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Preliminary investigations of deep ground water microbiology in Swedish grantic rock

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ABSTRACT

The intention with this study was to collect data that can support the planning of a project over the eventual impact between microbes and a Swedish HWL repository. The total numbers of bacteria, the numbers of aerobic and anaerobic bacteria and the most probable numbers of Thiobacilli and related bacteria were determined in three different boreholes at different levels. The highest total numbers of bacteria were registered in the EV01 borehole close to the Simpevarp nuclear power plants and were between 10^5 and 10^6 bacteria/ml. The higher of these two numbers refer to samples taken with a gas sampler. The gas sampler also gave the highest number of aerobic and anaerobic bacteria. The results presented here point out the gas sampler as being the sample site that gives the safest data describing the aquifer conditions with respect to microbiology. The first task that needs to be solved by a coming project is judged to be collection of data on numbers, species and activity of deep ground water microbial populations in Swedish granitic rocks.

APPENDIX 1

TABLE OF CONTENTS

SUMMARY

Natural occurring bacteria in ground water can adsorb and transport released radionuclids. The modelling of deep geological repositories for disposal of nuclear waste must consider the possibility of impact with microorganisms. The intention with this study was to collect data that can support the planning of a project over the eventual impact between microbes and a Swedish HWL repository.

Three testsites were studied. A 60 m deep well, which supplies a one family house, was used as a reference well. The second testsite was a borehole close to Simpevarp nuclear power plant called EV01. Water from the levels 635, 558, 522 and 420 meters was analysed. The boreholes V1 and V2 in the Stripa mine were also investigated. The total numbers of bacteria were analysed with fluorescence microscopy. The numbers of aerobic and anaerobic heterotrophic bacteria were analysed with plate count technique and a most probable number technique was used to assay the numbers of Thiobacilli and related bacteria. Sampling in EV01 were made both in the field laboratory and down in the borehole with a gas sampler and in the borehole sond.

The borehole sond and the gas sampler had significantly higher total numbers of bacteria/ml than the field lab. Moreover, the heterotrophic count was 100 times higher in the gas sampler than in the field lab sample. A possible explanation to this can be that the original population attaches to the tube walls or collapses for unknown reasons during pumping. The bacteria in the gas sampler and in the field lab could be two different populations. This remains to be investigated. The results presented here points out the gas sampler as being the sample site that gives the safest data describing the aquifer conditions with respect to microbiology.

The first task to be solved by a coming project is judged to be collection of data on numbers, species and activity of deep ground water microbial populations in Swedish granitic rocks. Secondly, models for the interactions between a nuclear waste repository and the developing microbial populations should be set up based on the collected data. Finally, specific studies should be performed on the effect on nuclid migration from bacteria judged by the models to be probable inhabitants in a Swedish nuclear waste repository.

1 INTRODUCTION

> "There is no field of human endeavour, whether it be in industry or agriculture or in the preparation of food or in connection with the problem of shelter or clothing, or in the conservation of human and animal health and the combating of disease, where the microbe does not play an important and often dominant role".
>
> Selman Waksman, 1942

Selman Waksman was a microbiologist who in 1952 received the Nobel Prize in Physiology and Medicine for the discovery of the antibiotic streptomycin. Though his statement of 1942 is a very powerful one, we might tend to think that because of Waksman's personal involvment with microbiology, his case is much overstated. But is it? In many respects Waksman's remarks are even more pertinent in todays world.

This is especially true for the field of microbial ecology. Since the time of Waksman's Nobel Prize, our understanding of the relationships between microbes and their environments has increased from almost nothing to the complex networks of microbial ecosystems. Still, new horizons of ecosystem as well as species complexity arise behind every new understanding.

The understanding of ground water microbiology has just begun and we know very little about these inhabitants below our feets. With respect to the enormous adaptability of different microbial populations to different environments, the presence of microbes in deep ground water can be expected. It remains to determine numbers, species and activity of these organisms.

Natural occurring bacteria in ground water can absorb and transport released radionuclids. Strandberg *et al.*, (1981) has neatly shown how *Pseudomonas aeruginosa* and *Saccharomyces cerevisiae* very efficiently accumulate uranium to concentrations of more than 15% of the cell dry weight. The modelling of deep geological repositories for disposal of nuclear waste must consider the possibility of impact with microorganisms. Several countries have initiated studies on this possibility. West *et al.*, (1985) summarized the British

geomicrobiology program on this topic. McKinley et al., (1985) gives an overview of the consequences of microbial activity in a Swiss HLW. Common for these reports and others as well are that the authors conclude quantitative assessment difficulty due to the lack of relevant data. The intention with this study was to collect data that can support the planning of a project over the eventual impact between microbes and a Swedish HLW repository.

