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Äspö Hard Rock Laboratory

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Microorganisms in buffer from the Canister Retrieval Test – numbers and metabolic diversity

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Abstract

”Canister Retriveal Test” (CRT) is an experiment that started at Äspö Hard Rock Laboratory (HRL) 2000. CRT is a part of the investigations which evaluate a possible KBS-3 storage of nuclear waste. The primary aim was to see whether it is possible or not to retrieve a copper canister after storage under authentic KBS-3 conditions. However, CRT also provided a unique opportunity to investigate if bacteria survived in the bentonite buffer during storage. Therefore, in connection to the retrieval of the canister microbiological samples were extracted from the bentonite buffer and the bacterial composition was studied.

In this report, microbiological analyses of a total of 66 samples at the C2, R10, R9 and R6 levels in the bentonite from CRT are presented and discussed. By culturing bacteria from the bentonite in specific media the following bacterial parameters were investigated: The total amount of culturable heterotrophic aerobic bacteria, sulphate-reducing bacteria, and bacteria that produce the organic compound acetate (acetogens). The biovolume in the bentonite was determined by detection of the ATP content. In addition, bacteria from the bentonite were cultured in different sulphate-reducing media. In these cultures, the presence of the biotic compounds sulphide and acetate was investigated, since these have potentially negative effect on the copper canister in a KBS-3 repository. The results were to some extent compared to density, water content, and temperature data provided by Clay Technology AB. The results showed that 10^0–10^2 viable sulphate-reducing and acetogenic bacteria and 10^2–10^4 heterotrophic aerobic bacteria g^{-1} bentonite were present after five years of storage in the rock. Bacteria with several morphologies could be found in the cultures with bentonite. The most bacteria were detected in the bentonite buffer close to the rock but in a few samples also in bentonite close to the copper canister.

When the presence of bacteria in the bentonite is investigated, it is important that the sample procedures are accurate. Oxygen is toxic to many of the bacteria that thrive in anaerobic groundwater; among them are the sulphate-reducing bacteria. To avoid underestimation of the number of sulphate-reducing bacteria in the bentonite it is important to exclude oxygen during sampling. Therefore, the bentonite was at all occasions except one put in an anaerobic chamber shortly after arrival to the laboratory in Göteborg a few hours after *in-situ* sampling. One bentonite block from the R9 level was not put in the anaerobic chamber until four days after sampling. Samples from this specific bentonite block were extracted 10–20 cm into the bentonite to, if possible, retain bentonite not exposed to oxygen. Another factor that may give underestimating results is desiccation. By wrapping the bentonite in sterile aluminium foil and plastic bags it was possible to avoid desiccation. The aluminium foil also served as protection against contamination of bacteria from the surroundings. The sampling procedures worked accurately. It was possible to sample the bentonite under sterile conditions, which was proved by the fact that bacteria only grew where the ambient conditions during storage in the CRT bentonite allowed this. Presence of sulphate-reducing bacteria proved that the bentonite was sampled without intrusion of oxygen in levels toxic to these organisms.

Sammanfattning

”Canister Retriveal Test” (CRT) är ett försök som startades upp vid Äspö Hard Rock Laboratory (HRL) 2000. CRT ingår i de undersökningar som utvärderar ett eventuellt KBS-3 förvar av använt kärnbränsle. Det primära syftet var att undersöka huruvida det är möjligt att återta en kopparkapsel som förvarats nere i berget under autentiska KBS-3 förhållanden, men CRT erbjöd även en unik möjlighet att undersöka ifall bakterier klarar sig i bentonitbufferten vid lagring. Därför togs i samband med återtaget av kopparkapseln mikrobiologiska prover ut från bentonitbufferten där den bakteriella sammansättningen studerades.

I denna rapport beskrivs mikrobiologiska analyser vad gäller totalt 66 punkter på C2, R10, R9 och R6 nivåerna i bentoniten från CRT. Genom att odla bakterier från bentoniten i specifika medier kunde följande bakteriella parametrar undersökas: Det totala antalet av odlingsbara heterotrofa aeroba mikroorganismer, sulfatreducerande bakterier och bakterier som producerar det organiska ämnet acetat (acetogener). Även biovolymen i bentoniten bestämdes genom att undersöka ATP innehållet. Dessutom odlades bakterier från bentoniten upp i syrefritt sulfatreducerarmedium. I dessa kulturer undersöktes närvaro av de biologiskt producerade ämnena sulfid och acetat, eftersom dessa potentiellt kan ha negativ inverkan på kopparkapseln i ett KBS-3 slutförvar. Resultaten jämfördes till viss del med data över densitet, temperatur och vatteninnehåll från Clay Technology AB. Resultaten visade att det fanns viabla sulfatreducerande och acetogena bakterier i storleksordningen 10^0–10^2 och odlingsbara aeroba bakterier i storleksordningen 10^2–10^4 g^{-1} bentonit på flera ställen i bentonitbufferten efter fem år nere i berget. Från dessa ställen kunde också bakterier med flera olika morfologier odlas upp. De flesta bakterierna återfanns nära berget i utkanten av bufferten men i några prover också nära kopparkapseln.

När förekomsten av bakterier undersöks i bentonit är det viktigt att den provtas på rätt sätt. Syre är giftigt för många bakterier som lever i syrefria miljöer, däribland sulfatreducerande bakterier. För att utesluta att antalet av dessa bakterier underskattas är det viktigt att inte syre tränger in i bentoniten. Därför slussades bentoniten vid alla tillfällen utom ett in i en box med syrefri atmosfär när den anlänt till laboratoriet i Göteborg några timmar efter *in-situ* provtagning. Ett prov från R9 nivån slussades inte in i anaeroboxen förrän fyra dagar efter provtagning. Prover från just detta bentonitblock togs 10–20 cm in i bentoniten för att i möjligaste mån utesluta att syre trängt in. En annan faktor som kan ge ett underskattande resultat är uttorkning. Genom att svepa in bentoniten i steril aluminiumfolie och plasta in den skyddades bentoniten mot uttorkning. Samtidigt skyddades den även mot kontaminering av bakterier från omgivningen. Protokollen för provtagning fungerade tillfredställande. Det var möjligt att provta bentoniten sterilt, vilket kunde visas genom att det inte växte i alla bentonitprover utan bara i de prover där bakterierna överlevde de omgivande förhållandena i bentoniten under lagringen i CRT. Att sulfatreducerande bakterier växte i odlingsmediet visar att bentoniten som planerat provtogs under syrefria förhållanden.

Contents

1 Methods

1.1 Sampling of CRT bentonite

Sampling

The bentonite from the C2, R10, R9 and R6 levels was sampled according to AP TD F69P3-06-002 Provtagning och analys av mikroorganismer and presence of bacteria was investigated. In Table 1-1, the sampling dates for each level are shown. Directly after sampling the bentonite was wrapped in sterile aluminium foil and plastic sheets and sent to the lab at Microbial Analytics Sweden AB in Göteborg. After arrival, approximately 5h after *in-situ* sampling, the bentonite blocks were wrapped in new sterile aluminium foil and plastic bags and placed in the anaerobic box (COY Laboratory Products, Grass Lake, MI, USA). Inside the box, a triplicate of bentonite pieces weighing 1–2 g each from points at various distances to the copper canister was sampled by means of a hammer, a sterile chisel and a sterile knife.

Preparation of enrichment culture media and inoculation

Bacteria from the bentonite were enriched in anaerobic growth media supplied with sulphate /Widdel and Bak 1992/. Two types of sulphate-reducing enrichment media were produced; One medium with an organic carbon and energy source and one with inorganic carbon and energy sources. Both media contained a basal salt solution (l^{-1} milli-Q water); 7 g NaCl, 1 g $CaCl_2 \times H_2O$, 0.67 g KCl, 1 g NH_4Cl, 0.15 g KH_2PO4, 0.5 g $MgCl_2 \times 6H_2O$, 3 g $MgSO_4 \times 6H_2O$. The salt solution was autoclaved and cooled under a N_2/CO_2 (80/20%) atmosphere for 1h. After that the following solutions were added: 10 ml trace element solution, 60 ml 1M $NaHCO_3$ solution, 10 ml yeast extract solution, 10 ml vitamin solution, 1 ml thiamine solution, 1 ml vitamin B_{12} solution, 5 ml iron stock solution, 2 ml resazurin solution, 10 ml cystein-HCl solution, and 10 ml Na_2S solution. Five ml of 50% lactate solution (l^{-1} milli-Q water) was added to the anaerobic enrichment media with organic energy and carbon source. The pH was set to 6.5–7.5 and the medium was added in 45 ml aliquots to N_2/CO_2 (80/20%) filled 120-ml serum flasks sealed with butyl rubber stoppers. The serum flasks and an additional set of sterile rubber stoppers were placed in an anaerobic box.

The serum flasks were opened inside the box and 1 g bentonite pieces from each sample point were put in the anaerobic media with and without lactate, respectively. The serum flasks were resealed with new stoppers and removed from the anaerobic box. To the cultures without lactate, H_2/CO_2 at 2 bars above atmospheric pressure (80/20%) was added as energy and carbon source. The enrichment cultures were left in room temperature overnight for the bentonite to disperse in the medium. After that they were vigorously shaken and incubated at 30°C for six weeks.

