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The Microbe-REX projekt

Microbial O_2 consumption in the Äspö tunnel

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April 1998

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ABSTRACT

The report presents data on microbial O_2 reduction activities by microorganisms obtained with different techniques: Winkler method, gas chromatography, most probable numbering, enrichment technique, inhibitor analysis and radiotracer measurements. The samples were collected from boreholes and open funnel ponds at Äspö in 1996-1998. The evaluation of the microbial activities in open ponds predicts the future microbial activities after the O_2 intrusion around the future repository. The metabolic potential of the microbial population inhabiting groundwater was evaluated on the basis of electron donors available and microbial 16S rRNA gene diversity. The contribution of different microbial groups to the O_2 reduction was elucidated using specific inhibitors selectively affecting different microbial groups.

Our experiments show that microbial O_2 reduction does occurs in deep groundwater. Carbon dioxide was produced concurrently with O_2 reduction confirming the biogenic nature of the reduction. The populations developed O_2 reduction rates and capacity depending on the initial concentration of dissolved O_2 reduction. Rates of O_2 reduction ranged from 0.32 to 4.5 µM/day. Depending on temperature and the type of groundwater the approximate time needed for consumption of 500 µM of dissolved O_2 ranged from 0.31 to 3.99 years. After approximately a 2 weeks period the microbial population *in vitro* was able consume O_2 both at 30°C and 60°C. At 16°C no delay in O_2 consumption was observed. Our results demonstrated that methanotrophs survive in deep groundwater and that they were induced by O_2. Some bacteria use H_2 or CH_4 as electron donor instead of organic matter, which means that microbial O_2 reduction will occur also in deep groundwaters where the availability of organic carbon is limited. Specific CH_4 oxidation rates ranged between 3.00 and 220 nM CH_4 per litre per day. Comparison of the total O_2 reducing activities by gas chromatography and radiotracer tests showed that CH_4 oxidation was responsible for 6.7% of the total O_2 reduction in the groundwater, for 9.1% of the total O_2 reduction on stones and 57% of total O_2 reduction in pond water. Most probable numbering showed that CH_4 oxidation organisms constituted 0.15% and 35% of the total cell population in the groundwater and pond water, respectively. Methane oxidation is proposed as one of the dominating microbial mechanisms for O_2 reduction. The contribution of hydrogen and organic carbon depending organisms remains to be studied. A kinetic model for microbial O_2 has been developed, and it may be applied to performance assessment calculations of nuclear waste repositories.

KEYWORDS: microbial diversity, ground water, microbial O_2 consumption model, CH_4 oxidation

SAMMANFATTNING

Rapporten presenterar bevis på mikrobiell O_2 reduktion i grundvatten baserade på olika metoder: våtkemisk syremätning, gaskromatografiska undersökningar, total och levande cell antal, anrikande och rena kulturer, inhibitor och radiotracer analyser. Proven togs från olika borrhål och öppna vattensamlingar i Äspö tunneln under 1996-1998. Mätningar av den mikrobiella aktiviteten i vattensamlingarna visar på omfattningen av mikrobiell syreförbrukning i det grundvatten som tränger in i slutförvaret efter förslutning. Den metaboliska potentialen hos de mikrobiella populationer som reducerar O_2 i djupa grundvatten har utvärderats i förhållande till tillgängliga elektron donatorer och art-diversitet. Bidraget från olika mikrobiella grupper till O_2 reduktionen har analyserats med hjälp av inhibitorer som selektivt slår ut de studerade mikrobiella grupperna.

Sammantaget visar undersökningen att mikrobiell O_2 reduktion sker i djupa grundvatten. CO_2 produceras samtidigt med O_2 reduktion vilket visar den biologiska naturen av den observerade O_2 reduktionen. De mikrobiella populationerna utvecklade O_2 reduktionshastigheter och kapaciteter som beror av start koncentrationerna av löst O_2. O_2 reduktionshastigheten varierade mellan 0.31 och 4.5 µM O_2 dag^{-1}. Beroende på temperatur och typ av grundvatten varierade den ungefärliga tiden som behövs för reduktion av 500 µM löst O_2 mellan 0.31 och 3.99 år. Efter ungefär två veckors adaptation reducerade de mikrobiella populationerna O_2 både vid 30°C och 60°C. Vid 16°C sker O_2 reduktion direkt utan något adaptation. Våra resultat visar också att metanoxiderandebakterier överlever i djup grundvatten och att deras metabolism aktiveras av O_2.

Vissa bakterier använder H_2 eller CH_4 som elektrondonator i stället för organiskt material, vilket innebär att mikrobiell O_2 reduktionen också kommer att ske på större djup där tillgången på organiskt material är begränsad. Den specifika metanoxidationshastigheten varierade mellan 3 och 220 nM CH_4 $liter^{-1}dag^{-1}$. Jämförelse av den totala O_2 reduktioner med gaskromatografi och radiotracer tester visade att CH_4-oxidation var ansvarig för 6.7% av O_2 reduktionen i grundvattnet, för 9.1% på sprickytor och 57% i de öppna vattensamlingarna. Antalet av levande metanoxiderandebakterier utgör upp till 0.15% av hela mikrobiella populationen i grundvattnet och upp till 35% i de öppna vattensamlingarna i tunneln. Metanoxidation utgör en av de dominerande mekanismerna för mikrobiell O_2 reduktion. Bidraget från vätgas och organiskt material beroende av mikroorganismer i grundvatten behöver studeras ytterligare. En kinetisk modell för mikrobiell O_2 reduktion har utvecklats och den kan användas för uppskattningar av mikrobiell syreförbrukning i slutförvaret efter förslutning.

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SUMMARY AND CONCLUSIONS

Total microbial respiration

Rates of microbial O_2 reduction in groundwater ranged from 0.31 to 4.5 μM/day and could be inhibited in groundwater. Carbon dioxide was produced concurrently with O_2 consumption confirming the biogenic nature of the O_2 disappearance. O_2 reduction rates and specific cell respiration were reproducible in different groundwater and with time.

The time for reduction of 500 μM O_2 ranged from 0.31 to 3.99 years depending on O_2 concentration, temperature and type of groundwater. O_2 consumption depended exponentially on time.

Diverse microbial populations capable of O_2 reduction was present both in oxic and anoxic groundwater. There was not a significant difference in O_2 reduction rates, capacities or efficiencies between the anoxic and oxic groundwater.

The O_2 reduction capacity of the tested groundwater reached 700 μM. The O_2 reduction efficiency ranged between 40 and 100%, depending on O_2 concentration and continuation of the tests.

At ambient temperature (15-16°C) O_2 reduction was observed both in oxic and anoxic groundwater without delay. The microbial population *in vitro* was able to start active O_2 reduction at 30°C and 60°C after 2 weeks. Thermophilic organisms were present in the groundwater. The adaptation period is expected to be longer at temperatures, which differ from the ambient groundwater temperatures.

The structure of the mixed population depends on the energy sources available. Chemical analysis of groundwater and gases may predict the dominating microbial groups, which will be activated by O_2.

Methane-oxidation in groundwater

Methanotrophs survive in deep groundwater at low numbers and they are active. Specific CH_4 oxidation rates ranged between 3.08 and 220 nmol CH_4 per litre per day. The percentage of CH_4-oxidising bacteria in the total cell counts in groundwater was up to 30%. Methane-oxidising bacteria may be inducted by oxidation of the groundwater. The attached CH_4-utilising populations appear to be more active than the free-living.

25 pure cultures actively oxidising CH_4 have been isolated from Äspö, affiliated to *Methylomonas, Methylosinus, Methylococcus, Methylobacter* and *Methylocystis.* Half of the isolates grow without copper. A new species

Methylomonas scandinavica, was described and studied. *M. scandinavica* develops at low temperature (4- 24°C) and high salinity (1 M).

A specific inhibitor of CH_4 oxidation, acetylene, reduced O_2 reduction on 16-70%. CH_4 oxidation was responsible at least for 2.7-6.67% of O_2 reduction in the groundwater and for 5.1-9.1% of O_2 reduction on peroxides rock surfaces. The GC test showed that CH_4 oxidation is responsible for 0.32-6.7% in anaerobic groundwater and 9.08-57% of total O_2 reduction in oxidised groundwater. The impact of CH_4 oxidation on the total O_2 reduction means that CH_4 oxidation is one of the dominant processes in microbial O_2 reduction. Efficiency of CH_4 oxidation ranged between 38 and 80%.

A model for microbial O_2 reduction

A model for microbial O_2 reduction is proposed. It is an approximation that reflects known factors affecting microbial respiration. For two substrates, namely O_2 and an energy source, the process may be approximated to the double Monod form. Multisubstrate Monod kinetics can describe the influence of microbial electron donors available in groundwater on the O_2 reduction capacity. In addition, stoichiometric and weight coefficients for each physiological group of microorganisms participating in the respiration are included.

Different microbial reactions impact O_2 reduction in accordance with available electron donors and viable competent organisms. Energy donors such as CH_4, H_2, acetate and formate were actively used in the presence O_2.

Temperature is a factor affecting solubility of O_2 and consequently its availability for microorganisms. Temperature may determine which groups of microorganisms are active reducing O_2.

Metabolic abilities of indigenous organisms like V_{max} E and K_m at *in situ* groundwater O_2 concentrations and similar kinetic parameters (K_{mo}) for O_2 reduction at *in situ* substrate content could provide the necessary data for testing the capacity model. Pure cultures of CH_4-utilising microorganisms were isolated. Kinetic characteristics were studied and used in the model testing. O_2 reduction rates at expense of CH_4 in pure culture and in the groundwater are in good agreement with the empirical values.

1 INTRODUCTION

Safety assessment of the SKB concept for high level nuclear waste disposal must consider maintenance of the redox stability of the engineered barriers (copper, bentonite and backfill) and the surrounding rock. If a copper canister corrodes, radionuclides may be released. Under oxic conditions some long-lived nuclides, especially Np, Pu, Tc, U, are more soluble and mobile, while under reduced conditions most radionuclides sorbe on particles, rock walls and on microorganisms adsorbing on their negatively charged cell walls. Copper corrosion is very slow under reduced conditions. Safety assessment calculations include the time for obtaining low redox conditions in the rock aquifers and other barriers after repository closure. Geochemical and biochemical reactions reducing O_2 in groundwater, on solid mineral phases and in backfill, determine the reducing capacity of the barriers. The redox experiment in detailed scale (REX) project deals with O_2 reduction by the geological medium and buffering capacity of rock against an oxygen disturbance (Puigdomenech at el., 1996). Biochemical reactions are catalysed by microorganisms. There is a need to define biochemical reactions in relation to the electron donor availability and microbial diversity in deep groundwater. Microbe-REX has investigated the microbial nutrients, organism diversity, microbial activity and the O_2 reduction potential. The Microbe-REX project defines and studies microbial reactions which may be responsible for biological O_2 reduction and production of reduced compounds that chemically reduce O_2.

1.1 HYPOTHESIS

Our concept of biological O_2 reduction in groundwater includes using O_2 as a terminal electron acceptor in different microbial metabolic reactions at gradient O_2 concentrations (Figure 1). The possibility of the reactions can be predicted from the presence and quantity of different electron donors in groundwater, dissolved gas and on solid phases. Theoretically, predicted respiration potentials for organic carbon and hydrogen dissolved in groundwater preclude that heterotrophic facultative anaerobes and methane oxidising bacteria may contribute to O_2 reduction at closure of a deep repository. It is hypothesised that microbial oxidation of organic carbon and methane are important subsurface processes that will reduce O_2 in and around a future HLW repository. Adding the catalytic abilities of microorganisms to the modelling of O_2 disappearance may reduce the inorganic 300 years scenario to an organic scenario lasting not more then months.

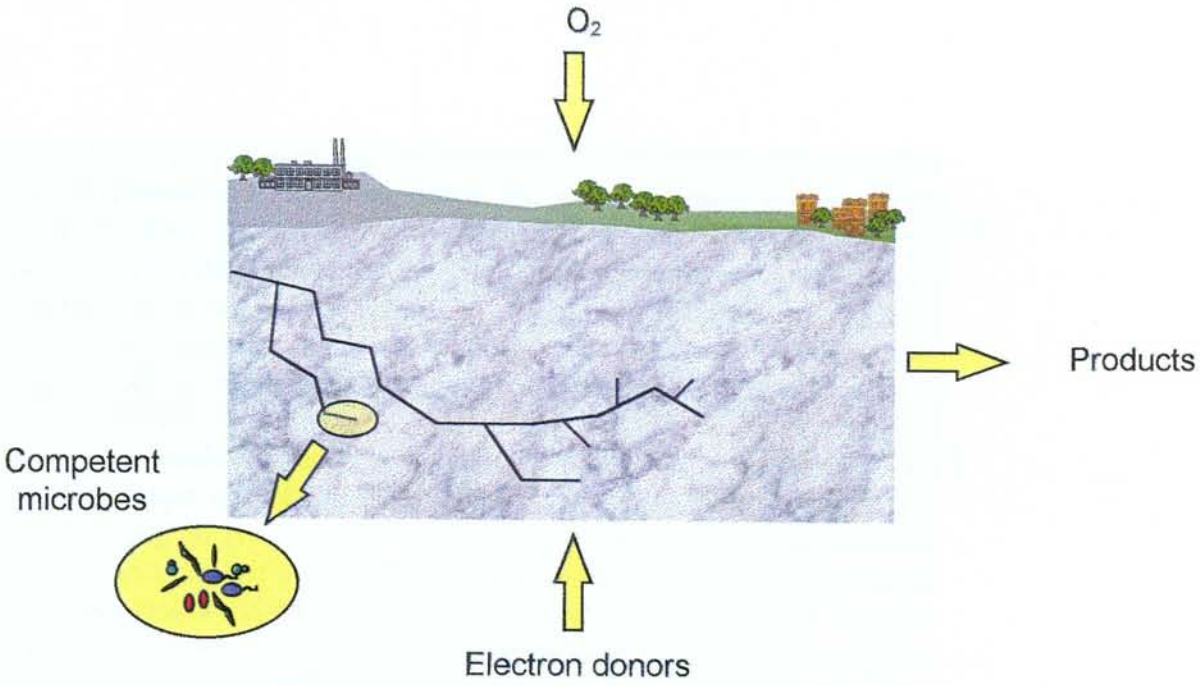


Figure 1 *Microbial oxygen reduction in deep subsurface.*

1.2 MICROBIAL ELECTRON DONORS IN GROUNDWATER AND IN MINERAL PHASES

The chemical characteristics of Äspö groundwater are shown in Table 1. Organic matter is dissolved in groundwater at concentrations of 0.3-18 mg/l (Table 1). The radiometric dating of organic carbon from the borehole KA2862A (depth 380 m) at Äspö yielded an age greater than 10.000 years (Smellie et al., 1995), which implies that the carbon does not originate from modern organic carbon degradation. Substrates such as fulvic acids contain aromatic hydrocarbons that commonly are non-degradable under anaerobic conditions but are expected to be degraded with O_2 as electron acceptor (Aragno et al., 1991, Busse et al., 1991, Hanson et al., 1996). The ability of microbes to use organic material as carbon and energy source is influenced not only by its concentration, but also by its degradability. Organic carbon in groundwater from shallow boreholes consists of a mixture of humic and fulvic acids (Wallin et al., 1995) and non-identified low molecular weight acids, which supposedly derived from partial decomposition of plant and bacterial debris (Mann and Wetzel, 1995). Fulvic acids are water soluble, while humic acids are alkali soluble and includes polymerised aromatic molecules, purines, pyrimidines and uronic acid polymers. High proportions of chemolithoautotrophs (microbes deriving carbon and energy from inorganic compounds), low-biodegradability of the high molecular weight organic compounds dissolved in groundwater and the high age of the carbon (^{14}C) compounds suggest that microbes inhabiting the deep hard rock environments most likely depend on chemolithoautotrophic ways for life. Both aerobic and anaerobic environments will be present in and around a

HLW repository. The pool of electron donors that may reach aerobic areas of a repository is not yet quantified. Such electron donors can be formate, acetate and lactate that are products of anaerobic degradation processes. These electron donors may be consumed with O_2 as terminal electron acceptor.

Table 1 *Characteristics of groundwater in Äspö. The concentrations are shown in mg/l (Nilsson, 1995).*

Borehole	Depth	Sampling date (D-M-Y)	SECUP	SECLOW	pH	T °C	HCO_3^-	SO_4^{2-}	Cl^-	NH_4^+	Fe^{+2}	Fe Total	DOC
Shallow Site Boreholes													
HBH02	15	12.11.93	21	32.4	6.1	n.d.	55	70	12	0.04	2.88	2.93	17.3
HBH01	45	05.09.94	31	50.6	7.2	n.d.	319	123	348	0.04	0.404	0.398	14.0
KR0015B	68	05.09.94	19.82	30.31	7.2	8.9	422	108	535	0.27	0.308	0.308	18.0
KR0013B	68	05.09.94	7.05	16.94	7.2	8.9	291	148	1740	0.24	0.360	0.363	11.0
KR0012B	68	05.09.94	5	10.57	7.4	9.3	326	137	532	0.32	0.185	0.200	11.0
SA813B	112	05.09.94	5.6	19.5	6.8	10.4	292	298	3110	n.d	5.02	6.350	7.1
SA1327B	179	15.10.92	6	20.3	7.4	11.0	252	225	3920	n.d.	2.150	2.308	6.5
SA1420A	192	06.09.94	6	50	7.1	11.4	199	308	2950	n.d.	1.604	1.60	2.6
KA2512A	345	13.12.94	0	37.27	7.2	11.8	197	101	4750	n.d.	n.d.	0.79	3.8
KA2858A	380	10.03.95	39.77	40.77	7.8	12.9	9	577	10300	n.d.	0.090	0.090	1.3
KA2862A	380	27.01.95	0	15.98	7.2	13.3	8	666	13300	n.d.	0.124	0.132	0.8
KA3005A	400	07.12.94	0	58.11	7.7	14.3	81	288	4870	n.d.	0.582	0.593	2.2
KA3010A	400	08.12.94	0	60.66	7.5	14.3	73	309	5580	n.d.	0.755	0.810	2.8
KA3067A	409	14.12.94	0	40.05	7.4	14.0	53	307	5650	n.d.	0.414	0.536	1.8
KA3105A	414	16.12.94	0	68.95	7.6	14.2	102	243	3960	n.d.	0.414	0.426	2.6
KA3110A	414	10.03.95	20.05	28.63	7.5	13.4	161	286	3940	n.d.	0.970	1.140	3.2
HD0025A	420	09.09.95	0	200	7.6	15.0	176	n.d.	3350	n.d.	n.d.	n.d.	n.d.
KA3385A	446	11.01.95	0	34.18	7.7	14.5	10	443	6650	n.d.	0.152	0.152	0.9

There are several electron donor alternatives to organic matter dissolved in groundwater, which can be oxidised by bacteria with molecular O_2. They are CH_4, H_2, CO, Fe^{2+}, Mn^{2+} and U^{4+}. CH_4 and H_2 are expected to act as inert gases in most geochemical systems, and are therefore usually overlooked and not analysed. Some data on CH_4 and H_2 in hard rock have been published (Sherwood Lollar et al., 1993a; 1993b). From 2.2 up to 1574 µM H_2 in groundwater from Canadian shield and Fennoscandian shield rocks were found. Dissolved CH_4 and H_2 have been found in Äspö groundwater (Kotelnikova and Pedersen, 1997). The results of gas analysis of Äspö groundwater in headspace of serum bottles are shown in Figure 2A, B. The concentrations of H_2 and CH_4 in Äspö groundwater, measured in bottles filled with the groundwater, were 45 nM -100 µM and 19 -980 µM, respectively (Figure 2A, B). Gas extraction data show evidences for 18-40 ml gas dissolved per litre of groundwater, containing N_2, He, H_2, CO_2, CO and CH_4 (Table 2). We received the data with method described earlier (Pedersen 1997, Appendix 1) (Table 2). H_2 was detected in all investigated boreholes except two shallow boreholes (KR0012B and KR0013B). CH_4 has been detected in all Äspö groundwaters investigated (Table 2).