2 MATERIALS AND METHODS

2.1 TESTSITES

2.1.1 Hindås

A 60 m deep well which supplies a one family house was used as a reference well. This well has been followed by our lab for several years primarily to study a population of Gallionella ferruginea that thrives in the well (Hallbeck and Pedersen, 1987). Hindås can be reached from our lab within 30 minutes which together with the very stable bacteria population in the well makes it a suitable reference well for testing methods and for performance of pilot experiments. The mean total number of bacteria determined with the fluorescence technique described under 2.3.1 is shown in Table 1-1.

Table 1-1 The total number of bacteria as determined with the fluorescence technique in the water from the 60 m deep reference well in Hindås. N = number of observations. S.D. = standard deviation.

Date	Cells ml/10^5	(N)	(S.D.)
860316	2.40	30	0.48
860902	2.88	30	1.50
860923	2.32	30	1.01
861027	2.73	30	1.34
870928	2.39	45	0.72
871014	4.74	15	1.2
871019	0.94	30	0.78
871110	1.78	30	0.39

2.1.2 Ävrö

During the investigations water was pumped from 4 levels (635, 558, 522 and 420 meters) in a borehole called EV01 at Ävrö close to the nuclear power plant at Simpevarp, Oskarshamn. The integrated mobile field laboratory described by Wikberg et al (1987) was used for water sampling and for the analysis of the samples immediately after sampling. Samples were taken in 1 l sterile bottles from the inside of the field laboratory, from the borehole sond and from the gas sampler. The

tubing from the sond to the surface was made of polyamid.

2.1.3 The Stripa mine

The Stripa mine was used to check if a tubing material could induce a microbial wall growth that has influence on the results. Two boreholes V1 and V2 were sampled (Nordström et al., 1985). The tubing from borehole V1 was made of polyamide and drained an artesian crush zone between 500-505.9 m at a flow rate of 500 ml/min. The tubings from borehole V2 drained three artesian levels. V2:1 = 559-800 m at 13 ml/min, V2:4 = 402-410 m at 65 ml/min and V2:5 = 389-397 m at 33 ml/min. The flow rates were measured in April 1987. All levels refers to the levels of the boreholes in the mine. By adding 360 m to V1 and 410 m to V2 the distances from the ground to the sampling levels are achieved.

2.2 WATER CHEMISTRY

The chemical composition of the ground waters studied was analysed as described by Wikberg et al., (1987) at the Ävrö test site and as described by Nordström et al., (1985) for the Stripa mine water. The reference wellwater has been analysed by our lab and by the water lab at Chalmers Technical University.

2.3 METHODS USED FOR ENUMERATION AND CULTURING OF BACTERIA

2.3.1 The total numbers of bacteria

Staining with acridine orange and counting in an fluorescence microscope was used to assay the total number of bacteria. Immediately after sampling portions of the water samples were filtered on Nucleopor membrane filters (0.2 µm, diameter = 13 mm). The volume filtered depended on the number of bacteria in the sample. Approximately 50 bacteria/sight field were aimed at. The samples were stained for 3 minutes with a solution of 10 mg acridine orange, 45 mg KH_2PO_4, 59 mg Na_2HPO_4 and 100 ml filtered and distilled water. The dried filters were examined under blue light (390-490 nm) in an fluorescence microscope (filter < 515 mm, Zeiss) at 1250 times enlargement and 15 sight fields were counted on each filter (1.1×10^{-4} cm^2/sight field).

2.3.2 The numbers of aerobic heterotrophs

The numbers of aerobic heterotrophs were determined with plate count technique on two media with

different total concentrations of the organic components, 1.5 g and 0.15 g, respectively. The media used was composed by peptone 0.5 g, yeast extract 0.5 g, glucose 0.25 g, starch 0.25 g, $CaCl_2 \cdot 2H_2O$ 0.2 g, $MgSO_4 \cdot H_2O$ 0.4 g, K_2HPO_4 0.1 g, NaCl according to the salinity of the ground water sampled, trace metals solution 1 ml, agar 15 g, distilled water 1000 ml, pH was adjusted to 7.5 after sterilization. This media is referred to as MA. The second media contained one tenth of the peptone, yeast extract, glucose and starch in MA and is referred to as MC.

The trace metal solution consisted of $ZnSO_4 \cdot 7H_2O$ 2.2 g, $CaCl_2 \cdot 2H_2O$ 7.34 g, $MnCl_2 \cdot 4H_2O$ 2.5 g, $CoCl_2 \cdot 6H_2O$ 0.5 g, $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ 0.5 g, $FeSO_4 \cdot 7H_2O$ 5 g, $CuSO_4 \cdot 5H_2O$ 0.2 g, Na_2 EDTA 50 g, NaOH for pH adjustment, distilled water 1000 ml, pH 4.0.