Sampling of bentonite for ATP determination

The third piece from each sampling point was put in a sterile 50-ml Falcon tube for ATP analysis. The tube was removed from the anaerobic box and 10 ml of B/S extraction solution (BioThema AB, Handen, Sweden) were added. The bentonite was dispersed in the extraction solution over night and frozen at –20°C until analysis.

Table 1-1. Sampling dates, inoculation start and density, temperature and water content for the bentonite samples from the CRT (extrapolated from Clay Technology AB data) at Äspö HRL.

Level	Sampling date	Inoculation date	Density (kg m^{-3})	Temperature (°C)	Water content (%)
C2	2006-02-03	2006-02-06	2,000–2,020	35–55	22–26
R10	2006-02-10	2006-02-13	1,890–2,080	35–55	20–26
R9	2006-02-21	2006-02-22	1,900–2,030	35–55	25–32
R6	2006-03-17	2006-03-20	1,970–1,985	50–85	26–29

1.2 Presence of bacteria in the CRT bentonite

The microbial metabolites sulphide and acetate were analysed in the enrichment cultures after six weeks of incubation. The enrichment cultures were also analysed with microscopy to examine the presence of bacteria and confirm that the metabolites were of biological origin.

Sulphide analysis

The sulphide concentration was determined in enrichment cultures with lactate and H_2/CO_2 according to /Widdel and Bak 1992/. Approximately 1 ml of the supernatant from each enrichment culture was carefully withdrawn with an anaerobic syringe and needle and 0.1 ml was added to 2 ml of 5 mM $CuSO_4$. If sulphide was present in the sample, a brown precipitate (CuS) was formed. The absorbance of the solution was measured spectrophotometrically (Genesys 10 UV, Thermo electron corporation, Waltham, MA, USA) at λ 480 nm and the concentration was determined with an external standard curve. The limit for a positive value was set to 109 mg l^{-1}, which always was higher than the background in medium without bacteria.

Acetate analysis

The acetate concentration was determined in the enrichment cultures with H_2/CO_2. A sample from the supernatant from each enrichment culture was carefully withdrawn with an anaerobic syringe and needle. The acetate concentration in the supernatant in the bentonite enrichment cultures was determined spectrophotometrically with a kit (Boehringer Mannheim, Mannheim, Germany) which detects acetate by an enzymatic method. The limit for a positive value was set approximately three times as high, 15 mg l^{-1}, as the background in medium without bacteria.

Microscopy

The supernatant from the enrichment cultures was diluted 20 times in sterile water and 0.1 ml was filtered onto 0.2 um pore size filters stained black (Osmonics, Minnetonka, MN, US) and stained with acridine orange (10 mg l^{-1}) for 7 min. Bacteria on the filters were observed in an inverted microscope (Nikon Diaphot 300, Teknooptik AB, Göteborg, Sweden) at 1,000 times magnification using blue light (390–490 nm) and photographed.

1.3 Enumeration of bacteria from the CRT bentonite

As mentioned above, the enrichment cultures were left at room temperature overnight after inoculation to allow the bentonite to disperse in the medium. After dispersion of the bentonite, the enrichment cultures were used as inocula for analysis of the number of viable culturable heterotrophic aerobic bacteria (CHAB) originating from the bentonite, as well as most probable number (MPN) of sulphate-reducing bacteria (SRB) and autotrophic acetogens (AA). AA are bacteria able to convert H_2 and CO_2 to acetate.

Culturable heterotrophic aerobic bacteria (CHAB)

In the CHAB analysis 0.1 ml from each of the enrichment cultures was spread on a triplicate of agar plates, which contained (l^{-1} milli-Q water); peptone 0.5 g, yeast extract 0.5 g, starch 0.25 g, Na-acetate 0.25 g, $CaCl_2 \times 2H_2O$ 0.2 g, K_2HPO_4 0.1 g, NaCl 10 g, trace metal solution 1 ml, agar 15 g. After 1 week of incubation at room temperature the number of colonies was counted. The mean and the standard deviation for the plates from both enrichment cultures from each sample point were calculated.

MPN analysis of sulphate-reducing bacteria (SRB)

In the MPN analysis of SRB 1 ml of the original enrichment cultures was inoculated into five anaerobic tubes with the sulphate-reducing media with lactate. After six weeks of incubation, the sulphide concentration in the MPN tubes was determined as described above. MPN of SRB was

calculated using a computer program described in /Klee 1993/ with 99% confidence levels according to Cornish and Fischer included in the program.

MPN analysis of autotrophic acetogens (AA)

In the MPN analysis of AA 1 ml of the original enrichment cultures was inoculated into five anaerobic tubes with sulphate-reducing media added with H_2/CO_2 but with the exclusion of 3 g $MgSO_4 \times 6H_2O$. After six weeks of incubation, the acetate concentration in the MPN tubes was determined as described above. MPN of AA was calculated using a computer program described in /Klee 1993/ with 99% confidence levels according to Cornish and Fischer included in the program.

1.4 Biovolume in the CRT bentonite

The ATP in the approximately 1 g of bentonite was extracted with 10 ml of B/S extraction solution. The ATP content in the extract was analyzed with the BioThema ATP Biomass Kit HS (BioThema AB, Handen, Sweden) and a Sirius FB12 luminometer (Berthold Detection Systems, Pfortzheim, Germany). The method used was modified from /Eydal and Pedersen 2007/ which has previously been described in TD-06-01. The ATP concentration was calculated based on the average of three measurements as follows:

$$amol\ ATP\ g^{-1}\ bentonite = \frac{I_{smp} - I_{bkg}}{I_{(std + smp - bkg)} - I_{(smp - bkg)}} \times DF/B \times SF$$

Where:

I: light intensity measured as relative light units
smp: sample
bkg: background
std: standard
B: g bentonite
DF/B: dilution factor to obtain amount of ATP g^{-1} bentonite = 100/B
SF: shift factor from pmol to amol = 10^6

The bentonite interfered with the ATP analysis. Therefore, a calibration curve of ATP measurements with B/S extraction solution, bentonite (1 g/10 ml) and five different known amounts of ATP was prepared and compared to B/S extraction solution with the same ATP amounts but without bentonite. Using this calibration curve, the measurements in the bentonite samples were translated to the corresponding ATP amounts without the bentonite interference.

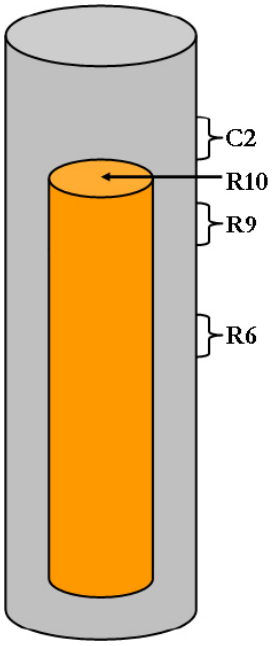
2 Results and evaluation

2.1 Sampling

Figure 2-1 schematically shows where the bentonite samples for microbiological analysis from the CRT were extracted and how many they were. Samples were taken from the C2, R10, R9 and R6 levels. The sample preparation was performed under anaerobic conditions with sterile equipment, which assured quality sampling of the bentonite. The extrapolated corresponding density, temperature and water content data for these positions are shown in Table 1-1 (from Clay Technology AB).

2.2 Presence of bacteria in the CRT bentonite

Enrichment cultures were prepared to examine if viable bacteria were present in the sampled bentonite from the C2, R10, R9 and R6 levels in the CRT bentonite. In Tables 2-1, 2-2, 2-3 and 2-4 presence of sulphide and acetate as well as bacterial growth detected by microscopy in these enrichment cultures are presented.



Bentonite samples from CRT				
Level	Rock interface	Bulk bentonite	Copper interface	Total
C2	7	11		18
R10			5	5
R9	13	7	13	33
R6	5		5	10

Figure 2-1. *Schematic picture over the location of the bentonite samples from the C2, R10, R9 and R6 levels in the CRT sampled in February–March 2006. The table compiles where the samples were taken and how many they were.*

Table 2-1. Comparison of temperature, density, and water content in the sampled bentonite and bacterial growth in enrichment cultures with C2 bentonite from the CRT after six weeks of incubation at 30°C. + growth – no growth.