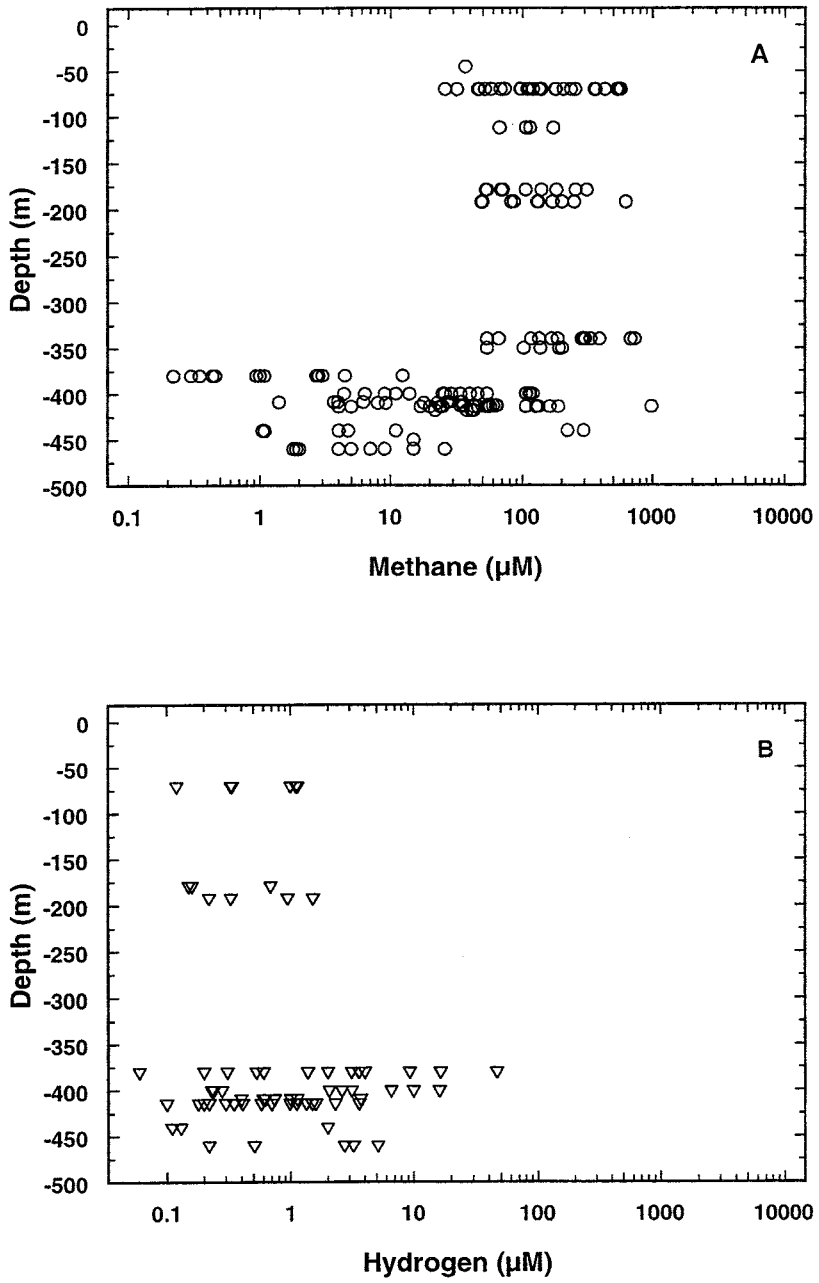


Figure 2 *CH_4 (A) and H_2 (B) concentrations in Äspö groundwater measured as gas in the head space of sample vessels and re-calculated to dissolved gas.*

The concentration of iron (total Fe^{2+}+Fe^{3+}) ranged from 0.15 to 5.00 mg/l (Table 1) and uranium ranged from 3.45 to 5.66 µg/l. The manganese ranged between 0.2 and 1.2 mg/l. The percentages of iron and manganese compounds in minerals from Äspö are shown in Table 3. The concentrations of H_2, CO and CH_4, Fe^{2+} and Mn^{2+} exceed the Michaelis-Menten K_m coefficients (Pedersen, 1997, section 2.1) of the competent microorganisms, oxidising these compounds (Table 4). This indicates that concentrations of these compounds are high enough to support the microbial growth.

Table 2 Composition (μM) of gas dissolved in Äspö groundwater (Pedersen, 1997a).

Borehole	Depth M	Analyses date	Total gas (ml)	N_2	He	H_2	CH_4	CO_2
KR0012B	68	971024	29	897	1.7	0.0000	42	246
KR0013B	68	971024	37	1020	4.5	0.0000	80	393
KR0015B	68	971024	50	542	130	2.5230	116	209
KA2862A	380	971104	52	1821	137	0.9840	0.92	0.81
KA3005A	400	970214	32.0	1114	127	0.0320	75	55
KA3010A	400	970612	32.6	1658	324	1.2637	2.25	5.81
KA3110A	414	970213	18.0	607	18	0.5878	38	75
ZEDEX	420	970313	22.0	719	87	0.0800	31	58

Table 3 *The concentration of iron and manganese in different Äspö granites and minerals (data from Tullborg, 1995 and West et al., 1997).*

Compound	Fe_2O_3 %	FeO %	MnO %
Äspö diorite	1.9	2.7	0.09
Ävro granite	2.2	1.3	0.06
Fine-grained granite	1.7	-	0.03
Chlorite	-	21.0 (80% of total iron is Fe2+)	0.50
Calcite	-	0.1	1.00
Epidote	14.0	-	0.10
Mixed-layer clay	-	2.0	-
Barite	-	0.06	n.d.
Pyrite	-	53.11	n.d.
Magnetite	68.7	31.08	0.048
Mica	-	19.26	0.587
Plagioclase	-	0.119	-
Diorite	2.26	2.705	0.11
Prehnite	0.07	10.40	-
Biotites	n.d.	17.3	0.60

Table 4 *Michaelis-Menten coefficients of different autotrophic aerobic organisms for their respective electron donor.*

Microorganisms	K_m (μM)	Electron donor
Iron oxidising bacteria	0.2-0.3	Fe^{2+}
Hydrogen oxidising bacteria	0.05-1.0	H_2
Carbon monoxide oxidising bacteria	0.005 -0.050	CO
Methane oxidising bacteria	0.6 -5	CH_4

1.3 MICROORGANISMS AND MICROBIAL PROCESSES FOUND IN ÄSPÖ GROUNDWATER

The presence of microorganisms in subterranean environments and particularly in Äspö is well documented (Figure 3, 4) (Pedersen 1993, 1996, 1997a). Numerous attached and unattached populations have been found (Kotelnikova and Pedersen 1997; Pedersen et al., 1996; Pedersen and Ekendahl 1990, 1992; Pedersen and Karlsson 1995). It has been shown that these populations are active, metabolising organic carbon and that they show autotrophic activity incorporating carbon dioxide (Figure 3) (Pedersen and Ekendahl, 1992). Their diversity is large and has been revealed using molecular methods; approximately 200 new, not earlier reported 16S rDNA sequences, have been found at different subterranean sites (Pedersen et al., 1996). Genera and species cultured from anaerobic Äspö groundwater identified with 16S rRNA sequencing are shown in Table 5. The groundwater was dominated by anaerobic and facultative anaerobic organisms that can perform different biochemical reactions (Table 6).

All living cells obtain their energy from a series of oxidations and reductions involving the transport of protons and electrons from a substrate to a final acceptor. *Aerobic* organisms use atmospheric O_2 as a terminal electron acceptor for respiration and have mechanisms defending their cell from the toxic effect of free O_2 radicals, which are obligate products of O_2 interaction with cell metabolites. However, microorganisms vary in their response to O_2, a difference that reflects differences in their respiratory and enzymatic systems. Organisms that require atmospheric O_2 for growth are termed *obligate aerobes*. Other organisms, termed *obligate anaerobes*, are inhibited by molecular O_2 and grow only in its absence. A third group, made up of the *facultative anaerobes*, includes those organisms that can grow anaerobically but grow better in the presence of O_2. The forth group, called *microaerophils* grows only at narrow and low O_2 gradient concentrations. They are sensitive to high O_2 concentrations and commonly oxidise compounds biochemically that may be inorganically oxidised spontaneously by molecular O_2.

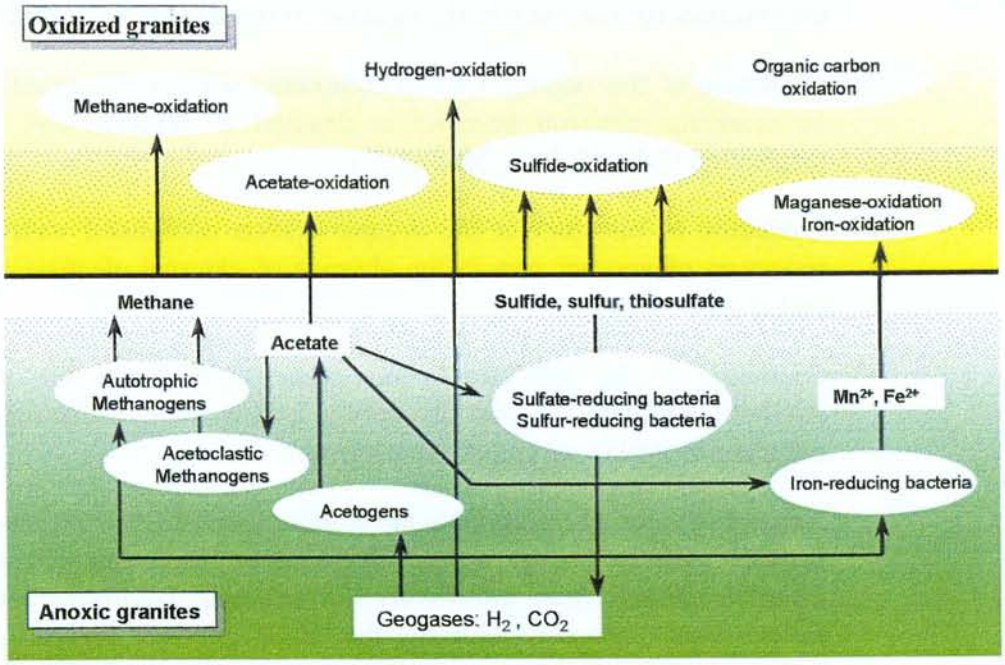


Figure 3 *Microbial production of the compounds for biogenic and abiogenic reduction of a radioactive waste repository.*

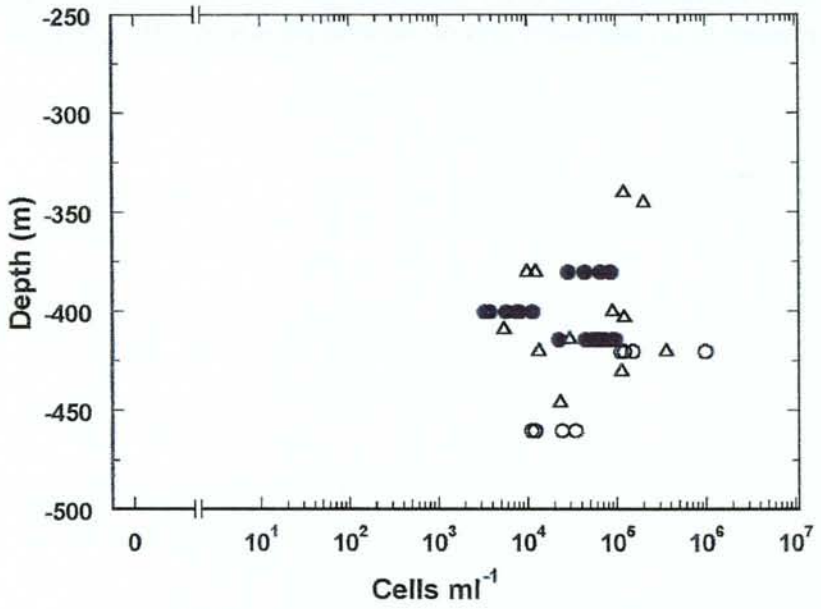


Figure 4 *Total cell counts in Äspö groundwater counted 1995 and 1997. Acridine orange direct counts in the anoxic groundwater 1995(Δ), 1997(•) and in oxygen containing groundwater from the tunnel 1997(o).*

Depending on the presence of certain electron acceptors and microorganisms, as well as the nature of their substrate, there are several possibilities for the microbial oxidation of organic carbon compounds:

Oxidation of the organic carbon compound with concomitant reduction of an inorganic electron acceptor is denoted as aerobic (O_2) or anaerobic respiration (NO, S^0, Fe^{3+}, Mn^{3+}, U^{6+}).

Oxidation of one part of the organic carbon compound with concomitant reduction of another part in the absence of external electron acceptors (so called fermentation).

Oxidation of the substrate in one organism with transfer of protons to another organism (proton accepting organisms: methanogens, sulfate-reducing bacteria) -so called syntrophism.

Anaerobic types of metabolism are of Microbe-REX interest since anaerobes provide reduced energy sources for aerobic respiration (Figure 3). They dissolve minerals non-specifically, for example, by production of organic acids. Anaerobes contribute to the redox process surviving in O_2-free microenvironments even under the oxic conditions and produce electron donating reduced compounds (CH_4, H_2, CO, acetate, S^{2-}, Fe^{2+}, Mn^{2+}, U^{4+}).

Subterranean food chains with H_2 as the energy base instead of the sun are inferred by published data from our laboratory (Pedersen, 1995a, 1996, 1997a, Kotelnikova and Pedersen, 1996, 1997, 1998b). Short summaries of the background concerning different facultative anaerobic, aerobic, microaerophilic and strict anaerobic groups which may use electron donors present in groundwater and on mineral phases as follow below.

1.4 REDOX RELATED MICROORGANISMS AND THEIR PRODUCTS

Table 5 *16S and 18S rRNA genes detected with sequencing and species of bacteria identified with isolation techniques in Äspö groundwater.*

Closest organism in the database	Similarity %	Carbon source	Metabolism type of the most closely related genus
Strict anaerobic 16S rRNA gene organisms			Anaerobic respiration and fermation
*Eubacterium (2)**	99.1	Org**, CO_2	Fermentation, reduction of CO_2 to acetate, butyrate, propionate with organic matter or H_2 as electron donors
Eubacterium Aspo-4	99.7		
Desulfovibrio (4)	97.3	Lactate CO_2	Reduction of SO_4, S_2O_3, S^o to sulphide with electrons from organic substances
Desulfovibrio aespoeensis sp.nov	98.5	lactate, pyruvate, acetate	Reduction of SO_4, S_2O_3, S^o to sulphide with H_2 and organic acids as electron donors
Desulfotomaculum	92.2	FA, Al**, CO_2	Reduction of SO_4, S_2O_3, S^o to sulphide with organic substances as electron donors
Clostridium	88.8	CO_2	Fermentation, reduction of CO_2 to acetate, propionate, butyrate with organic matter or H_2 as electron donors
Desulfobacula	96.5		
Desulfobacterium	95.0		
Desulfuromonas	84.6	CO_2	Reduction of sulphur to sulphide with H_2 or acetate as electron donors
Pelobacter	85.8	Org, FA	Fatty acid oxidation to CO_2 and H_2
Geobacter	83.2	acetate, FA, Al, CO_2	Reduction of Fe^{3+}, NO_3^-, Mn^{4+}, U^{4+} and sulphur with H_2 or acetate as electron donors
Methanogenium bavaricum	91.7	CO_2	Reduction of CO_2 to CH_4 with H_2 as electron donor
Acetobacterium woodii	72.5	CO_2	Fermentation, reduction of CO_2 to acetate, butyrate, propionate with organic matter or H_2 as electron donors
Rhodoferax fermentans	96.0	Org	Fermentation

Closest organism in the database	Similarity %	Carbon source	Metabolism type of the most closely related genus
Strict anaerobic pure cultures			
Desulfovibrio aespoeensis sp.nov	100	lactate, pyruvate, acetate	Reduction of SO_4, S_2O_3, S^o to sulphide with H_2 and organic acids as electron donors
Desulfomicrobium baculatum	99.5	formate, lactate, malate	Reduction of SO_4, S_2O_3, S^o to sulphide with electrons from organic substances as electron donors
Desulfovibrio	91.4	lactate, formate, Al, FA	Reduction of SO_4, S_2O_3, S^o to sulphide with electrons from H_2 and organic acids as electron donors
Methanobacterium subterraneum sp. nov.	100	CO_2	Reduction of CO_2 to CH_4 with H_2 as electron donor
Desulfovibrio	91.4	lactate, formate, Al, FA	Reduction of SO_4, S_2O_3, S^o to sulphide with electrons from H_2 and organic acids as electron donors
"Eubacterium" nov.	100	Org, CO_2	Fermentation, reduction of CO_2 to acetate, butyrate, propionate with organic matter or H_2 as electron donors
Microaerophilic 16S rRNA gene organisms			Aerobic respiration at gradient O_2 concentrations / reduction of O_2
			Autotrophic and mixotrophic growth
Thiomicrospira denitrificans	98.5	CO_2	NO_3 reduction with S_2O_3 as electron acceptor
Leptothrix	95.9	CO_2	Fe^{2+} oxidation
Gallionella ferruginea	93.2	S, CO_2	Fe^{2+} oxidation, glucose, fructose, sucrose oxidation
Methylococcus	77.0	CO_2	CH_4 oxidation
Methylomonas	77.0	CO_2	CH_4 oxidation
Alcaligenes eutrophicus	93.3	CO_2	Autotrophic H_2 oxidation, mixotrophy
Methylophilus	98.3	CO_2	CH_3OH and CH_4 oxidation
Methylotrophus			(oxidised groundwater from HD0025B)

Closest organism in the database	Similarity %	Carbon source	Metabolism type of the most closely related genus
Microaerophilic pure cultures			
Gallionella ferruginea	pure culture	CO_2	O_2 reduction with Fe^{2+}, glucose, fructose, sucrose as electron donors
Thiothrix sp. nov.	pure culture	Acetate, CO_2	Reduction of O_2, molecular sulphur or NO_3^- with acetate as electron donor
Facultative anaerobic 16S rRNA gene organisms			Fermentation, anaerobic or aerobic respiration
Bacillus megaterium	99.6	Org**	Reduction of Mn^{3+} NO_3^- O_2 with organic compounds as electron donors
Bacillus sp.	98,6	Org	Reduction Fe^{3+}, Mn^{3+} NO_3^- or O_2 with organic compounds as electron donors
*Acinetobacter (3)**	99.6	Org	Oxidation of aliphatic alcoholes, decarboxilic and fatty acids, unbranched hydrocarbons, sugars, recalcitrant
Acinetobacter junii	98.3	Org	Reduction of O_2 with aromatic compounds as electron donors (phenol, benzoate, mandelate, n-hexadecan, cyclohexanol, 2,3-butandiol)
Ewingella (2)	96.0	Org	
Plesiomonas (2)	96.0	Org, acetate	Reduction of NO_3, or O_2 with organic compounds as electron donors
Yersinia	96.7	Org	Reduction of NO_3, or O_2 with organic compounds as electron donors
P. necessarius	98.7	Org	
Rhodoferax fermentans	98.0	Org	
Pseudomonas sp. (8)	98.6-99.4	Org	Reduction of Mn^{3+} or NO_3^-,with aromatic hydrocarbon or organic carbon compounds as electron donors
Sphingomonas (2)	96.8		
Flavobacterium capsulatum (6)	95.4	CO_2, Org	isolated from distilled water
Thiobacillus	78.9	CO_2	Reduction of O_2, Mn^{3+} or NO_3^- with S^0 or S^{2-} as electron donors

Closest organism in the database	Similarity %	Carbon source	Metabolism type of the most closely related genus
Facultative anaerobic pure cultures			
Shewanella putrefaciens		lactate	Reduction of Fe^{3+}, Mn^{4+}, O_2, fumarate with organic compounds as electron donors
Pseudomonas vesicularis		Org	Nutritionally versaltile with regard to low-molecular-weight compounds, heterotrophic
Pseudomonas fluorescens		Org	
Eukaryotic 18S rRNA gene organisms			
Candida holmii (2)	99.7	S, Al	
Saccharomyces	97.8	S, Al	non-methylotrophic, fermentation, O_2 reduction

* -Number in parenthesis shows the frequency of the gene identification. **Org -organic matter, Al -alcohols, S-sugars, FA -fatty acids

Table 6 *Selected microbial reduction reactions. The free energy (ΔG) is given for a pH 7.0, unless it is specified otherwise in the left column. *K_m is half-saturation constant for substrate (Pedersen and Karlsson, 1995, section 2.1).*

Process	Energy giving reaction	ΔG (kkal/mol)	pH ranges	Examples of responsible organisms
Aerobic				
Respiration of glucose	$C_6H_{12}O_6 + 6O_2 \rightarrow 6\,CO_2 + 6H_2O$	-688	5.6-8.0	*Pseudomonas*
Nitrification	$NO_2^- + 0.5O_2 \rightarrow NO_3^-$ $NH_4^+ + O_2 \rightarrow NO_2^- + H_2O + 2H^+$	-14.9 -57.8	6.6-10.0 7.0-9.4	*Nitrobacter, Nitrococcus* *Nitrosomonas, Nitrosococcus*
Sulphur oxidation	$S^o + O_2 + H_2O \rightarrow H_2SO_4$ $S_2O_3^{2-} + 2O_2 + H_2O \rightarrow 2SO^{2-}_4 + 2H^+$	-141.2 -195.5	1.0-4.0 5.0-7.0	*Thiothrix, Thiobacillus,* *Thiothrix, Thiobacillus*
at pH 6.5	$S^{2-} + O_2 + H_2O \rightarrow SO^{2-}_4 + 2H^+$	-159.2	6.5-7.0	*Thiothrix, Thiobacillus*
Manganese oxidation, at pH 6.0	$2Mn^{2+} + 2H^+ + 0.5O_2 \rightarrow 2Mn^{3+} + H_2O$	-6.45		*Leptothrix, Artrobacter, Metallogenium*
Iron oxidation at pH 6.5	$2Fe^{2+} + 2H^+ + 0.5O_2 \rightarrow 2Fe^{3+} + H_2O$	-11.2 kJ	5.0-6.5 n.d.	*Gallionella ferruginea, Metallogenium, Leptospirillum,*

Process	Energy giving reaction	ΔG (kkal/mol)	pH ranges	Examples of responsible organisms
Iron oxidation at pH 2.0	$H_2S+4H_2O+8Fe^{3+} \rightarrow 8Fe^{2+}+SO_4^{2-}+10H^+$	-481.7	1.0-4.0	*Thiobacillus ferroxidans*
H_2 oxidation	$H_2+0.5O_2 \rightarrow H_2O$	-113.3	4.0-8.0	*Alcaliganes eutrophicum (K_m * 8-80 nM), Hydrogenobacter, Pseudomonas*
CO -oxidation	$2CO + O_2 \rightarrow 2CO_2$	-122.8	4.0-8.0	*Hydrogenomonas carboxidans (K_m 5-50 nM)*
Methane oxidation	$CH_4+2O_2 \rightarrow CO_2+2H_2O$	-195.4	6.5-7.5	*Methylomonas methanica (K_m15μM), Methylosinus Methylomonas methanica (K_m.7μM) Nitrosomonas europea (K_m 97 nM)*
Anaerobic				
Fermentation of sucrose, alcoholes, cellulose, proteins	Ethanol, alcoholes, FA, methylamines, methanol, lactate, formate, acetate, CO_2 + H_2	-5-60 -12-20 -3.1	6.5-8.5	*Yeast, Clostridium, Lactobacillus, Propionobacter, Syntrophomonas*
Denitrification	$NO_3^- \rightarrow NH_4^+$+, N_2, N_2O	-82.2 -112	7.0-9.0	*Geobacter metalloreducens Desulfovibrio, Clostridium Pseudomonas denitrificans*
Iron and manganese reduction	$Fe^{3+}+ e \rightarrow Fe^{2+}$ $2Mn^{4+}+ 8e \rightarrow 4Mn^{2+}$	-24.0 -94.5	7.0-9.0	*Geobacter metalloreducens Shewanella putrefaciens*
Sulphur and sulphate reduction without light at low temperatures	S^o, S_2O_3, SO_3^{2+}, $SO_4^{2+} \rightarrow S^{2-}$	-12 -18	6.5 - 10.5	*Desulfovibrio, Desulfotomaculum, Desulfomicrobium, Desulfuromonas, Campylobacter*
Reduction of sulphur at 70-95°C	$S^o + H_2 \rightarrow H_2S$ $SO_4^{2+} \rightarrow S^{2-}$	-14	5.0-7.5 6.0	*Thermotoga, Thermosipho, Pyrococcus, Pyrodictium, Thermoproteus, Archaeoglobus*
Methane production	$4H_2+CO_2 \rightarrow CH_4+2H_2O$ $CHOOH \rightarrow CH_4$ $CH_3COOH \rightarrow CH_4$ $CH_3OH \rightarrow CH_4$ $CH_3NH_3 \rightarrow CH_4$	-23.2 -23.2 -28.0	7.0 - 10.0	*Methanobacterium, Methanogenium, Methanohalobium, Methanothrix Methanosarcina*
Autotrophic acetate production	$4H_2+2CO_2 \rightarrow CH_3COOH +2H_2O$	-14.4	6.0-8.0	*Eubacterium, Clostridium, Acetogenium,*

1.4.1 Hydrogen-related bacteria

1.4.1.1 Hydrogen oxidising bacteria

Many bacteria can utilise H_2 and details can be found in Pedersen and Karlsson, (1995), sections 2.6.2, 2.6.3, and 3.6. Rather than being an inert gas it is very reactive in the biogeochemical environment, and H_2 will contribute to the reducing capacity of the deep groundwater system, constituting the energy base of subterranean microbial ecosystems.