For dilution of the samples prior to spreading a dilution media was used. It consisted of $CaCl_2 \cdot 2H_2O$ 0.2 g, $MgSO_4 \cdot 7H_2O$ 0.4 g, K_2HPO_4 0.1 g, NaCl according to the salinity of the ground water sampled, filtered (0.2 µm) distilled water 1000 ml.

The samples were serially diluted to a concentration corresponding to the inverse of the total number of bacteria as determined by the fluorescence technique and spread in triplets on the agar plates. The MA plates were incubated at 20°C for 7 days and the MC plates for 14 days. The theory and statistical treatment behind plate counts is well treated by Niemelä (1983) and was used here.

2.3.3 The numbers of anaerobic heterotrophs

The numbers of anaerobic heterotrophs were determined as described under 2.3.2, with the following additions. The media MA and MC were supplemented with 1 g of KNO_3/l as electron acceptor and are referred to as MB and MD, respectively. The plates were incubated in an anaerobic atmosphere at 20°C with 10% CO_2 and 90% N_2 (Oxoid anaerobic system) for 7 (MB) and 14 days (MD).

2.3.4 Most Probable Number (MPN) for Thiobacilli and related bacteria

A media that was designed for enrichment of facultative anaerobic Thiobacillus and related bacteria (Kuenen and Tuovinen, 1981) was used for MPN-technique. The media (TB) consisted of $Na_2S_2O_3 \cdot 5H_2O$ 5 g, yeast extract 0.3 g, KNO_3 2 g, NH_4Cl 1 g, KH_2PO_4 2 g, $NaHCO_3$ 2 g, $MgSO_4 \cdot 7H_2O$ 0.8 g, trace

metals solution 1 ml (see 2.3.2) and distilled water 1000 ml. The pH was adjusted to 7 after sterilization. The TB media was distributed on 20 ml screw cap tubes which were completely filled with media that was cooled off after the sterilization. The dilution media under 2.3.2 was used for serial dilution of the samples. The tubes were inoculated in five parallells with 2 ml in each tube from each dilution. After 14 days of incubation growth in the tubes were confirmed with outstreaks on agar plates made of the TB-media with 15 g agar/l (Merck 1613 hochrein). Growth on a plate indicated a positive MPN-tube. The MPN:s were calculated as described by Niemelä (1983).

2.4 FACTORS LIMITING GROWTH OF MICROBIAL POPULATIONS IN GROUND WATER

2.4.1 Oxygen

Two series of five 120 ml (±10 ml) Winkler bottles were filled with well water from the reference well (2.1.1). The bottles were flooded with more than 5 times their volume. One ml of water was withdrawn from the bottles in one of the series and replaced by air. The total numbers of bacteria in the bottles were followed over 144 h in 20°C.

2.4.2 Electron acceptors and energy sources

A series of 18 Winkler bottles were supplemented with 50 µm/l each of the following substances without letting air into the bottles: No addition, K_2SO_4, $NaNO_2$, KNO_3, NH_4Cl, $FeCl_2$, CaS, K_2SO_4 + NH_4Cl, K_2SO_4 + $FeCl_2$, K_2SO_4 + CaS, $NaNO_2$ + NH_4Cl, $NaNO_2$ + $FeCl_2$, $NaNO_2$ + CaS, KNO_3 + NH_4Cl, KNO_3 + $FeCl_2$, KNO_3 + CaS, and with nutrient broth 50 µg/l, and air 1 ml. The total numbers of bacteria were counted in the bottles after 120 h in 20°C.

2.5 INVESTIGATIONS IN THE EV01 BOREHOLE

2.5.1 Time course experiment

The water coming up from the 635 m level was sampled over time to check for patchiness in the wellwater due to natural variations and/or variations in the pumping system. Water from the system outlet in the field laboratory were sampled before the 0.45 micron filter every fifth minute between 12.30 and 12.55 on 870421.

2.5.2 Sampling site experiments

One sample was taken outside the field lab on 870421 after the control unit. The flow rate increased noticeably when the tubing was discon-

nected for sampling. On 870604 samples were taken in the field lab with and without the field lab electrode system in line to evaluate eventual variance from this part of the system. A sample was also taken on the water in the borehole sond when it was taken up on the ground for calibration and a shift of the pump level. On 870826 a sample was taken from the gas sampler when it was brought to the surface. The total number of bacteria/ml of all those samples were compared with the total numbers of bacteria 1 ml from the sampling site in the field laboratory sampled at the same times.