Measurement	Level	Carbon and energy source	Distance from r = 0 (cm)									
			88	88	87	87	86	86	86	60	60	60
			Rock interface							Bulk bentonite		
Temperature (°C) [a]	C2		35–55	35–55	35–55	35–55	35–55	35–55	35–55	35–55	35–55	35–55
Density (kg m^{-3}) [a]	C2		2,016	2,016	2,016	2,016	2,016	2,016	2,016	2,009	2,009	2,009
Water content (%) [a]	C2		26	26	26	26	26	26	26	24	24	24
Sulphide [b]	C2	Lactate	+	–	+	+	+	–	+	–	–	–
Growth of bacteria [d]	C2	Lactate	+		+	+	+		+		–	
Acetate [c]	C2	H_2/CO_2	+	–	+	+	+	+	+	–	+	+
Sulphide [b]	C2	H_2/CO_2	–	–	+	–	+	+	+	–	+	–
Growth of bacteria [d]	C2	H_2/CO_2	+		+	+	+	+	+		+	+

Measurement	Level	Carbon and energy source	Distance from r = 0 (cm)							
			60	59	59	59	33	32	32	32
			Bulk bentonite							
Temperature (°C) [a]	C2		35–55	35–55	35–55	35–55	35–55	35–55	35–55	35–55
Density (kg m^{-3}) [a]	C2		2,009	2,009	2,009	2,009	2,013	2,013	2,013	2,013
Water content (%) [a]	C2		24	24	24	24	22	22	22	22
Sulphide [b]	C2	Lactate	–	+	–	–	–	–	–	–
Growth of bacteria [d]	C2	Lactate		+						
Acetate [c]	C2	H_2/CO_2	–	+	–	+	–	+	–	–
Sulphide [b]	C2	H_2/CO_2	–	+	–	–	–	+	–	–
Growth of bacteria [d]	C2	H_2/CO_2		+		+		+		

[a] data from Clay Technology AB, [b] detection limit for sulphide 109 mg l^{-1}, [c] detection limit for acetate 15 mg l^{-1}, [d] analysed with microscopy.

Table 2-2. Comparison of temperature, density, and water content in the sampled bentonite and bacterial growth in enrichment cultures with R10 bentonite from the CRT after six weeks of incubation at 30°C. + growth – no growth.

Measurement	Level	Carbon and energy source	Distance from r = 0 (cm)				
			53	53	53	53	53
			Copper interface				
Temperature (°C) [a]	R10		35–55	35–55	35–55	35–55	35–55
Density (kg m^{-3}) [a]	R10		1,980	1,980	1,980	1,980	1,980
Water content (%) [a]	R10		22	22	22	22	22
Sulphide [b]	R10	Lactate	–	–	–	–	–
Growth of bacteria [d]	R10	Lactate					
Acetate [c]	R10	H_2/CO_2	–	–	–	–	–
Sulphide [b]	R10	H_2/CO_2	–	–	–	–	–
Growth of bacteria [d]	R10	H_2/CO_2					

[a] data from Clay Technology AB, [b] detection limit for sulphide 109 mg l^{-1}, [c] detection limit for acetate 15 mg l^{-1}, [d] analysed with microscopy.

Table 2-3. Comparison of temperature, density, and water content in the sampled bentonite and bacterial growth in enrichment cultures with R9 bentonite from the CRT after six weeks of incubation at 30°C. + growth – no growth.

Measurement	Level	Carbon and energy source	Distance from r = 0 (cm)									
			88	88	88	88	88	88	88	88	88	88
			Rock interface									
Temperature (°C) [a]	R9		35–55	35–55	35–55	35–55	35–55	35–55	35–55	35–55	35–55	35–55
Density (kg m^{-3}) [a]	R9		1,914	1,914	1,914	1,914	1,914	1,914	1,914	1,914	1,914	1,914
Water content (%) [a]	R9		32	32	32	32	32	32	32	32	32	32
Sulphide [b]	R9	Lactate	+	–	–	–	–	–	–	–	–	–
Growth of bacteria [d]	R9	Lactate	+									
Acetate [c]	R9	H_2/CO_2	+	+	–	–	–	–	–	–	–	–
Sulphide [b]	R9	H_2/CO_2	–	–	–	–	–	–	–	–	–	–
Growth of bacteria [d]	R9	H_2/CO_2	+	+								
Measurement	**Level**	**Carbon and energy source**	**Distance from r = 0 (cm)**									
			88	**88**	**87**	**80**	**75**	**70**	**70**	**65**	**60**	**55**
			Rock interface			**Bulk bentonite**						
Temperature (°C) [a]	R9		35–55	35–55	35–55	35–55	35–55	35–55	35–55	35–55	35–55	35–55
Density (kg m^{-3}) [a]	R9		1,914	1,909	1,909	1,909	1,909	1,909	2,025	2,028	2,028	2,028
Water content (%) [a]	R9		32	29	29	29	29	29	25	26	26	26
Sulphide [b]	R9	Lactate	–	–	–	–	–	–	–	–	–	–
Growth of bacteria [d]	R9	Lactate										
Acetate [c]	R9	H_2/CO_2	–	–	–	–	–	–	–	–	–	–
Sulphide [b]	R9	H_2/CO_2	–	–	–	–	–	–	–	–	–	–
Growth of bacteria [d]	R9	H_2/CO_2										

[a] data from Clay Technology AB, [b] detection limit for sulphide 109 mg l^{-1}, [c] detection limit for acetate 15 mg l^{-1}, [d] analysed with microscopy.

Table 2-3. Continued.

Measurement	Level	Carbon and energy source	Distance from r = 0 (cm)									
			54	53	53	53	53	53	53	53	53	53
			Copper interface									
Temperature (°C) [a]	R9		35–55	35–55	35–55	35–55	35–55	35–55	35–55	35–55	35–55	35–55
Density (kg m^{-3}) [a]	R9		2,016	1,995	1,995	1,995	1,995	1,995	2,016	2,016	2,016	2,016
Water content (%)[a]	R9		26	27	27	27	27	27	26	26	26	26
Sulphide [b]	R9	Lactate	–	–	–	–	–	–	–	–	–	–
Growth of bacteria [d]	R9	Lactate										
Acetate[c]	R9	H_2/CO_2	–	+	+	–	–	–	–	–	–	–
Sulphide [b]	R9	H_2/CO_2	–	–	–	–	–	–	–	–	–	–
Growth of bacteria [d]	R9	H_2/CO_2		+	+							

Measurement	Level	Carbon and energy source	Distance from r = 0 (cm)		
			53	53	53
			Copper interface		
Temperature (°C) [a]	R9		35–55	35–55	35–55
Density (kg m^{-3}) [a]	R9		2,016	2,016	2,016
Water content (%) [a]	R9		26	26	26
Sulphide [b]	R9	Lactate	–	–	–
Growth of bacteria [d]	R9	Lactate			
Acetate [c]	R9	H_2/CO_2	–	–	–
Sulphide [b]	R9	H_2/CO_2	–	–	–
Growth of bacteria [d]	R9	H_2/CO_2			

[a] data from Clay Technology AB, [b] detection limit for sulphide 109 mg l^{-1}, [c] detection limit for acetate 15 mg l^{-1}, [d] analysed with microscopy.

Table 2-4. Comparison of temperature, density, and water content in the sampled bentonite and bacterial growth in enrichment cultures with R6 bentonite from the CRT after six weeks of incubation at 30°C. + growth – no growth.

Measurement	Level	Carbon and energy source	Distance from r = 0 (cm)									
			88	88	88	85	85	55	55	53	53	53
			Rock interface					Copper interface				
Temperature (°C) [a]	R6		50–85	50–85	50–85	50–85	50–85	50–85	50–85	50–85	50–85	50–85
Density (kg m^{-3}) [a]	R6		1,970	1,970	1,970	1,970	1,970	1,985	1,985	1,985	1,985	1,985
Water content (%) [a]	R6		29	29	29	29	29	26	26	26	26	26
Sulphide [b]	R6	Lactate	–	–	–	–	–	–	–	–	–	–
Growth of bacteria [d]	R6	Lactate										
Acetate [c]	R6	H_2/CO_2	+	+	–	–	–	–	–	–	–	–
Sulphide [b]	R6	H_2/CO_2	–	–	–	–	–	–	–	–	–	–
Growth of bacteria [d]	R6	H_2/CO_2	+	+								

[a] data from Clay Technology AB, [b] detection limit for sulphide 109 mg l^{-1}, [c] detection limit for acetate 15 mg l^{-1}, [d] analysed with microscopy.

Figure 2-2 shows where sulphide was found in the bentonite enrichment cultures with addition of the organic carbon source lactate from the C2, R10, R9 and R6 levels. Elevated sulphide concentrations (109–997 mg l^{-1}) were detected in several samples. In the samples close to the rock at the C2 level six of 18 enrichments contained sulphide, as did one sample close to the rock at the R9 level. One culture with bentonite situated ~53 cm from r = 0 at the C2 level also contained sulphide (922 mg l^{-1}). In all the sulphide containing cultures, bacteria with various morphologies were found. These bacteria were very likely SRB. In Figure 2-3, images from microscopy analysis of two of these cultures are shown.

In Figure 2-4 the concentrations of acetate in the bentonite enrichment cultures with addition of the inorganic energy and carbon sources H_2/CO_2 are shown. Acetate, ranging from 20 to 328 mg l^{-1}, was found in 11 of 18 cultures with bentonite from the C2 level, four of 33 cultures from the R9 level and two of the ten cultures from the R6 level. No acetate was found in the cultures inoculated with bentonite from the top of the canister at the R10 level. Concomitant growth of bacteria with various morphologies was evident in all the samples containing acetate. Some of these bacteria were very likely acetogens. In Figure 2-6, images from microscopy analysis of these cultures are shown. In difference to the sulphide producing bacteria utilizing lactate showed in Figure 2-2, the acetogens were present in samples from several distances to r = 0, i.e. throughout the respective level.

