^3

* not analysed

Table 8-10 *Composition of culturable microbial population during* in situ *REX experiment (1998-1999).*

Microbial group	REX chamber				
	980707	980722	981029	990128	990312
Total number (cells ml^{-1})	7.7×10^5	5.0×10^5	5.5×10^5	1.8×10^5	5.3×10^5
Heterotrophic aerobic microbes (cells ml^{-1})	8.0×10^4	5.6×10^4	6.0×10^3	6.7×10^4	5.0×10^5
Heterotrophic microaerophiles (cells ml^{-1})	8.5×10^3	8.5×10^4	1.3×10^4	3.8×10^4	6.9×10^3
Heterotrophic anaerobes (cells ml^{-1})	na*	na	na	na	na
Methane-utilising bacteria (cells ml^{-1})	1.9×10^4	4.6×10^2	4.1×10^3	1.4×10^4	5.5×10^3
Hydrogen-utilising bacteria (cells ml^{-1})	3.0×10^5	8.8×10^4	4.1×10^4	4.9×10^4	1.0×10^5
Iron-reducing bacteria (cells ml^{-1})	2.8×10^4	4.8×10^4	6.9×10^3	1.3×10^3	6.9×10^2
Sulfate-reducing bacteria (cells ml^{-1})	1.8×10^3	2.4×10^2	7.6×10^1	5.5×10^1	6.9×10^1
Methanogens (cells ml^{-1})	4.6×10^2	3.1×10^3	0	0	0

* not analysed

Table 8-11 *Percentage representation of culturable microbial groups of the total number of cells during in groundwater from* in situ *REX experiment (1998-1999).*

Microbial group	KA2862A section (7.4-16.0 m)				REX chamber				
	980311	980707*	990128	990312	980707	980722	981029	990128	990312
Heterotrophic aerobic microbes	0.25	14.5	4.7	0	13.3	14.0	0.24	75.7	63.1
Heterotrophic microaerophiles	58.7	5.48	58	1.37	5.48	16.9	4.56	23.9	2.27
Methane-utilising bacteria	0.001	13.9	0.05	0.06	10.1	0.50	1.37	12.2	1.87
Hydrogen-utilising bacteria	0,19	12.4	0.07	0	52.2	39.7	13.3	27.6	32.1
Iron-reducing bacteria	na*	1.2	na	2.32	9.27	55.6	2.19	1.42	0.23
Sulfate-reducing bacteria	na	13.9	42.8	54.7	0.98	0.12	0.03	0.06	0.02
Methanogens	na	1.2	6.7	0	0.33	1.63	0.00	0.00	0.00

* not analysed

Table 8-12 *Shift of pH values of the groundwater during 3 weeks* in vitro *oxygen reduction tests in the REX chamber.*

Treatment	Sampling date									
	980707		980722		981029		990128		990312	
	pH	delta pH*	pH	delta pH	pH	delta pH	pH	delta pH	pH	delta pH
Ground water (GW)	6.67	1.13	6.94	0.86	6.62	1.08	9	0.77	8.35	0.64
GW+ inhibitors	6.35	1.45	6.41	1.39	6.45	1.35	7	2.77	6.93	2.84
GW+CH_4, 300 uM	6.68	1.12	6.72	1.08	6.43	1.37	9.1	0.67	8.54	1.23
GW+CH_4+inhibitors	6.08	1.72	6.4	1.4	6.45	1.35	9.06	0.71	7.36	2.41
GW+H_2. 1000 nM	6.57	1.23	6.76	1.04	6.45	1.35	8.75	1.02	8.54	1.23
GW+H_2 +inhibitors	6.16	1.64	6.54	1.26	6.05	1.75	6.78	2.99	7.11	2,66

Shift of pH values of the groundwater during 3 weeks in vitro oxygen reduction tests in groundwater from KA2832A section (7.4-16.0 m).