2.5.3 The total numbers of bacteria

The total numbers of bacteria were determined in the water from the sampling site in the field laboratory at the end of the pumping period of each level.

2.5.4 The numbers of aerobic and anaerobic heterotrophs

The numbers of aerobic heterotrophs were determined in the water from the field lab and from the gas sampler 870828. The number of anaerobic heterotrophs were determined 870923 in addition to the aerobic organisms. The media MA, MB, MC, and MD were used.

2.5.5 The MPN of Thiobacilli and related bacteria

The MPN of Thiobacilli and related bacteria was determined on well water sampled 870923. Five parallel tubes were inoculated from the sample and from each of 4 serial 10-fold dilution making a total of 25 tubes. Growth were confirmed on agar plates of the same media. For details see 2.3.4.

2.6 INVESTIGATIONS IN THE STRIPA MINE BOREHOLES V1 AND V2

2.6.1 The total numbers of bacteria

The total numbers of bacteria were determined in 1 1 samples from V1, V2:1, V2:4 and V2:5. After the samples were brought to the ground surface they were processed at once in a provisional lab set up in the old mine cottage hospital.

2.6.2 The numbers of aerobic heterotrophs

The numbers of aerobic heterotrophs were determined in all four samples as described under section 2.3.2.

2.6.3 The MPN:s of Thiobacilli and related bacteria

The MPN:s of Thiobacilli and related bacteria were determined in five parallell tubes inoculated from serial 10-fold dilutions up to the inverse of the total numbers determined.

2.7 Statistical treatment of data

Statistical treatment of data was executed with SAS Institute Inc., (Statistical Analysis System, 1985) on a personal AT-computer (LCS-286). The problem of determining whether among a given set of more than two samples there are means that differ significantly has to be solved by a procedure called the analysis of variance. Pedersen (1982) and Pedersen et al., (1986) demonstrates how this procedure is used with microbial populations. This work has used the same approach adapted for microscopic counts. The data obtained were processed with the GLM (General Linear Model) procedure which uses the method of least squares to fit general linear models (SAS Institute Inc., 1985). Ryan-Einot-Gabriel-Welsch multiple F-test was used to rank means when the analysis of variance indicated a significant treatment effect. All variates were transformed to the logarithms and the effects could then be assumed to be additative.

3 RESULTS

3.1 WATER CHEMISTRY

Table 3-1 shows the chemical composition of the Ävrö test site waters. Table 3-2 shows the chemical composition of the reference well water. Table 3-3 shows the chemical composition of boreholes V1 and V2 in the Stripa mine.

Table 3-1 The chemical composition of the ground water at the Ävrö test site, borehole EV01

Level	m	635	558	522	420
Date		870420/22	870602/03	870825	870922/23
pH		6.5	7.2	7.0	6.9
Cond.	mS/m	2660	1310	680	232
Oxygen	mg/l	0.05	0.09	-	0.2
TOC	mg/l	<0.5	3.9	-	9.6
Si	mg/l	4.0	5.1	5.8	4.9
Na	mg/l	3200	1500	750	255
K	mg/l	8	6	7.4	5.0
Li	mg/l	1.2	0.55	0.21	0.0051
Ca	mg/l	2800	1100	440	162
Mg	mg/l	31	60	42	29
Sr	mg/l	-	20	6.0	5.9
Al	mg/l	0.027	0.39	0.11	0.16
Mn	mg/l	0.18	1.7	2.4	3.1
Fe_tot	mg/l	0.438	1.02	2.23	1.68
Fe^{2+}	mg/l	0.430	1.02	2.23	1.68
HCO_3	mg/l	9.9	42.3	81	1.87
Cl	mg/l	9700	4300	1970	616
F	mg/l	1.4	1.8	2.2	2.6
Br	mg/l	72	24	8.9	3.0
I	mg/l	0.72	0.32	0.10	0.06
S^{-2}	mg/l	<0.01	0.81	1.20	0.59
PO_4	mg/l	<0.003	0.010	<0.005	0.001
SO_4	mg/l	400	220	118	47
NO_2	mg/l	<0.001	<0.001	<0.001	<0.001
NO_3	mg/l	<0.01	<0.01	<0.02	<0.01
NH_4	mg/l	-	0.080	0.06	0.08

Table 3-2 The chemical composition of the 60 m deep reference well water at Hindås. The pH was 6.5

Oxygen	mg/l	<0.1	Mn	mg/l	0.18
TOC	mg/l	1.4	Fe_{tot}	mg/l	4.2
Si	mg/l	7.0	Fe^{2+}	mg/l	-
Na	mg/l	7.2	HCO_3	mg/l	62.7
K	mg/l	3.5	Cl	mg/l	12
Ca	mg/l	16.8	PO_4	mg/l	0.067
Mg	mg/l	2.8	NO_2	mg/l	0.0024
Al	mg/l	0.24	NO_3	mg/l	0.019
			NH_4	mg/l	0.140

Table 3-3 The chemical composition of the ground water at the Stripa mine test site, borehole V1 and V2.