H_2 is consumed by aerobic bacteria hydrogen-oxidising bacteria in the presence of O_2. The hydrogen-oxidising bacteria are characterised by the ability to utilise gaseous H_2 as electron donor with O_2 as electron acceptor and the ability to fix carbon dioxide to organic carbon. They can grow in a chemolithoautotrophic manner or use organic matter. The presence of genes distantly related to those of H_2-oxidising bacteria in Äspö groundwater have been detected with molecular techniques (Table 5) (Pedersen and Karlsson, 1995).

1.4.1.2 Acetate producing bacteria

Acetate producing bacteria (Pedersen and Karlsson, 1995, section 3.8) have the capability to react H_2 with CO_2 to acetate. H_2 addition to groundwater from Äspö resulted in rapid acetate production (Kotelnikova and Pedersen, 1998b). Three acetogenic species have been isolated from the tunnel and are available for laboratory experiments. One of them is a Gram-positive, strictly anaerobic *Eubacterium* like species, see Pedersen et al. (1996a) for details. The acetate produced can be used by acetoclastic methanogens, sulphate reducing bacteria and other heterotrophic microorganisms. The presence and distribution of homoacetogens in the deep granitic rock groundwater from Äspö Hard Rock Laboratory were studied by Kotelnikova and Pedersen (1998b). Homoacetogens were found as viable cells in granitic groundwater in numbers from 10 to 3.6 x 10^4 cells/ml and they produced acetate autotrophically. It appears that deep granitic aquifers are inhabited by autotrophic acetogens, which may produce acetate at the expense of subterranean H_2 and bicarbonate.

1.4.1.3 Methane producing Archaea

Methane occurs frequently in subterranean environments all over the globe. Concentrations from 1 up to 181 µM of CH_4 in Swedish the groundwater have been published (Pedersen and Karlsson, 1995, Table 1.2). Recent data indicate up to 980 µM of CH_4 in Äspö groundwater. Sherwood Lollar et al. (Sherwood Lollar et al., 1993a-b) reported from 1.3 up to 18576 µM of CH_4 in the groundwater from Canadian shield and Fennoscandian shield rocks.

The methanogenic archaeas have the ability of producing CH_4 from H_2 and CO_2 (autotrophic methanogens), from acetate (acetoclastic methanogens) and from some organic sources (Pedersen and Karlsson, 1995, section 3.7). Data from our laboratory indicate that these methanogenic types are very abundant in the Äspö groundwater (Kotelnikova and Pedersen, 1997) and, by use of ^{14}C-tracers, that they are active and produce CH_4 with a rate of between 0.02 up to 3.10 µM per hour (Kotelnikova and Pedersen, 1998b). Different physiological groups of methanogens (autotrophic H_2/CO_2-consuming and heterotrophic acetate, methanol and trimethylamine consuming) were found at depths ranging from 68 to 446 m below the sea level in numbers from 12 to 4.5 x 10^5 cells/ml. *Methanosarcina*-like organisms were observed in groundwater from depths of 45-68 m and obtained in active enrichment cultures. A new species of alkaliphilic *Methanobacterium* was isolated from the depths of 68, 409 and 420 m and studied (Kotelnikova et al., 1997).

1.4.2 Methane-oxidising bacteria

A variety of bacteria, the methanotrophs, oxidise CH_4 readily, utilising it as source of electrons for the energy generation and as their sole source of carbon (Table 7). These bacteria are all aerobes, and are widespread in soils and water (Hanson and Hanson, 1996). They are also of a large diversity of morphological types, seemingly related only in their ability to oxidise CH_4. They are found wherever stable sources of CH_4 are present. There is some evidence that although CH_4 oxidisers are obligate aerobes, they are sensitive to O_2 and prefers microaerophilic habitats for the development (Hanson and Hanson, 1996). They are therefore often found concentrated in a narrow band between anaerobic and aerobic zones were CH_4 meets an oxygen containing system. Such environments will be common in future repositories during the open phase and for some time after closure. Once established, this group of bacteria will be active as long as there is O_2 present for the oxidation of CH_4 and they will oxidise available CH_4 with remaining O_2 after closure. Consequently, the system will rapidly go anoxic after closure if CH_4 is in excess. Recent DNA results of the microbial diversity during drilling of Äspö boreholes revealed 16S rRNA sequences distantly related to CH_4-oxidising bacteria (Pedersen, Karlsson, 1995) (Table 5).

***Table 7** Some physiological characteristics of CH_4-oxidising bacteria*

Property	**Characteristics**
Energy source	CH_4
Half-saturation constant for CH_4	0.6 -10 µM
Carbon source	CH_4
Microaerophilic	Yes
O_2 requirement	<4% in gas phase
Ability to survive under anaerobic conditions	Yes, more than 90 years
N_2-fixation	Yes

1.4.3 Heterotrophic facultative anaerobic microorganisms

The contribution of a heterotrophic microbial population to the O_2 reducing processes will be determined by the available pool of organic carbon in a groundwater system. Once organic material is produced from H_2, it can be fermented or respired under anaerobic conditions (Table 6) by other microbes. The final products of the anaerobic processes and organic matter that were not degraded under anaerobic conditions may be oxidised under oxic conditions. Such oxidations goes very efficient if O_2 is present (Table 6). The presence of 16S RNA genes related to facultatively anaerobic heterotrophic organisms of the genera *Pseudomonas, Acinetobacter, Bacillus* and yeast *Saccharomyces* and 18S rRNA genes of *Candida* (Table 5) suggests that the heterotrophic organisms may contribute to the O_2 reduction.

1.4.4 Iron-oxidising bacteria

The iron oxidising bacteria use ferrous iron as an energy source when growing with O_2. They can use organic substances or CO_2 as a source of carbon at close to neutral pH (5.0-6.5) e.g. *Metallogenium* and *Gallionella ferruginea* (Table 5, 6). Some can oxidise iron and sulphide at low pH (1.0-4.0) and grow autotrophically or mixotrophically as *Thiobacillus* (Table 6). *Gallionella* has been isolated in pure culture (Hallbeck and Pedersen, 1991). 16S rRNA genes distantly resembling *Gallionella* have been observed (Table 5). The microbial reaction of ferrous iron oxidation yields little energy and demands 1 mole of O_2 for 4 moles of ferrous iron. The reaction is not effective in relation to O_2 reduction unless the ferrous iron is renewed and supplied continuously. The potential source of ferrous iron may be the activity of bacteria reducing iron anaerobically or dissolving it from minerals. Anaerobic microenvironments may constitute a source of dissolved ferrous iron under oxic conditions (Grantham et al., 1997).

1.4.5 Iron-reducing bacteria

Iron(III)-reducing bacteria are affiliated to *Geobacter, Desulfuromonas, Geovibrio,* and *Shewanella* genera. They use ferric iron as the terminal electron acceptor for the oxidation of organic compounds to CO_2 or to other oxidised metabolites. They conserve energy for growth by electron transport phosphorylation. In the absence of ferric iron, many of these bacteria can use alternative electron acceptors, e.g. nitrate, fumarate, humic acids and elemental sulphur. Some iron-reducing bacteria can grow autotrophically and use H_2 as electron donor, e.g. *Geobacter sulfurreducens* and *Geovibrio ferrireducens.* One iron reducing bacteria has been isolated from the tunnel (*Shewanella* sp.). Dissolution of ferrous iron by one sulphate reducing and one iron reducing bacteria isolated from Äspö has been suggested (West et al., 1997). The process may contribute both to microbial and chemical O_2 reduction. Iron reducing bacteria may shuttle electrons from organic electron donors or hydrogen to ferric iron associated with minerals in the fractures, thereby producing ferrous iron available for iron-oxidation with oxygen as electron acceptor.

1.4.6 Sulphur oxidising bacteria

Sulphur oxidising bacteria are able to reduce O_2 with sulphide or thiosulfate as electron donors and with acetate or CO_2 as carbon source under microaerophilic conditions. Organism related to *Thiothrix nivea* has been enriched from the tunnel walls and ponds (Table 5). The microbial sulphur-oxidising reactions are energy rich (Table 6), but the contribution of the group is not expected to be significant since the sulphide concentration is seldom is above 1 mg/l. This is because sulphide easily precipitates on aquifer walls as pyrite or other minerals with metals.

1.4.7 Sulphate reducing bacteria

H_2 constitutes a part of the microbial redox system. H_2S has a biogenic signature in Äspö (Wallin, 1995) M; it is produced by sulphate-reducing bacteria (Table 5). These bacteria usually reduce sulphate with organic material as electron and carbon source and produce H_2S, CO_2 or acetate (Table 6). Details can be found in Pedersen and Karlsson, 1995, section 3.5 and Pedersen, 1995b. One of sulphate reducing bacteria isolated from Äspö has been described as a new species (Motamedi, Pedersen 1997). Some sulphate-reducing bacteria can oxidise acetate and the acetogenic bacteria (1.3.2.2), therefore, probably constitutes an important link between H_2 and sulphate reducing bacterial activity in deep groundwater environments.

1.4.8 Total microbial capacity for O_2 reduction

Total microbial capacity for O_2 reduction depends on the amount of electron donors dissolved in groundwater and emitting from minerals. The amount of molecular O_2 that may be reduced through the biochemical reactions may be predicted from the microbial reactions described in Table 6. Independently of "place of play" (oxic, transit or anoxic environments) and way of reduction, the reducing potential for the reduction of O_2 can simply be calculated as budgets of electrons available in groundwater. When this is done for some of the abundant microbial electron donors, a very interesting situation appears: many groundwaters seem to have enough reduced compounds to provide microbial growth and keep the post closure groundwater system reduced. This situation becomes even more promising if continuous new production of reduced microbial products (Table 6) is anticipated. Scooping calculations are shown in Table 8.

Table 8 Electron donors available in Äspö groundwater, bacterial groups and their theoretical O_2 reducing capacities. DOC - dissolved organic carbon.

Energy donor	**DOC**	**CH_4**	**Fe^{2+}**	**Mn^{2+}**	**U^{4+}**	**S^{2-}**	**H_2**	**CO**
Concentration in groundwater, µM	25-333	2.5-1000	2.7 -89	3.6-22	23.8	3 -78	1.26	4-50 nM
Aerobic microbial group name	Hetero-trophic	Methane-oxidising	Iron-oxidising	Manganese -oxidising	Uranium-oxidising	Sulphur-oxidising	H_2-oxidising	Monocarbon -oxidising Bacteria
Energy yielding coefficient*	6	2	0.25	0.25	1.0	1.0	0.5	0.5
O_2 consumed µM	150-1980	5.0 –2000	0.8-22	0.9-5.5	23.8	3-78	0.63	2-25 nM

*-The energy giving (stoichiometric) coefficients is predicted from the respiration reactions in Table 6.

The groundwater chemistry, gas data and microbiological analysis of the Äspö microbial community imply that:

- The microbial 16S rRNA gene diversity is large in the groundwater. There is a large metabolic potential for O_2 reduction (Table 5).
- Electron-donors for O_2 reduction by different bacterial groups are present in the groundwater (Tables 1, 2, 3) and they may be used for reduction of the system through the biological reactions (Table 6).
- DOC and CH_4 are effective O_2 reducers through biological reaction (Table 8).

Literature data on physiology of methanotrophs (Table 7) indicates that methanotrophs may be associated with groundwater and rock surfaces and that they survive under anaerobic conditions in dormant forms. O_2 may induce activity of a CH_4-oxidising population.

Previous approximations of the time needed for reduction of the repository environment gave figures of above 10 years (Puigdomenech et al., 1996). The study of a vertical fracture zone in the Äspö tunnel showed that O_2 in engrossed shallow water rapidly disappeared (Banwart et al., 1994). Microbial reduction of O_2 was suggested as mechanism responsible for reduction of the groundwater (Wallin et al., 1995).

The presence of H_2, CH_4 and active methanogens have been shown in Äspö groundwater (Kotelnikova and Pedersen, 1997), and it predicts that *de nova* CH_4 synthesis in anaerobic ground water is ongoing. Methane is a mobile compound easily diffusing in groundwater. It is chemically inert but biologically an active and powerful reducing agent (1 mole of CH_4 demands

8 electrons in biological reduction, when methanotrophs oxidise CH_4 with O_2: $CH_4 + 2O_2 \Rightarrow CO_2 + 2H_2O$. Methane is one of the dominating microbial electron and carbon donors that are dissolved in the groundwater. Methane may be consumed as donor of both energy and carbon in the course of CH_4-oxidising organism development and activity. Literature data on physiology of CH_4-oxidising bacteria indicate that change from oxic to anoxic conditions have little effect on the CH_4-oxidising potential (Hanson, Hanson, 1996). We hypothesised that methane-oxidising bacteria may be associated with groundwater, surviving anaerobic conditions in dormant or active forms. Introduction of O_2 to anoxic groundwater may induce increased activity of CH_4-oxidising populations in deep groundwater. The theoretically predicted respiration potentials for different electron donors in groundwater (Table 8) preclude that significant contributions to the process of rock reduction may be expected from CH_4-oxidising bacteria. The experiments reported here allowed us to estimate total microbial O_2 reduction and, particularly, CH_4-depending reducing capacity of unattached and attached biota in different types of groundwater.

2 OBJECTIVES

We presume that the microbial O_2 reduction ability may greatly reduce the time of restoration of anoxic conditions compared to a non-biological scenario. The electron donors dissolved in groundwater determine the microbial respiration potential. Significant contribution to O_2 reduction may be expected from organic carbon and CH_4-oxidising bacteria. To test the hypothesis the following objectives have been studied:

- Investigations of microbial O_2 reduction in Äspö HRL environments.
- Elucidation of the structure of microbial populations in deep groundwater.
- Quantification of the dominating electron donors and microbial activities in deep groundwater.
- Demonstration of the presence and activity of a CH_4-utilising microbiota and other O_2-reducing microorganisms in deep groundwater.
- Development of model for microbial O_2 reduction, which can be used for prediction of the time, it will take for the repository to return to anoxic conditions during the post closure phase.

3 INVESTIGATONS OF MICROBIAL O_2 REDUCTION IN ÄSPÖ GROUNDWATER

3.1 ÄSPÖ TUNNEL SITES SAMPLED FOR MICROBIAL REX DURING 1996-1997

Most activities described here were performed from March 1996 to July 1998. At different steps of the investigation groundwater and stones were sampled from several sites in the tunnel at the depths between 414 and 460 meters including the backfill experiment tunnel and the REX KA2861A (Table 9). A description of the drilling procedure that included measures to avoid microbial contamination of boreholes during drilling (Winberg et al., 1996) is given by Pedersen et al. (1997b). The experiments performed at the sites sampled are listed in Table 9.

3.2 STRUCTURE OF THE MICROBIAL COMMUNITY IN ÄSPÖ GROUNDWATERS

The structure of microbial communities was determined in the groundwater from borehole KA2861A and KA2862A. Sampling was done the March, 11 1998 from borehole KA2862A and the inner section (7.38-15.98 m depth), the outer section (6.82-6.92 m depth), and from the REX borehole KA2861A, the inner section (8.50-9.80 m depth) and the outer section (0-8.50 m depth). At the next step (070798-220798) the groundwater was sampled from the REX chamber, which was installed in the KA2861A borehole between 9.30 and 9.80 m depth. The water was collected into sterile empty bottles, inoculated in liquid media and on solidified medium for most probable counting 10-30 min later.

Total acridine-orange count showed $2.4x10^3$-$7.5x10^5$ cells per ml of groundwater. The lowest microbial number ($2.4x10^3$ cells/ml) was observed in the inner section of KA2862A borehole.

The structure of the microbial populations in the inner section (7.38–15.98) groundwater of KA2862A is shown in Figure 5. We were able to cultivate up to 62% of the total microbial population. We expressed most probable number of different microbial groups as percentages related to the total count.

The flow from the inner section of KA2861A during sampling was 2-3 ml/h, from the outer section of KA2861A-20 ml/h, from the outer section of KA2862A -2 ml/min and from the inner section of KA2862A -250 ml/min.

Table 9 The experiments performed with groundwater and stones from Äspö access tunnel during June 1996 to July1998.