Figure 2-5 shows the concentrations of sulphide in the bentonite enrichment cultures with addition of the inorganic energy and carbon sources H_2/CO_2. Elevated levels of sulphide were present in seven of the 18 enrichment cultures from the C2 level. The sulphide in these samples was, like acetate, present in cultures enriched on bentonite from several distances from r = 0. Since acetate is a carbon source used by many sulphate-reducing bacteria /Madigan et al. 2000/ and acetate was produced in the bentonite enrichment cultures (Figure 2-4, Table 2-1), the sulphide in these cultures might have originated from acetate metabolism by SRB. Another possibility for SRB to gain carbon and energy is to use the inorganic energy and carbon sources H_2/CO_2. Taking these two life strategies into consideration, some of the bacteria shown in Figure 2-6 from the cultures growing on H_2/CO_2 are also very likely to be SRB.

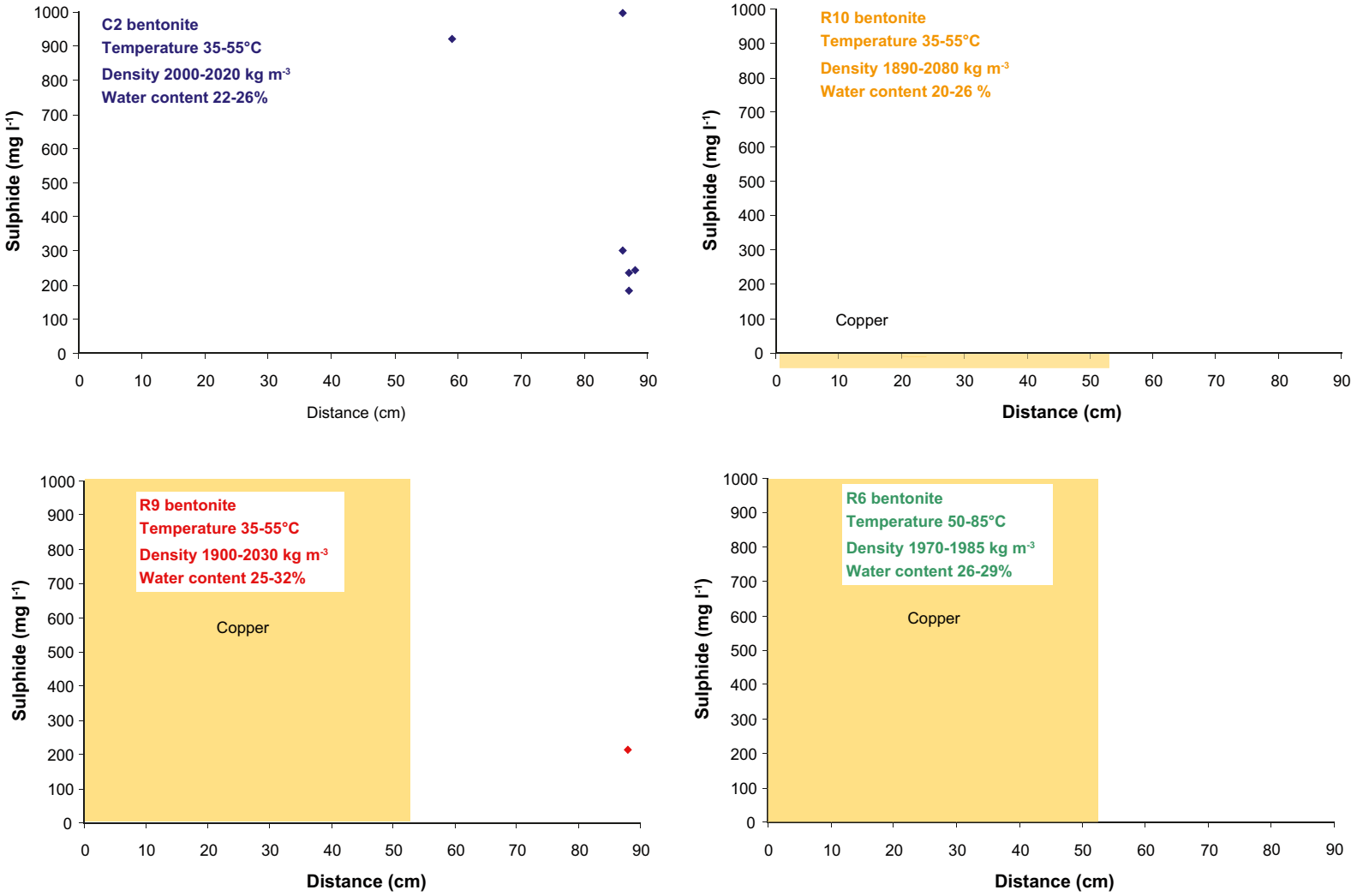


Figure 2-2. *Sulphide concentrations in the anaerobic enrichment cultures inoculated with C2 bentonite (blue), R10 bentonite (orange), R9 bentonite (red) and R6 bentonite (green) from the CRT experiment. The medium for the enrichments contained lactate as carbon and energy source. The bentonite pieces inoculated in the cultures were sampled from different distances from r = 0 and incubated six weeks at 30°C prior to analysis.*

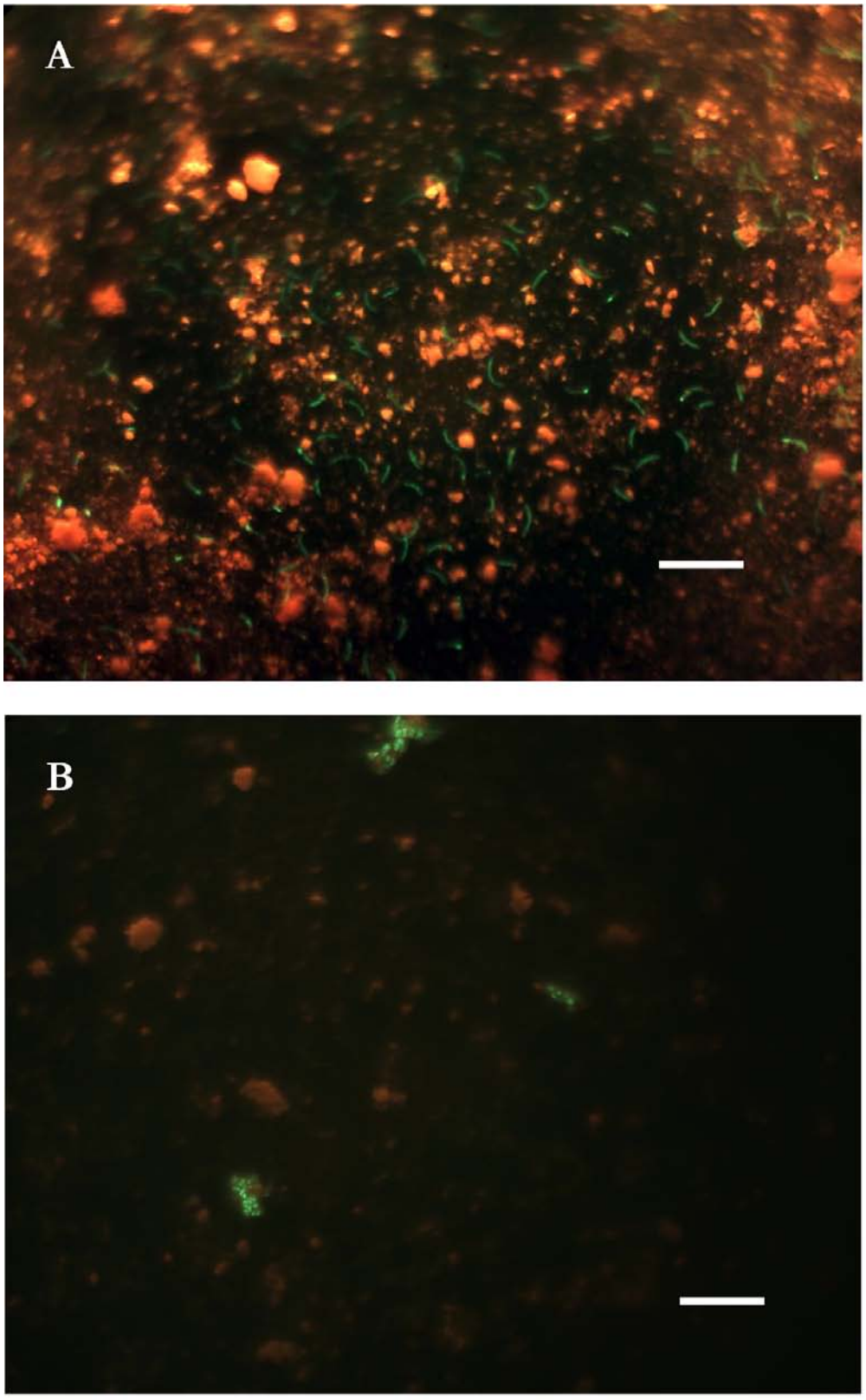


Figure 2-3. *Images from the bentonite enrichment cultures with lactate added as energy and carbon source. A) Bacteria in an enrichment culture from bentonite at the C2 level situated ~53 cm from r = 0. The enrichment culture contained ~900 mg l^{-1} sulphide. B) Bacteria in an enrichment culture from bentonite at the R9 level situated ~88 cm from r = 0. The enrichment culture contained ~200 mg l^{-1} sulphide.*