Borehole		V1	V2:1	V2:4	V2:5
Level		505-505.9	559-822	402-410	389-397
Date		870212/0423	870212/0423	870212/0423	870212/0423
pH		9.42	10.24	9.37	9.55
Cond. µS		1460	1180	1664	425
Oxygen	mg/l	-	-	-	-
TOC	mg/l	-	-	-	-
Si	mg/l	-	-	-	-
Na	mg/l	230	210	210	87
K	mg/l	1.2	0.47	0.60	0.19
Li	mg/l	0.064	0.043	0.045	0.018
Ca	mg/l	200	110	230	31
Mg	mg/l	0.25	0.047	0.30	0.10
Sr	mg/l	1.6	1.0	1.9	0.27
Al	mg/l	0.007	0.036	0.027	0.031
Mn	mg/l	-	-	-	-
Fe_{tot}	mg/l	0.006	0.017	0.008	0.004
Fe^{2+}	mg/l	<0.005	0.015	<0.005	<0.005
HCO_3	mg/l	-	-	-	-
Cl	mg/l	580	510	700	180
F	mg/l	5.3	5.4	3.0	4.8
Br	mg/l	-	-	-	-
I	mg/l	0.15	0.29	0.19	0.030
S^{-2}	mg/l	<0.01	3.4	0.15	<0.01
PO_4^-	mg/l	-	-	-	-
SO_4	mg/l	82.4	35.6	80.9	4.83
NO_2	mg/l	0.002	<0.001	<0.001	<0.003
NO_3	mg/l	<0.010	<0.010	<0.010	<0.010
NH_4	mg/l	<0.010	<0.010	0.010	<0.010

3.2 INVESTIGATIONS OF FACTORS LIMITING OF MICROBIAL POPULATIONS IN GROUND WATER

3.2.1 Oxygen

Figure 3-1 shows that inlet of oxygen induced growth of microorganisms in the ground water. The total no of cells increased from $4.74 \cdot 10^5$ cells/ml to $1.42 \cdot 10^6$ cells/ml after 144 h. There was no significant change in total number of bacteria in the control bottles. The influence of oxygen was significant at pr > F = 0.0001.

3.2.2 Electron acceptors and energy sources

Table 3-4 shows the effect from different additions. The result is ranked after increasing effect. A growth factor is calculated that shows the increase in the total number of cells as compared to the bottles whith no addition. The following two criteria of classification analysis of variance model was used to evaluate the effect from the additions: Aghi = m + Ag + Fgh + Eghi where A is the total number of bacteria from the ith sight-field on the hth filter from the ght addition, m is the over all mean. Ag is the effect from the gth addition, Fgh is the effect from the hth filter from the ght addition. Eghi is the random sampling effect (residual). Table 3-5 shows the results of the analysis of variance. The effect from the additions is significant while the filter effect is not.

3.2.3 The number of aerobic and anaerobic heterotrophs

The number of aerobic heterotrophs in the reference well 871014 was $0.15 \cdot 10^5$ bacteria/ml and the number of anaerobic heterotrophs was $0.018 \cdot 10^5$ bacteria/ml.

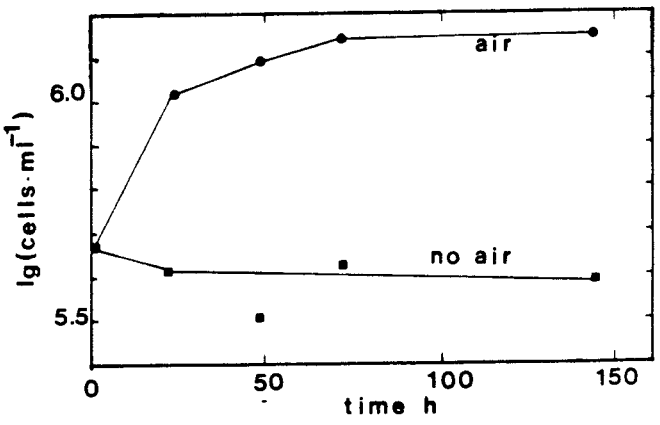

Figure 3-1 The effect from oxygen on the microbial population in the reference well water

Table 3-4 The effect from different additions on the total number of bacteria/ml in ground water after 120 h. N is the number of observations on which the mean is based. Means with the same letter of grouping are not significantly different. GF shows the increase of cells for each addition compared with no addition