Designation of sampled location	Gases in the groundwater	Microbes on solid phase	Structure of microbial community	Effect of temperature	Total respiration	Enrichment of CH_4 oxidising	MPN of CH_4 oxidising	Aerobic mineralisation of carbon and energy sources	Inhibitor experiment	Effect of O_2 on respiration
KA2861A		X	X		X	X	X			
(REX site)	X	X	X		X	X	X		X	
KA2862A	X	X								
KA3105A	X				X		X			
KA3010A	X	X			X	X	X	X		X
KA3110A	X									
KA3067A	X	X			X	X	X			
KA3005A	X	X				X	X		X	
2200m	X			X						
Backfill		X			X	X	X			
tunnel, 3190		X				X	X			
3450m		X				X	X			
3500m										

Table 10 *Microbial numbers and activities found in the groundwaters of KA2861A and KA2862A 980311. Section length is shown below borehole designations.*

Analysis	KA2861A 6.82-6.92 m	KA2861A 8.50-9.80 m	KA2862A 6.82-6.92 m	KA2862A 7.38-15.98 m
Total microbial count, cells ml^{-1}	746 000	344 300	132 000	2 400
Most probable count, cells ml^{-1}				
Heterotrophic microaerophiles, cells ml^{-1}	55 000	42 000	77 000	0
Heterotrophic aerobes, cells ml^{-1}	38 000	9 300	333	0
Methane-oxidising bacteria				
Group I, cells ml^{-1}	80 000	77 000	42	0
Group II and X, cells ml^{-1}	42 000	42 000	5	5
H_2-oxidising bacteria, cells ml^{-1}	77 000	28 000	250	0
Total microbial respiration, $\mu M\ O_2\ liter^{-1}\ day^{-1}$	1.34	1.26	1.62	0
Total microbial respiration with 5 w% of minerals, $\mu M\ O_2\ day^{-1}$	1.39	n.d.	2.78	0
Carbon dioxide production, $\mu M\ day^{-1}$	0.42	3.7	3.23	0
Carbon dioxide production with 5 w% of minerals, $\mu M\ day^{-1}$	4.68	n.d.	4.92	0
Methane oxidation, $nM\ day^{-1}$	10.00-11.00	2.95-8.12	0.25	0.24

n.d.: no data

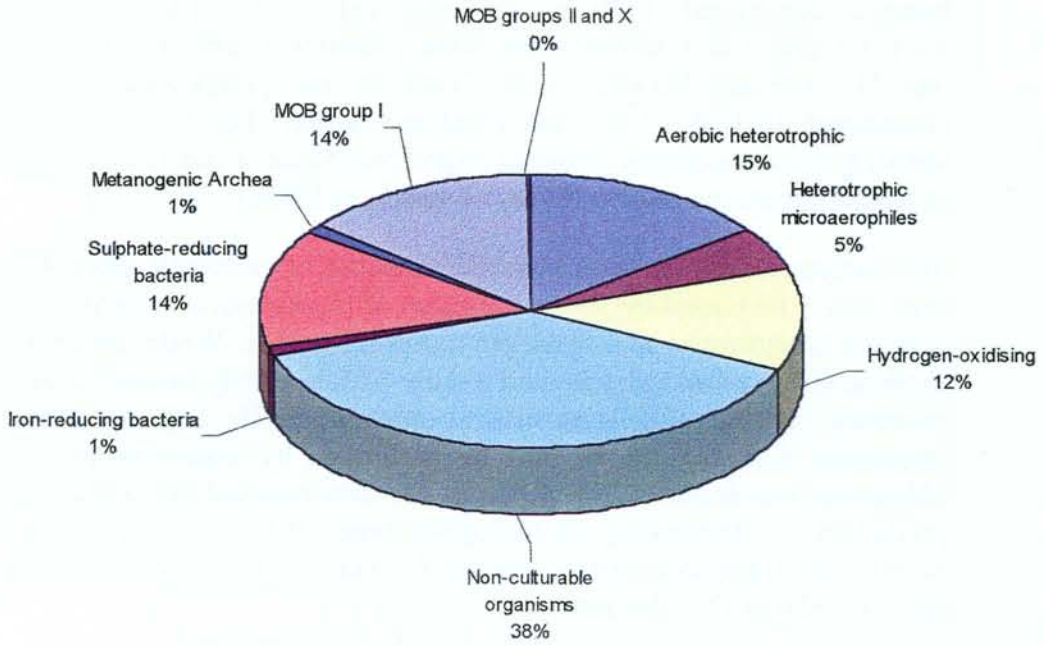


Figure 5 *Structure of the microbial population in KA2862A groundwater, 07.07.98. MOB–methane-oxidising bacteria.*

The structure of the microbial populations in the two studied (KA2861A and KA2862A) boreholes differed (Table 10). The highest number of all microbes tested was observed in the outer section of KA2861A. Both CH_4-oxidising and H_2-oxidising bacteria were found in KA2861A and KA2862A groundwaters, but they dominated KA2861A borehole compared to KA2862A. H_2-and CH_4-oxidising organisms constituted 10-23% of the microbial population in KA2861A groundwater. As anticipated for the outer section of KA2862A, the contribution from heterotrophic aerobes to total cell number was more than from CH_4- and H_2-oxidising bacteria (Table 10). In KA2861A heterotrophic microaerophilic and aerobic organisms were present at numbers comparable with CH_4- and H_2-oxidising bacteria. The fraction of H_2-oxidising bacteria was 9-10% in the anaerobic groundwater from KA2861A. Fracture of CH_4-oxidising bacteria in the inner section of KA2861A was below 1% while it was up to 20% in the outer section of this borehole. Heterotrophs constituted up to 58% in the outer section of KA2862A and 13-16% in the inner and outer sections of KA2861A. High counts of microaerophilic and aerobic heterotrophs in the outer section of KA2862A agrees with high CO_2 release rates in our batch experiment (Table 10) in this borehole. Aerobic organisms comprised a high fraction of the microbial population in the outer sections of the outer section of KA2861A and the outer section of KA2862A, which possibly indicates in-leakage of air.

Analysis of the microbial population included most probable viable counting of strict anaerobic bacteria the 980707 in the ground water originating from the inner section of KA2862A placed in REX chamber (Figure 5). The fractions of strict anaerobic methanogens and sulphate reducing bacteria were 1.24% and 14% in the inner section of KA2862A. Iron-reducing bacteria constituted 1.2% of the total cell count. The heterotrophic microaerophilic and aerobic population constituted 20%. Cultivable CH_4 and H_2-oxidising bacteria were found in the groundwater and they constituted 12-14% of the microbial population. The fraction of CH_4-utilising bacteria counted earlier in Äspö groundwater constituted up to 30% of whole population (Kotelnikova and Pedersen 1998a).

The analysis based on most probable counting of culturable physiological groups may be biased by possible overlap of physiological capacities of the detected heterotrophs and hydrogen-oxidising groups. While the ability to produce CH_4, reduce sulphate and oxidise CH_4 is strictly limited to specific microbial groups, capabilities such as oxidation of H_2, organic matter and respiration with iron or O_2 may be performed by organisms possessing ubiquitous metabolisms. Nevertheless, the data received are useful for the prediction of dominating physiological types that can reduce O_2. The number of organisms able to respire O_2 may be used for evaluation of specific cellular O_2 reduction.

Comparative analysis of different physiological microbial groups in the studied Äspö groundwater showed that DOC, CH_4 and H_2-oxidising organisms were the dominant culturable groups.

3.3 MICROBIAL O_2 REDUCTION IN ÄSPÖ GROUNDWATER

Total microbial O_2 reduction was analysed in batch experiments (Table 9) followed by gas-chromatographic determination of O_2, CH_4 and CO_2 in headspace of serum vials as described in 6.2. The groundwater from KA3110A, KA3105A, KA2862A, KA2861A and the 2200 m pond site in the accession tunnel was sampled and analysed. Killed controls were added with inhibitors. The results for KA3105A and KA2200 m pond site are described in Kotelnikova and Pedersen (1998a).

The results of the bath experiments with groundwater from KA3110A showed that the O_2 reduction in the non-inhibited samples exceeded the O_2 reduction in killed controls (Figure 6A, B). An O_2 concentration below 4 μM was the minimal concentration of dissolved O_2 at which microbial O_2 reduction could be observed. The half-saturation constant for oxygen for KA3110A groundwater (K_{mO2}) was 130 μM. An increase of the O_2 concentration stimulated the microbial O_2 reduction rate. The experiment was conducted with the groundwater, which contained a mixed microbial community. The different microorganisms present had probably different K_m for O_2 and could be active at different O_2 concentrations. Rates of O_2 reduction ranged between 0.14 and 4.02 μM/day. We did not observe any delay (or adaptation phase) in the reduction process. O_2 reduction capacities reached 80 μM. O_2 reduction rate and capacity increased with the O_2 concentrations in variants with addition of BESA (Figure 7). The O_2 reduction was the reaction of 1-st order and best fitted with an exponential curve. Similar relations were shown at 5 other initial concentrations of O_2. The rates of O_2 reduction related to the initial O_2 concentration for groundwater from KA3110A and modelled curve are shown in Figure 14.

Carbon dioxide was produced with rates that correlated with the O_2 reduction rates (Figure 6B), which implies that O_2 was consumed in biological reactions, since carbon dioxide often product of microbial O_2 reduction. The pH of the groundwater was not changed significantly under the experiment.

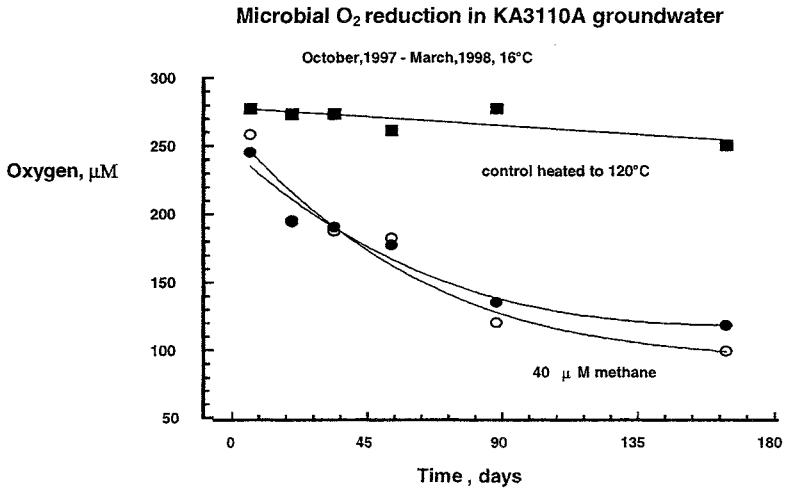
Methane oxidation was observed in the experiments with addition of CH_4. Addition of CH_4 stimulated O_2 reduction in KA3110A (Figure 6B). During 3 month laboratory experiment we did not observe any effect from added sterile granite on the O_2 reduction rates. Autoclaving of the groundwater, addition of sodium aside and formaldehyde hindered O_2 reduction. Both acetylene and cycloheximide inhibited the O_2 reduction as well (Figure 6B, 7). See also section 3.2, 3.3 and 3.5 of (Kotelnikova and Pedersen, 1998a).

Results from the batch experiments with KA2861A and KA2862A groundwaters showed that microbial O_2 reduction took place in the inner and outer section of KA2861A and the outer sections of KA2862A ground water. The microbial O_2 reduction rates ranged between 1.26 and 1.62 μM per day (Table 10). Both microbial O_2 reduction and CO_2 production in the outer sections of KA2861A and KA2862A were stimulated by the presence

of 5w% of crushed granite from KA2861A core (Table 10). Microbial O_2 reduction results are in good agreement with direct count and MPN observations (Table 10). The fact that microbial O_2 reduction in ground water with the lowest microbial total number was not observed without addition of substrate shows the importance of microbial processes in O_2 reduction. Microbial O_2 reduction was observed in the ground water where O_2 competent microbial groups could be cultured, consuming organic matter, CH_4 or H_2 (Table 10).

CO_2 production was observed simultaneously with O_2 reduction. CO_2 production was higher in the outer section of KA2862A than in the outer section of KA2861A while microbial O_2 reduction did not differ significantly. Aerobic microbial O_2 reduction accounted for 50% of the total carbon dioxide production in the outer section of KA2862A. Relatively low CO_2 yield in the outer section of KA2861A agrees with high count of hydrogen-oxidising bacteria in this ground water (Table 10). H_2-oxidising bacteria use CO_2 as carbon source.

A



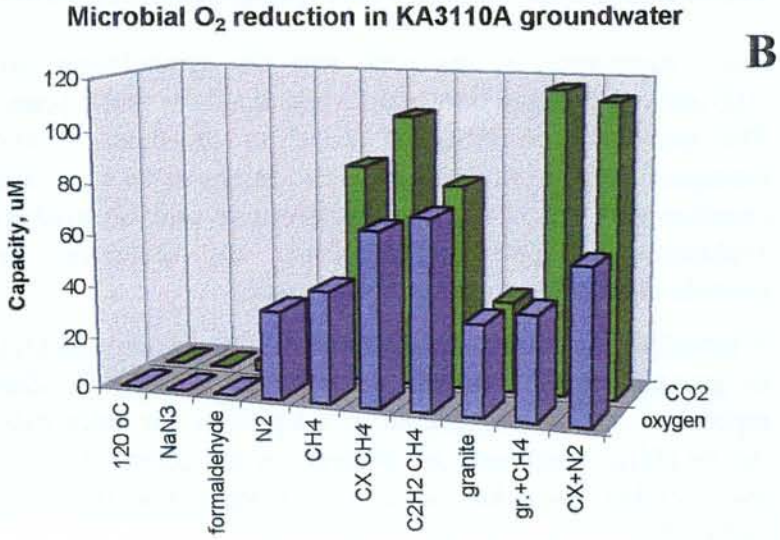


Figure 6 *Effect of heating (A) and different additions (B) on microbial O_2 reduction in KA3110A groundwater at 16℃. Sodium aside (1%), granite: mineral: water ratio 20:80, CH_4 : 1% CH_4 in head space, CX: cycloheximide (1%), N_2: no additions, air in head space, C_2H_2: acetylene, 5% and formaldehyde (2%) were used. 120℃: the samples were heat autoclaved at 120℃ for 20 min, gr.+ CH_4 : mineral: water ratio 20:80+1% CH_4 in head space*

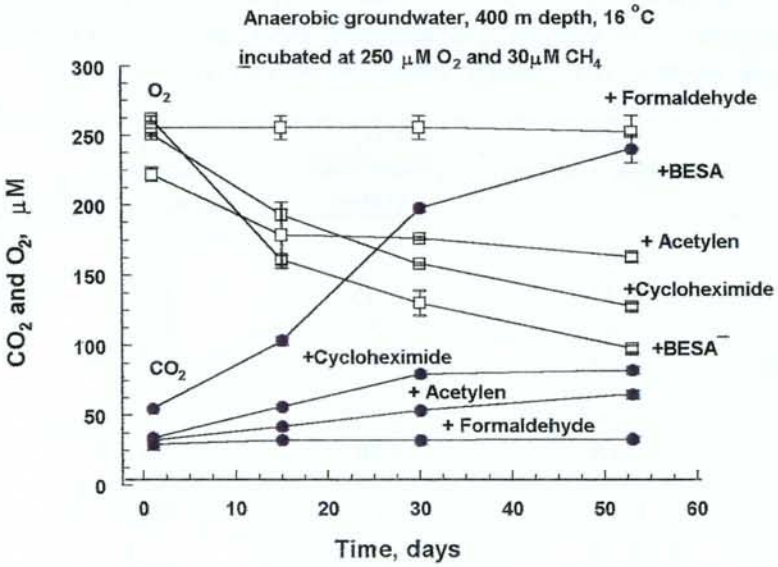


Figure 7 *Effect of inhibitors on O_2 reduction and CO_2 production in groundwater sampled from KA3110A and incubated with 250 µM O_2 and 30 µM CH_4 at 16℃. Points show average of 4 replicates.*

3.4 TEMPERATURE EFFECT ON MICROBIAL O_2 REDUCTION

The temperature of the rock and the groundwater around the future repository will range between 15 and 80°C for many years after deposition. The potential for biogenic reduction processes should, therefore, be estimated both at psychrotrophic conditions at 15-16°C and at thermophilic conditions at 40-60°C. Since temperature and O_2 gradients are expected, evaluation of potential microbial O_2 reduction activity demands consideration of these parameters *in situ*.

A modelling approach includes estimation of microbial O_2 reduction activity in groundwater at ambient temperatures (15-70°C). Zones close to the repository can be considered as auspicious for microbial O_2 reduction if thermophilic organisms are present. In the current research we considered the microbial O_2 reduction activity at the temperatures 15°C, and at 30°C and 60°C.

Incubation temperatures of the duplicate sample bottles were 15, 30 and 60°C. The groundwater was sampled from the pond at 2200 m tunnel length (depth 300 m). For each temperature, 8 sterile 115 ml-bottles were filled with 20 ml groundwater and 10 ml CH_4. Two of them got no additions, two of them were added with 0.1 ml of 2 M BESA, two - with 5% of acetylene and two - with 0.25% of formaldehyde. All variants (24 totally) were analysed three times; one day after inoculation and 17 and 34 days after inoculation (Table 11, below).

Table 11 *O_2 reduction rates, capacities and efficiencies and CO_2 production rates at different temperatures in aerobic groundwater from depth of 300 m. The rate were calculated as the mean of two parallel measurements in two replicates.*

Inhibitor	O_2 reduction, μM/day	CO_2 production, μM/day	O_2 reduction capacity, μM	O_2 reduction efficiency, %
16° C				
no inhibitor	7.22	1.31	246	87
+BESA	4.73	10.15	164	61
+Acetylene	7.87	2.54	264	98
+Formaldehyde	3.23	4.51	59	25
30° C				
No inhibitor	2.95	4.31	47	19
+BESA	3.69	6.60	126	41
+Acetylene	3.02	3.71	104	42
+Formaldehyde	2.04	2.01	69	28
60° C				
No inhibitor	5.55	3.81	179	69
+BESA	5.81	3.84	198	64
+Acetylene	3.68	3.35	125	41
+Formaldehyde	4.06	1.61	133	48

Carbon dioxide production is of interest because it provides an estimate of microbial organic matter degradation in groundwater. Furthermore, it has a significant impact on the water quality and secondary porosity of the hydrological system because CO_2 generated by microbial metabolism drives carbonate and silicate dissolution. Carbon dioxide was produced in all

variants (Table 11). The selective inhibitors used did not effect O_2 reduction and CO_2 production in the tested concentrations (Table 11). O_2 was consumed at 20-98% at all tested temperatures during 35 days. Although mesophilic and thermophilic aerobic organisms were present in the tested water, O_2 was used at higher rates at 16°C than at 30°C or 60°C (Table 11). The O_2 reduction capacities and efficiencies were higher at 16° C than at 30 and 60°C. The time delay of the active O_2 reduction in the experimental vials is called the adaptive phase. It was shorter at 16°C than at 30°C or 60°C. The adaptation phase at the temperatures 30°C and 60°C was 17 days, implying that the optimal temperature for O_2 reduction is close to the current temperature of groundwater in the tunnel (16°C). At temperatures of 30°C or 60°C, the microbial community will be activated after approximately two weeks. In the studied groundwater (2200 m) CH_4 oxidation was not detected during the experiment. The inhibitor of CH_4 production, acetylene, did not effect at used concentrations (Table 11). Temperature between 16 and 60°C did not significantly effect the rates of O_2 reduction, CO_2 production or O_2 reduction efficiency.

3.5 MICROBIAL O_2 REDUCTION EVALUATED WITH CHEMICAL METHOD

Section 4.3 described results obtained using a gas chromatograph technique. This section deals with O_2 reduction measured in the groundwater with the Winkler method in *in vitro* experiments (Paerl, 1997). Samples were collected aerobically in March and June 1996 from KA3105A, KA3010A, KA3005A, KA3110A, KA2862A, KA3067A and KA3010A. Dissolved O_2 was analysed with the Winkler method (Paerl, 1997) 3 times over 115 days in March-May 1996. The groundwater was sampled one more time and analysed 3 times over 45 days in June-August 1996. Energy sources were not added during those experiments. The resulting average O_2 reduction is shown in Figure 8 A and B. The rates are shown in Table 12. On the basis of the rates the time needed for reduction of 500 µM of dissolved O_2 at 17°C was calculated (Table 12). The curves in Figure 8 show the O_2 reduction during the experiment.

Table 12 *O_2 reduction by microbial communities in various Äspö groundwaters (June-October 1996).*

Borehole	Average O_2 reduction rate*(µM/day)	The time for reduction of O_2 (500 µM) (years)**	Total O_2 reduction capacity, (µM)	RSD %
KA3110A	0.65	1.43-4.17	145-345	27
KA2862A	0.83	0.54-2.84	145-345	31
KA3010A	2.44	0.29-1.15	220-413	70
KA3110A	2.03	0.46-2.11	215	40
KA3067A	2.11	0.54-0.73	165	3
KA3105A	0.55	1.71-3.03	260	4

* - Rate was calculated as $R = \ln 2(O_2)_2-(O_2)_1)/Time_2-Time_1$ ** -The time was calculated as $(O_2)/365R$, where (O_2) -concentration 500 µM.

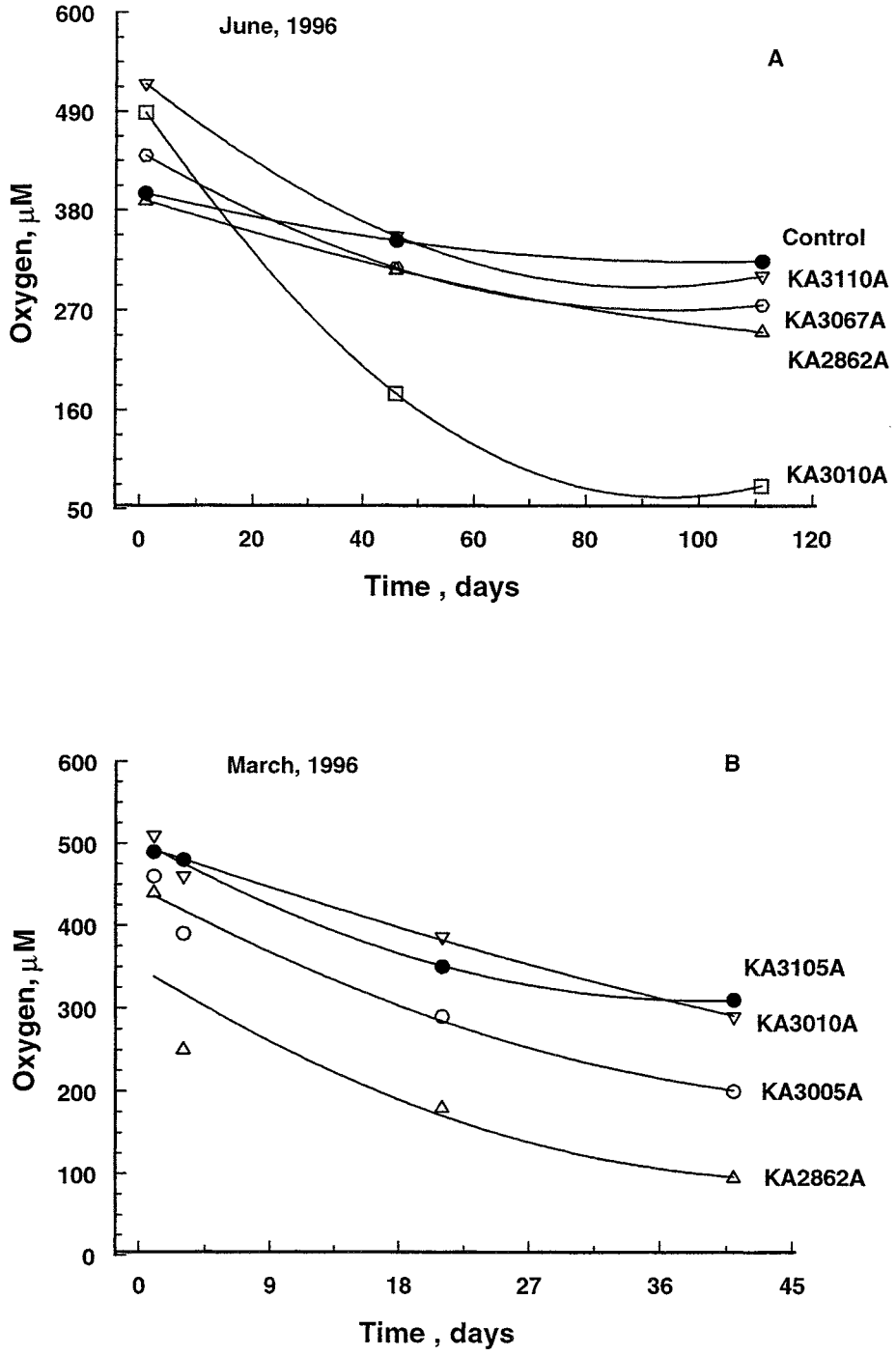


Figure 8 *O_2 reduction in Äspö groundwater determined with the Winkler method in June (A) and March (B) 1996. No electron donors were added. The samples were incubated at 17°C during 45 and 115 days.*

3.6 UPTAKE OF CARBON AND ENERGY SOURCES FOR MICROBIAL RESPIRATION

Uptake of different substrates used in microbial reactions was studied in groundwater from KA2862A sampled from the REX chamber after 28 days of circulation after O_2 pulse (Table 13). The groundwater was sampled and processed as described in section 6.8, 6.9 and 6.10. The experiment aimed at an elucidation of *in vitro* activities of both aerobic and anaerobic microorganisms.