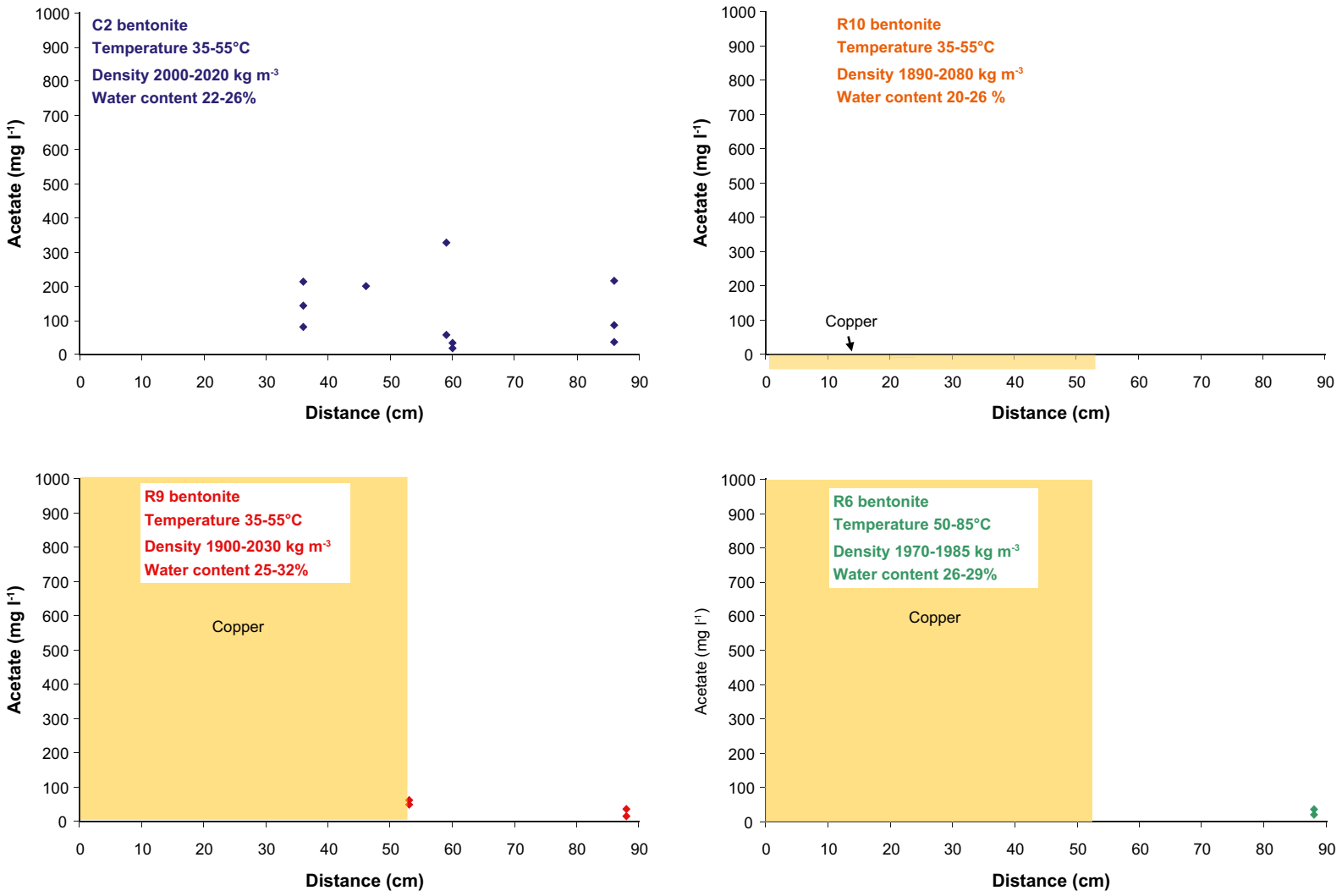


Figure 2-4. *Acetate concentrations in the anaerobic enrichment cultures inoculated with C2 bentonite (blue), R10 bentonite (orange), R9 bentonite (red) and R6 bentonite (green) from the CRT experiment. The medium for the enrichments contained CO_2/H_2 as carbon and energy sources. The bentonite pieces inoculated in the cultures were sampled from different distances from r = 0 and incubated six weeks at 30°C prior to analysis.*

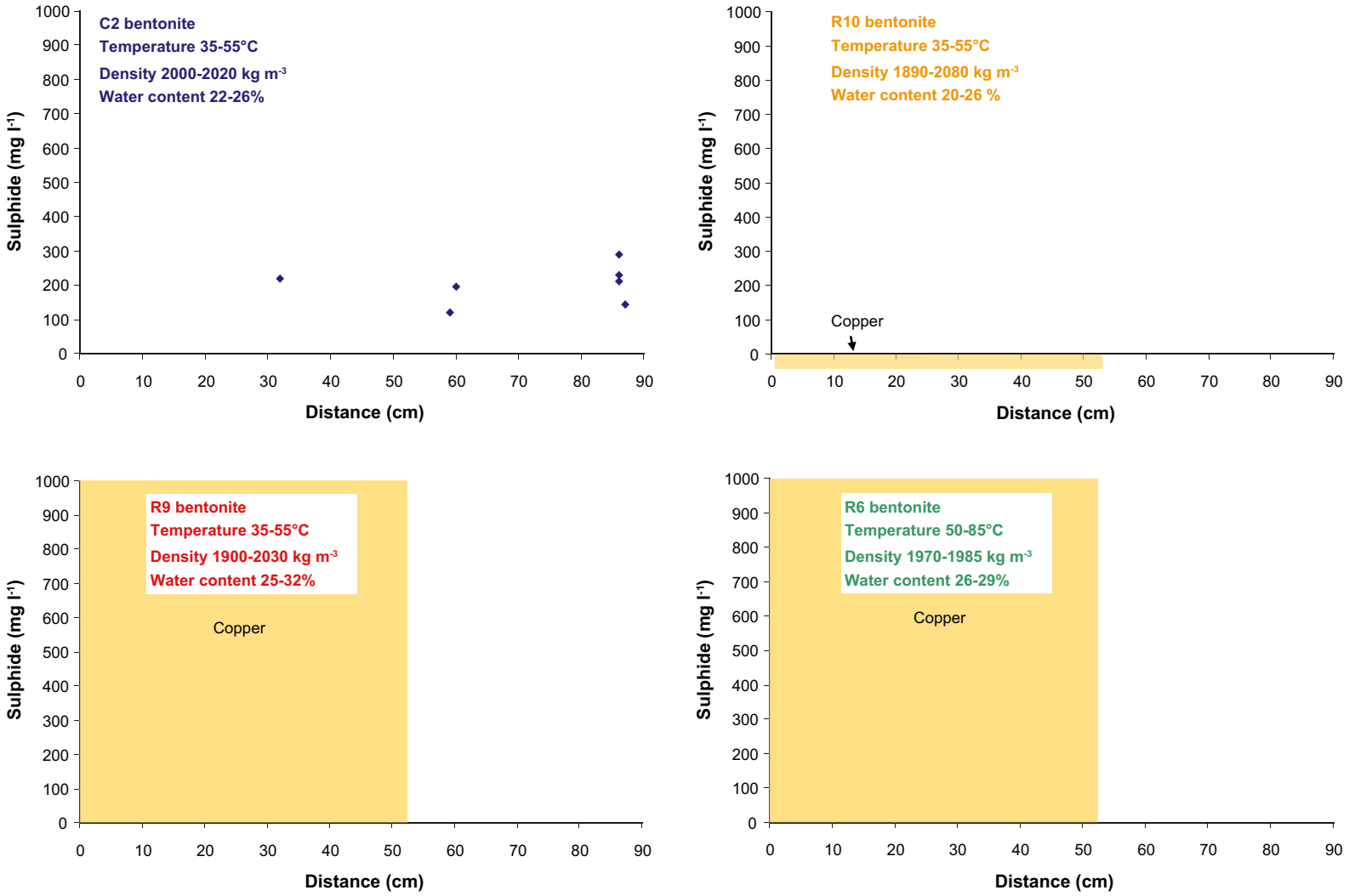


Figure 2-5. *Sulphide concentrations in the anaerobic enrichment cultures inoculated with C2 bentonite (blue), R10 bentonite (orange), R9 bentonite (red) and R6 bentonite (green) from the CRT experiment. The medium for the enrichments contained CO_2/H_2 as carbon and energy sources. The bentonite pieces inoculated in the cultures were sampled from different distances from r = 0 and incubated six weeks at 30°C prior to analysis.*

