SKB

TECHNICAL REPORT 96-01

Bacteria, colloids and organic carbon in groundwater at the Bangombé site in the Oklo area

Karsten Pedersen (editor)

Department of General and Marine Microbiology,
The Lundberg Institute, Göteborg University,
Göteborg, Sweden

February 1996

SVENSK KÄRNBRÄNSLEHANTERING AB
SWEDISH NUCLEAR FUEL AND WASTE MANAGEMENT CO

P.O.BOX 5864 S-102 40 STOCKHOLM SWEDEN
PHONE +46 8 665 28 00 TELEX 13108 SKB
FAX +46 8 661 57 19

BACTERIA, COLLOIDS AND ORGANIC CARBON IN GROUNDWATER AT THE BANGOMBÉ SITE IN THE OKLO AREA

Karsten Pedersen (editor)

**Department of General and Marine Microbiology,
The Lundberg Institute, Göteborg University,
Göteborg, Sweden**

February 1996

Bacteria, colloids and organic carbon in groundwater at the Bangombé site in the Oklo area

Karsten Pedersen (editor)	Department of General and Marine Microbiology The Lundberg Institute Göteborg University Medicinaregatan 9C 413 90 Göteborg Sweden tel. +46-31-7732578 fax +46-31-7732599 E-mail pedersen@gmm.gu.se
Johanna Arlinger	Department of General and Marine Microbiology
Roland Bruetsch	PSI, Villigen, Switzerland
Claude Degueldre	PSI, Villigen, Switzerland
Lotta Hallbeck	Department of General and Marine Microbiology
Marcus Laaksoharju	GeoPoint AB, Sollentuna, Sweden
Théophile Lutz	TATrace analytic SA, Morges, Switzerland
Catharina Pettersson	Department of Water and Environmental Studies Linköping University 581 83 Linköping, Sweden

FEBRUARY 1996

Key words: 16S rRNA, bacteria, Bangombé, colloids, diversity, DNA sequencing, humic substances, Oklo, phylogeny, organic carbon.

ABSTRACT

Natural analogues are investigated to understand long-term geological processes as part of the task to develop safe and reliable concepts for disposal of radioactive waste. Among many different repository aspects that must be assessed are stability of the waste and the engineered barriers, behaviour of the geological system that hosts the repository, the potential migration of radionuclides in the geosphere as well as the influence of microorganisms, colloids, and organic matter on repository performance. The Oklo region contains the only known examples of natural fission reactors and is therefore, perhaps, one of the best known natural analogues for the geological disposal of radioactive waste. This report describes how microorganisms, colloids and organic matter were sampled from groundwater in 1993 and 1994 from six boreholes at the Bangombé site in the Oklo region and subsequently analysed.

For analysis of microrganisms, DNA was extracted from groundwater, amplified and cloned and information available in the ribosomal 16S rRNA gene was used for mapping diversity and distribution of bacteria. The results showed that this site was inhabited by a diversified population of bacteria. Each borehole was dominated by species that did not dominate in any of the other boreholes; a result that probably reflects documented differences in the geochemical environment. Two of the sequences obtained were identified on genus level to represent *Acinetobacter* and *Zoogloea*, but most of the 44 sequences found were only distantly related to species in the DNA database. The deepest borehole, BAX01 (105 m), had the highest number of bacteria and also of total organic carbon (TOC). This borehole harboured only Proteobacteria beta group sequences while sequences related to Proteobacteria beta, gamma and delta groups and Gram-positive bacteria were found in the other four boreholes. Two of the boreholes, BAX02 (34 m) and BAX04 (10 m) had many 16S rRNA gene sequences in common and they also had similar counts of bacteria, content of TOC, pH and equal conductivity, suggesting a hydraulic connection between them.

The colloid sampling at Bangombé was conducted from four boreholes in July 1994 and the analyses comprised: colloids on membrane for scanning electron microscopy (SEM) analysis, colloids on membrane for ICP-MS analysis, and groundwater samples in bottles for single particle analysis. The results from the investigations carried out by the 3 analytical procedures were consistent. The colloid concentration in these Na-Mg-Ca-HCO_3 type waters of pH 6-7 and slightly negative Eh was rather low, about 20-100 ppb. This low colloid concentration was a consequence of relative concentrations of calcium, magnesium and sodium in the water which reduce colloid concentration because these cations act as a colloid cement (aggregation, sticking) in the aquifer. However, the presence of Fe(II) induces a large potential of artefact material. Trace element results show that transition metals and some heavy metals are associated with the colloid phase. Iodine, sulphur and selenium may be trace components of the organic colloids.

Sulphur and selenium may be associated with transition (Cu, Zn, Fe, Ni, Pt, etc.) and heavy metals (Pb) in the colloid phase. Distribution coefficients of trace elements between the water and colloid phases (K_p) were estimated. For example, for uranium, an average of 200 pg ml^{-1} was detected in the water, and 40 pg ml^{-1} was detected in the colloid phase. For uranium, a K_p value of $2 \cdot 10^6$ ml g^{-1} was calculated considering [colloid] = 100 ng ml^{-1}. With this large K_p value, it is likely that uranium is not only sorbed but also associated with groundwater colloids.

Groundwater samples were collected for analysis of the concentration of organic carbon (TOC), humic substances and metals associated with the humic substances. Humic substances and associated metals were isolated on a weak anion exchange resin. TOC varied in the range 4-14 mg l^{-1} in BAX01, BAX02 and BAX03 whereas BAX04 had a TOC of <1.5 mg l^{-1}. The result of the isolation procedure indicated that humic substances comprised only a minor fraction (<3%) of the TOC which is in agreement with results obtained in studies performed with groundwater from granitic bedrock where, however, the TOC in general is only a few mg/l. The molecular weight distribution, determined with gel filtration, indicated that the humic matter consisted of fractions with different molecular weights. The presence of a low molecular weight fraction suggests ongoing subsurface processes in which the humic substances are decomposed. The metal speciation study indicated that a large fraction, i.e. 8-67%, of uranium (U) was bound to the humic matter compared to the fractions of Ca and Fe (<0.4% and 0.02-10%, respectively). The largest fraction of U associated with humic substances was found in BAX03, i.e. in the reactor.

SAMMANFATTNING

Naturliga analoger studeras för att öka förståelsen av geokemiska processers inverkan på slutförvar av utbränt kärnbränsle. Många olika egenskaper hos ett sådant slutförvar måste utvärderas innan förvaret byggs. Stabiliteten hos avfallet och de konstruerade barriärerna, det omgivande bergets egenskaper samt rörligheten hos olika radionuklider i förvarsmiljön och i det omgivande berget är alla exempel på viktiga egenskaper. Radionukliders rörlighet kan påverkas av de mikroorganismer, kolloider och organiska ämnen som finns i grundvatten och förekomsten av dessa komponenter måste därför också studeras. De naturliga kärnreaktorerena i Oklo är det enda kända exemplet där kärnklyvning skett utan mäniskans medverkan. Man anser att detta område är väl lämpat för studier av hur ett förvar kan komma att påverkas av sin omgivning i ett långsiktigt geologiskt tidsperspektiv. Denna rapport beskriver hur mikroorganismer, kolloider och organiska ämnen i grundvatten från 6 borrhål (BAX01-05 och 07) kring Bangombé i Oklo-området provtogs och analyserades.

Förekomst av mikroorganismer anayserades genom att DNA (arvsmassa) extraherades från grundvattnet, mångfaldigades, klonades och sekvensbestämdes. Genen för ribosomalt RNA, 16S rRNA, utnyttjades för bestämning av diversitet och fördelning av mikroorganismer i Bangombés grundvatten. Resultaten visar att dessa grundvatten innehöll en rad olika arter av bakterier. Varje borrhål dominerades av en egen bakterieart som inte dominerade något av de andra undersökta borrhålen; ett resultat som troligen speglar skillnaderna i den geokemiska miljö varje borrhål utgör. Två av de funna sekvenserna kunde identifieras såsom tillhöriga släktena *Acinetobacter* och *Zoogloea*, men de flesta av de funna 44 olika sekvenserna var bara avlägset besläktade med de sekvenser som finns deponerade i internationella DNA databaser. Det djupaste borrhålet, BAX01 (105 m), hade flest bakterier per volym och hade också den högsta halten organiskt kol (TOC). I detta borrhål fanns bara skevenser som grupperade med Proteobakteriernas betagrupp, medan de övriga fyra studerade borrhålen också innehöll sekvenser som grupperade med Proteobakteriernas beta- gamma- och deltagrupper samt med Grampositiva bakterier. Två av borrhålen, BAX02 (34 m) och BAX04 (10 m) hade många gemensamma sekvenser, lika stora totalantal av bakterier samt snarlika värden på TOC, pH och konduktivitet, vilket kan tolkas som om dessa två borrhål står i nära hydraulisk kontakt med varandra.

Provtagning för analys av kolloider i grundvatten runt Bangombé gjordes i fyra av borrhålen under juli 1994. Följande analyser utfördes: Kolloider provtogs på membran för undersökning i svepelektronmikroskop (SEM) och ICP-MS samt i flaskor för partikelanalys. Resultaten från de tre olika tillvägagångsätten för kolloidanalys var samstämmiga. Halterna av kolloider var relativt låga, ca 20-100 ppb. De låga kolloidhalterna förklaras troligen av det fanns förhållandevis höga koncentrationerna av kalcium, magnesium och natrium i vatten, vilka tillsammans fungerar som ett kolloidalt cement (aggregatbildning och vidhäftning) i

aquifärerna. Förklaringen är dock osäker eftersom höga halter av järn(II) kan resultera i felaktiga värden. Spårelementanalyserna visar att övergångsmetaller och vissa tungmetaller förekom i förening med kolloidfasen. Jod, svavel och selen förekom, troligen också tillsammans med övergångs- (Cu, Zn, Ni, Pt, etc.) och tungmetallerna (Pb), i kolloidfasen. Fördelningskoefficienterna för spårelement mellan grundvatten och den kolloidala fasen (K_p) beräknades för olika metaller. För uran uppmättes ett medelvärde på 200 pg ml^{-1} i vattnet och 40 pg ml^{-1} i den kolloidala fasen. Med dessa ingångsvärden och en kolloidhalt på 100 ng ml^{-1} beräknades ett K_p värde på $2 \cdot 10^6$ ml g^{-1}. Detta höga värde indikerar att uran inte är ytsorberat på kolloiderna utan istället utgör en genomgående fraktion av kolliderna.

Grundvatten provtogs också för analys av totalhalten organiskt kol (TOC), humusämnen och av halten metaller i förening med humusämnena. Humus och metaller isolerades på en svag jonbytare. TOC varierade mellan 4 och 14 mg l^{-1} i BAX01, BAX02 och BAX03, medan BAX04 hade ett TOC-värde som understeg 1.5 mg l^{-1}. Resultaten pekar på att humus utgjorde en mycket liten del av TOC (<3%). Molekylviktfördelningen bestämdes med gelfiltrering och visade att humusämnena utgjordes av flera fraktioner. Den låga halten humus tolkas som ett resultat av pågående humusnedbrytning i aquifärerna. Metall-sorptionsmätningarna tyder på att en stor del av uranet i grundvattnet (8-67%) var bundet till humusämnen och att endast en mindre del var bundet till kalcium och järn (<0.4 och 0.02-10% respektive). Störst mängd uran bundet till humus påträffades i BAX03, dvs. i själva reaktorn.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

SUMMARY AND CONCLUSIONS

Long-lived radioactive waste, produced by the nuclear energy industry and from other sources, must find safe disposal. Many countries are planning to build underground repositories for the disposal of such waste. Among all different repository aspects that must be assessed are stability of the waste and engineered barriers, migration of radionuclides in geological environments, behaviour of the geological system that surrounds the repository as well as the influence of groundwater components such as microorganisms, colloids, humic substances and organic matter on repository performance. Long-term predictions due to these components acting on repository functions such as radionuclide retardation, are as difficult to make as most other predictions of long-term behaviour of engineered systems in natural environments. Natural analogues are consequently valuable, so as to predict the possible effect from microbial activity in a future radioactive waste repository. The Oklo region contains the only known examples of natural fission reactors and is therefore, perhaps, the best known natural analogue for geological disposal of radioactive waste.