Radiolabelled substrates of known concentration and specific activity were added to the groundwater and then incubated. The radiotracer technique for measurement of *in situ* growth and activity of microorganisms is based on the premise that the metabolism of the examined organisms remains as *at situ* level during incubation with the radiolabelled substrate. The incubations were initiated with radiotracers immediately after sampling. Natural conditions were simulated by keeping the samples in sealed serum bottles, in dark, without agitation, at temperature of 16°C and using substrate concentrations close to ambient. The incubation time, 17 days, allowed adaptation of aerobic and microaerophilic organisms to oxic conditions. The aerobic metabolic processes studied included CH_4 and H_2-oxidising activities and the heterotrophic activities of glucose, acetate and formate respiration.

Groundwater dissolved organic carbon (DOC) is a complex mixture of substances derived from biotic and abiotic reactions. It is both consumed and produced by microorganisms. This study of DOC consumption with O_2 was motivated by the role of DOC in the microbial reduction. Glucose is a very easily respirable substrate, but it is normally not found in groundwater. Acetate is a key intermediate in anaerobic metabolism of organic matter both in marine and freshwater environments, being the main product of anaerobic DOC degradation. Formate is also a very important intermediate in anaerobic syntrophic reactions. Formate may be present as a transit molecule in metabolic pathways. Groundwater DOC consists of high molecular weight (HMW) molecules, namely, fulvic acids, and low molecular weight (LMW) aliphatic hydrocarbons, substituted alcohols and organic acids (Atlas and Bartha, 1997). The LMW fraction is suggested to be recycled by microorganisms and partly deriving from microbial activity transformation of deep kerogens (Murphy et al., 1992, Aravena et al., 1993). Low ^{14}C values observed for LMW have been explained by participation of autotrophic acetogenesis (Gelwicks et al., 1989, Preuss et al., 1989). Active homoacetogens have been found in Äspö groundwaters, producing acetate from H_2 and CO_2 (Kotelnikova et al., 1997, 1998b). These considerations determined the choice of organic substrates to be tested for the study of microbial O_2 reduction in the deep groundwater.

Table 13 Selected microbial respiration reactions studied in groundwater.

Process	Energy giving reaction	ΔG (kJ/mol)	pH ranges	Example of performing organisms
Aerobic				
Respiration of glucose	$^{14}C_6H_{12}O_6 + 6O_2 \rightarrow 6\ ^{14}CO_2 + 6H_2O$	-688	5.6-8.0	*Pseudomonas*
Respiration of Acetate	$^{14}CH_3H^{14}COO + O_2 \rightarrow 2^{14}CO_2 + 2H_2O$	-419.4	7.0-7.5	
Respiration of Formate	$2^{14}CH_2OO + O_2 \rightarrow 2^{14}CO_2 + 2H_2O$	-86.9	7.0-7.5	
H_2 oxidation	$H_2 + 0.5O_2 + ^{14}CO_2 \rightarrow$ ^{14}C-biomass + H_2O	-113.3	4.0-8.0	*Alcaliganes eutrophica Hydrogenobacter, Pseudomonas*
Methane oxidation	$^{14}CH_4 + 4O_2 \rightarrow ^{14}CO_2 + 2H_2O$	-195.4	6.5-7.5	*Methylomonas methanica Methylocinus*
Anaerobic				
Fermentation of glucose	$^{14}C_6H_{12}O_6 + O_2 \rightarrow ^{14}CO_2 + H_2$	-5-60	6.5-8.5	*Yeast, Clostridium, Lactobacillus, Propionebacter*
Methane production	$4H_2 + ^{14}CO_2 \rightarrow ^{14}CH_4 + 2H_2O$	-23.2	7.0 -10.0	*Methanobacterium, Methanogenium*
Autotrophic acetate production	$4H_2 + 2^{14}CO_2 \rightarrow ^{14}CH_3COOH + 2H_2O$	-14.4	6.0-8.0	*Eubacterium, Clostridium Acetogenium*

The free energy change is given for pH 7.0, unless it is specified in the left column.

Although the rates of different microbial processes in the laboratory experiment probably were different from *in situ* rates, they allowed estimation of comparative aspects of these processes among the microbial reactions taking place in the groundwater (Figure 9).

The observed rates of $^{14}CO_2$ production from $^{14}CH_4$ were lower than from ^{14}C-acetate, nevertheless it exceeded the CO_2 production rates from ^{14}C -glucose and ^{14}C -formate (Table 14). H_2 was oxidised aerobically and carbon from ^{14}C-bicarbonate was detected in the biomass. ^{14}C-carbon dioxide was measured as the final product in the case of organic-oxidation, but it was

used as a carbon source in the case of autotrophic H_2 oxidation. ^{14}C biomass incorporation values resulting from CH_4 and H_2 oxidation were similar.

Use of radioisotopic ^{14}C-labelled heterotrophic substrates showed production of $^{14}CO_2$ and ^{14}C-biomass from ^{14}C-glucose and ^{14}C-acetate in the presence of O_2 in the groundwater, which had been in contact with O_2 for 28 days (Table 14). ^{14}C-formate was transformed aerobically and ^{14}C-carbon was detected only in the biomass. Interestingly, metabolic rates of aerobic ^{14}C-acetate and ^{14}C-formate oxidation were significantly faster than ^{14}C-glucose oxidation (Figure 9). The laboratory incubations with added [^{14}C]-acetate, glucose and formate probably overestimated the *in situ* rates of the microbial metabolism in the subsurface, since the dissolved concentrations of the substrates in the experiment and the long incubation period may have altered the natural conditions. However, the acetate oxidation observed during the experiment indicates the presence of an active acetate competent microbial group. Thus, the radiotracer test showed that acetate respiration is expected to be an important microbial process consuming O_2 in the groundwater if acetate is present. Acetate oxidation activity is followed by formate oxidation and fermentation of glucose. Aerobic heterotrophs compose a significant group of microbes in subsurface. For example, aerobic heterotrophs dominated the microbial community in Middedorf sedimentary rocks (Murphy et al., 1992, Phelps et al., 1994).

Our results agree with the observed abundance of heterotrophic, H_2 and CH_4-utilising bacteria in the groundwater (Table 10). KA2862A groundwater harboured organisms possessing ubiquitous metabolic features to oxidise H_2, formate, and acetate with O_2 or ferment glucose. The broad metabolic magnitude can be an essential survival strategy for microorganisms inhabiting oligotrophic, H_2-, CH_4- and iron-rich environments.

Our results agree with our the previous observations. Experiments characterising uptake of different substrates in oxic and anoxic deep groundwater have been performed earlier in USA (Chapelle et al., 1988, Chapelle et al., 1990, Stevens et al., 1993), at Äspö (Pedersen et al., 1992), and in Canada (Jain et al., 1997). However, aerobic consumption of lactate dominated over acetate and glucose at all tested depths in Äspö groundwaters (Pedersen et al., 1992). Acetate, lactate and glucose assimilations demonstrated presence of heterotrophic bacteria. Incubation in air decreased the assimilation of CO_2, formate, lactate and leucine by attached and unattached bacteria (Pedersen et al., 1992). The uptake of acetate significantly exceeded uptake both of lactate and glucose in Canadian experiments. Formate and CO_2 were consumed at lower rates then glucose (Jain et al., 1997). It is appears, that acetate is widely distributed energy and carbon source in deep groundwater.

We studied uptake and metabolism of H_2 and CH_4 not only in the presence of O_2 but also anaerobically. Methanogenesis, homoacetogenesis and anaerobic CH_4 oxidation were tested in the same groundwater. The purpose of this test was to demonstrate the extent of the anaerobic activities in deep groundwater, which has been oxygenated.

The radiotracer test demonstrated a minor role for anaerobic autotrophic CH_4 and acetate production in the studied groundwater that is in a good agreement with MPN results. These processes are however expected to be of major importance in anaerobic groundwater or in close proximity to biofilms.

The nature and origin of DOC in groundwaters is not yet completely understood. HMW DOC is hardly degradable for microbes. Microbial growth rates are affected by the availability of DOC components, the structure of the microbial population and the physiological state of the competent groups. Thus, in natural systems, measurement of total DOC nutrient concentrations may not provide an adequate predictor of growth or respiration potential. The results of the radiotracer test demonstrated the necessity for consideration of the role of acetate, formate, CH_4 and H_2 oxidisers in the modelling of microbial respiration.

Table 14 *Uptake and mineralisation of carbon substrates by microorganisms in ^{14}C radiotracer experiment with the KA2862A groundwater injected in REX chamber 980707 and circulating there until 980724.*

Microbial processes	**Rate of ^{14}C-substrate incorporation**	
	Product, μM day^{-1}	**Biomass, μM day^{-1}**
Aerobic processes		
Glucose oxidation	0.05	642
Acetate oxidation	12030	9470
Formate oxidation	0	8880
Aerobic CH_4 oxidation	2.07	40
H_2 oxidation	n.d.	30
Anaerobic processes		
Glucose fermentation	1135	740
Homoacetogenesis	0.64	n.d.
Methane production	0.03	n.d.
Anaerobic CH_4 oxidation	0	0.5

Values in the table are the average of four replicates. The activities in killed controls were subtracted from the activities in the non-inhibited samples.

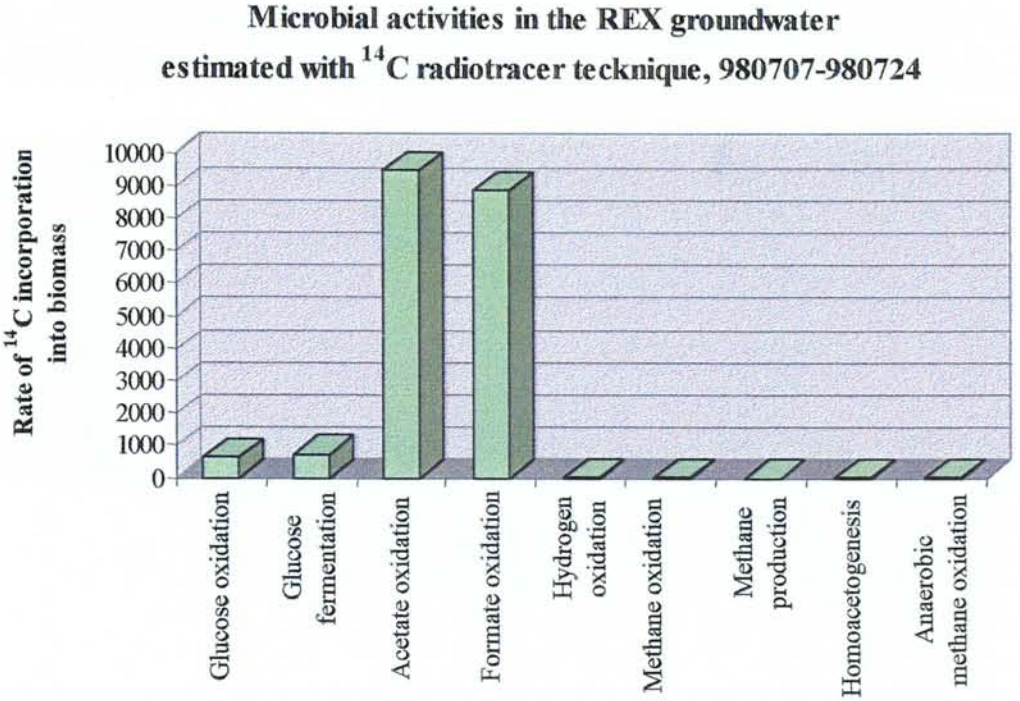


Figure 9 *Microbial activities with different electron donors and electron acceptors in the REX KA2862A groundwater, incubated in the experiment chamber for 17 days.*

4 INTERPRETATION OF RESULTS AND MODELLING CONCEPTS

4.1 MICROBIAL EFFECTS ON GROUNDWATER CHEMISTRY

The presence of active aerobic microbial communities has repeatedly been shown in different types of subsurface environments (White et al., 1983, Balkwill et al., 1989, Hicks et al., 1989, Phelps et al., 1989a-b, Stevens et al., 1993). Recharging rainwater in basalts of Savannah River Basin (USA) was oxidised (Madsen et al., 1989). Deep granitic groundwater in Canada were highly reduced, while small amounts of oxygenated, $^{18}O_2$ depleted spring-melt water penetrated the bedrock to depths of 200 m (Gascoyne et al., 1997). They also observed a high count of microorganisms in groundwater containing high levels of DOC and dissolved O_2 but low counts in DOC-and O_2 deficient groundwaters. The dissolved O_2 concentrations were low but detectable (0.1-0.25 mg/l) (Gascoyne et al., 1997). O_2 has not been found in Finland granitic groundwater (Sherwood Lollar et al., 1993a, 1993b).

Granitic hydrogeological systems supporting microbial populations is very heterogeneous. Different physiological types of microorganisms may exist in aquifers, with different mineral phases (e.g. iron oxides, metal sulphides or carbonates), different types of organic matter at different redox potentials due to the hydrological conductivity and supply rate of electron acceptors and donors. This heterogeneity exists from millimetre to kilometre scale. The scale of heterogeneity is important for understanding of the system behaviour. Hydrological properties define the nutrient fluxes and amount of available electron donors for microbes.

The tendency of microbial electron-accepting processes to proceed from O_2 to ferrous iron reduction to sulphate reduction to methanogenesis and homoacetogenesis in the groundwater is a defined characteristic of subterranean aquifers (Figure 10). This behaviour follows directly from the abundance of a potential electron acceptor (O_2) relative to other renewable electron acceptors (Mn^{4+}, Fe^{3+}, SO_4^{2-}, CO_2). The microbial population structure and activities observed after oxidation of KA2862A groundwater in the REX chamber indicated that the microbial system follows the behaviour described above. The main tendency of the groundwater chemistry alteration is a drop of O_2 concentrations followed by reduction of other available electron acceptors.

Our results showed that the main energy sources used for O_2 reduction appear to be acetate, CH_4 and H_2. Introduction of O_2 changes the environment drastically, stimulates aerobic metabolisms and induces additional electron acceptors (Figure 11). These changes select for the most adapted organisms and initiate a complex chain of events (Figure 10). This succession of different microbial groups is time and a redox potential dependent process. The succession model is an approximation. The real situation appears to be more complicated, since the succession of microbial groups is affected by many physical and chemical factors in the environment.

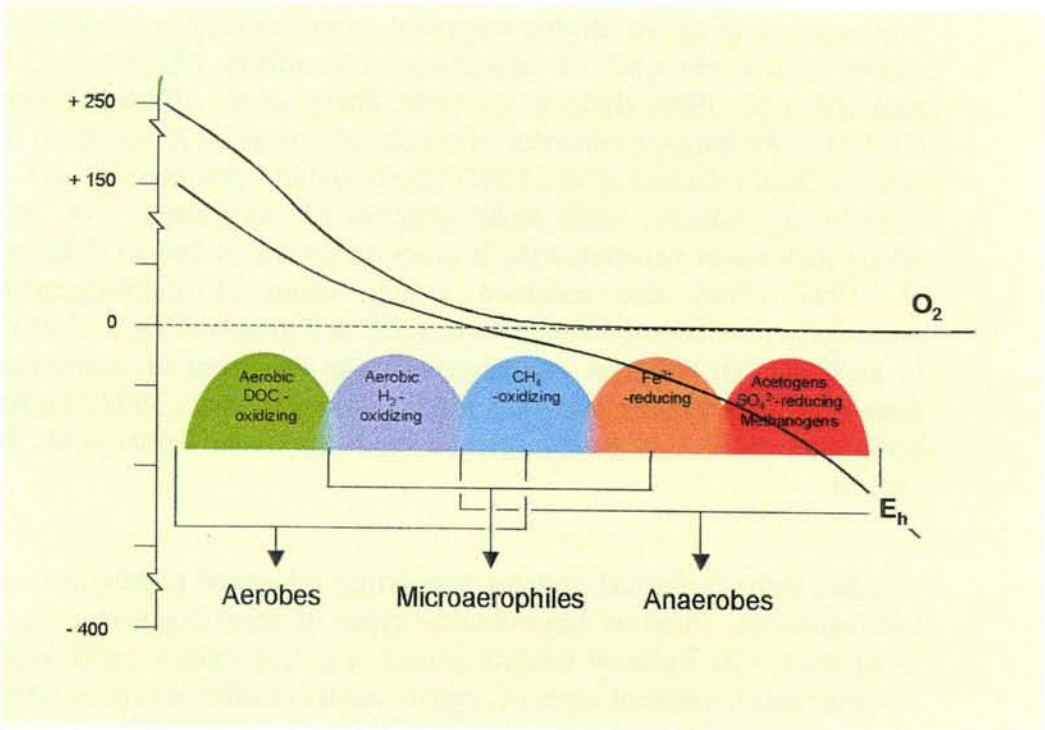


Figure 10 *Conceptual scheme of succession of microbial groups in a cause of O_2 reduction and redox evolution. The y-axis shows concentration of O_2 (μM) and redox (mV). See microbial reduction reactions in Table 6.*

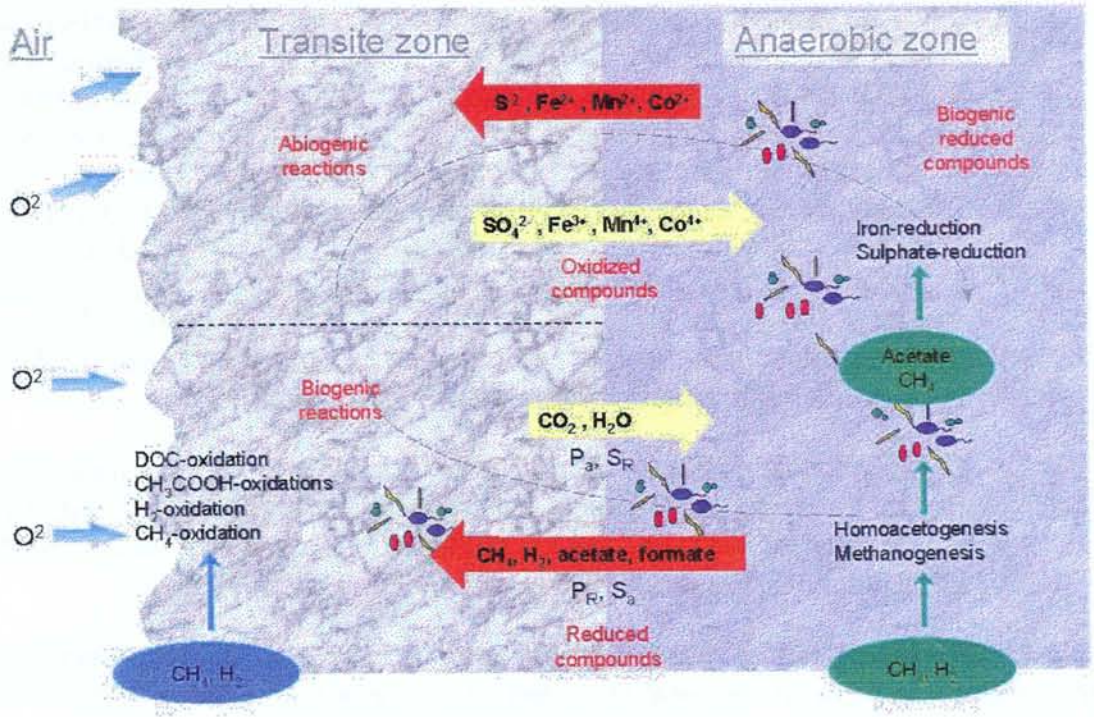


Figure 11 *Microbe mediated processes in groundwater. Renewal of some inorganic electron acceptors is a result of autooxidation.*

A conceptual succession model of microbial activity around the HRL repository must focus on the near field inventory (bentonite, backfill material and the surrounding rock with groundwater). The first aspect of the model is the rock-groundwater potential for microbial activity and defines to what extent nutrients and the electron donors are available for microbial biomass production and respiration. Another aspect of the model is a priory consideration of diverse microbial communities present in the groundwater and on the rock surrounding the future HRL. This aspect includes the need of knowledge about the potential for different microbial processes (or demonstration of the presence and the activity of the specific microbial groups). The model is expected to predict the biochemical behaviour of the system around the repository. Our current model of the microbial O_2 reduction is based on investigations of microbial O_2 consumption. It considers types and concentrations of electron donors available for microbes in the ground water and diversity of the microbial community. The diversity of the microbial community defines potential of different microbial metabolisms.