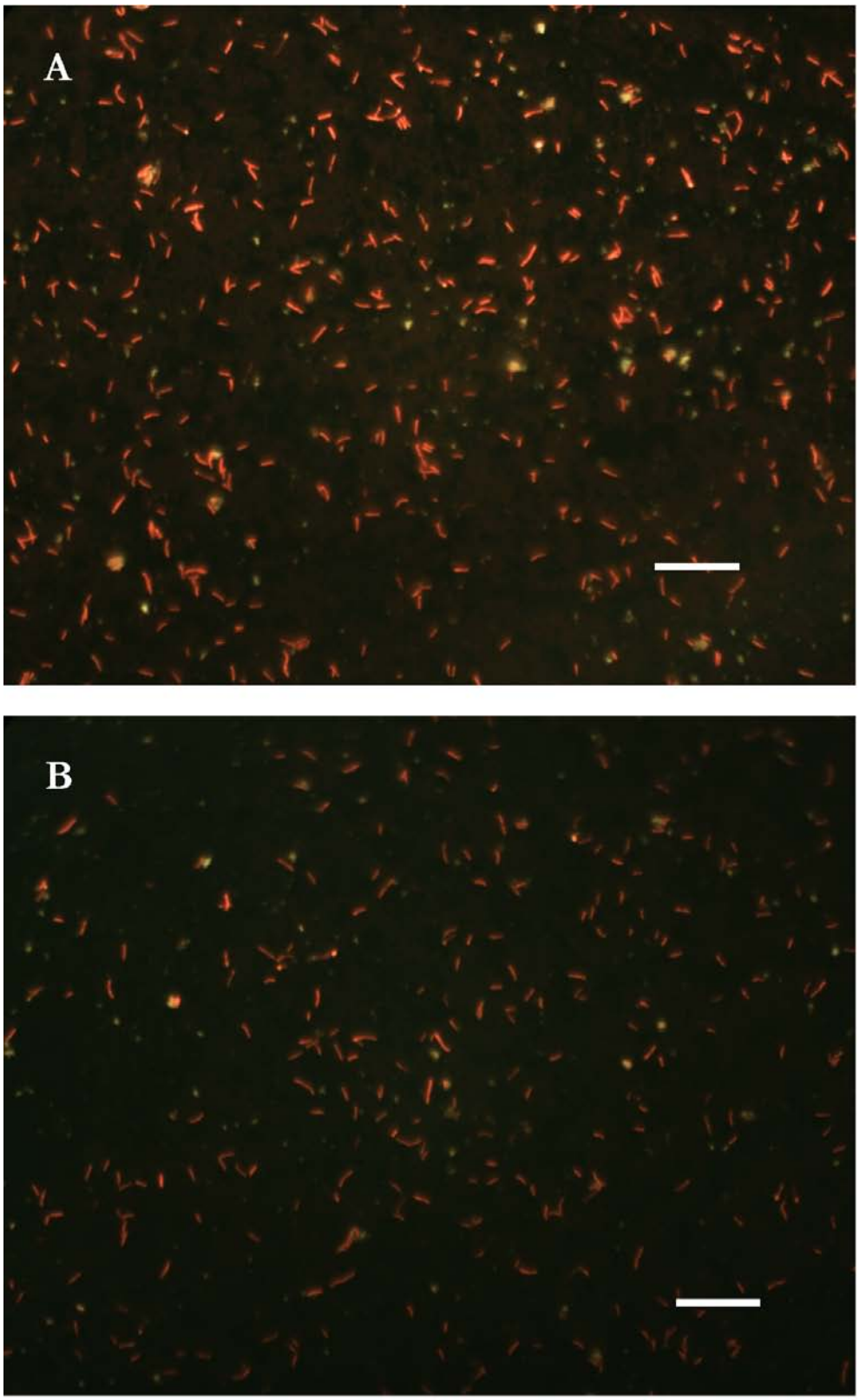


Figure 2-6. *Images from the bentonite enrichment cultures with H_2/CO_2 added as energy and carbon sources. A) Bacteria in an enrichment culture from bentonite at the C2 level situated at ~32 cm from r = 0. The enrichment culture contained ~200 mg l^{-1} sulphide and ~200 mg l^{-1}acetate. B) Bacteria in an enrichment culture from bentonite at the C2 level situated ~53 cm from r = 0. The enrichment culture contained ~40 mg l^{-1} acetate.*

2.3 Enumeration of bacteria from the CRT bentonite

The bacteria in the CRT bentonite were also enumerated by MPN analysis of SRB and AA, and by plate counting of CHAB. These data are compiled in Tables 2-5, 2-6, 2-7 and 2-8. Figure 2-7 shows the CHAB numbers and Figures 2-8 and 2-9 the MPN of AA and SRB in the C2, R10, R9 and R6 levels. Growth was not always detected in the in the tubes in the MPN analysis even if presence of sulphide and acetate with concomitant growth of bacteria was found in the CRT bentonite enrichment cultures. Theoretically, the numbers of SRB in these samples would be ~1–5 g^{-1} bentonite. These samples are illustrated with brighter squares in Figures 2-8 and 2-9.

Approximately 10^0–10^2 AA and SRB g^{-1} bentonite were found. Compared to the groundwater in the area where 10^1–10^3 ml^{-1} AA and SRB can be detected /Pedersen 2000/, these numbers are lower. Nonetheless, it is obvious that both AA and SRB were present in several of the bentonite samples.

2.4 Biovolume in the CRT bentonite

ATP measurements showed the living bio-volume in a sample. All samples with bentonite from the CRT bentonite except one from the R6 level close to the copper canister contained less than 5×10^4 amoles ATP g^{-1} (Tables 2-5, 2-6, 2-7 and 2-8). This number corresponds to 10^4–10^6 cells g^{-1}, if the ATP content in the cells in the bentonite is regarded to be the same as in anaerobic bacteria in deep granitic groundwater. These bacteria have been shown to contain 0.1–1 amoles ATP $cell^{-1}$ /Eydal and Pedersen 2007/. The ATP content in the R6 bentonite with a value above detection limit was quite high, 1,400,000 ± 70,000 amoles g^{-1}. This corresponds to 10^7–10^8 cells g^{-1}. Such high levels of ATP could imply a possible contamination at some stage during sample preparation, especially so since the CHAB number was below detection in this sample. However, it can not be completely ruled out that many bacteria were present in this sample.