Understanding the microbial part of biogeochemical processes which may have influenced radionuclide migration from the Oklo natural analogue requires information about the diversity and distribution of present bacteria. Traditional studies of the diversity of microbial analogue communities have been incomplete because of an inability to identify and quantify all contributing populations. New methods in molecular biology are now being used to overcome this problem. Part of this report describes how groundwater was sampled, DNA extracted, amplified and cloned and how information available in the ribosomal 16S rRNA gene was used for mapping diversity and distribution of subterranean bacterial populations at Bangombé in the Oklo region. The total number of bacteria was also determined.

Colloid studies using systems operative over long time spans are useful because they clarify phenomena which can never be tested under laboratory conditions. The colloid study of the Poços de Caldas project enabled a discussion of the potential of colloids within much more extensive space and time scales than those described in laboratory tests. The advantage of the Oklo system with Bangombé in this context is that it deals with a different type of groundwater, more reducing than the Poços de Caldas waters and with a well defined $^{235}U/^{238}U$ signature. One aim of this study was to assess the role of the colloid in transport of radionuclides in the Bangombé system with emphasis on the depleted uranium isotopic signature provided by nuclear criticality two billion years ago.

To estimate complexing and transporting capacity of the humic substances in different environments it is necessary to analyse concentration and composition of humic matter under natural conditions. The Bangombé natural reactor and its surroundings provide an opportunity to estimate complexing capacity for humic matter in an environment with increased concentrations of actinides. In this report sampling and analysis of humic matter and organic substances have been

described as well as how the results were used to estimate the role of natural organic matter for mobilisation and transport of metals and radionuclides. Unfortunately, the methods used in this study cannot separate the organic content from the inorganic content in the colloids.

The following results and conclusions were obtained during this investigation:

Bacteria

The 16S rRNA gene sequencing, of DNA extracted from the subterranean environment at the Bangombé site, showed that it was inhabited by a diversified microbiota. Each borehole was dominated by species that did not dominate any of the other boreholes; a result that probably reflects documented differences in the geochemical environment. Two of the boreholes, BAX02 and BAX04 contained many common 16S rRNA gene sequences in common and they also had similar bacterial counts of TOC, pH and equal conductivity, suggesting that these boreholes are hydrologically connected. The Bangombé natural analogue for a radioactive waste repository was inhabited by many different bacteria. This supports the idea that bacteria will also inhabit constructed repositories. The next step will be to gather information about the predominating species and their in situ activity. Culture media can now be directed towards these bacteria and the species of interest may be selected using nucleic acid probes and optical tweezers as described recently. The influence of bacterial activity on their environment must be studied further important parameters to study relating to radionuclide mobility, are redox effects, production of complexing agents and mobility of bacteria and uptake of radionuclides by bacteria.

Colloids

The results from three analytical procedures SEM/EDS, ICP-MS and Single Particle Counting were consistent. Particle counts from BAX02 and BAX04 groundwaters with a single particle spectrometer correspond to SEM particle counts on membranes. Evaluation of mass concentration with density 2 (average for SiO_2 and clay) from both particle counting and SEM analysis was consistent and a concentration in the range of 20-60 $ng \cdot ml^{-1}$ is acceptable for a size range of 10-1000 nm. The obtained concentration from the elementary analysis of colloid cakes with oxides was smaller because Si is not determinate. However, locally, one large particle (no preliminary separation at 1000 nm) may increase the mass concentration. Pareto coefficient b (<4) indicates that the mass concentration increases with colloid size. However, there must be an upper size cut-off dictated by sedimentation in the borehole during pumping. Trace element results show that transition metals and some heavy metals were associated with the colloid phase. Iodine, sulphur and selenium may also be associated with organic colloids. Sulphur and selenium were associated with transition (Cu, Zn, Fe, Ni, Pt etc.) and heavy metals (Pb) in the colloid phase. Because of an expected low Eh, sulphate results may be due to sulphide oxidation.

TOC and humic substances

TOC varied in the range 4-14 mg l^{-1} in BAX01, BAX02 and BAX03 whereas in BAX04 TOC was <1.5 mg l^{-1}. Humic substances comprised only a minor fraction (<3%) of the TOC. The humic substance from BAX02, BAX03 and BAX04 consisted of fractions with different molecular weights, whereas the humic matter from BAX01 had a more homogeneous character. Traces of LMW organic acids were detected, although they could not be identified nor verified. A large fraction, i.e. 8-67%, of the uranium was bound to the humic matter compared to the fractions of Ca and Fe (<0.4% and 0.02-10%, respectively). The largest fraction of U associated to humic substances was found in BAX03, i.e. in the reactor.

1 INTRODUCTION

The discovery in 1972 of isotopic anomalies in the Oklo uranium deposit in Gabon, Africa, was an important scientific event which revealed the existence of natural nuclear reactions in a distant past (Bodu et al, 1972). The Oklo reactors became critical about 2 billion years ago and operated intermittently for some 10^5 to 10^6 years. Roughly 1000 to 2000 tonnes of uranium were initially present as fuel of which about 6-12 tons consisted of ^{235}U that underwent fission and produced some 4 tonnes of plutonium. The reactors operated at temperatures of up to 600°C and pressures of 800 to 1000 bar (Miller et al, 1994).

Long-lived radioactive waste, produced by the nuclear energy industry and from other sources, must find safe disposal. Many countries are planning to build underground repositories for the disposal of such waste. Although it may be possible to make reasonably accurate predictions of the outcome of single processes in a repository, the long-term behaviour of the total geological environment is very complex. Therefore, natural analogues, or natural systems, are investigated to gain an understanding of long-term geological processes as part of the task to develop safe disposal concepts (Miller et al, 1994). The generally accepted definition of the term 'natural analogue' is "An occurrence of materials or processes which resemble those expected in a proposed geological radioactive waste repository".

Among all different repository aspects that must be assessed are stability of the waste and engineered barriers, migration of radionuclides in geological environments, behaviour of the geological system that surrounds the repository as well as the influence of groundwater components such as microorganisms, colloids, humic substances and organic matter on repository performance (Pedersen and Karlsson, 1995). Long-term predictions due to these components acting on repository functions such as radionuclide retardation, are as difficult to make as most other predictions of long-term behaviour of engineered systems in natural environments. Natural analogues are consequently valuable (Brandberg et al, 1993), so as to predict the possible effect from microbial activity in a future radioactive waste repository. The Oklo region contains the only known examples of natural fission reactors and is therefore, perhaps, the best known natural analogue for geological disposal of radioactive waste. It has been investigated by multi-disciplinary consortia of researchers and new achievements were recently published (Blanc and Maravic, 1995).

1.1 BACTERIA

Understanding the microbial part of biogeochemical processes which may have influenced radionuclide migration from the Oklo natural analogue requires

information about the diversity and distribution of present bacteria. Traditional studies of the diversity of microbial analogue communities have been incomplete because of an inability to identify and quantify all contributing populations. New methods in molecular biology are now being used to overcome this problem (Amann et al, 1995; Ekendahl et al, 1994). Genotypic identification of bacteria using sequence information available in ribosomal RNA genes has become a very important complement to phenotypic identification and classification techniques (Boivin-Jahns et al, 1995). Part of this report describes how groundwater was sampled, DNA extracted, amplified and cloned and how information available in the ribosomal 16S rRNA gene was used for mapping diversity and distribution of subterranean bacterial populations at Bangombé in the Oklo region. The total number of bacteria was also determined, concomitant with analysis of the concentration of total organic carbon (TOC).

1.2 COLLOIDS

Increasing evidence indicates that, under some subsurface conditions, components dispersed in water may exist as colloids and suspended particles which are transported with the flowing groundwater. Contact of radionuclides with this mobile phase may enhance the rate of radionuclide transport (McCarthy and Zachara, 1989). Accurate assessment of radionuclide migration requires fundamental understanding of the potential role of colloidal particles in possible radionuclide transport in the subsurface.

In models developed to describe radionuclide transport by a colloid facilitated mechanism, it was recently demonstrated that for a fractured crystalline rock, the colloid effect on radionuclide transport may be negligible even when colloids' attachment onto the host rock is nonexistent; if the product of the colloid concentration and the sorption coefficient of the radionuclides onto the colloids is less than 1 (Smith and Degueldre, 1993). This study was carried out on fractured crystalline bedrock in Switzerland with about a 1 km pathway from the near field to the biosphere. The model assumes a reversible radionuclide sorption on the colloids. However, when sorption is not reversible, the situation is more complex, and, worse still, when sorption is totally irreversible, attachment of the colloids on the rock must be taken into account. This behaviour induces colloid retention which can slow down a contaminated colloid front. These phenomena are too complex to study under laboratory conditions since, with a far field of one kilometre, time constants relate to decades, centuries or even longer time scales. Colloids on which nuclides were sorbed irreversibly were considered to carry the activity in the groundwater. This is the most conservative way to estimate the doses transported by the colloids in the geosphere.

Colloid studies using systems operative over long time spans are useful because they clarify phenomena which can never be tested under laboratory conditions. The colloid study of the Poços de Caldas project enabled a discussion of the potential of colloids within much more extensive space and time scales than those

described in laboratory tests (Miekeley et al, 1992). The Poços de Caldas system is, however, not entirely similar to a Swedish far-field because pH of this K-SO_4 groundwater is lower than that expected in a Swedish far field and in addition the host rocks are highly weathered and no longer crystalline. A debate concerning colloid transport was actually based on the rare earth element signatures. Even if these elements are not directly relevant for safety analysis, they are, however, analogues of trivalent actinides. The advantage of the Oklo system with Bangombé in this context is that it deals with a different type of groundwater, more reducing than the Poços de Caldas waters and with a well defined $^{235}U/^{238}U$ signature. One aim of this study was to assess the role of the colloid in transport of radionuclides in the Bangombé system with emphasis on the depleted uranium isotopic signature provided by nuclear criticality two billion years ago.

1.3 ORGANIC CARBON

Organic carbon in groundwater can be divided into dissolved, particulate and colloidal organic matter. Some of these fractions are important in processes involved in mobilisation and transport of metals. Complex formation occurs between metals and charged groups on organic matter, primarily carboxylic and phenolic functional groups. Thus, organic acids of different size are of special interest. In groundwater, low-molecular-weight (LMW) organic acids often comprise the largest fraction (i.e. 50%) of organic matter while high-molecular-weight (HMW) organic acids like humic substances comprise about 15% (Malcolm, 1991). Humic substances form very strong complexes with highly charged metal ions, e.g. the actinides (Choppin and Allard, 1985) but also LMW organic acids form complexes with the metals, although those complexes are weaker (Brynhildsen, 1991). LMW organic acids are produced by microorganisms as exudates or formed during decomposition of humic substances originating from ground surface. Colloids consist partly of organic matter, especially humic substances, which has an impact on the surface charge and thus influences their ability to bind metals (Ledin, 1993). Besides competition between metals for available binding sites on organic acids, the ability to form complexes varies for different metals depending on e.g. charge and size. Therefore, to estimate complexing and transporting capacity of the humic substances in different environments it is necessary to analyse concentration and composition of humic matter under natural conditions. The Bangombé natural reactor and its surroundings provide an opportunity to estimate complexing capacity for humic matter in an environment with increased concentrations of actinides. In this report sampling and analysis of humic matter and organic substances have been described as well as how the results were used to estimate the role of natural organic matter for mobilisation and transport of metals and radionuclides. Unfortunately, the methods used in this study cannot separate the organic content from the inorganic content in the colloids.

2 MATERIALS AND METHODS

2.1 SITE DESCRIPTION

The Bangombé reactor was discovered in the 1980's and is located some 30 km south of the Oklo deposit. This reactor zone occurs at a depth of 16 m and the investigated boreholes were drilled during 1992 to evaluate the geological, hydrogeological and hydrochemical surroundings of the reactor as shown in Fig. 2.1. The boreholes were packed-off at different depth sections and a submersible pump was used to bring groundwater up to ground surface. Several borehole volumes were pumped for a day or two before sampling to ensure clean and representative aquifer water.

Details of planning strategy, drilling, hydraulic testing and groundwater sampling of the boreholes are given in Smellie & Winberg (1992), Smellie et al (1993), Blanc (1993), Winberg and Smellie (1993). An overview of the Bangombé study, including initial results and discussion of groundwater chemistry, is given by Toulhoat et al (1994) and also by Louvat (1994). Preliminary details of mineralogy, trace element geochemistry and isotope geochemistry of the Bangombé reactor zone and host rock environment are summarised and edited by Gauthier-Lafaye (1994).