Previous and recent microbiological research has shown the presence of sulphate-, iron-reducing and acetate-producing bacteria and methanogens in Äspö groundwater and the presence of the potential electron donors and acceptors for them (Kotelnikova and Pedersen 1996a-b; Pedersen *et al* 1996). Our results of gas analysis of dissolved groundwater gases showed an abundance of CH_4 (up to 1000 μM) and H_2 (up to 10-100 μM). H_2 is used as an energy source for chemolithoautotrophic anaerobes. The acetate produced

by homoacetogens is in turn an energy and carbon source for the acetate consuming anaerobes. On the basis of the results a model of the subsurface microbial community was built. The community exists at the expense of H_2 and CH_4 (Figure 3,11). The schemes show that production of chemical reducing species is ongoing in deep groundwater. Anaerobic organisms contribute to the reducing character around a HLR, producing H_2S, Fe^{+2}, acetate and CH_4, thereby providing energy sources and electron donors for O_2 reduction by aerobic organisms (Figure 11). The important advantage of microbial catalysis is a continuous production of new reducing powers in anaerobic zones (like Fe^{+2}, Mn^{+2},S^{-2}, S^o, CH_4) and new electron acceptors (Fe^{+3}, SO_4, S^o, S_2O_3, CO_2) in oxidised zones. Thus, all processes mediated by microbes lead to reduction of a system.

Our experimental data demonstrate that microorganisms were responsible for the observed O_2 reduction in the investigated groundwater. O_2 reduction rates ranged between 0.32 and 4.02 µM/day for groundwater and 1.73-4.50 µM/day for oxic groundwater (Table 15).

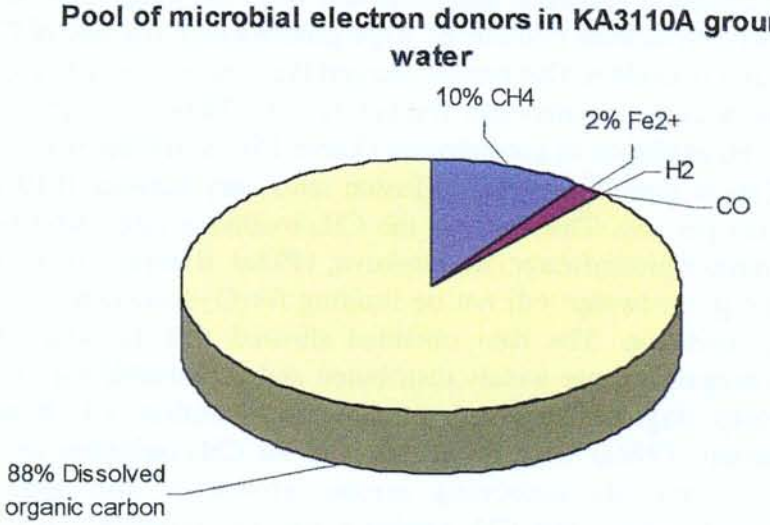
Table 15 *Compilation of the results of O_2 reduction, O_2 capacity and contribution of CH_4-oxidising bacteria to the total O_2 reduction in groundwater by unattached and attached microorganisms.*

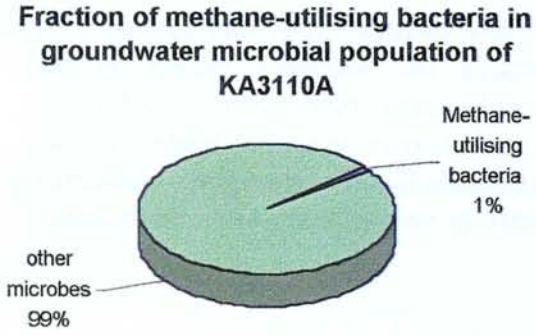
Sampling place	**Depth (m)**	**Total O_2 reduction capacity* (µM/litre)**	**O_2 reduction rate (µM/day)**	**Percent of O_2 reduction at the expense of CH_4 oxidation, %**	**The reduction time (Years)**
Groundwater					
KA2862A, 17°C	380	55	0.83	5.8	1.65
KA2861A, 17°C	380	n.d.	0.31	2.9	3.99
KA3105A, 17°C	414	180	0.550	n.d.	2.49
KA3010A, 17°C	400	11	1.92-3.78	0.11	0.71
23°C		227	3.78	0.32	0.36
KA3110A, 17°C	414	5-700	2.03-4.02	0.73-6.00	0.31
23°C		470	2.06	4.19	0.61
KA3067A, 17°C	409	38	1.60	n.d.	0.86
KA3005A, 23°C	400	100	0.32	1.88-6.66	3.96
Pond water					
2200 m, 15°C	300	205	1.73-4.00	19-57	0.32
2200 m, 36°C		85	0.91	32.00	1.39
2200 m, 60°C		188	1.50	n.d.	0.84
3500 m water	460	n.d.	0.95	20.83	n.d.
3500 m granite		n.d.	1.4	28.57	0.98
3450 m granite	460	n.d.	3.8	9.38	0.36
3190 m, granite	420	n.d.	3.12	3.04	0.44
3190 m, 17°C		211	3.12	2.11	0.41
3190 m, 23°C		236	4.50	0.72	0.30

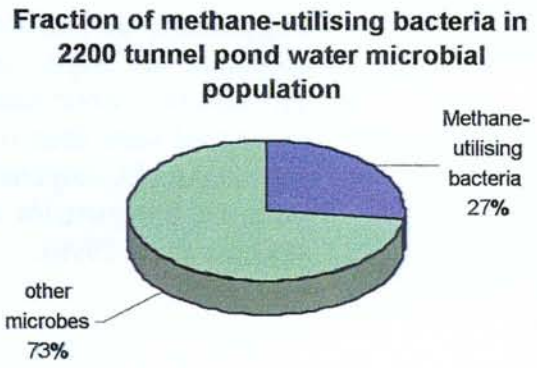
*Total capacity was calculated as difference of initial and final O_2 concentrations during the experiments

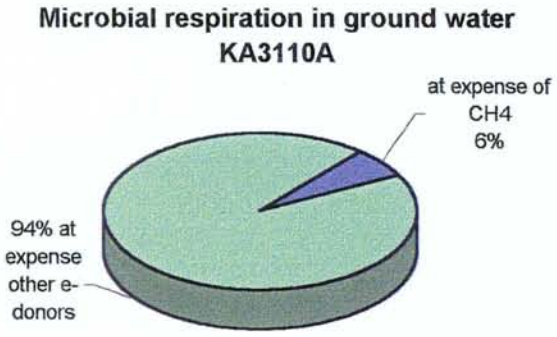
Different microbial populations are expected to be active at different O_2 concentrations. Which are the most important microbial groups to be studied with regard to reduction processes and why? As CH_4 is one of main energy and carbon sources in oxidised Äspö groundwater, the role of CH_4-oxidising bacteria is evident. Our results showed the presence of active CH_4-oxidising bacteria in the groundwater (Figure 12, 13). There is a significant potential for CH_4 oxidation in groundwater (Table 15). At the ambient concentrations of CH_4 in groundwater the diffusion rates vary between 0.19 and 1.45 μM per cm per day. That exceeds the CH_4 oxidation rates determined in Äspö subsurface groundwater (Kotelnikova, 1998a). It implies that the gas transfer in the groundwater will not be limiting for O_2 reduction at the expense of CH_4 oxidation. The data obtained showed that H_2 and CH_4 oxidising microorganisms are widely distributed and responsible for O_2 consumption in deep oligotrophic Äspö groundwaters (section 3.1, Kotelnikova and Pedersen, 1998a). Significant potential for CH_4 oxidation in environments not continuously supporting aerobic growth is also shown. The data collected suggest that CH_4 oxidation can be stimulated by mineral phases (Kotelnikova and Pedersen, 1998a).

Supposedly, our activity data could underestimate the activities of CH_4 oxidation in Äspö, since we measured the activities mostly in the groundwater, while methanotrophs may be much more active in a biofilm associated state than in a free state. It is known from the literature, that methanotrophs, responsible for CH_4-oxidation in soils, are tightly associated with the soil particles and lose the activity very quickly after dissociation (Prieme et al., 1996).









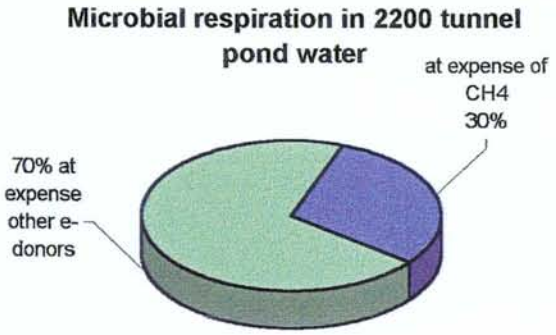


Figure 12 *Comparative diagrams showing relation between microbial electron donor sources, numbers of viable CH_4-oxidising bacteria and the impact of CH_4 oxidation on total microbial respiration.*

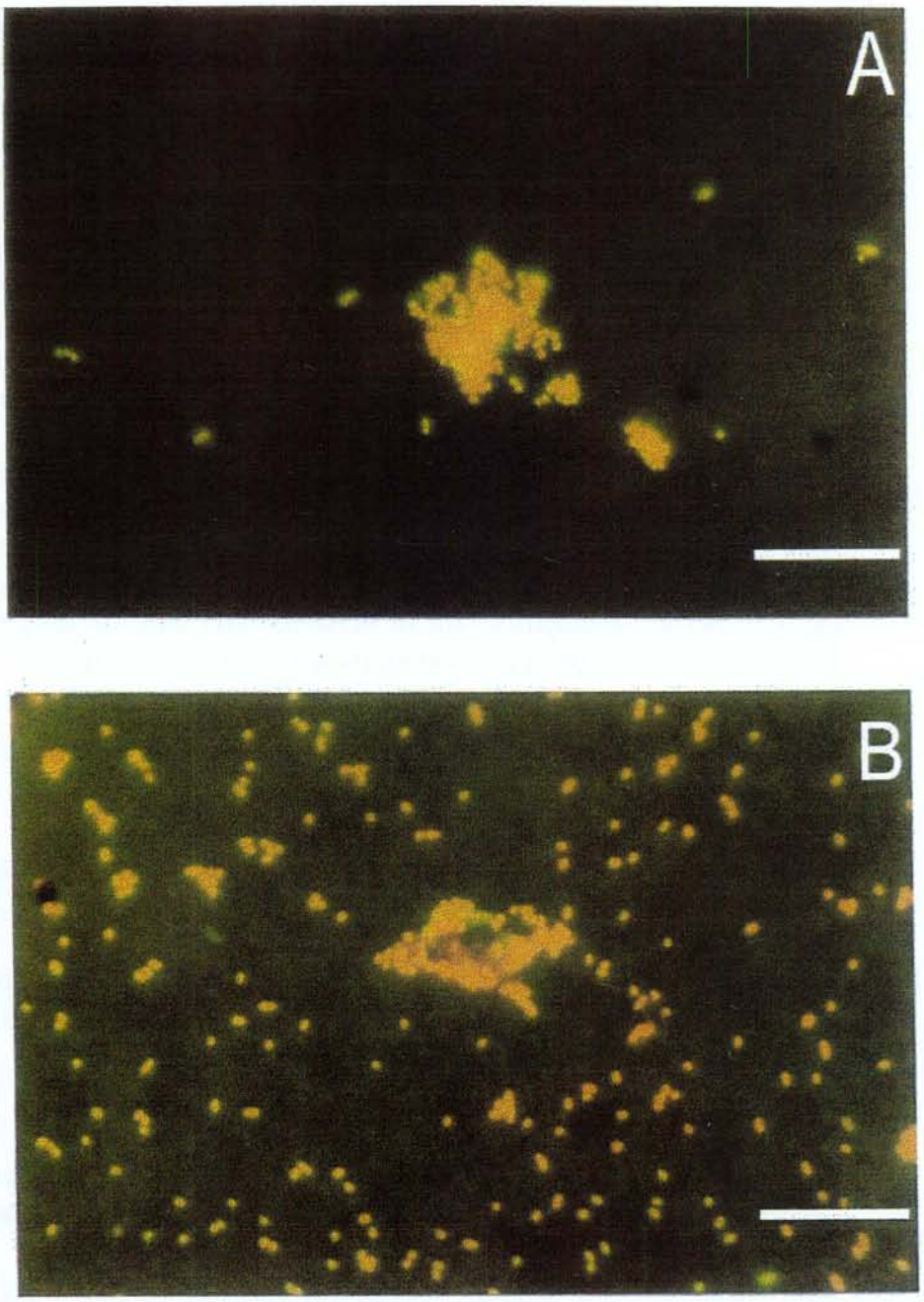


Figure 13 *Phase-contrast micrographs of methane-utilising bacteria isolated in pure cultures from KA3105A (A) and KA3110A groundwater, grown in NMS medium (A) and sterile ground water from KA3110A (B) with CH_4 and O_2. Bars, 10 μm.*

Our tests showed that CH_4 oxidation contributed to about 30% of the total microbial respiration. Which microbial processes do contribute the remaining 70%? We observed H_2-oxidising and heterotrophic bacteria in the groundwaters. Heterotrophic bacteria were earlier shown to be present and active in Äspö (Pedersen and Ekendahl 1990, 1992). They consumed glucose, lactate, formate, leucine and acetate. The anaerobic attached and unattached heterotrophic activities exceeded aerobic ones by approximately 100-70% (Pedersen and Ekendahl 1992). The concentration of DOC in Äspö groundwater varies from 25 to 333 µM of carbon. Organic compounds, which are hardly degradable under anaerobic conditions, may be accessed by microbes in the presence of O_2. It means that the flux of groundwater passing through the repository is a source of a certain quantity and quality of nutrients and an energy sources for aerobic respiration. Neutrophilic autotrophic *Gallionella* mediated iron-oxidation occurs on tunnel walls. High pH groundwater excludes the possibility of development of iron-oxidising bacteria *Thiobacillus*, as the most known iron-oxidising *Thiobacillus* are acidophilic. All of these bacteria will probably participate in accordance with substrate availability and the O_2 concentration.

4.2 KINETIC MODEL FOR MICROBIAL O_2 REDUCTION

4.2.1 Predicted O_2 concentrations in the groundwater

Evaluation of expected ambient O_2 concentrations includes calculations of solubility of O_2 in groundwater at ambient temperatures, (15-80°C) (50 atm) salinities and pressure. The concentration of dissolved O_2 in repository groundwater air saturated at atmospheric pressure will be approximately 310 µM at 15°C. At 40°C it will be 198 µM. The concentration will decrease with increasing temperature.

4.2.2 General model

Reduction of O_2 in groundwater will be affected by the diffusion rate, the advection flow, the microbial respiration potential and the chemical O_2 reduction:

$$\frac{d[O_2]}{dt} = \text{Diffusion + Flow advection - (Microbial } O_2 \text{ reduction + mineral } O_2 \text{ reduction)}$$

$$\frac{d[O_2]}{dt} = D\frac{d[O_2]}{dX} + V\frac{d[O_2]}{dt} - \left(\sum V_s + \eta\frac{dJ}{dt}\right)$$

D: diffusion coefficient
V: flow rate
V_s: specific microbial reduction rate
J: mineral O_2 uptake
η: structural mineral coefficient

We analysed individual species, physiological groups and the whole ground water community. The analysis was synthesised as the model. The conceptual approach is focused on the microbial activity near the repository. The model relies on confident expectation that microbial diversity is large

and organisms, able to oxidise electron donors, which are present in the groundwater, can be activated in the presence of O_2. The electron donors available for the microbial O_2 reduction define particular microbial activities. Concentrations of the electron donors in a groundwater define contribution of a certain microbial metabolism or structure of microbial community. The physiological structure of the mixed microbial population provides weight coefficients for each participating group. The total respiration capacity may be constituted by the sum of contributing capacities of diverse microbial metabolisms. It may be used in the model as the sum of additives yielding the total O_2 uptake capacity in a case when the microbial populations are non-competing.

4.2.3 Kinetics of O_2 and substrate consumption

The experimental data received for KA3110A groundwater (Kotelnikova and Pedersen, 1998a) were interpreted by using techniques developed for purified enzyme systems to describe O_2 reduction catalysis by whole microbial populations.

The basis of the approach relies on the Michaelis-Menten theory, in which rate constants (k1, k2, k3) describe single substrate (S) interaction with the enzyme (E), formation of an intermediate complex (ES), and an eventual product formation (P).

$$S + E \longrightarrow ES \longrightarrow P + E$$

S: substrate
E: enzyme
ES: enzyme-substrate complex
P: product

$$Substrate + O_2 \xrightarrow{Microbes} Product + CO_2$$

This theory is described by the relationship of reaction velocity with substrate concentration as shown in Figure 14 as a Monod relationship by means of the Michaelis-Menten equation:

$$v = V_{max} \frac{[S_0]}{Km + [S_0]}$$

where v is reaction velocity at a specific substrate concentration, V_{max} is the maximum velocity when the enzyme is substrate saturated and K_m is substrate concentration when enzyme velocity is ½ of the maximum (or half-saturation constant).

Over the whole range of O_2 concentrations tested from low to high $[O_2]$, the variation of (v) with respect to $[O_2]$ follows the empirical Monod equation (Michaelis-Menten kinetic used for microbial growth):

$$v = V_{max} \frac{[O_2]}{Km_0 + [O_2]}$$

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 O_2 reduction velocity is ½ of the maximum.

To use the Monod equation, it is necessary to determine the values for V_{max}, the maximal reduction rate and K_m. We used linear transformations of the Monod equation for KA3110A groundwater microbial reduction to determine V_{max} and K_m (Figure 15).

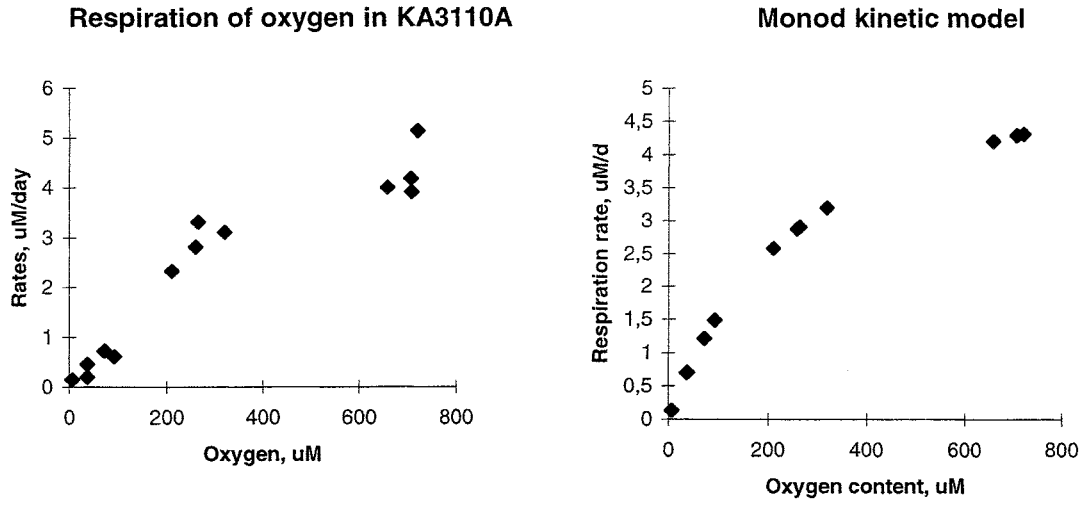


Figure 14 Microbial reduction of O_2 in KA3110A groundwater and Monod modelled reduction rate.

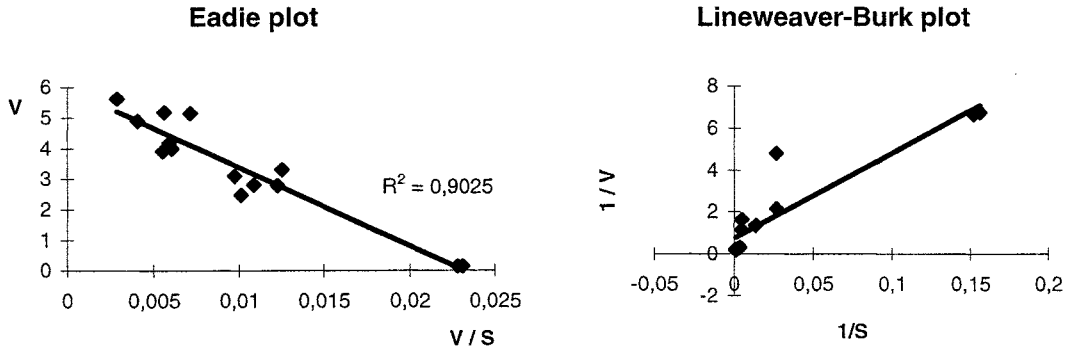


Figure 15 Linear transformations of the Monod equation for microbial O_2 respiration in KA3110A Äspö groundwater. Velocity v, is expressed as µM/day, O_2 concentration S is expressed as µM.

The constants became: V_{max} = 6 µM day^{-1}, K_m = 280 µM. These constants have subsequently been used for modelling of microbial O_2 reduction process in a groundwater (Figure 14). The correlation of the experimental data of O_2 reduction to the proposal model was $R^2 = 0.86$.