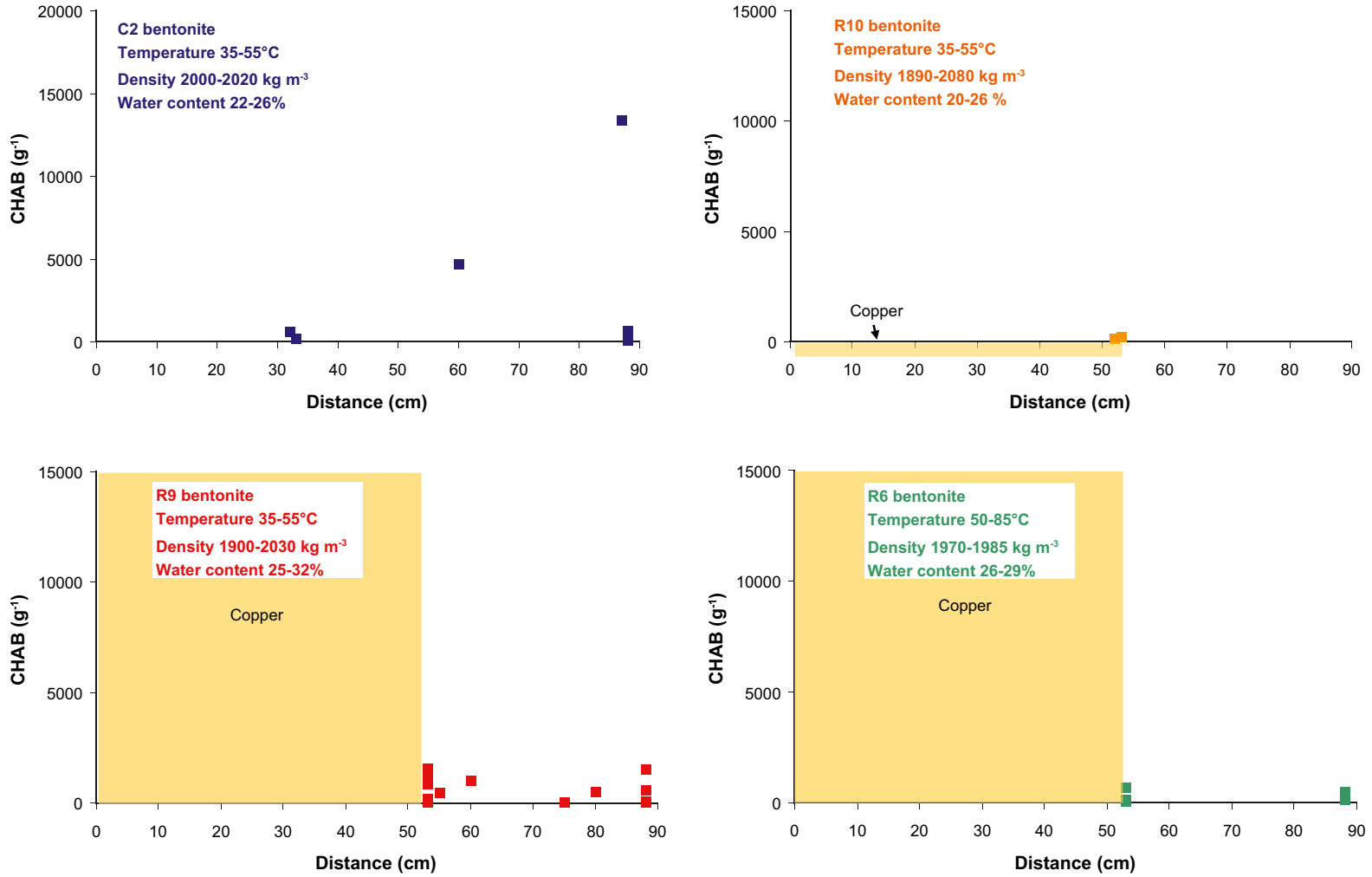


Figure 2-7. *Culturable heterotrophic aerobic bacteria (CHAB) in the C2 bentonite (blue), R10 bentonite (orange), R9 bentonite (red) and R6 bentonite (green) from the CRT experiment after one day of dispersion of the bentonite in the anaerobic media. The numbers are means of the counts from the bentonite enrichment cultures with lactate and those with addition of CO_2/H_2 (n=2).*

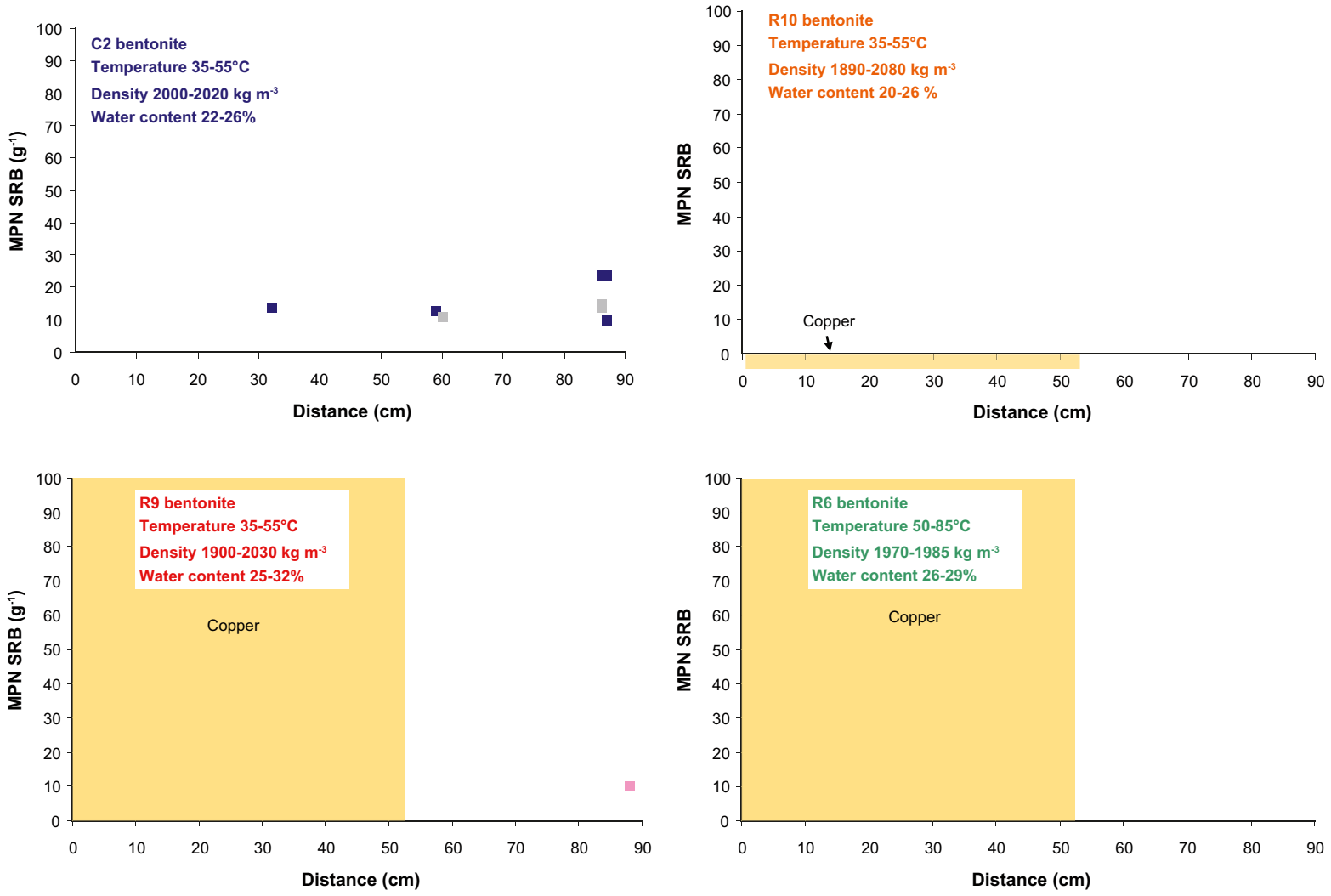


Figure 2-8. *MPN of sulphate-reducing bacteria (SRB) in the the C2 bentonite (blue), R10 bentonite (orange), R9 bentonite (red) and R6 bentonite (green) from the CRT experiment. The brighter squares show samples where sulphate-reducing bacteria were present in the enrichment culture used as inoculum but not in the MPN tubes and represents the highest theoretical number of sulphate-reducing bacteria in the bentonite sample.*

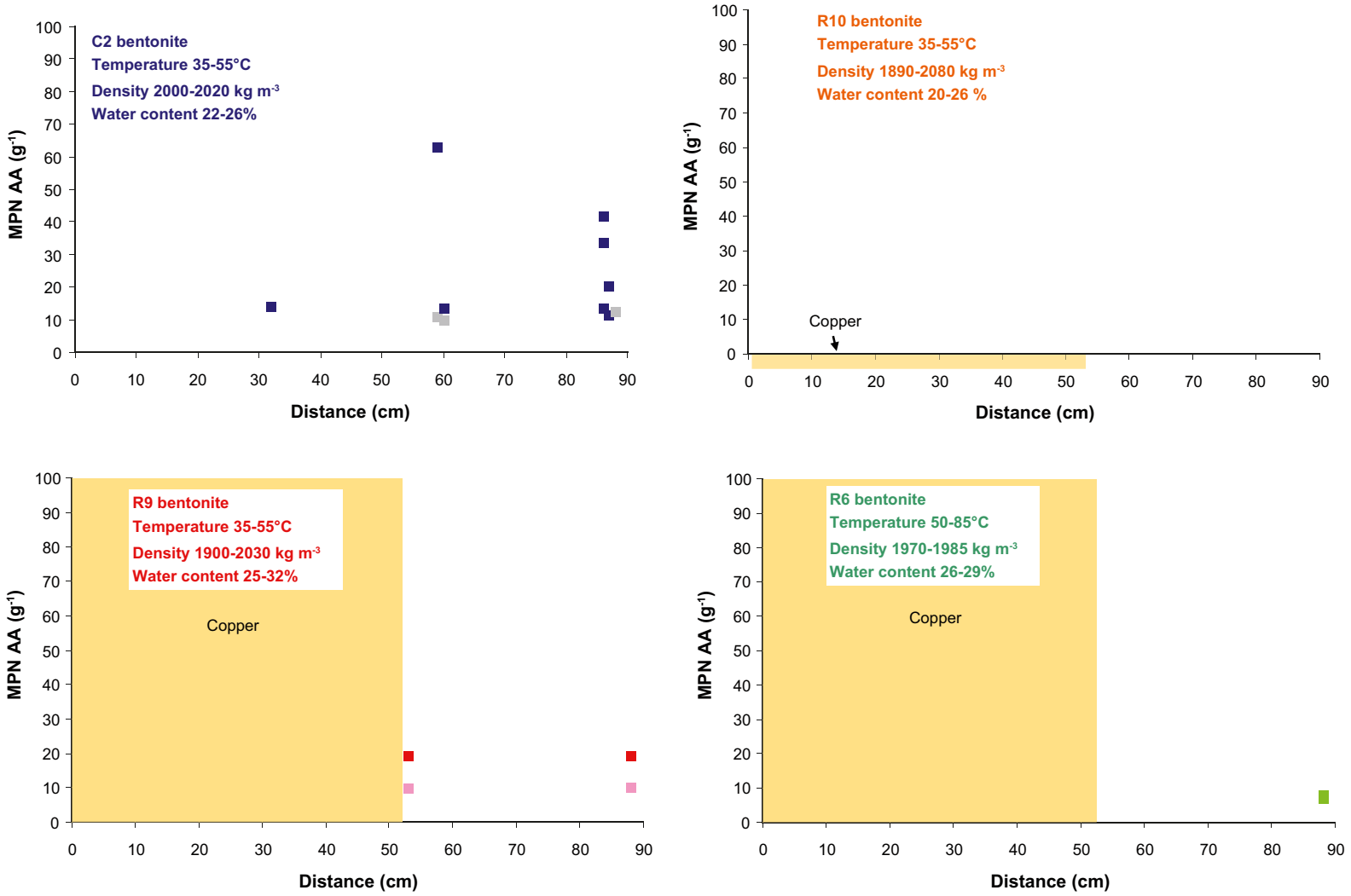


Figure 2-9. *MPN of autotrophic acetogens(AA) in the the C2 bentonite (blue), R10 bentonite (orange), R9 bentonite (red) and R6 bentonite (green) from the CRT experiment. The brighter squares show samples where sulphate-reducing bacteria were present in the enrichment culture used as inoculum but not in the MPN tubes and represents the highest theoretical number of sulphate-reducing bacteria in the bentonite sample.*

Table 2-5. Enumeration of culturable heterotrophic aerobic bacteria (CHAB), sulphate-reducing bacteria (SRB), autotrophic acetogens (AA) and determination of the ATP content in the C2 bentonite from the CRT.