Table 2.1 presents borehole information and groundwater chemistry data that provide a background for interpretation of the obtained bacteria, colloid and organic matter data.

Table 2.1 Borehole information and groundwater chemistry data for the Bangombé nuclear reactor zone.

Borehole	Screened section, m	Sample date	Temperature °C	E_h mV	pH	Conductivity mS/m
BAX01	96-105	06-03-1993	28.6	74	7.0	272
		09-07-1994	27.6	12	6.5	227
BAX02	27.2-33.9	03-03-1993	28.3	35	6.2	89
		12-07-1994	27.1	67	5.7	64
BAX03	11.9-12.5	03-03-1993	29.0	26	6.7	162
		10-07-1994	29.0	132	6.0	104
BAX04	8.9-10.2	03-03-1993	26.1	130	5.9	67
		13-07-1994	28.3	104	5.5	61
BAX05	23.8-31	06-03-1993	26.1	147	6.6	89
		14-07-1994	26.7	-36	6.0	112
BAX07	4.5-6.5	11-07-1994	28.0	69	4.8	15

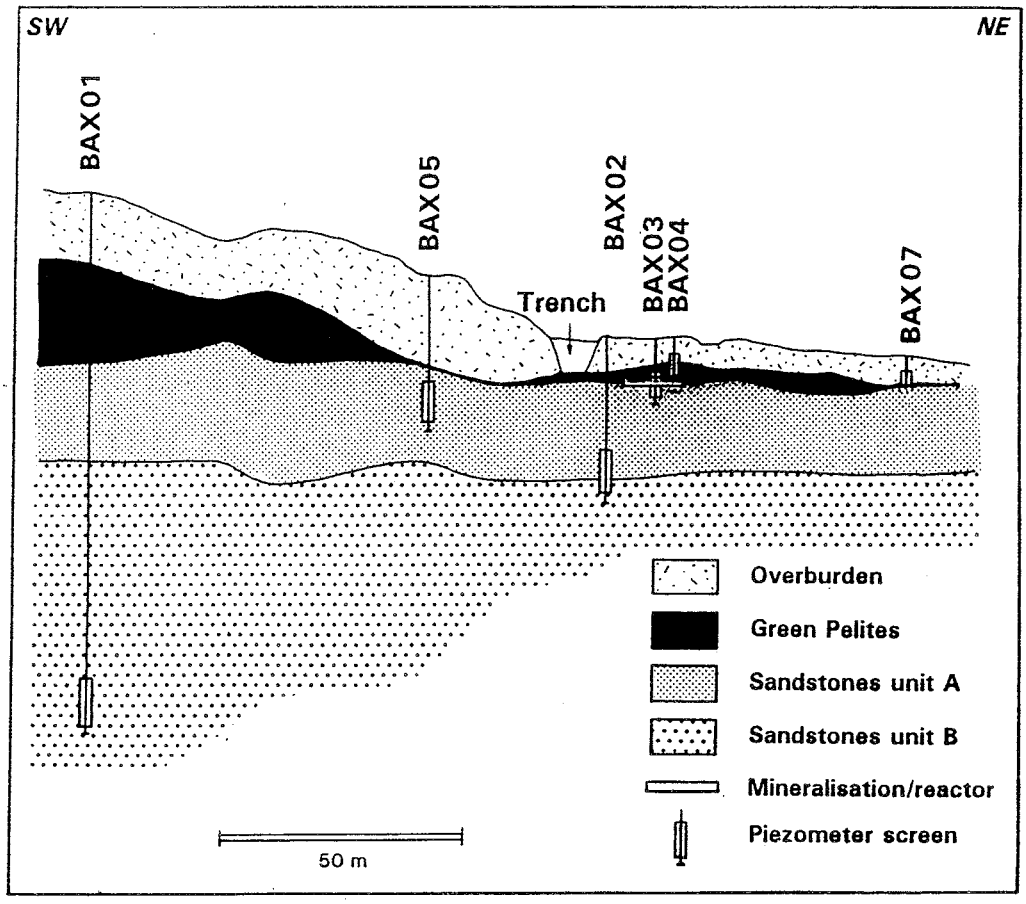


Figure 2.1 Conceptual geological section of the Bangombé reactor showing the location of the boreholes and screen positions. The depths of the boreholes are as follows, BAX01, 105 m; BAX02, 33.9 m; BAX03, 12.5 m (reactor zone); BAX04; 10.2 m, BAX05, 31m; BAX07, 6,5m.

The major rock types of the Bangombé site under the overburden are green pelites, sandstone unit A and sandstone unit B. The reactor is located between the green pelites and the sandstone unit A. It is characterised by a high concentration of uranium (mostly uraninite, 20-60% U) and by fission products whose end-member isotopic compositions are very different from those of the natural elements. The reactor ores are usually, but not always, surrounded by a clay halo; illite + Mg-Al chlorite adjacent to core, with the chlorite becoming more Fe-rich towards the surrounding sediments, quartz is largely absent. The clay features have been formed by conversion of the original sandstone during thermal convective circulation of water, initiated by heat released from radionuclear fission reactions (Gauthier-Lafaye and Weber, 1989).

2.2 SAMPLING FOR DETERMINATION OF THE TOTAL NUMBER OF BACTERIA

Samples of 40 ml from BAX01, BAX02, BAX03, BAX04 and BAX05 (only 1993) were preserved with formaldehyde (2% final concentration) and transported to the laboratory in Göteborg. During the July 1994 expedition, sampling was repeated three times with intervals of approximately one hour.

2.2.1 Determination of the total number of bacteria

Acridine orange direct count (AODC) (Hobbie et al, 1977) was used to determine the total number of cells in the water. Nuclepore filters of 0.2 μm pore size and 13 mm in diameter were pre-stained with Sudan-black and air dried. All solutions were filter sterilised (0.2 μm). The sample was diluted two-fold its volume with 0.1% oxalic acid and vigorously shaken to reduce clogging of the filters. A portion of the sample was filtered onto a pre-stained Nuclepore filter at -20 kPa and stained for 6 minutes with acridine orange. The number of bacteria was counted using blue light (390-490 nm) under an epifluorescent microscope (Olympus BH-2). Two filters were counted for each water sample. Between 500 and 600 cells or a minimum of 15 fields (0.0064 mm^2 each) were counted on each filter. This procedure should predict a sample mean with a precision of 5 % (Hallbeck and Pedersen, 1990, Niemelä, 1983). The results were calculated as mean values of two filters from each sample.

2.2.2 16S rRNA gene extraction, DNA-amplification, cloning and sequencing

Groundwater samples from BAX01, BAX02, BAX03, BAX04 and BAX07 were collected directly from the boreholes using a syringe at the outflow of the tubing connected to the submersed borehole pump; 10 ml samples filtered onto sterilised 0.2 μm pore-sized Nuclepore filters. The filters were deep frozen with dry ice (approximately -25°C) and transported deep frozen to the laboratory in Göteborg. The sampling was repeated three times with intervals of approximately one hour concurrently with sampling for the total number of bacteria and TOC. BAX07 was sampled once only.

The protocol for DNA extraction used in this report is based, with minor modification, on the procedure described by Marmur, (1961) and Wallace, (1987). The filters were resuspended in 760 μl of 20 mM Tris-HCl, pH 8.0; 20 mM EDTA; 0.35 M sucrose and incubated with 2 mg ml^{-1} lysozyme (Sigma) at 37 °C for 1 h to destroy cell walls. Thereafter the cells were lysed by adding 40 μl 20% Sodium Dodecyl Sulphate (SDS) and proteins were digested with 200 μg ml^{-1} proteinase K (Sigma) during an additional incubation at 60 °C for 1 h. The mixture was extracted with an equal volume phenol: chloroform: isoamyalcohol (25:24.1), and thereafter 3 extractions with chloroform: isoamylalcohol (24:1, called chisam) so that no cell debris was visible. The DNA obtained was precipitated with 1/3 volume of 10 M NH_4Ac (final concentration 2.5 M) and 2.5 volumes of 99% ethanol. To ensure complete precipitation, 50 μg tRNA was added as a coprecipitant and the mixture was incubated at -70 °C overnight. The precipitate was washed with 100 μl 70 % ethanol (v/v) and dried in vacuum for 30 s, dissolved in TE-buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) overnight and stored at -20 °C.

One μl of the extracted DNA solution was added to a mixture of 10 μl of 10xPCR buffer (Polymeras Chain Reaction) (Stratagene), 0.2 mM of each nucleotide triphosphate, 0.25 μM of each primer and double distilled water to a final volume

of 100 µl. The samples were treated with 10 mg ml^{-1} of RNase A (Sigma) for 15 minutes at 37°C and incubated at 95°C for 5 minutes, before addition of 1 µl Pfu DNA polymerase (Stratagene) and coating with 100 µl mineral oil (Sigma). A total of 30 cycles were performed at 95°C (30 s), 55°C (1 min), 72° C (2 minutes) followed by a final incubation at 72°C for 10 minutes.

The 5' and 3' primers used matched the universally conserved positions 519-536 and 1392-1404, *E. coli* Brosius numbering (Brosius et al, 1978). These were chosen to ensure that bacterial, archaeal and eukaryal species could be amplified. The amplification products were purified with the QIAEX agarose extraction kit (Qiagen) following the manufacturer's specifications and were finally diluted in 20 µl TE-buffer and stored at -20°C.

The purified samples were cloned with the pCR-Script SK(+) cloning kit (Stratagene) following the manufacturer's specifications. From each DNA extraction, a total of 10 white colonies containing the insert was randomly picked. The colonies were inoculated in 3.5 ml LB (Luria Broth)+Ampicillin overnight at 37°C. From each culture 0.5 ml was suspended in 0.5 ml of concentrated glycerol and stored at -80°C. The recombinant plasmids were extracted from the bacteria with the Wizard Magic miniprep kit (Promega).

The sequencing was accomplished using an Autoread Sequencing kit (Pharmacia Biotech) following the manufacturer's instructions. All clones were sequenced using the 3' primer 907-926 (Ekendahl et al, 1994) labelled with fluorescein. Gel electrophoresis was performed on an ALF DNA Sequencer (Pharmacia Biotech). Sequences obtained from the three separate sampling occasions were pooled and compared directly on the monitor of the ALF manager OS/2 computer, using the ALF manager program, version 2.5. Clones that were identical were put in the same group.

2.2.3 Sequence analysis

The 16S rRNA gene clones were compared to sequences available in the European Molecular Biology Laboratory (EMBL) database using the FastA procedure in the GCG program package (Genetic Computer Group, Wisconsin, USA). This procedure calculates identities between an unknown sequence (clone) and sequenced bacteria in the database. The phylogenetic analysis was performed by using programs contained in the PHYLIP version 3.5c package (Felsenstein, 1989) compiled for a PC. Nucleotide positions that could be unambiguously aligned for all clones were included in the analysis. The final data set comprised 353 nucleotide positions, position no. 542 - 883 (*E.coli* Brosius numbering, but not including 12 inserts in the analysed data set, [Brosius et al, 1978]). The distances were calculated using the DNADIST program and a tree was built running the KITCH program with contemporary tips. KITCH was run with a randomised input order of data with 10 jumbles and during execution, 171,833 trees were examined. The tree was drawn by using a drawing program, DRAWTREE, also available in the PHYLIP package.

2.3 COLLOID SAMPLING

The colloid sampling at Bangombé in July 1994 was conducted in four boreholes: BAX01, BAX02, BAX03 and BAX04 (Fig. 2.1). The sampling took place at the well head according to a protocol derived from studies by McCarthy & Degueldre (1993) and Laaksoharju et al (1994). Since their previous studies demonstrated the potential of artefact production by oxidation, particular attention was paid to flow optimisation and artefact-free sampling. Artefact-free sampling refers to a sampling procedure where known in-situ, on-line or offline disturbances on the colloid concentration have been minimised.