Treatment	Sampling date					
	980311		990128		990312	
	pH	delta pH*	pH	delta pH	pH	delta pH
Ground water (GW)	6.13	1.53	7.5	0.2	6.69	1.01
GW+ inhibitors	6.96	0.7	7.23	0.47	6.48	1.22
GW+CH_4, 300 uM	na**		7.69	0.01	6.54	1.16
GW+CH_4+inhibitors	na		7.23	0.47	6.64	1.06
GW+H_2, 1000 nM	na		7.6	0.1	7.15	0.55
GW+H_2 +inhibitors	na		7.29	0.41	6.81	0.89

na** not analysed,
*delta pH was calculated as difference between initial and averaged final pH values in the incubation vials

Table 8-13 *Microbial oxygen uptake, carbon dioxide production and methane oxidation in KA2862A groundwater from section 7.4-16.0 m incubated under* in vitro *conditions.*

Treatment	Date	Oxygen consumption rate μM day^{-1}	pM oxygen cell^{-1} day^{-1}	Carbon dioxide production μM day-1	Methane oxidation μM day-1	delta pHh
KA2862A	990128					
Ground water (GW)		1.23	0.01	0.15	0	-0.46
GW+CH4, 7000 uM		0		0	0	-0.24
GW+H_2, 1000 nM		0		0	0	-0.33
RSD,%		0.75		58	1.4	
KA2862A	990312					
Ground water (GW)		0.06	0.0008	2.04	0	-0.9
GW+CH4, 300 uM		0	0		0	-1
GW+H_2, 1000 nM		0	0		0	-0.45
RSD, %		0.7	1.1		1.1	

The rates are presented as difference between rates in non-inhibited sample and killed control. The killed control contained 2% formaldehyde, 1% sodium aside, 1 mg/l mercury chloride, 400 mg/l streptomycin and 0.58 mg/l bacitracin

Table 8-14 *Microbial oxygen uptake, carbon dioxide production and methane oxidation in the REX chamber groundwater incubated under* in vitro *conditions.*

Treatment	Date	Oxygen consumption rate μM day^{-1}	pmol oxygen $cell^{-1}$ day^{-1}	Carbon dioxide production μM day^{-1}	Methane oxidation μM day-1	delta pH
Ground water (GW)	980707	0.725	0.00095	0.7	0	-0.79
GW+CH_4, 500 nM		2.16	0.00282	1.4	1.08	-0.37
GW+H_2, 1000 nM		1.58	0.00206	2.8	0	-0.87
RSD,%*		25		20	25	
Ground water (GW)	980722	0.72	0.00143	0	0	-0.48
GW+CH_4, 500 nM		0.5	0.00099	0.4	2.03	-0.25
GW+H_2, 1000 nM		0.49	0.00097	0.5	0	-29
RSD,%		25		19	12	
Uranin, 1 mg/l	981029	1.17	0.00213	0.6	0	0
Ground water		0.46	0.00084	0.6	0.58	-0.2
GW+CH_4, 500 nM		0.49	0.00089	0.8	2.22	-0.1
GW+H_2, 1000 nM		0.97	0.00177	0.5	0	-0.2
RSD,%		31		1.6	12	1.5
Ground water (GW)	990128	0.21	0.00012	0	0	-1.2
GW+CH_4, 7000 uM		3.33	0.01891	0.44	1.65	-0.64
GW+H_2, 1000 nM		2.44	0.01386	0.67	0.08	-0.92
RSD,%		4.1		22	0.41	
Ground water (GW)	990312	0.77	0.00145	1.5	0	-1.35
GW+CH_4, 300 uM		1.06	0.00200	2.79	0.94	-1.16
GW+H_2, 1000 nM		0.47	0.00089	1.58	0.02	-1.16
RSD,%		3.2		5.8	2.6	

The oxygen uptake were counted as (O2)-(O2)/time. The uptakes are presented as difference between of samples and killed controls. Killed controls contained 2% formaldehyde, 1% sodium aside, 1 mg/l mercury chloride, 400 mg/l of streptomycin and 0.58 mg/l of bacitracin.