4.2.4 O_2 consuming rate and the electron donors

As observed in the Äspö subterranean environment, multiple substrate utilisation is the rule owing to the low nutrient levels found in the groundwater. Äspö groundwater is saturated with gases under 30-40 atm over pressure. The fact that we performed experiments with groundwater at atmospheric pressure suggests an underestimation of the O_2 consuming capacity since the amount of groundwater gases dissolved at atmospheric pressure is lower than what can be dissolved at 40 atm. To estimate the electron donor pool, which may be potentially used for O_2 reduction, we included the microbial substrate uptake kinetic into the model. Multisubstrate Monod kinetics can be used to describe the utilisation of many substrates. For two substrates, namely O_2 and an electron donor, this takes the double Monod form:

$$S_1 + O_2 \rightarrow X$$

$$v = V_{max} E \frac{[S_0]}{Km + [S_0]} \cdot \frac{[O_2]}{Km_0 + [O_2]}$$

where V_{max} is maximal uptake velocity of an electron donor (S) by a competent microorganism present in groundwater, E is the efficiency coefficient, K_m is half-saturation constant of the organisms for S, S_0 is initial electron donor concentration in the groundwater; O_2 is the initial O_2 concentration in the groundwater, K_{mo} is half-saturation constant of the organism of O_2. The kinetic constants K_m, K_{mo}, V_{max} and E reflect biochemical capacities of individual physiological species.

In this equation just one electron donor for microbial O_2 is considered, in addition it is assumed that the biomass within the system remains essentially constant and that the groundwater behaves as a well-mixed continuous flow. In reality, many microbial groups contribute to O_2 reduction (Figure 10, 11).

The sum of O_2 uptaking rates of different contributing non-competing microbial groups at expense of the electron donors available in the groundwater will result in the total respiration velocity. The O_2 reduction of separate physiological group is an additive in the summary respiration.

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

a, b, c, d, e, f – structural coefficients characterising representation of specific physiological metabolisms types in the whole microbial population.

Each additive represents individual and non-competing types of microbial metabolisms reducing O_2, for example, CH_4-oxidation, H_2-oxidation, acetate-oxidation or any other kind of O_2 reducing metabolism. Basic elements of the model include the initial electron donor concentration $[S_n]$, initial O_2 concentration $[O_2]$ and the constants characterising metabolic abilities of participating microorganisms: K_m, K_{mo}, E and V_{max}.

Thus, knowledge about microorganisms inhabiting the deep groundwater environment can provide information about rates of specific electron donor transformations. For example, methanotrophs have K_m values of 97 nM-15 µM for CH_4, and H_2 consuming bacteria have K_m values of 8 to 80 nM. The

V_{max} range remains to be determined for organisms isolated from the Äspö environment. Metabolic abilities of indigenous organisms like V_{max} and K_{mo} E at *in situ* groundwater O_2 concentrations and K_o at the *in situ* electron donor contents could provide the necessary data for testing of the model. CH_4-utilising bacteria were enriched and isolated in pure cultures.

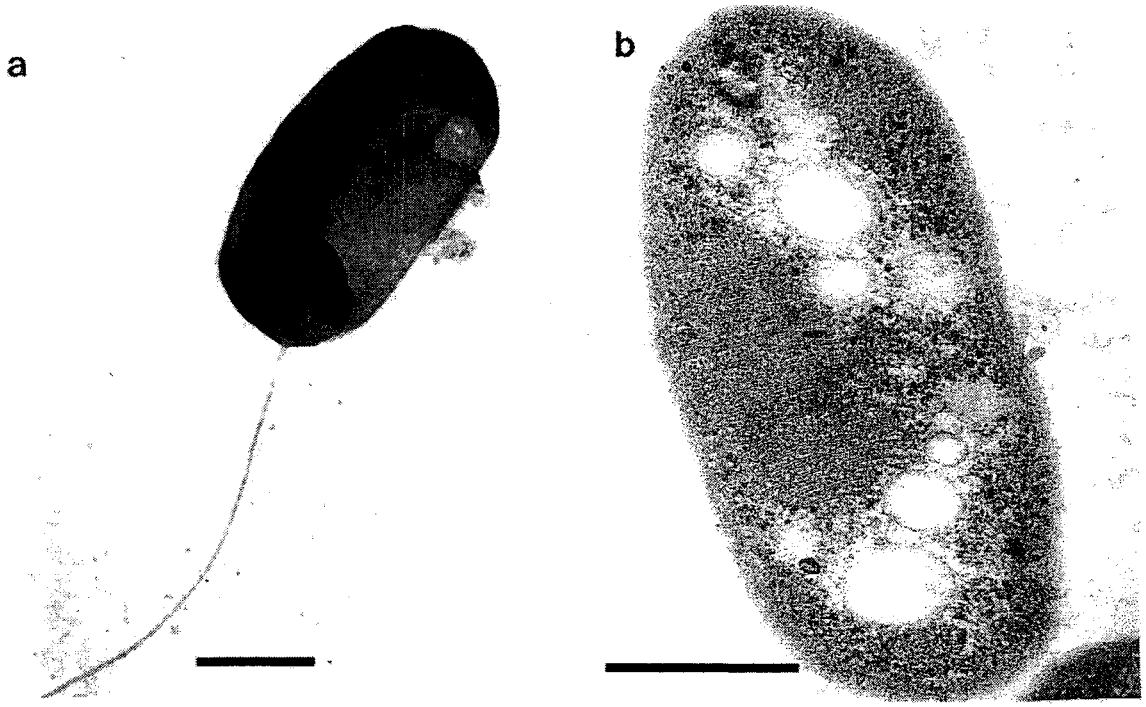


Figure 16 *Cell morphology and ultrastructure of Methylomonas scandinavica strain SR5. Negatively stained cell with single flagellum (A), thin section of the cell showing Type I intracytoplasmic membranes and electron dense inclusions (B). Bars 0.5 μm*

Physiology of the organisms isolated from the groundwater defined the eligibility of a certain organisms in the testing of model. Physiological characteristics of the CH_4-utilising bacteria isolated from Äspö environments (Figure 16) (Kalyuznaya et al., 1999; Kotelnikova et al., 1999) showed that these organisms are highly adapted to the groundwater conditions and may be regarded as typical representatives of CH_4-utilising population in the groundwater. Kinetic characteristics of the isolates have been studied. The coefficient K_m for CH_4 32.5 μM was determined for *Methylomonas scandinavica* strain SR5 isolated from the Äspö environment (Kalyuznaya et al., 1999). The coefficient K_{mo} for O_2 1.5 μM was determined for *Methylomonas scandinavica* strain 3500. The V_{max} of *Methylomonas scandinavica* strain 3500 ranged from 6.0 to 22 μM O_2 per litre per day (Kotelnikova et al., 1999). We used these kinetic features (V_{max} and K_m, K_{mo}) for CH_4 and O_2, (E) efficiency coefficient for CH_4-dependent O_2 reduction and CH_4 content in the ground water to test the proposed model. For example, the rate of microbial CH_4 oxidation at exponential growth follows the Monod kinetic:

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

where [CH_4] is CH_4 content in groundwater (Table 2), E is CH_4 oxidation efficiency (Table 11), and [O_2] is dissolved O_2 in the groundwater. The calculated estimates were close to the empirical values. For example, O_2 reduction rates observed experimentally ranged between 1.43 and 4.02 μM/day in KA3110A groundwater (Table 12, 15). O_2 reduction at the expense of CH_4 was calculated using the proposed model:

$$v = 6\mu M / day 0.46 \frac{[38\mu M]}{32.5 + [38\mu M]} \cdot \frac{[250\mu M]}{1.5 + [250\mu M]} = 1.51\mu M / day$$

We tested the model using chemical and microbial kinetic data for other groundwaters. The results are shown in Table 16. Similar calculations can be done for H_2 oxidation or for any other substrate found in a groundwater.

Table 16 *Application of the model for calculation of O_2 reduction at expense of CH_4-oxidation in Äspö groundwaters.*

Site	Content of CH_4 in groundwater	E CH_4	K_m [CH_4]	K_m [O_2]	V_{max}	Experimental rate of O_2-microbial reduction	Modelled rate of O_2-reduction at expence of CH_4-oxidation
	μM	%	μM	μM	μM/day	μM/day	μM/day
KR0013B	80	*0.54*	32.5	1.5	6	n.d.	*2.29*
KR0015B	116	*0.8*	32.5	1.5	6	n.d.	*3.72*
KA2862A	0.92	0.8	32.5	1.5	6	1.62	0.13
KA3005A	75	0.46	32.5	1.5	6	2.02	1.91
KA3010A	2.25	0.54	32.5	1.5	6	4.46	0.21
KA3110A	38	0.47	32.5	1.5	6	2.07	1.51
ZEDEX	31	*0.5*	32.5	1.5	6	n.d.	*1.45*
KR0012B	42	*0.5*	32.5	1.5	6	n.d.	*1.68*
3190	n.d.	0.32	32.5	1.5	6	4.81	
KA2861A	1.13	0.8	32.5	1.5	6	0.31	*0.32*
KA3105A	43	*0.5*	32.5	1.5	6	0.55	*1.70*
KA3067A	28	*0.5*	32.5	1.5	6	1.6	*1.38*

n.d. = no data

We studied just one part/step in the microbial successive reduction, namely, CH_4 oxidation. The parameters of a CH_4-utilising pure cultures were used in the application of the model. We were able to calculate the O_2 reduction rate for CH_4 oxidation. The rest of microbial groups participating O_2 reduction in the groundwater remain to be studied to apply the proposed model.

4.2.5 O_2 reduction capacity mediated by microorganisms

Because we observed active consumption of several substrates and the presence of different physiological groups of microorganisms in the groundwater tested, the apparent kinetics of this process can be approximated by similar equation for each substrate used for O_2 reduction. Specific O_2 reduction capacity of aerobic cells in the groundwater is included as a variable in the model. In fact, total O_2 reduction capacity is the sum of particular O_2 reduction capacities:

$$C = Y_1 a C_1 + Y_2 b C_2 + Y_3 c C_3 + Y_n z C_n$$

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 expense of specific electron donor at 100% substrate consuming efficiency (Table 6, 8).

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.

$$C_n = E[S_n]$$

where E is a O_2 reduction efficiency, and $[S_n]$ is concentration of an electron donor in a groundwater.

The chemical content of groundwater and gases predict dominating microbial groups in a given period of time. Metabolic potential is determined by physiological features of the organisms inhabiting the rock environments. The physiological structure of a mixed microbial population provides weight coefficients for each participating group. Diversity of population is expressed by number of additives. The real diversity is enormous. The model simplifies the situation by accounting only physiological groups dominating in the population at certain circumstances. The weight coefficient of non-active microbial group is low. As soon as the weight coefficient diminishes the additive approaches zero.

As example, the O_2 reducing capacity mediated by microorganisms may be calculated for KA2862A groundwater. We observed that culturable aerobic and microaerophilic heterotrophic organisms constituted around 20% of whole population (a=0.2), H_2-oxidising bacteria constituted 12% (b=0.12), and CH_4-utilising - 14.3% (c=0.143) (Figure 5). Stoichiometric coefficients for dissolved organic carbon, H_2 and CH_4 may found in Table 8 (DOC, 6.0; H_2, 0.5 and CH_4, 2.0). Concentrations of the energy sources [S] may be found in Table 1 and 2 (DOC - 67 μM, H_2 - 0.98 μM, CH_4 - 0.92 μM). Efficiency coefficients were not determined, but they may be approximated to 1.0 since we use a structural parameter based on culturable cell counts. Thus, the O_2 reduction capacity mediated by known microorganisms in KA2862A groundwater may be calculated as:

$$C = 6x0.2x67 + 0.5x0.12x0.98 + 2x0.143x0.92 = 80.72\mu M$$

4.2.6 Effect of temperature on microbial O_2 reduction

The effect of temperature on respiration rates was studied during Microbial REX (Kotelnikova and Pedersen, 1998a). The observed rates did not differ significantly at different temperatures. The microbial population in the groundwater adapted to higher temperatures after 14 days. This period of time is far too short in comparison with repository time to be considered as an important factor. Thus, the temperature factor between 16 and 60°C may be neglected as a model term. Temperature seems to be a more important physical factor affecting the solubility of O_2, and consequently its aquatic concentrations and availability for microbes. The solubility of O_2 decreases with increasing temperature. Thus, the temperature has to be considered in the dissolved O_2 concentration calculations.

5 PERFORMANCE ASSESSMENT RECOMMENDATIONS

The facts experimental results obtained indicate that microbial activity will greatly contribute to O_2 reduction in groundwater in a HLW repository. Such microbial activity will have a positive influence on the performance of a HWL repository and reduce the risk for corrosion of the canisters

O_2 reduction is affected by different microbial groups depending on available electron donors and viable competent organisms. Substrates such as CH_4, H_2, acetate and formate will be actively oxidised in the presence O_2. Gases dissolved in a groundwater (CH_4 and H_2) will add to the reducing capacity. This process will be mediated by microorganisms. Methane oxidation will be one of the dominant processes for microbial O_2 reduction.

The biochemical O_2 reduction depends on the microbial diversity in the groundwater. The structure of mixed population depends on the electron donors available. Therefore, the ***qualitative chemical analysis of groundwater and gases*** may predict dominating microbial groups, which will be activated by O_2. While ***quantitative chemical analysis of groundwater and gases*** may predict the extend of these microbial processes. ***Specific kinetic parameters of microorganisms*** (K_m, V_{max}, K_{mo2}, V_{maxO2}, E) quantify the ability of microbiota inhabiting the groundwater to use electron donors and reduce O_2. The model proposed in this report is based on the above listed characteristics. The model may be used to predict the O_2 reduction capacities and rates of microorganisms in the groundwater.

6 DESCRIPTION OF THE EXPERIMENTAL PROCEDURES

6.1 ANALYSIS OF GAS EXTRACTED FROM THE GROUNDWATER AND IN HEADSPACE OF THE SERUM BOTTLES

6.1.1 Injection and subsampling technique

Gas was extracted from groundwater using special equipment described in details in Pedersen 1997, Appendix 1. Subsampling 50 ml-syringes were used to transfer extracted gas from the extraction system to the gas chromatograph. High precision Hamilton syringes of small volume (10-50 µL) were used for sampling of gas from the headspace of the serum bottles and for in-column injections. The injection volumes varied between 2 and 20 µL. Gas chromatographic measurements of O_2 reduction, CH_4 oxidation and CO_2 production were made on the headspace of serum bottles.

6.1.2 Gas chromatography conditions

Methane, ethane and propane were determined with a Varian GC-3700 chromatograph equipped with a 2 m 1/8 inch steel column packed with Porapak Q and a flame ionisation detector (FID) with N_2 as a carrier gas. The injector, column and detector temperatures were isothermal 100, 100 and 200°C. Methods of gas chromatography are summarised in Table 17.

O_2 and nitrogen were analysed with the column packed with Molsieve 5A mesh 60/80, 1/8 inch, length 5 m, thermal conductivity detector. Filament, detector and column temperature were isothermal, 300, 120 and 60°C, respectively. As the carrier gas for the O_2 analysis helium was used. The minimal concentrations of CH_4 and O_2 which could be determined were 4.5 nM and 45 nM, respectively.

Table 17 Methods of the gas analyses.

Gas	Designationof column	Length of column	Column T °C	Injector T °C	Detector T °C	Carrier gas flow rate (ml/min)	Retention time (apx. min)	Sensitivity (nm)	Instrument
Methane	Porapack Q80/1002 x 1/8	2 m	100	100	FID B 200 +	N_2, 30	0.6	4.5	GC3700
Hydrogen	Mol Sieve 5A	6 m	35	100	TCD120fil 300 -	Nitrogen30	1.52	45	GC3700
Helium	Mol Sieve 5A	6 m	35	100	TCD120fil 300 -	Nitrogen30	1.23	45	GC3700
Oxygen	Mol Sieve 5A	6 m	60	100	TCD120fil 300 +	Helium30	2.85	0.4	GC3700
Carbon monoxide	Hayesep Q 80/1002 m x 1/8	2 m	40	100	FID A200 C	Nitrogen30	0.64	5.0	GC3400
Carbon dioxide	Hayesep Q 80/1002 m x 1/8	2 m	40	100	FID A200 C	Nitrogen30	1.56	2.0	GC3400
Methane	Hayesep Q 80/1002 m x 1/8	2 m	40	100	FID A200 C	Nitrogen30	0.81	5.0	GC3400
Nitrogen	Mol Sieve 5A	6 m	60	100	TCD120fil 300 +	Helium30	5.94	90	GC3700
Ethane	Porapack Q80/1002 x 1/8	2 m	100	100	FID B 200 +	N_2, 30	1.5	4.5	GC3700
Propane	Porapack Q80/1002 x 1/8	2 m	100	100	FID B 200 +	N_2, 30	2.3	4.5	GC3700

H_2 and helium were determined with Molsieve 5A column, mesh 60/80, 1/8 inch, length 5 m, nitrogen as carrier gas (30 ml/min). TCD filament, body and column temperature was isothermal, 300, 120 and 33 °C, respectively.

Carbon dioxide was analysed with methanising catalytic system on a gas chromatograph STAR 3400CX equipped with Hayesep packed 2 m length 1/8 steel column, FID and with nitrogen as carrier gas (30 ml/min flow).

6.1.3 Calibration and integration of peaks

Calibration, integration of peaks and calculations of the gas concentration was done with Star Chromatography Workstation, Varian, version 4.01 Software, daily. In Table 17, detailed information on methods used for analysis of gases is described. Calibration for each gas was done with two different standard mixtures and pure gas. The results were consistent. Gas mixture of a precise composition (AGA) was used as standard. Headspace measurements were corrected for gases remaining in water using Henry's law. Gas consumption and production data are reported as micromoles per litre per day of incubation (µM/day) or nanomoles per litre per day (nM/day). The CO_2 concentrations were not corrected for bicarbonate and pH.

6.1.4 Error of O_2 analyses with the gas chromatography

To be able to create the planned concentrations of O_2 in the experimental bottles the following experiment was installed. Ten serum bottles were filled with 15 ml of boiled distilled water, flushed with O_2-free nitrogen, and closed with butyl rubber stoppers. Different volumes of the air were injected into the bottles and O_2 concentrations were measured using the gas chromatography. In the case of volumes exceeding 0.1 ml, the removed volume of gas was replaced with the injected volume. The sensitivity level for O_2 with the gas chromatography was 30-40 nM. Relative standard deviation (RSD) of measurements was 14-44%, and it was higher when the O_2 concentration was below 1%. The standard deviation at calibration step varied between 0.12 and 1.35% of O_2 and did not depend on the concentration. The response of the TCD detector to O_2 was linear. Sampling contamination with O_2 was not significant during repetitive injections. The concentration of O_2 was reproducible (RSD 0.69-0.70%) after 10 times of sampling from the same bottle. Thus, gas chromatography could be successfully used for O_2 measurements in headspace of bottles containing groundwater.

The experimental concentration of O_2 in closed bottles filled with gas mixture ranged between 17 and 22 % (230-265 µM dissolved at 1 atm and 16°C). Solubility of O_2 at 25°C and 1 atm in water saturated with air is 258.3 µM. As soon as the O_2 was consumed, microaerophilic conditions were created.

6.2 DETERMINATION OF TOTAL MICROBIAL O_2 REDUCTION

6.2.1 Sampling and experiment procedure

The groundwater from each borehole was placed in 6 of 25 ml-serum bottles in 5 ml portions. Killed controls were added with 2,5% formaldehyde, 1% sodium aside, 1 mg/l of mercury chloride, 400 mg/l of streptomycin and 0.58 mg/l of bacitracin. Sample set did not contain any additions. All bottles were closed with butyl rubber stoppers and aluminium rings and contained air in the headspace. The sample bottles were cultivated laying horizontally, in the dark, without shaking. The incubation temperature was 16°C. Rock samples were placed into groundwater and incubated in the same way as the groundwater. The experiment was proceeded for 90 days. The gases (CO_2, O_2, CH_4, H_2) in headspace of the experimental vessels were analysed with a GC system every 10-th day. The GC tests were followed by pH measurements. The initial concentration of O_2 in closed bottles filled with air was 20 %. Solubility of O_2 at 16°C and 1 atm in water saturated with air was 310 μM. In the experiments estimating the O_2 reduction rates and activities we used O_2 concentrations calculated for atmospheric pressure as start concentrations.

6.2.2 Calculations

To calculate O_2 reduction capacities the final O_2 concentrations were subtracted from the initial ones. The O_2 reduction rates were calculated as

$$V = \ln 2 \frac{[O_2]}{dt}$$

The O_2 reduction rates in killed controls were subtracted from non-inhibited samples to give the biogenic reduction. The efficiency coefficient was calculated as the percent of O_2 consumed of the initial O_2 contamination. The rates of CH_4 oxidation were calculated as the difference between the CH_4 concentrations in the different analysing times and divided by time. At a 17% CH_4 concentration and at atmospheric pressure the maximum possible rates (or first-order kinetic) of CH_4 oxidation is expected. All points were calculated as a mean of four independent replicates. The efficiency coefficient was calculated as the percent of CH_4 consumed of the initial CH_4 concentration. To calculate the percent of O_2 reduction at the expense of CH_4 oxidation the molar ratios of gases consumed (CH_4, O_2) and produced (CO_2) were estimated considering that one mole of consumed CH_4 demands 2 moles of O_2.