Addition	Cells/ml·10^5	N	Grouping	GF
no addition	4.57	30	A	1.00
S^{2-}	4.57	30	A	1.00
Fe^{2+}	4.80	30	A	1.05
NH_4^+	4.88	30	A	1.07
SO_4^{2-}	6.07	30	B	1.32
$NH_4^+ + SO_4^{2-}$	6.22	30	D	1.36
NO_2^-	6.92	30	C D	1.51
$S^{2-} + SO_4^{2-}$	6.96	30	E C D	1.52
$Fe^{2+} + SO_4^{2-}$	7.42	30	E C	1.62
NO_3^-	7.60	30	E C	1.66
$NH_4^+ + NO_2^-$	7.99	30	E F	1.75
$NH_4^+ + NO_3^-$	8.65	30	F G	1.89
$NO_2^- + Fe^{2+}$	9.48	30	H G	2.07
$NO_3^- + Fe^{3+}$	10.6	30	H	2.32
$NO_3^- + S^{2-}$	12.1	30	I	2.64
$NO_2^- + S^{2-}$	12.6	30	I J	2.75
Oxygen (Air)	14.0	30	J	3.06
Nutrient broth	213	30	K	46.6

Table 3-5 Results from the analysis of variance on the addition experiment results. The total number of observations was 540 distributed over 36 filters from 18 additions. The overall mean was $9.04 \cdot 10^5$ cells/ml. The model explained 96.5 % of the total sum of squares

Source	Degrees of freedom	Sum of squares	Mean square (MS)	F-test	F	pr >F
Addition (A)	17	71.394	4.1996	$\frac{MS(A)}{MS(R)}$	837.1	0.0001
Filter (F)	1	0.01526	0.01526	$\frac{MS(F)}{MS(R)}$	3.03	0.0823
Residual (R)	521	2.6318	0.00500			

3.3 RESULTS FROM THE INVESTIGATIONS IN THE EV01 BOREHOLE

3.3.1 Time course experiment

Table 3-6 shows the effect from different sampling times in the field lab. There was no effect from different sampling times as can be seen in Table 3-7.

3.3.2 Sampling site experiment

Table 3-8 shows that sampling outside the field lab, in the borehole sond and in the gas sampler resulted in significantly higher numbers of bacteria. There was no effect observed from the field lab electrode system.

3.3.3 The total numbers of bacteria

The total numbers of bacteria in the field lab are shown in Table 3-9. There was an increase in the number with decreasing level. Stalked bacteria like Hyphomicrobium and Caulobacter were frequently observed.

3.3.4 The numbers of aerobic and anaerobic heterotrophs

The numbers of aerobic and anaerobic heterotrophs cultured on two media with different organic content are shown in Table 3-10. In general, except for the gas sampler, the numbers were about 100 times lower than the total number of bacteria. This factor was 10 for the gas sampler.

3.3.5 The MPN for Thiobacillus and related bacteria

The MPN for Thiobacillus and related bacteria indicated 460 bacteria/ml with 80 bacteria/ml as lower and 1470 bacteria/ml as upper 95 % confidence limits.

Table 3-6 The effect from different sampling times 870421 in EV01, level 635 m. N is the number of observations on which the mean is based. Means with the same letter of grouping are not significantly different

870421	cells/ml·10^5	N	Grouping
12.30	1.63	15	A
12.35	1.08	15	A
12.40	1.05	15	A
12.45	1.44	15	A
12.50	1.49	15	A
12.55	1.29	15	A

Table 3-7 Results from the analysis of variance on the time course experiment. The total number of observations was 90 distributed over 6 sampling times. The overall mean was 1.31 · 10^5 cells/ml. The one criteria of classification model explained only 6 % of the total sum of squares

Source	Degrees of freedom	Sum of squares	Mean square (MS)	F-test F	pr >F
Time (T)	5	0.45195	0.09039	$\frac{MS(T)}{MS(R)}$ 1.14	0.3449
Residual (R)	84	6.6502	0.07917		

Table 3-8 The effect from different sampling sites at borehole EV01. N is the number of observations on which the mean is based. Means with the same letter of grouping are not signficiantly different. pr > F is the probability of getting an F-value smaller than the obtained for the model effect

Sampling site	Date	Cells/ ml · 10^5	N	Grouping	pr > F for model
Field lab	870421	1.30	105	A	0.0001
Outside field lab	870421	4.00	15	B	
Field lab	870604	3.82	45	A	
Field lab without the electrode system	870604	3.97	30	A	0.0001
Borehole sond	870604	9.97	15	B	
Field lab	870826	3.31	60	A	0.0001
Gas sampler	870826	41.2	15	B	