* RSD indicates maximal percent of relative standard error in the analytical procedure based on repeated mesurements of duplicated samples

Table 8-15 *Microbial oxygen uptake* in vitro *in groundwater from the REX chamber and modelled oxygen uptake by attached and unattached microflora.*

Date of sampling	**Time days**	**Initial O_2 μM**	**Final O_2 μM**	**AODC cells ml^{-1}**	**Microbial oxygen uptake**	**Microbial oxygen reduction coefficient* (MORC) μM $day^{-1}cell^{-1}$**	**Number of cells in the REX system****	**Modelled total oxygen uptake μM day^{-1}**	**The uptake observed *in situ***
980707	17	270.2	254.94	7.66×10^5	2.43	1.17×10^9	6.85×10^{10}	8.03	n.d.
	17	269.5	247.52	7.66×10^5	2.43	1.69×10^9	6.85×10^{10}	11.56	n.d.
980722	29	277.9	256.2	5.03×10^5	2.44	1.49×10^9	6.85×10^{10}	10.18	n.d.
	29	277.5	254.8	5.03×10^5	2.44	1.55×10^9	6.85×10^{10}	10.65	n.d.
981029	34	280	259	3.14×10^5	2.45	1.96×10^9	6.85×10^{10}	13.45	13.44
	34	280	253	3.14×10^5	2.45	2.53×10^9	6.85×10^{10}	17.30	13.44
990312	27	280	249	5.30×10^5	2.45	2.19×10^9	6.85×10^{10}	14.85	12.80
	27	280	250	5.30×10^5	2.45	2.1×10^9	6.85×10^{10}	14.37	12.80
Average									

* - Microbial oxygen reduction coefficient was calculated as $Rs=[(O_2)_1-(O_2)_2]/[timexAODC]$, (umol oxygen per cell per day)

**- the number of microbial cells in REX system was calculated as sum of the MPN viable cells attached on the inside tubing surfaces of the PEEK, drill core and gold cap and unattached in 2 liters of the REX groundwater., Table 8-18,

Table 8-16 *Oxygen reduction by methanotrophs isolated from Äspö groundwater.*

Isolate designation	Time days	Initial O_2 µM	Final O_2 µM	AODC cells ml^{-1}	Microbial oxygen reduction rate				MORC		Number of cells in the REX chamber	MORC	
					Linear µM day^{-1}	Logarithm initial O_2 µM	Logarithm final O_2	Logarithm µM day^{-1}	Linear µM day^{-1} $cell^{-1}$	Logarithm $cells^{-1}$		Logarithm µM day^{-1}	Linear
3500:k	1	41.79	37.15	1.25×10^7	4.64	1.62	1.57	0.051	3.7×10^{10}	4.07×10^{12}	8.7×10^9	0.0354	3.22
3500:k	1	15.93	13.99	1.25×10^7	1.94	1.20	1.15	0.056	1.55×10^{10}	4.5×10^{12}	8.7×10^9	0.0391	1.34
3500:k	1	37.62	34.88	1.25×10^7	2.74	1.58	1.54	0.033	2.18×10^{10}	2.62×10^{12}	8.7×10^9	0.0228	1.90
KA3110A:1	1	42.37	33.78	1.10×10^8	8.59	1.63	1.53	0.098	7.75×10^{11}	8.88×10^{13}	8.7×10^9	0.0077	0.67
KA3110A:1	1	45.63	44.22	1.10×10^8	1.41	1.66	1.65	0.014	1.27×10^{11}	1.23×10^{13}	8.7×10^9	0.0011	0.11
KA3110A:1	1	44.76	40.64	1.10×10^8	4.12	1.65	1.61	0.042	3.72×10^{11}	3.78×10^{13}	8.7×10^9	0.0033	0.32
KA3105A:1	1	39.57	37.63	4.6×10^6	1.94	1.60	1.58	0.022	4.19×10^{10}	4.71×10^{12}	8.7×10^9	0.0410	3.64
KA3105A:2	1	32.15	31.93	4.6×10^6	0.22	1.51	1.50	0.003	4.75×10^{11}	6.44×10^{13}	8.7×10^9	0.0056	0.41
KA3105A:3	1	41.48	39.46	4.6×10^6	2.02	1.62	1.60	0.022	4.36×10^{10}	4.68×10^{12}	8.7×10^9	0.0407	3.79
3500 vk	1	41.75	37.98	1.17×10^7	3.77	1.62	1.58	0.041	3.2×10^{10}	3.49×10^{12}	8.7×10^9	0.304	2.79
3501 vk	1	43.93	37.46	1.17×10^7	6.47	1.64	1.57	0.069	5.5×10^{10}	5.88×10^{12}	8.7×10^9	0.0511	4.78
3502 vk	1	35.01	32.22	1.17×10^7	2.79	1.54	1.51	0.036	2.37×10^{10}	3.07×10^{12}	8.7×10^9	0.0267	2.06