The sampling strategy took the following considerations into account:

- The sampling was performed with minimum modification of water chemistry from in-situ downhole conditions (in the aquifer) when pumping towards the ground surface (at well head). Modifications of the water flow rate in the water conducting zone during sampling were estimated in order to avoid any kind of particle or macromolecule presence which was not associated with the natural fluid phase. This natural flow rate was estimated by considering the hydraulic conductivity in the borehole section connected to the aquifer. This limited the pumping rate to less than 200ml min^{-1}.
- Particular attention was paid to artefact generation possibilities such as tubing interaction with groundwater (which can generate colloids by dissolution or because of tubing material allowing strong groundwater colloids to stick) and groundwater degassing (from in-situ to the surface) causing colloid generation due to chemistry changes of the groundwater. It was finally identified that any oxidation of this reducing water may induce strong colloid generation (iron oxy-hydroxide) while no significant pH changes and complementary calcite precipitation were to be expected.
- The sampling was carried out at the well head, within a glove-bag under N_2. Table 2.2 summarises the conditions under which the colloid samples were prepared. The following samples were prepared:
- Colloids on membrane for SEM analysis. The membranes were Amicon (1.2 cm^2 active surface) XM50. They consisted of a polyacrylamide surface membrane with 3 nm pores, carried on a cellulose support. After filtration the colloid phase was washed with a minimum of demineralised water, dried, and carefully set in a clean container prior to transport and SEM sample preparation.
- Colloids on membrane for ICP-MS analysis. These membranes were used without further treatment. After colloid collection, the colloid cake was washed with a minimum (1ml) of pure water to remove salt precipitation. The samples were dried on site and placed in small clean containers ready for transport.
- Fluid samples (groundwater in bottles) for single particle analysis. The fluid samples were obtained by diluting (50%-50%) groundwater with ultra-pure water. This treatment minimises the risk of calcite precipitation during transport. The blank consisted of pure water transported to site and returned to the laboratory.

Table 2.2 Samples prepared on site.

Borehole	Membrane type	Cut-off nm	Water volume ml
BAX01	Amicon	3	20
	Nucleopore	100	90
	Nucleopore	50	30
BAX02	Amicon	3	25
	Nucleopore	100	90
	Nucleopore	50	20
BAX03	Amicon	3	10
	Nucleopore	100	25
BAX04	Amicon	3	25
	Nucleopore	100	180
	Nucleopore	50	60

2.4 DETERMINATION OF TOC AND HUMIC SUBSTANCES

Samples for studies of organic carbon and humic substances were collected on two occasions, in March 1993 and July 1994, together with sampling for total numbers and 16S rRNA gene sequencing (c.f. 2.2.1 and 2.2.2). The samples were deep frozen (without any preservation) in plastic bottles immediately after sampling and kept cold during the transport to Linköping.

2.4.1 Total organic carbon (TOC)

The concentration of total organic carbon (TOC) was analysed on a Shimadzu TOC-5000 analyser utilizing catalytic combustion and IR-detection of produced carbon dioxide. The water samples were acidified to pH 3 and inorganic carbon was removed by flushing the sample with pure air prior to analysis.

2.4.2 UV-absorbance

The absorbance of the samples was measured at 254 nm on a Beckman DU-8 spectrophotometer. Since humic substances have a strong absorption at this wavelength, this analysis shows the extent of humic substances in the sample.

2.4.3 Low-molecular-weight organic acids

Capillary electrophoresis (Quanta 4000, Waters) was used in an attempt to analyse the LMW organic acids in the samples.

2.4.4 Humic substances

Humic substances were collected on a weak anion exchange resin, called diethylaminoethyl-cellulose (DEAE-cellulose), on site. No pretreatment of the groundwater is needed in this procedure; a detailed description of the procedure is presented elsewhere (Pettersson *et al,* 1990; Pettersson, 1992). During the campaign in 1993 organic isolation was achieved using a batch procedure. In 1994, a bacteria collecting filter and a filter tube filled with the DEAE resin were connected in series to the borehole outlet. Humic substances were desorbed with 0.1 M NaOH and neutralized with HCl. The eluate was analysed with respect to TOC and UV absorbance in order to estimate the concentration of humic substances. The calibration graph used for determining concentrations was based on humic substances isolated from other sources. The molecular weight distribution was determined by using gel filtration on a HPLC equipment (Waters) with a TSK 2000SW column (7.5 · 300 mm) and precolumn (7.5 · 75 mm); mobile phase was 0.05 M phosphate buffer (pH 6.8). Polystyrene sulphonates of known molecular weight were used as reference substances.

2.4.5 Metals associated with the humic fraction

Metals associated with the humic substances were analysed on an ICP-MS (SGAB Analys, Luleå) on samples collected in 1994 and were focused on a few elements: Ca, Fe, Th and U. Concentrated humic solutions desorbed from DEAE-cellulose were used in these analyses.

3 RESULTS

3.1 BACTERIA

3.1.1 Total number of bacteria and TOC

A comparison of samples from the two expeditions shows that between 1.4 and 5.3 times more bacteria were found in samples from March 1993 than in samples from July 1994. The opposite situation was found for the TOC values which had increased in July 1994 to between 1.2 and 2.9 times the concentrations found in March 1993 (Table 3.1). Each of the boreholes (BAX01-04) was sampled three times during approximately three hours and Fig. 3.1 shows a minor decrease in total numbers from first to last sampling time. The numbers were eventually stabilized at the end of a sampling period. The deepest borehole, BAX01, had the highest number of bacteria as well as of TOC during both sampling periods. There was a good correlation between the total number of bacteria assayed and the content of TOC (Fig. 3.2). The quotients of total bacterial counts from both the 1993 and the 1994 dates and TOC sampled at the same time (Table 3.1) were fairly uniform with the exception of the total number quotient in BAX01 and the TOC quotient in BAX02 and BAX04.

3.1.2 Distribution and diversity of bacteria analysed by 16S rRNA gene analysis

A total of 130 clones were sequenced, 30 from each borehole BAX01, BAX02, BAX03 and BAX04 and 10 from BAX07. The clones examined were allocated into 44 specific clone groups; those that were identical were placed in the same clone group, and each such group, together with clone sequences appearing only once, was given a clone group name, ranging from G1 to G44. Fig. 3.4 depicts a phylogenetic tree for 39 of the clone group sequences. Five distinct clusters of phylogenetically related bacteria were found (Woese, 1987); the beta, gamma and delta groups of the Proteobacteria, Gram-positive bacteria and a cluster (denoted V) with sequences only very distantly related to known and sequenced bacteria reported to the EMBL database. The Proteobacteria beta group was the largest phylogenetic cluster, to which 65 of the clones found belonged (50%) and had the highest diversity, (20 different clone groups) as shown in Table 3.2. There was one exception: only 16S rRNA genes belonging to the Proteobacteria beta group could be demonstrated in BAX01, while the other boreholes harboured 16S rRNA genes from 3 to 5 of the listed phylogenetic clusters. Between 11 and 16 different 16S rRNA genes were detected in each borehole with this sequencing method. Fig. 3.3 demonstrates the relationship between the number of specific clone groups obtained as the sequences from sample extraction No. 1, 2 and 3 are added.

Generally, each set of 10 sequences added new clone groups to the results. However, from 2 up to 6 of the 10 sequences were common when different extractions were compared. There were only 10 clones sequenced from BAX07 and the number of 6 clone groups from this very shallow borehole can therefore not be directly compared with the other boreholes.

Table 3.1 The total number of bacteria and the concentration of total organic carbon (TOC) determined in Bangombé groundwater samples collected in March 1993 and July 1994. Reduction in total number between March 1993 and July 1994 and increase in TOC for the same period are shown as quotients. N is the number of independent water samples analysed; numbers in brackets show standard deviations, nd = not determined.

	Total number of bacteria · 10^5 ml^{-1}			**TOC mg l^{-1}**		
Borehole	**March 1993 (N=1)**	**July 1994 (N=3)**	**Total number March 1993 / July 1994**	**March 1993 (N=1)**	**July 1994 (N=3)**	**TOC July 1994 / March 1993**
BAX01	5.8	4.03 (0.73)	1.4	6.6	14.2 (0.4)	2.2
BAX02	2.9	0.80 (0.28)	5.3	1.7	4.9 (0.8)	2.9
BAX03	5.5	1.29 (0.5)	4.2	4.1	8.3 (2.1)	2.0
BAX04	2.4	0.45 (0.24)	5.3	1.2	1.4 (0.05)	1.2
BAX05	1.7	nd	-	nd	nd	-
BAX07	nd	nd	-	nd	4 (N=1)	-

Some of the clone groups have several identical clone sequences, while others represent only a few or one clone sequence. The distribution of the 7 most commonly occurring clone sequences for the sampled boreholes, and the number of identical clone sequences these clone groups represent, are shown in Table 3.3. Typically, each clone group dominates in a separate borehole. Five of them are represented in all three extractions in the borehole they dominate (underlined). The closest species in the database for the dominating clones in Table 3.3 are shown in Table 3.4 (c.f. Fig. 3.4). They correspond to 56 % of the sequenced clones (73 clones). Several identical clones appeared when different boreholes were compared (Table 3.5). The boreholes BAX01-04 shared between 2 and 4 identical clone sequences with the exception of the BAX02 and BAX04 boreholes, which shared 11 identical clone sequences distributed over 5 different clone groups.

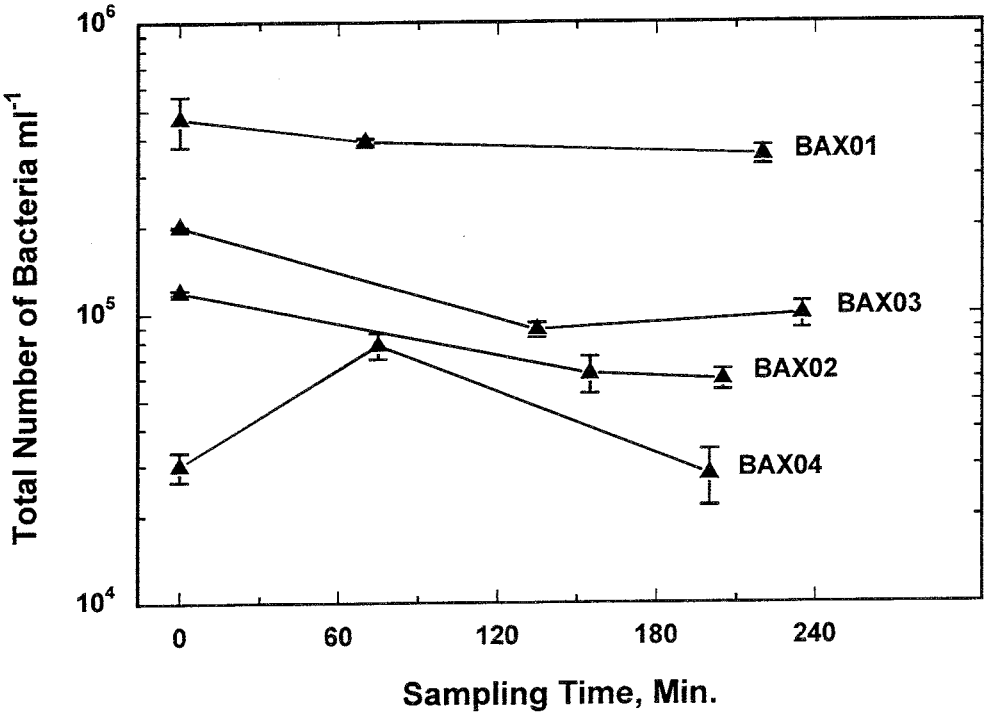


Figure 3.1 The total number of bacteria in flowing (pumped) groundwater from the Bangombé boreholes BAX01 to BAX04, sampled in series at three different times during July 1994. Bars denote the standard deviation of the two filters counted for each water sample.