Table 3-9 The total number of cells in the field lab

Date	Level	Cells/ml · 10^5	N
870421	635 m	1.30	105
870604	558 m	3.82	45
870826	522 m	3.31	30
870923	420 m	16.6	45

Table 3-10 The number of aerobic and anaerobic heterotrophs in EV01. MA-MD refer to the media composition. The numbers within parentheses indicate the distribution error under Poisson assumption of distribution

Date level	Cells/ml · 10^5 aerobes		aerobes		anaerobes	
	field lab MA	MC	gas sampler MA	MB	field lab	MD
870828 522 m	0.0219 (±0.0008)	-	2.09 (±0.797)	-		-
870923 420 m	0.0249 (±0.0009)	0.0233 (±0.0008)	-	0.0347 (±0.001)		0.0034 (±0.0003)

3.4 THE STRIPA MINE INVESTIGAITON

3.4.1 The total number of bacteria

The total number of bacteria in the boreholes V1 and V2 are shown in Table 3-11. Except for V2:1 the numbers were significantly lower than obtained in the EV01 borehole.

3.4.2 The numbers of aerobic heterotrophs

The numbers of aerobic heterotrophs are shown in Table 3-12. All V2 borehole levels contained relatively large amounts of moulds indicating close surface contact of the ground water in this borehole.

3.4.3 MPN for Thiobacillus and related bacteria

MPN for Thiobacillus and related bacteria gave the following results: V1 had 80 bacteria/ml (16, 265;· 95% confidence limits) V2:1 and V2:4 had < 10 while V2:5 had < 40. The numbers given for V2 are the upper 95% confidence limit.

Table 3-11 The total number of bacteria/ml in the V1 and V2 boreholes in the Stripa mine, sampled 870917. Mean with the same letter of grouping are not significantly different

Level		Cells/ml · 10^5	N	Grouping
V1	500-505.9 m (+ 360 m)	0.060	30	A
V2:1	559-822 m (+ 410 m)	2.26	15	B
V2:4	402-410 m (+ 410 m)	0.061	15	A
V2:5	389-397 m (+ 410 m)	0.097	15	C

Table 3-12 The number of aerobic heterotrophic bacteria and moulds in the V1 and V2 boreholes, sampled 870917. The numbers within parentheses indicate the distribution error under Poisson assumption of distribution

level	Cells/ml · 10^5			
	aerobic bacteria		moulds	
	MA	MC	MA	MC
V1 500-505.9 m (+ 360 m)	0.0005 (±0.0002)	0.0006 (±0.0002)	0	0
V2:1 559-822 m (+ 410 m)	0.0072 (±0.0008)	0.009 (±0.0009)	0.0063 (±0.0008)	0.0023 (±0.0005)
V2:4 402-410 m (+ 410 m)	0.0028 (+0.0005)	-	0.0017 (±0.0004)	-
V2:5 389-397 m (+ 410 m)	0.0051 (±0.0007)	0.0058 (±0.0008)	0.0017 (±0.0004)	0.0013 (±0.0004)

4 DISCUSSION

4.1 SAMPLING

The time course experiment showed that there was no patchiness in the total number of bacteria over times ≤ 25 minutes. The content of bacteria seems constant over time. The reference well has shown a similar stable total number over a period of 1.5 years (Table 1-1). This is consistant with the theory that predicts the number of bacteria in a system to be dependent on limiting factors. A ground water with a stable chemical composition should have a stable microbial population. This will be further developed under 4.4.

Attachment and growth of bacteria to surfaces are well known processes in microbial ecology (Pedersen 1982). Bacterial growth on the tube walls will result in a sloughing of the developed biofilms. When the EV01 system was disconnected outside the field lab. before the control-unit, there was an increase in the flow velocity due to the pressure release. This resulted in visable particles in the sample bottle and increased significantly the total number of cells registered. Sloughing of the biofilms inside the tube had occurred.

The biofilm growth on the polymid tubing surface could theoretically be enhanced by the plasticizer in the polyamide tube. Such processes are well known (Pedersen et al., 1986, Schoenen and Schöler 1985). The Stripa mine investigation was performed to elucidate if tubing material leaking organic substances could be shown to influence the results. This was not the case. V1 and V2 are considered to be boreholes draining the same ground water (Nordström et al., 1985). Still, V1 with polyamide tubing exhibited one of the two lowest total numbers and V2:1 and V2:5 with teflon tubing had significantly higher total numbers.

The tube that drains EV01 is approximatly 800 m long with a diameter of 6 mm. The water sampled in the field lab. has then passed 15 m^2 of surface area during the 2 hours of time it takes to reach the field lab. There is a large uncertainty whether the microbial populations reaching the surface are equivalent to the populations at the level drained.