Table 8-17 *Oxygen uptake observed* in situ *REX experiment.*

Oxygen pulse *in situ* interval	Initial O_2	Final O_2	Time	Initial O_2	Final O_2	O_2 reduction rate	Inhibition
	ppm	ppm	days	μM	μM	μM day^{-1}	%
980910 - 980915	1.3	0.6	4	41.6	19.2	6	0
981029 - 981102	1.2	0.15	2.5	38.4	4.8	13	0
981124 - 981130	0.9	0.2	2.5	28.8	6.4	9	0
981209 - 981218	5	1	5.5	160	32	23	0
990115 - 990128	4.5	1	5	144	32	22	0
990129 - 990206	5.5	1	5.5	176	32	26	0
990218 - 990301	7.5	1.9	5.5	240	60.8	33	0
990302 - 990311	7.8	1.8	5.5	249.6	57.6	35	0
990312 - 990315	1.6	0.4	3	51.2	12.8	13	0
990511 - 990519	30	8	8	960	256	88	25
990519 - 990531	30	12	12	960	384	48	50
990601 - 990608	10	6	5.5	320	192	23	

Table 8-18 *Total cell count in the REX system.*

Sample	Cells per unit	unit	No of units		Total count	Percentage
PEEK tubings	1360	mm^{-2}	4.6×10^7		6.18×10^{10}	91.2
Groundwater	77000	ml^{-1}	1.0×10^3		7.65×10^8	1.2
Drill core surface	35500	mm^{-2}	6.59×10^4		2.34×10^9	3.4
Gold cap surface	56700	mm^{-2}	4.89×10^4		2.77×10^9	4.2
				Sum	6.77×10^{10}	100

Table 8-19 *Microbial numbers in REX chambers groundwater during the* in situ *experiment.*

ACRIDINE ORANGE DIRECT COUNTS (AODC)

Numbers of cells		**980707**	**980722**	**981029**	**990128**	**990312**
Average	cells ml^{-1}	7.6×10^5	5.0×10^5	5.4×10^5	1.8×10^5	5.3×10^5
Minimum	cells ml^{-1}	7.7×10^5	5.0×10^5	5.6×10^5	1.6×10^5	5.3×10^5
Maximum	cells ml^{-1}	7.5×10^5	5.0×10^5	5.3×10^5	1.9×10^5	5.2×10^5
RSD,%		1.85	0.00	4.11	13.98	1.08

AEROBIC MICROORGANISMS

MPN of heterotrophic microorganisms counted in modified liquid R2A medium

Numbers of cells		**980707**	**980722**	**981029**	**990128**	**990312**
Average	cells ml^{-1}	8.5×10^3	8.5×10^4	1.3×10^4	3.8×10^4	6.9×10^3
Minimum	cells ml^{-1}	3.3×10^3	3.3×10^4	1.7×10^3	3.3×10^4	1.7×10^3
Maximum	cells ml^{-1}	4.2×10^4	4.2×10^5	2.5×10^4	4.2×10^4	1.2×10^4
Percent of total number, %		5.48	16.88	4.56	23.86	2.27

Viable heterotrophic microorganisms counted on modified solid R2A medium

Numbers of cells		**980707**	**980722**	**981029**	**990128**	**990312**
Average	cells ml^{-1}	8.0×10^4	5.6×10^4	6×10^2	6.7×10^4	5.0×10^5
Minimum	cells ml^{-1}	1.5×10^4	1.3×10^4	4.4×10^2	1.3×10^3	6.7×10^5
Maximum	cells ml^{-1}	1.0×10^5	7.1×10^4	7.5×10^2	1.3×10^3	3.3×10^5
Percent of total number, %		13.3	14	0.24	75.73	63.07