Analysis	Level	Distance from r = 0 (cm)									
		88	88	87	87	86	86	86	60	60	60
		Rock interface							Bulk bentonite		
CHAB (CFU[a] × 10^2 g^{-1} ± stdev)	C2	7 ± 5	2 ± 1	130 ± 90	–	–	–	–	47 ± 33	–	–
MPN SRB (g^{-1} with 99% confidence limits[b])	C2	–	–	10 (4–63)	24 (4–75)	1–15	14 (5–81)	24 (4–89)	1–11	–	–
MPN AA (g^{-1} with 99% confidence limits[b])	C2	–	13 (5–68)	12 (5–70)	21 (4–75)	34(6–124)	14 (5–81)	42 (5–109)	14 (5–75)	1–10	–
ATP (amole × 10^5 g^{-1} ± stdev)	C2	–	–	–	–	–	–	–	–	–	–

Analysis	Level	Distance from r = 0 (cm)							
		59	59	59	33	32	32	32	32
		Bulk bentonite							
CHAB (CFU[a] × 10^2 g^{-1} ± stdev)	C2	–	–	–	2 ± 1	6 ± 2	–	–	–
MPN SRB (g^{-1} with 99% confidence limits[b])	C2	13 (5–76)	–	–	–	1–14	–	–	–
MPN AA (g^{-1} with 99% confidence limits[b])	C2	63 (8–154)	1–11	–	–	14 (6–85)	–	–	–
ATP (amole × 10^5 g^{-1} ± stdev)	C2	–	–	–	–	–	–	–	–

[a] CFU – colony forming units, [b]determined using the method of Cornish and Fischer described in /Klee 1993/.

Table 2-6. Enumeration of culturable heterotrophic aerobic bacteria (CHAB), sulphate-reducing bacteria (SRB), autotrophic acetogens (AA) and determination of the ATP content in the R10 bentonite from the CRT.

Analysis	Level	Distance from r = 0 (cm)				
		53	53	53	53	53
		Copper interface				
CHAB (CFU[a] × 10^2 g^{-1} ± stdev)	R10	2 ± 2	3 ± 0.6	–	–	–
MPN SRB (g^{-1} with 99% confidence limits[b])	R10	–	–	–	–	–
MPN AA (g^{-1} with 99% confidence limits[b])	R10	–	–	–	–	–
ATP (amole × 10^5 g^{-1} ± stdev)	R10	–	–	–	–	–

[a] CFU – colony forming units, [b]determined using the method of Cornish and Fischer described in /Klee 1993/.

Table 2-7. Enumeration of culturable heterotrophic aerobic bacteria (CHAB), sulphate-reducing bacteria (SRB), autotrophic acetogens (AA) and determination of the ATP content in the R9 bentonite from the CRT.

Analysis	Level	Distance from r = 0 (cm)									
		88	88	88	88	88	88	88	88	88	88
		Rock interface									
CHAB (CFU[a] × 10^2 g^{-1} ± stdev)	R9	16 ± 22	1 ± 2	6 ± 2	1 ± 2	–	0.7 ± 1	–	–	–	–
MPN SRB (g^{-1} with 99% confidence limits[b])	R9	–	–	1–10	–	–	–	–	–	–	–
MPN AA (g^{-1} with 99% confidence limits[b])	R9	19 (3–71)	–	1–10	–	–	–	–	–	–	–
ATP (amole × 10^5 g^{-1} ± stdev)	R9	–	–	–	–	–	–	–	–	–	–
Analysis	**Level**	**Distance from r = 0 (cm)**									
		88	**88**	**87**	**80**	**75**	**70**	**70**	**65**	**60**	**55**
		Rock interface			**Bulk bentonite**						
CHAB (CFU[a] × 10^2 g^{-1} ± stdev)	R9	–	–	–	5 ± 7	0.7 ± 0.9	–	–	–	10 ± 13	5 ± 7
MPN SRB (g^{-1} with 99% confidence limits[b])	R9	–	–	–	–	–	–	–	–	–	–
MPN AA (g^{-1} with 99% confidence limits[b])	R9	–	–	–	–	–	–	–	–	–	–
ATP (amole × 10^5 g^{-1} ± stdev)	R9	–	–	–	–	–	–	–	–	–	–

[a] CFU – colony forming units, [b]determined using the method of Cornish and Fischer described in /Klee 1993/.

Table 2-7. Continued.

Analysis	Level	Distance from r = 0 (cm)									
		54	53	53	53	53	53	53	53	53	53
		Copper interface									
CHAB (CFU[a] × 10^2 g^{-1} ± stdev)	R9	–	16 ± 13	13 ± 3	9 ± 10	2 ± 2	0.6 ± 0.8	2 ± 0.8	0.8 ± 1.1	0.6 ± 0.9	–
MPN SRB (g^{-1} with 99% confidence limits[b])	R9	–	–	–	–	–	–	–	–	–	–
MPN AA (g^{-1} with 99% confidence limits[b])	R9	–	10 (4–59)	19 (3–71)	–	–	–	–	–	–	–
ATP (amole × 10^5 g^{-1} ± stdev)	R9	–	–	–	–	–	–	–	–	–	–

Analysis	Level	Distance from r = 0 (cm)		
		53	53	53
		Copper interface		
CHAB (CFU[a] × 10^2 g^{-1} ± stdev)	R9	–	–	–
MPN SRB (g^{-1} with 99% confidence limits[b])	R9	–	–	–
MPN AA (g^{-1} with 99% confidence limits[b])	R9	–	–	–
ATP (amole × 10^5 g^{-1} ± stdev)	R9	–	–	–

[a] CFU – colony forming units, [b]determined using the method of Cornish and Fischer described in /Klee 1993/.

Table 2-8. Enumeration of culturable heterotrophic aerobic bacteria (CHAB), sulphate-reducing bacteria (SRB), autotrophic acetogens (AA) and determination of the ATP content in the R6 bentonite from the CRT.

Analysis	Level	Distance from r = 0 (cm)									
		88	88	88	85	85	55	55	53	53	53
		Rock interface					Copper interface				
CHAB (CFU[a] × 10^2 g^{-1} ± stdev)	R6	–	–	5 ± 4	1 ± 1	–	1 ± 0.7	2 ± 1	–	7 ± 5	–
MPN SRB (g^{-1} with 99% confidence limits[b])	R6	–	–	–	–	–	–	–	–	–	–
MPN AA (g^{-1} with 99% confidence limits[b])	R6	1–7	1–8	–	–	–	–	–	–	–	–
ATP (amole × 10^5 g^{-1} ± stdev)	R6	–	–	–	–	–	–	–	14 ± 0.7	–	–

[a] CFU – colony forming units, [b]determined using the method of Cornish and Fischer described in /Klee 1993/.

3 Concluding remarks

AA and SRB were present in the CRT bentonite after five years of storage. The numbers of AA and SRB were low compared to the surrounding groundwater, which can be expected because of the harsh conditions in the bentonite. Nevertheless, they could be found repeatedly in many of the CRT bentonite samples. The results show that the bacteria from various distances from the rock and the inside the CRT bentonite were viable and had a potential to produce both sulphide and organic carbon in form of acetate, also in bentonite with a density over 2,000 kg m^{-3}. Especially, this was evident in bentonite where the maximum temperature was not too high. The elevated temperature in the R6 bentonite where the maximum temperature reached 85°C during some stage of the experiment obviously made it more difficult for the bacteria to survive compared to the sampled C2 and R9 bentonite where the maximum temperature reached 55°C.

The sampling procedures and analysis protocols in this experiment worked properly. The only cultured bacteria in the samples were the ones intrinsic in the bentonite during sampling. The origin of the bacteria in the bentonite is still to be determined but probably do they originate both from the surrounding groundwater and from the bentonite itself.

4 References

SKB's (Svensk Kärnbränslehantering AB) publications can be found at www.skb.se/publications.

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