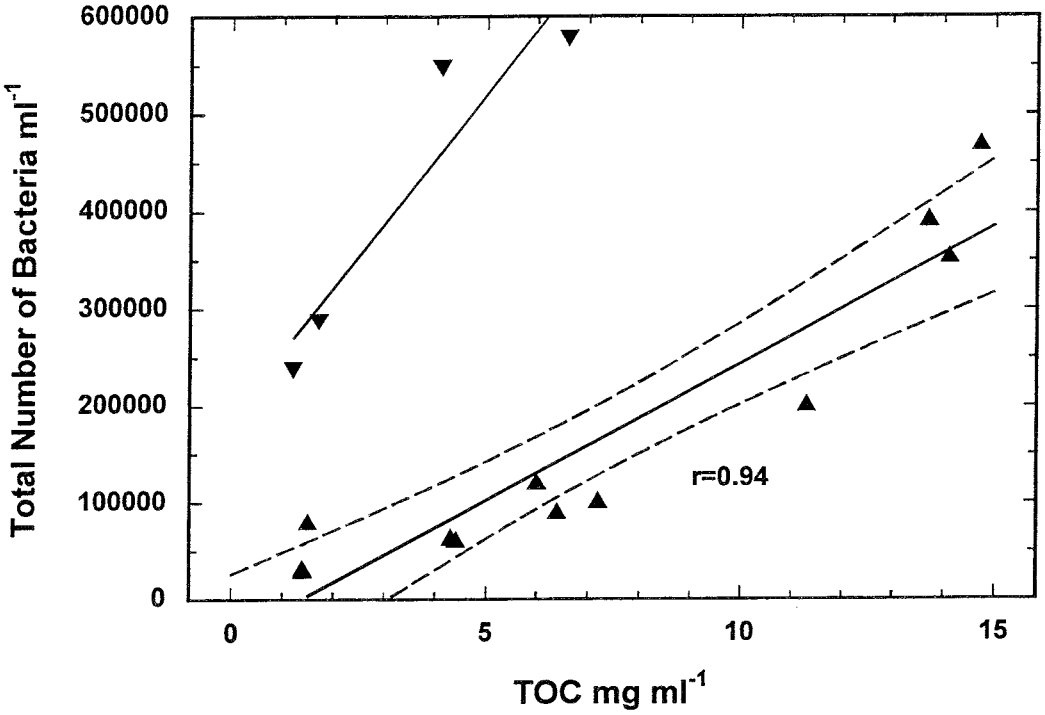


Figure 3.2 The correlation between the total number of bacteria and TOC content in flowing (pumped) groundwater from the Bangombé boreholes BAX01 to BAX04, sampled in March 1993 and in series at three different times during July 1994. ▼ = March 1993 ▲ = July 1994. The lines of dashes show the 95% confidence interval, the correlation coefficient, r, is 0.94.

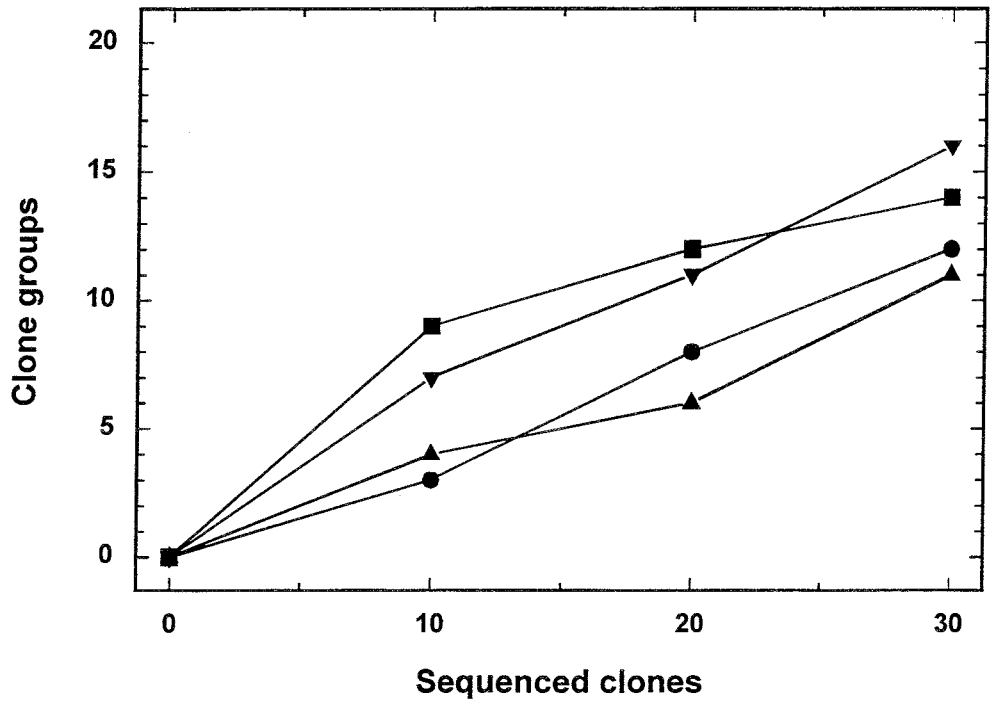


Figure 3.3 The number of clone groups found as a function of the number of sequenced clones from different boreholes. ■ = BAX01; ● = BAX02; ▲ = BAX03; ▼ = BAX04.

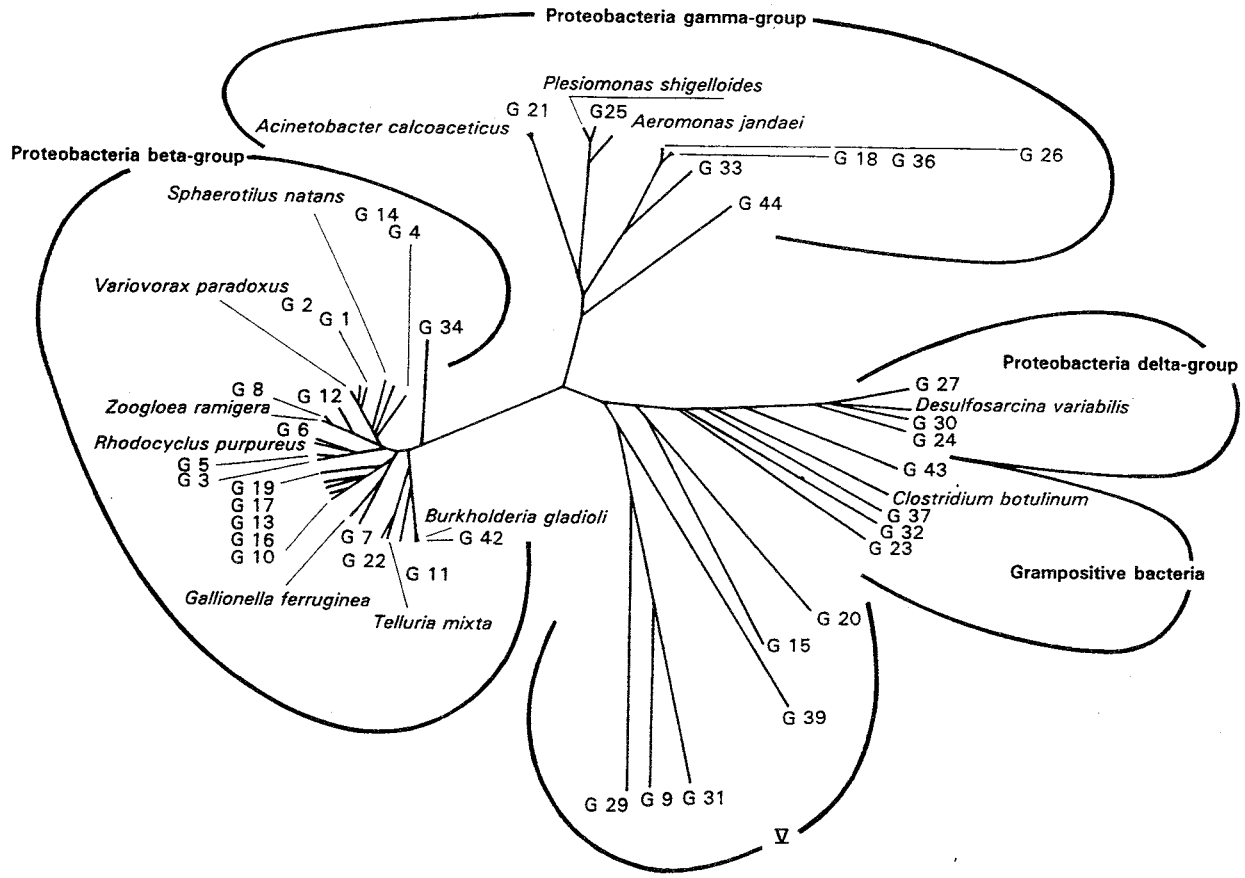


Figure 3.4. Evolutionary distance tree based on the 16S rRNA gene sequences of clones from boreholes in the geological surroundings of the Bangombé nuclear reactor. Major phylogenetic clusters of bacteria have been designated with their generally accepted names. Cluster V comprises clone sequences only very distantly related to known, sequenced and reported bacteria. As references, some 16S rRNA gene sequences of known bacteria from the EMBL database have been added to the tree and they are indicated with their Latin names. The tree shows 39 of the total of 44 specific clone groups found; 5 sequences were too short to be incorporated in this analysis.

Table 3.2 The number of clones that were affiliated in different major branches of the phylogenetic tree by PHYLIP as shown in fig. 3.4, distributed over the sampled boreholes. The total number of sequenced clones as well as the number of clone groups with a specific sequence are shown for each borehole. Note that some or several of the specific clone group sequences reported for each borehole may occur in several boreholes thereby resulting in a smaller sum of specific clone sequences, than the sum of specific clone group sequences for each borehole.

Phylogenetic branch in Fig. 3.4	**Number of clones and clone groups**											
	BAX01		**BAX02**		**BAX03**		**BAX04**		**BAX07**		**Sum of clones and clone groups**	
	Clones	**Groups**	**Clones**	**Groups**	**Clones**	**Groups**	**Clones**	**Groups**	**Clones**	**Groups**	**Clones**	**Groups**
Proteobacteria beta group	29	13	14	4	4	2	11	7	7	3	65	20
Proteobacteria gamma group	0	0	7	4	5	2	9	5	2	2	23	9
Proteobacteria delta group	0	0	1	1	2	2	0	0	0	0	3	3
Gram-positive bacteria	0	0	1	1	15	2	1	1	1	1	18	4
Remaining sequences (V)	1	1	7	2	4	3	9	3	0	0	21	8
Sum of clones and groups	30	14	30	12	30	11	30	16	10	6	130	44

Table 3.3 The dominating bacterial clone groups screened from Bangombé groundwater and their distribution over the sampled boreholes. The total number of clones in each clone group is followed, in parentheses, by the numbers achieved in the extractions from sample 1, 2 and 3 respectively. The numbers for clone groups that occur in all three extractions are underlined for clarity.

Clone group	BAX01	BAX02	BAX03	BAX04	BAX07
G4	2 (0+0+2)	4 (0+4+0)	0	1 (0+1+0)	0
G5	8 (2+3+3)	0	3 (2+1+0)	2 (0+1+1)	0
G8	7 (1+3+3)	0	0	0	0
G16	0	8 (4+2+2)	0	1 (0+1+0)	0
G21	0	2 (0+1+1)	4 (3+0+1)	2 (1+0+1)	1
G23	0	1 (0+0+1)	14 (4+6+4)	0	0
G15	0	6 (5+0+1)	0	7 (1+4+2)	0

Table 3.4 Comparison of the identity between some of the sequenced and most commonly occurring Bangombé clone groups and 16S rRNA sequences in the EMBL database by September 1995. The identity shows the percent identity between the obtained sequence and the closest related bacterium in the database.

Phylogenetic group	Clone group	No. of identical clone sequences	EMBL Accession No. for clone sequences	Closest species in the EMBL database	EMBL Accession No. for closest species	Identity %
Proteobacteria beta group	G4	7	X91175	*Sphaerotilus natans*	Z18534	94.8
	G5	13	X91176	*Rhodocyclus purpureus*	M34132	94.5
	G8	7	X91180	*Zoogloea ramigera*	D14257	98.3
	G16	9	X91188	*Rhodocyclus tenuis*	D16209	91.7
Proteobacteria gamma group	G21	9	X91273	*Acinetobacter junii*	X81658	99.4
Gram-positive bacteria	G23	15	X91275	*Afipia felis*	M65248	91.6
Group V	G15	13	X91187	*Thiomicrospira denitrificans*	L40808	87.0

Table 3.5 The numbers of identical clone sequences and clone groups shared between the different boreholes sampled from the subterranean environment of the Bangombé nuclear reactor.