MPN of hydrogen-oxidising microorganisms counted in HOM medium

Numbers of cells		**980707**	**980722**	**981029**	**990128**	**990312**
Average	cells ml^{-1}	3.0×10^5	8.8×10^4	4.1×10^4	4.9×10^4	1.0×10^5
Minimum	cells ml^{-1}	1.0×10^5	4.2×10^4	6.6×10^3	1.3×10^4	3.2×10^4
Maximum	cells ml^{-1}	4.0×10^5	2.0×10^5	7.5×10^4	8.4×10^4	1.7×10^5
Percent of total number, %		52.22	39.72	13.68	47.71	32.10

Table 8-20 *Microbial numbers in KA2862A groundwater sampled during REX experiment.*

ACRIDINE ORANGE DIRECT COUNTS(AODC)

Groundwaters were sampled from KA2862A boreholes

		980707*	990128	990312
	cells ml^{-1}	2.5×10^6	1.1×10^5	9.3×10^4
	cells ml^{-1}	1.6×10^5	1.1×10^5	5.3×10^4
AODC	cells ml^{-1}	2.0×10^5	1.1×10^5	7.3×10^4
RSD. %		32.69	2.73	38.04

AEROBIC MICROORGANISMS

MPN of heterotrophic microorganisms counted in modified liquid R2A medium

		980707	990128	990312
Average	cells ml^{-1}	2.9×10^3	4.1×10^4	5.6×10^2
Minimum	cells ml^{-1}	1.1×10^3	6.6×10^3	1.1×10^2
Maximum	cells ml^{-1}	9.9×10^3	7.5×10^4	1.0×10^3
Percent of total number, %		5.48	66.76	1.37

Viable heterotrophic microorganisms counted on modified solid R2A medium

		980707	990128	990312
Average	cells ml^{-1}	2.4×10^4	5.2×10^3	0
Minimum	cells ml^{-1}	1.4×10^4	5.1×10^3	0
Maximum	cells ml^{-1}	2.9×10^4	5.2×10^3	0
Percent of total number, %		14.54	4.65	0.00

MPN of hydrogen-oxidising microorganisms counted in HOM medium

		980707	990128	990312
Average	cells ml^{-1}	4.6×10^3	5.0×10^1	0
Minimum	cells ml^{-1}	1.8×10^3	2.5×10^1	0
Maximum	cells ml^{-1}	2.5×10^4	7.5×10^1	0
Percent of total number, %		12.39	0.07	0.00

MPN of methane-oxidising bacteria of group I in NMS+Cu

		980707	990128	990312
Average	cells ml^{-1}	1.5×10^4	3.1×10^1	2.1×10^1
Minimum	cells ml^{-1}	1.8×10^3	0.7×10^1	0.2×10^1
Maximum	cells ml^{-1}	2.8×10^4	5.4×10^1	4.1×10^1
Percent of total number, %		13.88	0.05	0.06

ANAEROBIC MICROORGANISMS

MPN of methane-producing microorganisms in ASPM with formate, acetate, hydrogen, and methanol

		980707	990128	990312
Average	cells ml^{-1}	4.6×10^2	4.1×10^3	0
Minimum	cells ml^{-1}	1.8×10^2	6.6×10^2	0
Maximum	cells ml^{-1}	2.5×10^3	7.5×10^3	0
Percent of total number, %		1.24	6.68	0.00

MPN of iron-reducing bacteria in ASPM added with acetate, hydrogen and ferrous iron

		980707	990128	990312
Average	cells ml^{-1}	1.3×10^3	1.0×10^3	1.0×10^3
Minimum	cells ml^{-1}	1.7×10^2	3.2×10^2	3.2×10^2
Maximum	cells ml^{-1}	2.5×10^3	1.7×10^3	1.7×10^3
Percent of total number, %		1.24	1.51	2.32

MPN of sulphate-reducing bacteria in ASPM with lactate, acetate and hydrogen

		980707	990128	990312
Average	cells ml^{-1}	1.8×10^3	2.5×10^4	2.9×10^4
Minimum	cells ml^{-1}	1.8×10^3	1.8×10^3	1.9×10^4
Maximum	cells ml^{-1}	2.8×10^4	4.8×10^4	4.0×10^4
Percent of total number, %		13.88	42.73	54.69