6.3 ANALYSIS OF O_2 WITH THE WINKLER METHOD

Each sampled groundwater (100 ml) was placed in 16 125-ml Winkler bottles (in four replicates for each O_2 determination) for incubation and subsequent measurement of microbial O_2 reduction. As a control for abiotic reduction formaldehyde (2%) inhibited samples treated in the same way were used. O_2 was analysed by a standard titration protocol (Paerl, 1997). Briefly, 1 ml of a KI solution (KI 600 g/l + NaOH 320 g/l) and 1 ml of $MnSO_4$ x H_2O (500 g/l) were added simultaneously with syringes + needles into each bottle. The sample bottles were incubated under water at 16°C. At the end of incubation, 1 ml 9 M H_2SO_4 was added to each bottle and titration was performed with 0.016 M $Na_2S_2O_3$, standardised against 0.01 M KIO_3. 0.5 ml of thyodene indicator was used to detect the end titration point. The Winkler method had the detection limit 4.5 µM in solution with 45% RSD for replicates.

6.3.1 Calculations

The reduction rates were calculated by fitting a logarithmic function for O_2 content for all sampling time points:

$$V = \ln 2 \frac{[O_2]}{dt}$$

where $d(O_2)$ is difference of O_2 concentrations during time period dt. The result was a mean of two measurements for each of 4 replicates for each groundwater type. The rates of O_2 reduction in killed controls were subtracted from the samples. Total O_2 capacities were calculated as O_2 consumed during the tested time interval.

6.4 INHIBITION OF MICROBIAL O_2 REDUCTION, CH_4 OXIDATION, AND METHANOGENESIS IN GROUNDWATER

Serum bottles (115 ml) were filled with 50 ml of groundwater. The sampling sites are specified in the corresponding result sections. Four different variants were proceeded: The first set contained no additions. Another set was treated as the first one, but added with 5% of acetylene. In this variant CH_4 oxidation supposed to be inhibited. The inhibited controls here were subtracted from total O_2 reduction rates to give non-CH_4 consuming O_2 reduction. The third set was added with BESA to inhibit carbon dioxide reduction by methanogens. Formaldehyde inhibited (0.25%-2%) samples were supposed to be used as controls for abiogenic reduction.

Killed controls in the respiration experiments with KA2826A and KA2861A groundwater (were added with formaldehyde (2% final concentration), sodium aside (1%), streptomycin (400 mg/l) and bacitracin (0.58 mg/l). Sample set contained no additions.

6.4.1 Incubation

The sample bottles were cultivated laying horizontally, in the dark, without shaking. The incubation temperatures and times are specified in the corresponding result sections. Rock samples were placed into groundwater, closed with butyl rubber stoppers and aluminium rings and incubated in oxidised groundwater.

6.5 METHANE IN *IN VITRO* EXPERIMENTS

6.5.1 Methane concentrations

The concentrations of dissolved gases under experimental conditions should be as close as possible to their ambient concentrations under repository conditions. The actual concentrations of CH_4 dissolved in Äspö groundwater collected from the depths 420-440 m are 0.95-980 µM (Figure 1A). *Under the repository conditions* after closure, the gases will be dissolved under 50 atm pressure and temperatures of 15 - 70°C. To create similar concentrations of dissolved CH_4 *in vitro*, the sample bottles must be filled with a gas mixture containing CH_4 in the gas phase at 1 atm pressure and 20°C (293°K). Methane is chemically inert, thus the Henry Law can be applied for the gas. Solubility of CH_4 in distilled water can be calculated from the following equation:

$$S = P_{CH4}\frac{[H_2O]}{H_{CH4}}$$

where S = solubility (µM), P_{CH4} = partial pressure of CH_4, H_{CH4} = Henry constant, for CH_4 it is 618.4 mol^{-1} atm litre of water/mole at 25°C at 1 atm.

$$P_{CH4} = C\frac{RT}{V}$$

where R = 8.2 x 10^{-2} litre atm K^{-1} mol^{-1}, T = temperature in Kelvins, V = volume of gas phase in litres, C = molar concentration of CH_4, RT/V = 2.45 atm /K mole (H_2O), mole (H_2O) = molar concentration of water in the analysed volume (for example, in 1 litre that is equal to 1000/18.02 = 55.49 moles)

Solubility of CH_4 is 1.34-1.68 mM at *in situ* salinity in Äspö groundwater. Thus, pressurised groundwater is not saturated with CH_4. To get the concentration of CH_4 in the groundwater close to the ambient at normal pressure it is enough to have 17-18% of CH_4 in the gas phase of cultivation bottles.

6.6 DETERMINATION OF THE TOTAL NUMBERS OF MICROORGANISMS

Water samples of 40 ml were collected from each section of the borehole, preserved with (0.2 ml) formaldehyde (2% final concentration), (0.2 ml) sodium aside (1%) and transported to the laboratory in Göteborg for counting. The total number of bacteria in the groundwater was determined by the acridine orange direct count (AODC) method. Each water was filtered in 4 repetitions at different volumes onto a Sudan-black stained Nuclepore filter of 0.22 µm pore size and 13 mm in diameter at-20 kPa and stained for 10 min with acridine orange. All solutions were filter sterilised (0.22 µm). Four filters were counted for each water sample. The number of bacteria was counted using blue light (390-490 nm) under an epifluorescence microscope (Olympus BH-2).

6.7 DETERMINATION OF MOST PROBABLE VIABLE MICROBIAL NUMBERS

6.7.1 Heterotrophic microorganisms

The viable count of heterotrophic aerobic and microaerophilic bacteria was analysed in liquid R2A medium and as plate counts on a nutrient R2A agar.

R2A modified medium contained (g per litre): NaCl 5.0, peptone 0.25,Casamino acids 0.25, Yeast Extract 0.25, Glucose 0.25, Soluble Starch 0.25, K_2HPO_4 0.15, $MgSO_4x7H_2O$ 0.3, Sodium Pyruvate 0.3. The ingredients were mixed in double distilled water. pH was adjusted to 7.35. The R2A medium was dispensed in 4.5 aliquots, closed with rubber stoppers and plastic screw caps and autoclaved. The first dilution was inoculated aerobically with 0.5 ml of the non-diluted groundwater in the first dilution, diluted in the same rate with syringes. Most probable numbering of viable heterotrophic aerobic and microaerophilic bacteria was done after 14 days incubation of the serial dilution (5-7 dilution, in triplicate) with optical density and CO_2 production control in the gas phase with the GC method. Controls were added with inhibitors (as described in section 6.2.1) and processed in the same way as the samples.

The medium added with 2.0 % agar was dispensed in Petri plates. All dilutions of one dilution seri in the liquid medium (0.1 ml) were spread in triplicate on the agar plates.

CFU were counted after 3 and 14 days of incubation at 16°C in the dark.

6.7.2 Methanotrophic bacteria

The technique for cultivation of microaerophilic microorganisms was followed. The Nitrate Mineral Salts medium (NMS) with modifications was used to cultivate CH_4-utilising bacteria (per litre):

NaCl-5.0 g, KNO_3-1.0, $MgSO_4x7H_2O$-1.0 g, $CaCl_2x2H_2O$-0.2 g, traces element solution-0.5 ml, Na_2HPO_4-2.0 ml, and NaH2PO4-2.00 ml. The buffer contained a mixture of 3.6 g of Na_2HPO_4, and 1.4 g of NaH_2PO_4 in 100 ml of distilled water, pH 6.8-7.0. First trace element solution contained, per 1 litre: $ZnCl_2$-10 mg, $MnCl_2x4H_2O$-3 mg, H_3BO_4, 4 mg, $CaCl_2x2H_2O$-20 mg, $CuCl_2x2H_2O$-1 mg, $NiCl_2x6H_2O$-2 mg, $Na_2MoO_4x2H_2O$-3 mg, pH 4.0. Second trace element solution contained the same components except $CuCl_2x2H_2O$. The sterile medium was mixed with sterile phosphates (Na_2HPO_4-0.23 g/l or 2 ml/l, NaH_2PO_4-0.07g/l or 2 ml/l) in a 1 litre volume, cooled to 60°C, phosphate buffer was added, pH was adjusted to 7.2-7.4 and the medium was distributed in sterile tubes (3 ml in 17 ml tubes). Two different variants of NMS were added with microelement solution (0.5 ml/l): with (for methanotrophs of group I) and without copper (for methanotrophs of group II and X).

Most probable numbering of viable CH_4-utilising bacteria was done by serial dilution of water in the liquid medium by 10x serial dilution. (5-7 dilution, in triplicate) with CH_4 reduction control in the gas phase. The sterile medium was dispensed into the 28 ml tubes (3 ml each), inoculated aerobically with 0.3 ml of the non-diluted groundwater in the first dilution, diluted in the same rate and closed with sterile rubber stoppers and plastic screw caps. Then 12 ml of air was drawn out with a 20 ml syringe and 12 ml of pure filter sterilised (Dyna Guard filter, 0.2 μm pore diameter) CH_4 were injected into each tube. The start points of the CH_4 and CO_2 concentration were analysed on the inoculation day by drawing 10 μL of gas from the gas phase, subsequently analysed by GC. Then the tubes were incubated horizontally to improve gas-exchange, without shaking, in the dark, at room temperatures. Each tube was checked by the GC after 10 and 75 days of incubation. Controls were added with inhibitors (as described in section 6.2.1) and processed in the same way as the samples. Tubes with both CH_4 oxidation and CO_2 production were counted as positive.

6.7.3 H_2-oxidising bacteria

The medium selective for H_2-oxidising bacteria from KA2861A was used (g/l): NaCl-3.28, NH_3Cl-1.0, $MgSO_4x7H_2O$-0.038, $CaCl_2x2H_2O$-0.01, trace element solution-1.0 ml, K_2HPO_4-0.03. $CaCl_2x2H_2O$ solution as 0.4% stock solution was autoclaved separately. The bicarbonate buffer was sterilised separately as stock solution (1.19M) 10g in 100 ml of distilled water. The trace element solution contained, per 1 litre: HCl (25%-7.7 M) 12.5 ml, $ZnSO_4$-144 mg, $MnCl_2x4H_2O$-100 mg, H_3BO_4, 30 mg, $CuCl_2x2H_2O$-2.0 mg, $CoCl_2x6H_2O$-190 mg, $NiCl_2x6H_2O$-24 mg, $Na_2MoO_4x2H_2O$-36 mg,

$FeSO_4x7H_2O$ 2100 mg, pH 4.0. The sterile medium was cooled to 50°C and mixed with 5.0 ml of sterile bicarbonate buffer, 2.5 ml of calcium solution in a 1 litre volume, pH was adjusted to 7.2-7.4. The medium was distributed in sterile tubes (4.5 ml in 13.5 ml tubes) and closed with butyl rubber stops and plastic screw caps in anaerobic box (10%CO_2, 5% H_2, 85% N_2). Controls were added with inhibitors (as described in section 6.2.1) and processed in the same way as the samples.

The tubes were inoculated with 0.5 ml of groundwater at rate 10x in the anaerobic box or with syringes. After inoculation provide H_2:CO_2 mixture (15:2) by flushing with gas containing H_2 at pressure 1.5 bar (2 bar at manometer) and 0.2 bar CO_2 (4.5 bar at manometer). 1 ml of pure filter sterilised (Dyna Guard filter, 0.2 µm pore diameter) air was injected into each tube (final O_2 content 2.5%).

The start point of the H_2 and CO_2 concentration was analysed on the inoculation day by drawing 10 µL of gas from the gas phase, subsequently analysed by GC. Then the tubes were incubated horizontally to improve gas-exchange, without shaking, in the dark, at 16°C. Each tube were checked by the GC after 10 and 75 days of incubation. Tubes with both H_2-oxidation and biomass production were counted 0x to 5x-10x with MPN to calculate cultivable cells as described before.

6.7.4 Methanogens and sulphate-reducing bacteria

The anaerobic technique described by Hungate, 1969 was practised. The ASPM medium Kotelnikova et al., 1997 (per litre): 0.4 g NH_4Cl, 0.03 g $MgCl_2$, 0.45 g NaCl, 0.5 g K_2HPO_4, 0.003 g $FeCl_3 \cdot 7H_2O$, 10 ml trace element solution Wolin et al., 1963 and 1 mg resazurine. The following carbon and energy sources were used (per litre): 2.0 g formate, H_2 + CO_2 (80:20%, 1,5 atm.), 2.0 g methanol. The pH was adjusted with 0.1 M NaOH or 0.1 M HCl to pH values corresponding to those of the groundwater used for inoculation (7.25-7.5). 2.5 ml portions of this medium were distributed into gas-tight, anaerobic culture tubes (Bellco glass, Inc, type no. 2047, 17 ml) under stream of O_2-free nitrogen. Then, the following reagents (per litre): 5 ml vitamin solutions Wolin et al., 1963, 1 mg coenzyme M, 2.0 g $NaHCO_3$ 0.25 g cysteine-HCl and 0.25g Na_2S x $9H_2O$ as sterile, anoxic solutions were added to each tube. The tubes were added with 2.5 ml of filter-sterilised groundwater and inoculated with 0.5 ml of non-sterile groundwater within 2 h from sampling. They were incubated at room temperature for up to 1 months. Control variants contained the same components and be added with bromethansulfonic acid (BESA), 20 mM, to inhibit methanogenesis. Increase of CH_4 in head space of the tubes was followed with gas chromatographic technique. The number of viable methanogens was estimated by an MPN method.

To count sulphate-reducing bacteria principally the same procedure for the medium preparation was applied, but the ASPM was added with sodium sulphate (2 g/l) and methanol was replaced with sodium lactate (5 g/l). Two weeks after inoculation the tubes were checked for sulphide production by modified Kline´s method, described below. Killed controls contained the same components and added with inhibitors (as described in section 6.2.1).

6.7.4.4 Analysis of H_2S, S^{2-}, HS^-

Reagents: (1) $Zn(CH_3COO)x2H_2O$, 24 g/l; Acetic acid, 1 ml/l. First, acetic acid was added into the water. The solution was kept in close bottle at room temperature. (2) N,N-dimethyl-p-phenilendiamine oxalate, 2.0 g and $FeCl_3$, 3.0 g were dissolved in 500 ml of 37% HCl. The solution was kept in dark at 5°C.

First, 5 ml of the acetate Zn reagent (1) was placed in the 50 ml tubes, added with 0.1-1.0 ml of sample (0-2.5 mM sulphide concentration) and mixed gently. Then, 20 ml of distillate water was added and mixed. Next, 2.5 ml of the diamine reagent (2) was mixed in and holds for 20 min. Finally, the volume was adjusted to 50 ml with the water and mix properly.

Absorption of the mixture was measured at 670 nm in 1 cm cuvett. The spectrophotometer was calibrated with the mixture added with inhibited control. Concentration of sulphide was calculated as: C=1.68AxD, where C-concentration of sulphide, A-absorption of the sample at 670 nm, D-number of dilution of the sample (calibration curve was received with 1 ml of standard solutions).

6.7.5 Iron-reducing bacteria

The ASPM medium was used for cultivation of Fe-reducing bacteria. After autoclaving, sterile $Fe(OH)_3$-amorphous, $NaHCO_3$ and L-Cystein (as reducer) were added to final concentrations of 5.0, 2.5 and 0.04 g/l respectively with sterile syringes. The pH of the autoclaved medium was 7.35. The number of viable iron-reducing organisms was estimated by an MPN method. Ferrozin method for determination of the dissolved ferric iron was used as positive growth indicator Lovely et al., 1986, 1988.

6.7.5.5 Preparation of Fe(III)

Amorphous Fe (III) oxyhydroxide was made by neutralising 1 M solution of $FeCl_3$ to pH of 7 with NaOH. The Fe(III) precipitate was washed by deionised, sterile water three times. This preparation of amorphous Fe(III) oxyhydroxide was done by using sterile techniques. The sterile stock solution was transferred into 100 ml serum bottles and bubbled with N_2 gas for 10 minutes. Each bottle was sealed with a butyl rubber stopper and an aluminium crimp.

6.8 AEROBIC MICROBIAL PROCESSES IN GROUNDWATER EVALUATED WITH A RADIOTRACER METHOD

6.8.1 *In vitro* CH_4 and H_2 oxidation

Groundwater was placed in 5 ml portions into 10 sterile 25 ml-serum bottles and flushed with sterile air to flush out non-labelled CH4 and closed with butyl rubber stoppers and aluminium crimp seal rings.

Four killed controls were added with inhibitors (as described in section 6.2.1) and radioactive (^{14}C)CH_4 (2) or (^{14}C)bicarbonate plus H_2 (2).

The second triplicate contained radioactive (^{14}C)CH_4 and serve for determination of the CH_4 oxidation rate. (0.1 ml of radiolabelled $^{14}CH_4$ stored over a saturated solution of NaCl containing 0.5 M NaOH, was added to give an activity of 0.25 µCi/ml (0.2 ml of 127.63 µM stock CH_4 gas CFQ 8591, Amersham, Sweden). Radioactive CH_4 was added to the gas phase at low concentration, which did not increase the *in situ* pool. Non-radioactive CH_4 was not provided. The third triplicate was added with (^{14}C)bicarbonate plus H_2.

The bottles were incubated for 14 days at 16°C in the dark. Then 0.5 ml of 1M NaOH was added with syringes to each repetition to stop all metabolic processes and to absorb the $^{14}CO_2$ evolved. After an additional incubation of 12 h, 6 portions of 0.5 ml were taken from each bottle.

One duplicate was counted in 10 ml of Ready Safe scintillation cocktail with a Beckman Scintillation Counter in automatic mode, average count time 5 min and automatic subtraction of blank counts.

The second 2 duplicates were placed into eppendorf tubes, with 0.05 ml of 5 M HCl, incubated overnight and then counted.

1 ml of the acidified overnight cell suspension was filtered through Nucleapore filters 25 mm diameter and 0.22 µm pore size, washed with sterile buffer twice and counted in 10 ml of Ready Safe scintillation cocktail.

6.8.2 Heterotrophic O_2 respiration

The groundwater was placed in 16 sterile 25 ml-serum bottles in 5 ml portions. The first duplicates were added with (mg/l): 0.25 of radiolabelled (^{14}C)-acetate, the second-with 0.1 of (^{14}C)-formate and the third-0.1 of (^{14}C)-glucose (Amersham, Sweden). Killed controls contained the same components and added with inhibitors (as described in section 6.2.1). The bottles were incubated for 24 hours at 16°C in the dark. Then 0.1 ml of 1M NaOH was added to each repetition with syringe to stop all metabolic processes and to absorb the $^{14}CO_2$ evolved. After an additional incubation of 12 h, 6 portions of 0.5 ml were taken from each bottle. Then the samples were treated as described in previous section.

6.9 ANAEROBIC MICROBIAL PROCESSES IN GROUNDWATER EVALUATED WITH A RADIOTRACER METHOD

6.9.1 *In vitro* autotrophic CH_4 production

The formation of $^{14}CH_4$ from $NaH^{14}CO_3$ was followed in 5 ml of groundwater placed in a separate set of sterile and nitrogen flushed 55 ml serum bottles equipped with new aluminium crimp sealed butyl rubber stoppers. Four bottles per borehole were injected with 18 ml groundwater collected from 15 boreholes at July, 7, 1998. Two of them had inhibitors (as described in section 6.2.1) Finally, $NaH^{14}CO_3$ was added in to final activities of 1.48 μCi ml^{-1}, corresponding to 164 μM (Sodium bicarbonate, CFA3, Amersham, Sweden). The bottles were subsequently supplied with 0.1 bar H_2 and incubated for 17 days at 16°C. The corresponding concentration of the dissolved H_2 in the medium became approximately 10 μM. The formation of $^{14}CH_4$ in the gas phase from $NaH^{14}CO_3$ was repeatedly measured after periods of 3-5 days by transferring 1 ml of gas from the bottles to a toluene-based scintillation cocktail trap (Quickscint 501, Zinsser Analytic, Frankfurt, Germany, Scint Varuhuset AB) with 1M NaOH for the trapping of CH_4 as described by Zehnder et al., 1979. A Beckman Scintillation Counter was used for the measurement of radioactivity with an average count time of 5 min and automatic subtraction of blank count.

6.9.2 *In vitro* autotrophic acetate production

Autotrophic acetate formation was studied in groundwater using an liquid scintillation technique and the procedures as describe above for autotrophic CH_4 formation ($NaH^{14}CO_3$, 1.48 μCi ml^{-1}) with the following modifications. Groundwater was added with BESA (50 mM) in two replicates with two killed controls and incubated for 17 days at 16 °C. The samples were subsequently acidified over night and filtered (0.2 μm). The amount of radioactive acetate, produced from $NaH^{14}CO_3$ in groundwater, was determined in the filtrate as described elsewhere Scnurer et al., 1994. Liquid scintillation was performed on 100 μl of filtered groundwater in 10 ml of Ready Safe scintillation cocktail (Beckman) using a Beckman Scintillation Counter as described above.

6.10 RATE CALCULATIONS

Methane, carbon dioxide and acetate formation rates were calculated from the observed increase in the concentration of respective labelled product over time. The amount (nM) of respective ^{14}C-labelled product formed were calculated as:

Concentration = disintegrations min^{-1} in sample-disintegrations min^{-1} in control/ specific activity of the C^{14} substrate

The total amount of a product in the radiotracer experiments was calculated as follows. The substrate concentrations, which equalled the sum of the *in situ* pool of substrate and the amount of non-labelled substrate and ^{14}C-labelled substrate added, was divided by the ^{14}C-labelled substrate concentration, giving a coefficient denoted K. This coefficient shows the ratio of unlabelled to labelled substrate. The incorporation rates (μM product h^{-1}) of labelled carbon in CH_4 from [1, $2^{-14}C$]-acetate or from $NaH^{14}CO_3$ or in acetate from $NaH^{14}CO_3$ were calculated as:

$$V = K\frac{(C_2 - C_1)}{t_2 - t_1}$$

where C_1, C_2 is the concentration of radio labelled product after time 1 and time 2. Standard deviations calculated for repetitions and results from different sampling dates did not exceed 30 %.

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