Borehole	No. of shared clone sequences and clone groups							
	BAX01		BAX02		BAX03		BAX04	
	Sequences	Groups	Sequences	Groups	Sequences	Groups	Sequences	Groups
BAX01	30	14	2	1	3	1	3	3
BAX02			30	12	4	3	11	5
BAX03					30	11	4	3
BAX04							30	16

3.1.3 EMBL accession numbers

The nucleotide sequence data reported in this report appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession numbers: G1, X91173; G2, X91179; G3, X91174; G4, X91175; G5, X91176; G6, X91177; G7, X91178; G8, X91180; G9, X91181; G10, X91182; G11, X91183; G12, X91184; G13, X91185; G14, X91186; G15, X91187; G16, X91188; G17, X91611; G18, X91612; G19, X91271; G20, X91272, G21; X91273, G22; X91274, G23; X91275, G24; XX91276, G25; X91277, G26; X91278, G27; X91279, G28; X91280, G29; X91281, G30; X91282, G31; X91283, G32; X91284, G33; X91285, G34; X91286, G35; X91287, G36; X91288, G37; X91289, G38; X91290, G39; X91291, G40; X91292, G41; X91293, G42; X91294, G43; X91295, G44; X91296.

3.2 CHARACTERISATION OF COLLOIDS

The Bangombé colloids were characterised after oxygen-free sampling and transfer to the laboratory. Characterisation was performed as follows: colloids isolated onto membrane filters were analysed using a) scanning electron microscopy for the morphologic investigations; b) energy dispersive X-ray analysis for the elementary semi-quantitative analysis of major components of large colloids or colloid aggregates; and c) ICP-MS for the quantitative elemental and isotopic analysis of the colloid phase. In addition, fluid samples were analysed with a particle counting unit.

3.2.1 CHARACTERISATION BY SEM/EDS

Membrane filters (Amicon XM50, 3 nm pore size, polyacrylamide membrane) loaded with colloids and prepared on site by filtration according to the above-mentioned protocol were treated for SEM investigation. The samples were visually controlled for the presence of local inhomogeneities on the active surface and for the absence of red spots due to iron(II) oxidation. The membranes were first dried under vacuum and then coated with a 20 nm platinum film. The advantage of the Pt film was that it allowed a better coating with smaller Pt clusters compared to gold: No iron colloids were observed. The colloid population was homogeneously dispersed onto the membrane allowing evaluation of colloid numbers. Rough counts allow estimations of the colloid concentrations (>100 nm): $2 \cdot 10^7$ pt·ml^{-1} including some silica and iron oxide colloids (some may be artefacts) in BAX01; about $1 \cdot 10^7$ pt·ml^{-1} including mostly clay colloids in BAX02;. $2 \cdot 10^7$ pt·ml^{-1} including silica, clay and iron oxide colloids in BAX03; and $2 \cdot 10^6$ pt·ml^{-1} including some large aggregates (mostly SiO_2 and clay colloids) in BAX04.

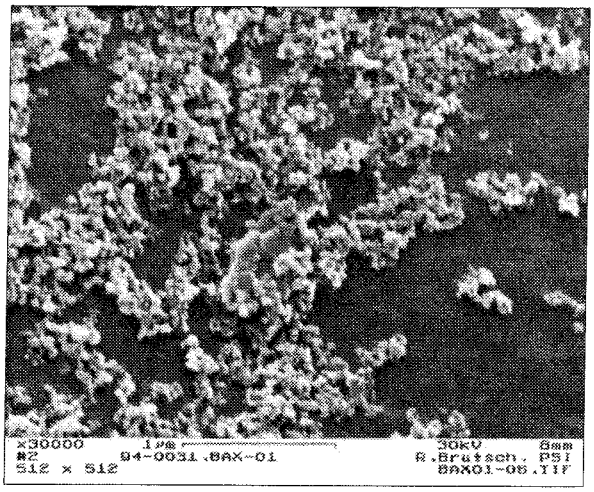
Fig. 3.5 a, b, c and d illustrate some of the typical micrographs obtained. The elements detected were predominantly Si and Si associated with Al, corresponding to silica/quartz colloids or particles, and clay colloids respectively. The following elements were also occasionally detected: Mg, S, Fe (locally), Mn, Ti, V, Cu and Ca. The analysed element concentrations of C, Pt and Cl were not considered. Carbon was the major component of the membrane, Cl was present as a component of the copolymer of the filter, and Pt was due to the coating film.

3.2.2 CHARACTERISATION BY ICP-MS

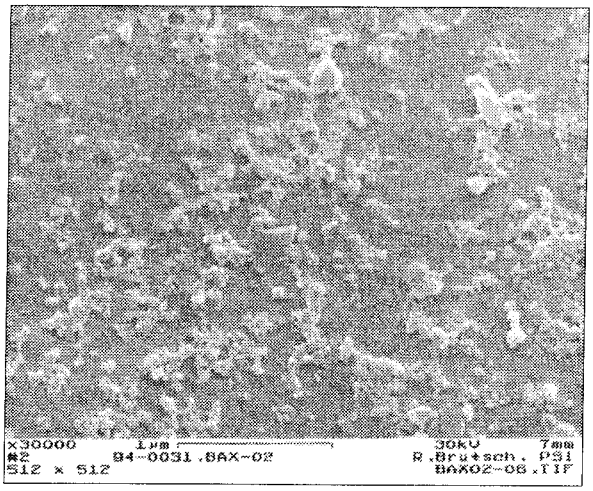
Membrane filters (Nucleopore 100 and 50 nm pore size, cellulose carbonate), prepared on site by the above-mentioned protocol were treated for ICP-MS analysis. The samples were visually acceptable without local heterogeneities. These membranes (including blanks for each pore size) were allowed contact with 0.5 ml concentrated HNO_3 for colloid dissolution. After dissolution of material loading the membrane, the volume was topped up to 5 ml. These solutions were analysed with a VG ICP-MS plasmaquad 2 plus unit. Internal standardisation was used by additions of iodine and standard additions of the element to be analysed; this was subsequently carried out with fully quantifiable results.

Colloid elements detected were Al, Fe (major components), as well as B, P, Mg, Ti, Cu, Se, Zn, Sr, I, Ba, La and U (minor components). Si cannot be measured by ICP-MS because of changes in the atmospheric pressure. These elements were detected earlier by EDS, with the exception of Zn which is an artefact caused by leaching from plastic in the container. Iodine and selenium concentrations may not be quantitative because of the nitric acid treatment. It must be noted that Th is nearly absent from the colloid phase (except some Th detected in the BAX01 sample). Hf, Ta, Ge and Pt may be present as traces associated with the colloid phase. Table 3.6 shows the concentration of elements detected as components of the colloids. Al may be used as a signature for clay, Fe may be present as oxide and/or sulphide.

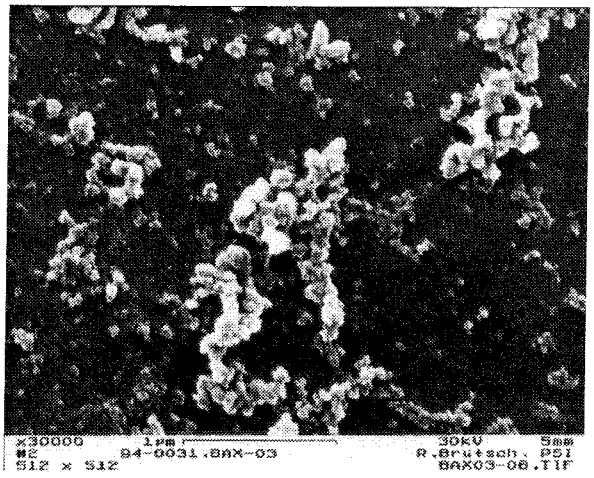
a)



b)



c)



d)

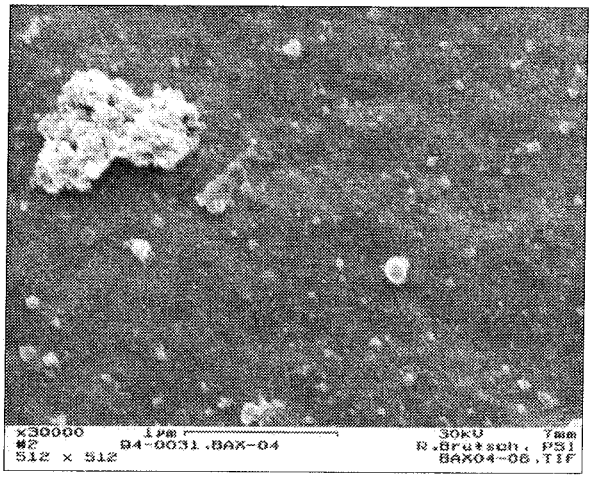


Figure 3.5 SEM micrographs of Bangombé colloids a) BAX01, b) BAX02, c) BAX03, d) BAX04. Filtered volumes were: BAX01 = 20 ml, BAX02 = 25 ml, BAX03 = 10 ml and BAX04 = 25 ml, active surface was 1.2 cm^2. Bar denotes 1 μm.

Table 3.6 Concentration of major and trace elements associated with the colloid phase ng ml^{-1} analysed with ICP-MS.

Element	Cut-off/nm							
	BAX01 100	BAX01 50	BAX02 100	BAX02 50	BAX03 100	BAX04 100	BAX04 50	Error estimated
Be	0.22	2.50	0.65	2.01	0.08	0.13	0.29	±0.05
B	-	21	18	28	5.8	12.8	55.8	±4
Mg	-	-	-	-	4.3	-	13	±6
Al	1.2	24	-	36	-	-	33	±10
P	1.15	11	0.6	8.6	9.0	1.49	23	±0.01
Ti	-	17	-	5	-	-	8	±6
Mn	0.6	9.2	0.2	22	3.2	1.0	3.25	±0.4
Fe	31	120	2.1	25.8	94	8.4	64	±6
Ni	0.08	2.1	0.14	0.9	1.4	0.004	1.2	±0.02
Cu	0.14	100	1.1	1.35	119	3.4	10,7	±0.01
Se	-	2.6	1.1	-	-	0.1	-	±0.01
Sr	-	0.25	0.06	0.08	0.18	0.01	0.07	±0.01
Pd	0.02	0.22	-	0.26	0.35	-	0.02	±0.01
Ag	0.9	-	0.07	1.0	0.8	0.1	-	±0.2
Sb	0.04	0.02	-	0.01	0.15	0.02	0.05	±0.05
I	1.5	5.2	0.03	4.2	1.2	0.29	-	±0.01
Ba	0.5	3.6	0.33	2.2	1.3	0.18	0.89	±0.1
La	-	0.04	0.02	0.08	-	0.03	-	±0.01
Pb	-	10	0.5	1.0	8	1.1	0.7	±0.5
Th	0.105	0.113	-	-	-	-	-	±0.005
U	0.040	2.22	0.025	0.048	0.114	0.314	0.350	±0.005
Total as oxide	50	300	30	100	250	30	100	±10

3.3 CHARACTERISATION OF COLLOIDS BY SINGLE PARTICLE COUNTING

Blank samples (ultra-pure water transported to site) as well as defined diluted samples (e.g. dilution factor 1/1) were analysed after extra dilution in a millipore water flow. The single particle spectrometer used was a HORIBA PLC311, which allows absolute concentration measurement of particles for sizes ∅ = 100, 200 and 500 nm under standard conditions. The pure water flow rate was 400 ml·min^{-1} and the injection rate of the groundwater samples was 200 ml·h^{-1} corresponding to a dilution of 133 times. The direct count is the number of particles counted in 0.1 ml (i.e. per min) in the flow of millipore water and groundwater sample mixture. Table 3.7 shows the results of cumulative counting obtained for the blank (i.e. pure water used for dilution), BAX02, BAX03 and BAX04. It must be noted that both BAX01 and BAX03 samples showed an abnormal artefact presence (red-brown precipitate) which was probably due to iron(II) oxidation (BAX01 was not counted). Trial tests carried out under N_2 to eliminate these artefacts were unsuccessful. The first test was an attempt to reduce the iron hydroxide precipitate deposit by hydrazine. The second test was to complex the iron hydroxide solid phase by EDTA. Both elimination tests failed. This abnormal artefact production could have occurred because it was impossible to perform sampling on the last sampling day, because the glass stoppers in the glass bottles used were not 100% tight, and because of long and difficult transport conditions. It was suggested that a double barrier strategy (e.g. a bottle with the sample in a container full of water) could be used. This was not done because of practical difficulties. However, results obtained for BAX02 and BAX04 are invaluable because they are artefact-free and may be used for further modelling.