This assumption is supported by the fact that both the borehole sond and the gas sampler had significantly higher total numbers of bacteria/ml than the field lab. Moreover, the heterotrophic count was 100 times higher in the gas sampler than in the field lab. sample. A possible explanation to this can be that the original population attaches to the tube walls or collapses for unknown reasons during pumping. New populations develop on the tube walls and re-enter to the flow. The bacteria in the gas sampler and the field lab. could be two different populations. This remains to be investigated.

The results presented here regarding sampling point out the gas sampler as being the sample site that gives the safest data describing the aquifer conditions with respect to microbiology.

4.2 ENUMERATIONS OF MICROORGANISMS

The total number of bacteria were about 100 times as high as the heterotrophic counts with exception for the gas sampler that had a 10 times higher total count. There are several possible reasons for this. Firstly, far from all bacteria will grow on the media used. This is a common problem in microbiology and it is impossible to suit all the different demands of different microorganisms with one media. The media used was an allround media composed to suit as many as possible. Secondly, the flourescence microscopy cannot distinguish between living (active) and dead (inactive) bacteria. Such enumeration methods are denoted "total" in microbiology terms. The results presented here show the importance of having a "total" method. Serious underestimations of bacterial numbers can be the result from investigations that rely only on plate counts.

The most probable number technique used is one example of how certain species specifically can be counted. The results presented suggest the method to be useful when expected species are looked for. The method can be further adapted to the study of ground water microbiology.

4.3 MICROBIAL SPECIES IN DEEP GROUND WATER

Deep ground water is very poor in organic nutrients. It can therefore be assumed that an indigenous microbial flora to a large extent is based on oligotrophic and/or chemosynthetic metabolisms. Several groups of bacteria solely depend on inorganic reduced energy sources, carbondioxide, inorganic electron acceptors and inorganic salts. As those organisms grow, they produce organic mate-

rial that can be used by heterotrophic bacteria. There is an almost complete lack of data concerning whether or not such microbial processes exsist and are of importance for nuclear waste repositories in deep geological formations. The findings of Thiobacilli, Caulobacter and/or Hyphomicrobium in the waters studied support the assumption above. Thiobacillli are chemolitotrophic organisms while the other two are specialists on very nutrient pore environments.

Drilling of holes in rocks usually introduces drilling fluids in the aquifer system unless a dry drilling technique is used. This, together with the fact that many bore holes stand open in contact with surface waters, introduces the question whether the observed microorganisms are indigenous or introudced? It is partly a question of accademic interest because building of repositories certainly will introduce a new flora as well as alter the environment locally in the ground. A question of greater importance then will be which microbial ecosystem will those new circumstances result in and what activity can be expected?

4.4 MICROBIAL ACTIVITY IN DEEP GROUND WATER

The cell density of continuously cultered bacterial (e.g. chemostat grown) is regulated by a limiting factor which can be the energy source, electronacceptor or any substance necessary for growth. The growth rate is regulated by the flux of substances needed for growth and have to be replenished to keep a microbial population active. If not, there can very well exsist a high number of bacteria but they are inactive. The experiment with additions in Hindås (Table 3-4) exemplifies this discussion. Introduction of a small amount of organic nutrients (nutrient broth) started a rapid growth of the anaerobic heterotrophs. Oxygen induced growth of Gallionella ferruginea registered by a heavy stalk production. A combination of an oxidized electron acceptor (e.g. NO_3) and a reduced energy source (e.g. S^{2-}) induced an increase in the cell number of chemolitotrophs close to what oxygen did. It becomes obvious that the ground water investigated harbours many different microbial species each with their own specific demands on limiting factors.

The nuclear waste repository undoubtably will change the ambient environmental conditions in the rock. A very important research area will be how this change affects the activity of the indigenous as well as the introduced microbial flora, and in turn, how this activity will influence the waste repository and migration processes. The gas samp-

ler offers a very good observation technique. It will, for instance, be possible to follow the activity of a sampled microbial population *in situ* by incubation on site in the borehole with suitable radioactive labelled substances. Uptake and turn-over rates can be studied.

4.5 TASKS TO BE SOLVED

- Data on numbers, species and activity of deep ground water microbial populations in Swedish granitic rocks have to be collected.

- Models for the interactions between a nuclear waste repository and the developing microbial populations should be set up. Actual data collected in Swedish granitic rocks should be used, merged with studies on the interactions between the backfill materials and bacteria.

- Specific studies should be performed on the effect on nuclid migration from bacteria judged by the models to be probable inhabitants in a Swedish nuclear waste repository.

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