*- the groundwater from KA2862A was placed in REX chamber and then sampled 07.07.98.
The groundeater was not oxidized or subjected to oxygen pulse

Table 8-21 *Total and viable counts of attached and unattached microorganisms in the REX chamber drill core, gold cap and KA2862A groundwater 990510-990811.*

Groundwaters	**ACRIDE ORANGE DIRECT COUNTS (AODC)**			
	REX chamber		**KA2862A**	**KA2862A**
	990519	**990608**	**990519**	**990608**
cells/ml	$6.67x10^5$	$1.04x10^5$	$1.38x10^5$	$9.05x10^4$
	$6.58x10^5$	$2.08x10^5$	$9.83x10^4$	$1.78x10^5$
			$8.25x10^4$	$9.26x10^4$
AODC	$6.63x10^5$	$1.56x10^5$	$1.06x10^5$	$4.16x10^4$
RSD, %	0.98	47.14	26.62	148.41

AEROBIC MICROORGANISMS

MPN of heterotrophic microorganisms counted in modified liquid R2A medium

cells ml^{-1} or cells mm^{-2}	**REX chamber**		**KA2862A**	**KA2862A**	**REX PEEK tube**		**Drill core**	**Gold cap**
	990519	**990608**	**990519**	**990608**	**990510**	**990608**	**990811**	**990811**
Average	$3.02x10^3$	0	$5.50x10^3$	$1.34x10^2$	$1.1x10^1$	$1x10^1$	$1.71x10^2$	$2.27x10^3$
Maximum	$5.50x10^3$	0	$9.90x10^3$	$2.50x10^2$	$2.2x10^1$	$1.5x10^1$	$2.18x10^2$	$1.84x10^4$
Minimum	$5.50x10^2$	0	$1.10x10^3$	$1.7x10^1$	$5x10^{-1}$	$1x10^{-1}$	$7.1x10^1$	$8.05x10^2$
Percent of total number, %	0.83	0.00	1.03	0.04	n.d.	n.d.	n.d.	n.d.

Viable heterotrophic microorganisms counted on modified solid R2A medium

cells ml^{-1} or cells mm^{-2}	**REX chamber**		**KA2862A**	**KA2862A**	**REX PEEK tube**		**Drill core**	**Gold cap**
	990519	**990608**	**990519**	**990608**	**990510**	**990608**	**990811**	**990811**
Average	$3.40x10^3$	$2.04x10^3$	$5.50x10^3$	$2x10^2$	$1.32x10^2$	$4.5x10^1$	$5.5x10^1$	$1.05x10^3$
Maximum	$6.26x10^3$	$3.86x10^3$	$9.90x10^3$	$2.50x10^2$	$1.66x10^2$	$7.4x10^1$	$9.5x10^1$	$1.25x10^3$
Minimum	$5.50x10^2$	$2.30x10^2$	$1.10x10^3$	$1.50x10^2$	$9.8x10^1$	$1.7x10^1$	$1.5x10^1$	$8.50x10^2$
Percent of total number, %	0.51	1.31	5.17	0.48	n.d.	n.d.	n.d.	n.d.

MPN of hydrogen-oxidising microorganisms counted in HOM medium

cells ml^{-1} or cells mm^{-2}	**REX chamber**		**KA2862A**	**KA2862A**	**REX PEEK tube**		**Drill core**	**Gold cap**
	990519	**990608**	**990519**	**990608**	**990510**	**990608**	**990811**	**990811**
Average	$5.5x10^3$	$4.08x10^2$	$2.27\ 10^3$	$3.0x10^1$	$9x10^0$	$2x10^0$	$1.91x10^4$	$1.91x10^4$
Maximum	$9.90x10^3$	$7.50x10^2$	$4.20x10^2$	$5.5x10^1$	$15x10^0$	$3.27x10^0$	$3.50x10^4$	$3.50x10^4$
Minimum	$1.10x10^3$	$6.6x10^1$	$3.3x10^1$	$4.6x10^0$	$2.74x10^0$	$3.3x10^{-1}$	$3.17x10^3$	$3.17x10^3$
Percent of total number, %	0.83	0.26	0.21	0.07	n.d.	n.d.	n.d.	n.d.