The original particle counting is averaged over a minimum of 8 runs allowing calculation of the standard deviation of the order of 5-10%. Each sample run may be used to calculate the original sample colloid concentration by multiplying the counts by an operational factor of 10 (counting time 1 min) and the operational dilution factor, i.e. ultra-pure water flow rate (400 ml·min^{-1}), divided by the fluid sample injection rate (200 ml·h^{-1}). The global correction factor (counting and dilution correction) of 1200 allows for a colloid concentration calculation of the sample. The determination of the original colloid concentration in the groundwater is obtained by

$$[\text{coll}] = \frac{\{[\text{coll}]_{\text{sample}} - [\text{coll}]_{\text{bl}} \cdot P_{\text{bl}}\}}{P_{\text{gw}}}$$

where:[coll] is colloid concentration in the groundwater, $[\text{coll}]_{\text{sample}}$ is colloid concentration in the sample, and $[\text{coll}]_{\text{bl}}$ is colloid concentration in the blank water. Both P_{bl} and P_{gw} are the portion of each blank and groundwater fluid volumes (0.5 for both components). Starting with counts obtained for the groundwater samples (Table 3.8) colloid concentration in the original groundwater could be calculated (as shown in Table 3.9).

Table 3.7 Bangombé sample original countings.

Borehole	Particle counts size (nm)		
	>100	**>200**	**>500**
Blanks	1716±190	191±15	47±8
BAX02	4983±265	481±42	135±19
BAX04	2197±175	358±26	133±15
BAX03[a]	12038±610	5598±524	3108±304

[a] This sample may contain artefacts; real numbers should be smaller.

Table 3.8 Bangombé colloid number concentrations.

Borehole	Cumulative colloid concentration (coll) ($pt\text{-}ml^{-1}$) Size (nm)		
	>100	**>200**	**>500**
BAX02	$9.9 \cdot 10^6$	$9.3 \cdot 10^5$	$2.7 \cdot 10^5$
BAX04	$3.2 \cdot 10^6$	$6.3 \cdot 10^5$	$2.6 \cdot 10^5$
BAX03[a]	$2.7 \cdot 10^7$	$1.3 \cdot 10^7$	$7.4 \cdot 10^6$

[a] This sample may contain artefacts; real concentration is smaller.

The colloid size distribution may be analysed further using the Pareto law (e.g. Degueldre, 1990, 1994):

$$\frac{\delta[coll]}{\delta \varnothing} = A \cdot \varnothing^{-b}$$

[coll] is given in $pt \cdot ml^{-1}$ and both A and b parameters may be determined as shown in Table 3.9.

Table 3.9 Bangombé colloid distribution parameters.

Borehole	b	A (pt-ml^{-1}·nm^{-1})
BAX02	3.19	$9.9 \cdot 10^{6}$
BAX04	2.53	$2.97 \cdot 10^{9}$
BAX03[a]	1.79	$9.81 \cdot 10^{8}$

[a] This sample may contain artefacts, real concentration is smaller.

The equation for the calculation of the mass concentration takes into account the shape of the colloid (spherical) and an average density (2 g·cm^{-3}).

$$\frac{\delta[coll]'}{\delta\varnothing} = \frac{\pi \cdot \varnothing^3}{6} \cdot \rho \cdot \frac{\delta[coll]}{\delta\varnothing}$$

[coll]' is given in ng·ml^{-1}. Table 3.10 reports the mass colloid concentrations. Note: mass is integrated up to 1000 nm

Table 3.10 Bangombé colloid mass concentrations.

	Cumulative colloid concentration [coll]' (ng·ml^{-1})		
	Size (nm)		
Borehole	**>100**	**>200**	**>500**
BAX02	51.3	44.2	26.0
BAX04	53.1	49.8	35.1
BAX03[a]	916.0	872.6	510.1

[a] This sample may contain artefacts, real concentration is smaller.

The surface complexation capacity can be estimated using model assumptions. Here, again, colloids are supposed to be spherical. Two hypotheses of site distributions were suggested: the first one assumed that complexation occurred at the surface of spherical colloids with a site density of 3 sites per nm^2; the second considered spherical colloids as nanoporous entities and with a site density of 3 sites per nm^3. Both minimum and maximum capacities were evaluated using the following equations (Degueldre, 1995):

$$\frac{\delta[\text{site}]}{\delta\varnothing} = \pi \cdot \varnothing^2 \cdot \Delta \cdot \frac{\delta[\text{coll}]}{\delta\varnothing} \quad \text{and} \quad \frac{\delta[\text{site}]'}{\delta\varnothing} = \frac{\pi \cdot \varnothing^3}{6} \cdot \Delta' \cdot \frac{\delta[\text{coll}]}{\delta\varnothing}$$

[site] : minimum complexation capacity, [site]': maximum complexation capacity, Integration is extended up to 1000 nm. The results are given in Table 3.11 and the Bangombé colloid size distributions are graphically shown in Fig. 3.6. Larger slopes were observed for BAX02 and BAX04, compared to BAX03. BAX02 and

BAX04 were artefact-free while BAX03 was affected by artefacts due to Fe (II) oxidation yielding iron hydroxide colloids which aggregated.

Table 3.11 Bangombé colloid complexation capacities.

Borehole	Cumulative colloid concentration ($pM \cdot ml^{-1}$) Size (nm)		
	>100	>200	>500
	min-max	min-max	min-max
BAX02	2.1 - 130	1.4 - 110	0.5 - 65
BAX04	1.7 - 130	1.4 - 120	0.7 - 87
BAX03[a]	49 - 4800	45 - 4700	30 - 3800

[a] This sample may contain artefacts, real concentration is smaller.

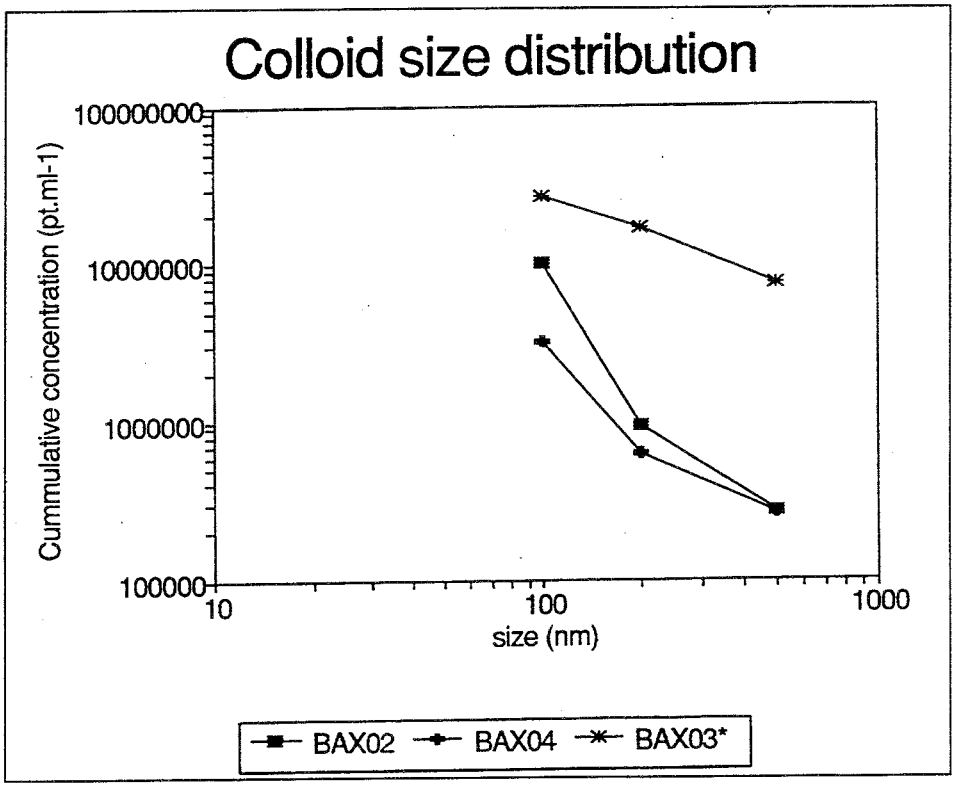


Figure 3.6. Bangombé colloid size distributions.

Because of a low uranium concentration in the colloid fluid phases, it was not possible to measure the isotopic ratio $^{235}U/^{238}U$ for colloids from the ICP-MS test. Even when the ICP-MS counting for aliquot of 0.5 ml was integrated, it was still not possible to acquire more than 500 counts for the mass $^{235}U/^{238}U$ thus diminishing the usefulness of this test. Thermal ionisation mass spectroscopy (TIMS) tests were then performed. However, they were unsuccessful.

3.4 TOC AND HUMIC SUBSTANCES

The analyses of the TOC in the BAX-samples on both sampling occasions show that TOC had more than doubled between March 1993 and July 1994 and the absorbance at 254 nm of the water had increased even more in three of the waters (BAX01, BAX02 and BAX03; Table 3.12). This indicates that the original water has been mixed with waters of a different origin.

The ratio between absorbance at 254 nm and TOC-values for the samples was approximately 0.01 or less which suggests that humic substances comprise only a minor fraction of the organic matter. Generally, the ratio is 0.04-0.05 in surface waters which corresponds to a humic fraction of at least 50% of the organic matter. The absorbance of humic substances is, however, dependent on the molecular weight of the substance; i.e. lower molecular weight results in a decrease in absorbance. Since the molecular weight of the humic fraction was relatively low in these samples (see below) the measured absorbance might give an underestimation of the humic fraction size. Moreover, the spectrophotometric analyses combined with TOC determinations of the elutes showed that only 0.5-3% of the organic material in the water was isolated as humic substances on DEAE-cellulose.

An attempt to analyse the LMW acids (e.g. formic acid, acetic acid, lactic acid) on capillary electrophoresis was unsuccessful. Only a few traces were detected, but they could not be identified nor verified. Possibly those acids are fairly rapidly consumed by microorganisms and therefore have a rather short life span which makes analysis of them almost impossible under the prevailing sampling conditions.

3.4.1 Molecular weight distribution

Gel filtration of the samples collected in 1994 showed that all samples contained humic substances with an average molecular weight that was slightly lower than the average molecular weight of surface water humic substances. The molecular weight of the humic substances sampled in 1993 was even lower which could be an effect due to a lower TOC content on that occasion. Other studies show that the molecular weight of humic substances in surface waters increases with increasing TOC (see e.g. Pettersson, 1992). The chromatograms obtained in the analyses of BAX02, BAX03 and BAX04 had split peaks caused by fractionation in the column due to different molecular sizes of the organic matter (Fig. 3.7) whereas BAX01 resulted in a fairly homogenous peak (not shown). The different peaks represent organic fractions of different molecular weights. The longer the retention time, the lower the molecular weight. The molecular weight distributions are different in BAX02 compared to BAX03 and BAX04, i.e. the LMW fraction is smaller in BAX02. The molecular weight distribution of humic matter in BAX03 and BAX04 is similar despite large differences in TOC content and the fact that there is no demonstrated hydrological connection between these two sites. Normally, gel filtration of humic substances from surface waters results in homogeneous peaks where no fractionation of the sample is observed.

Table 3.12 Concentration of total organic carbon (TOC) and UV absorbance at 254 nm on samples from Bagombé (1993, 1994). Three samples were taken on each site (except BAX07) in the 1994 sampling.

Sample	Date	Time (min)	TOC (ppm)	UV-absorbance (254 nm)
BAX01	March -93		6.6	0.07
BAX01	940714	0	14.7	0.13
		65	13.7	0.14
		155	14.1	0.12
BAX02	March -93		1.7	0.001
BAX02	940712	0	6.0	0.03
		170	4.3	0.01
		205	4.4	0.02
BAX03	March -93		4.1	0.02
BAX03	940710	0	11.3	0.10
		100	6.4	0.06
		220	7.2	0.07
BAX04	March -93		1.2	0.001
BAX04	940713	0	1.4	0.02
		75	1.5	0.01
		205	1.4	0.01
BAX07	940711		4	0.06

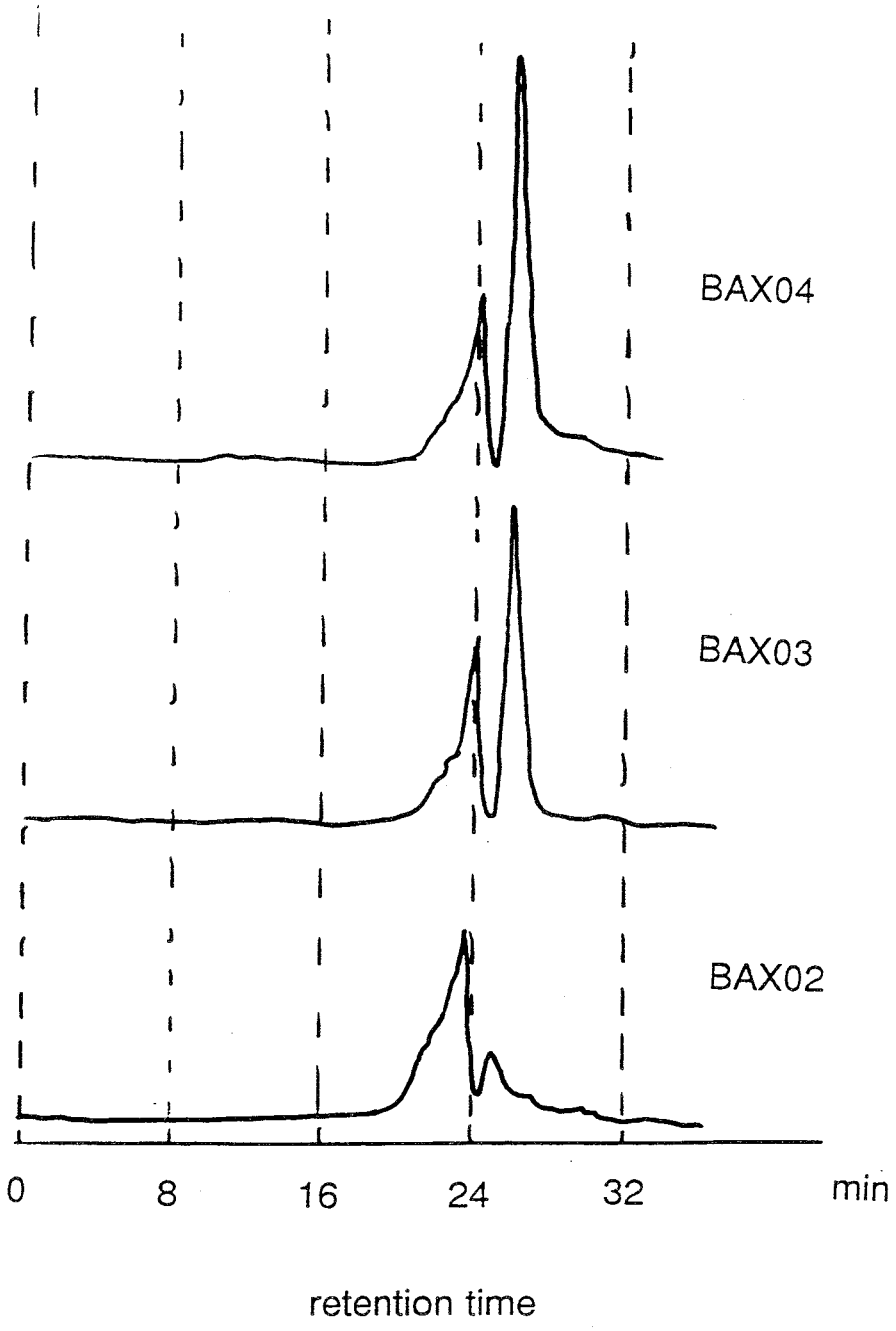


Figure 3.7 Gel filtration chromatograms of BAX02, BAX03 and BAX04. An increase in retention time indicates a decrease in the molecular weight of the material. Fractions of the humic matter with different molecular weights resulted in the peaks splitting.

The LMW of the humic fraction indicates ongoing subsurface processes in which humic substances are decomposed. This decomposition can be due to microbial activity or chemical reactions. High molecular weight material can also be removed from groundwater by adsorption onto the solid phases, thus resulting in a decrease of the molecular weight. LMWs of humic substances have earlier been observed in other groundwaters, e.g. at Cigar Lake, Canada (Pettersson et al, 1994).

3.4.2 Metals associated with the humic substances

Some metals associated with the humic fraction were analysed in the eluates, i.e. a concentrated solution of isolated humic substances from July 1994. For a few of these metals (Fe, Ca and U) the total concentration was analysed in water sampled in March 1993. Results from these analyses are presented in Table 3.13, where Th is also included although the analysis of the total concentration was not made. To estimate the humic bound fraction of metals it is necessary to know the total metal concentration. Since the content of organic matter was different on the two occasions, one might suspect that the concentration of metals or at least the fraction of the metals that formed complexes with the humic matter also varied.

Table 3.13 Metal concentration in the water: Total metal concentration in March 1993 and concentration of metals associated to the humic fraction in July 1994.

	Borehole			
Metal	**BAX01**	**BAX02**	**BAX03**	**BAX04**
Fe-total (mg/l)	1.8	7.1	5.4	3.0
Fe-humic (µg/l)	0.5	0.07	2	0.01
Ca-total (mg/l)	32	3.6	4.0	1.9
Ca-humic (µg/l)	5	100	20	200
Th-total	n.a.	n.a.	n.a.	n.a.
Th-humic (µg/l)	$2 \cdot 10^{-4}$	$7 \cdot 10^{-4}$	$6 \cdot 10^{-4}$	below det. limit
U-total (µg/l)	2.1	0.4	0.3	11.0
U-humic (µg/l)	0.3	0.03	0.2	3.5

The metal concentrations varied greatly between the four boreholes, particularly for Ca and U. The largest concentration of U was observed in BAX04 which is situated above the reactor whereas U concentrations in the reactor (BAX03) and below the reactor, (BAX02) were equal and much lower than above the reactor. Although great care must be taken in interpreting these results, they do indicate that a large fraction (8-67%; see Table 3.14) of U was bound to humic substances, compared to the fractions of Ca and Fe (<0.4% and 0.02-10%, respectively). Thus, the largest fraction of U associated to humic substances was found in BAX03, i.e. in the reactor. The high concentration of U combined with a low TOC content in BAX04 gave a high ratio between the total concentration of U and TOC and resulted in a large fraction of U associated with humic substances.

Table 3.14 The fraction of U associated with humic substances (U-humic/U-total) and the ratio between total concentration of U and TOC (U-total/TOC). The calculations are based on the values presented in Table 3.13.

	Borehole			
Fraction	**BAX01**	**BAX02**	**BAX03**	**BAX04**
U-humic/U-total (%)	14	8	67	32
U-total/TOC (μg/mg)	0.2	0.07	0.03	8

4 DISCUSSION

4.1 TOTAL NUMBER OF BACTERIA AND TOC

The total number of bacteria obtained in the Bangombé groundwater was in the range of other previously measured groundwater environments; from 10^3 up to 10^7 bacteria per ml (Pedersen, 1993a-b, Pedersen and Ekendahl, 1990, Pedersen and Karlsson, 1995) (Pedersen in press). The contribution of these bacteria to the TOC can be calculated assuming an average weight of a bacterium to be $2.8 \cdot 10^{-13}$ g of which 50 % is carbon (Neidhardt et al, 1990). This was done for BAX01; in March 1993, BAX01 water had $5.8 \cdot 10^8$ bacteria per litre (Table 3.1) which corresponds to a bacterial contribution of 0.081 mg l^{-1} to the TOC. Comparing this number with the TOC analysed on the same occasion (6.6 mg l^{-1}), suggests that the bacteria are responsible for approximately 1.25 % of the TOC in this sample. This proportion was in agreement with the other samples; consequently, bacterial biomass constitutes only a minor part of the TOC content.

A positive correlation between the TOC and the total number of bacteria has been demonstrated for deep granitic groundwater (129 m down to 860 m) sampled during the pre-investigation phase of the Äspö Hard Rock laboratory (HRL) in SE Sweden (Pedersen and Ekendahl, 1990). The Bangombé samples were from more shallow levels (4.5 m down to 105 m) and had higher TOC than those in the deep Äspö groundwater. Despite this, there was a very good correlation between the TOC and the total number of bacteria obtained during both sampling periods (Fig. 3.2). Increasing the TOC seems to stimulate bacterial growth, which is a likely conclusion, since the TOC may serve as a nutrient and a carbon source for heterotrophic bacteria. However, it is important to note that, at least in these cases, the relationship is not absolute, but relative (c.f. Fig. 3.2). This relative correlation of the TOC and bacterial number may be due to differences such as variations in degradability of the TOC, species of bacteria present, the ratio between attached and unattached bacteria and also the rate with which the TOC is supplied to the ecosystem.

4.2 DIVERSITY AND DISTRIBUTION OF BACTERIA

Nucleic acid probes for 16S rRNA sequences can be used to detect bacteria and their distribution in various ecosystems (Amann et al, 1991, Braun-Howland et al, 1992, Risatti et al, 1994). Use of such probes requires genetic information about the 16S rRNA sequences of a bacterial population to be studied, otherwise the specific probes cannot be constructed. Therefore, when probes are to be used in new environments with unknown bacteria such as those in Bangombé, the first step must be to analyse the genetic diversity of the 16S rRNA gene of the inhabiting populations, as was done here.

The 16S rRNA gene sequencing method was applied to five very different subterranean habitats; the Stripa research mine and Äspö hard rock laboratory in Sweden, Maqarin groundwater in Jordan, sand/bentonite buffer clays from a full-scale nuclear waste canister experiment performed at the Atomic Energy Canada Limited (AECL) underground research laboratory, North of Winnipeg, Canada, and finally the Bangombé environment reported here. Descriptions and comparisons of these habitats as well as comprehensive discussions about the limitations and possibilities of the method are published elsewhere (Pedersen, in press, Pedersen and Karlsson, 1995; Ekendahl et al, 1994). These investigations comprise more than 600 partially or totally sequenced 16S rRNA gene clones from 60 independent samples. More than 175 specific clone group sequences have been found during the investigations and 44 of them came from Bangombé.

When PCR amplification is used for determination of species diversity, the result may be biased due to methodological problems, such as uneven extraction of DNA and biased PCR due to differences in genome size (Farrelly et al, 1995). One of the most important biases is that organisms belonging to the Archaean domain have been found to have only one or a few gene copies of the 16S rRNA gene while eubacteria can have several copies, 5-7 or more, which will bias towards eubacteria (Ward et al, 1992). The results presented in this paper were obtained using universal primers only and should, for reasons discussed above, be expected to reveal mainly eubacterial diversity and distribution.

Ekendahl et al (1994) has shown that there is a relation between the numbers of clone groups obtained from a sample and the numbers of different species in the sample. The clone groups with many identical sequences that are listed in Table 3.4 then correspond to eubacteria that occurred in relatively large numbers. Different dominating clone groups were found in different boreholes (Table 3.4) indicating that among bacteria one or a few specific species dominate in each borehole. An extrapolation of figure 3.3 suggests that sequencing additional clones would have resulted in the discovery of additional clone groups. Therefore, the absolute number of different bacterial species in Bangombé must be larger. However, sequencing 30 clones per boreholes certainly was enough to find the predominating eubacterial sequence (Figure 3.3). A similar observation was made earlier with this method on attached populations in two levels of the Stripa borehole V2 (Ekendahl et al, 1994). Differences in groundwater chemistry, geology, minerals etc. in each borehole obviously favour different bacteria, a situation not unlike surface ecosystems.

There is no accepted value of % identity at which two 16S rRNA genes can be concluded to belong to the same genus or species. It can be quite different for different genera. It has been suggested (based on a comparison of rRNA sequences and on DNA-DNA reassociation), that a relationship at species level does not exist below 97.5% level of identity whitin the 16S rRNA gene (Stackebrandt and Goebel, 1994). At higher identity values, the species identity must be confirmed using DNA-DNA hybridisation (Fox et al, 1992). Accepting that this level approximately identifies a sequence on the genus level, some conclusions can be made about the sequences reported here. A comparison of 16S rRNA gene sequences obtained with EMBL sequences in the database, reveals 10

sequences that were related on a species level (not shown). The other 34 sequences had such a low identity (below 97.5%) that they should be regarded as either unknown or not reported to the database. This finding of many new and unknown bacterial 16S rRNA sequences in a natural environments is consistent with other studies (Giovannoni et al, 1990, Ward et al, 1990, 1992)

Sphaerotilus (G4), *Rhodocyclus* (G5), *Zoogloea* (G8) and *Acinetobacter*-like (G21) sequences predominated in one or several boreholes (Table 3.4). There were also three predominating sequences, G15, G16 and G23, that were so distantly related to known species that they must be regarded as new, at least when compared to the DNA database. Two other clones, G8 and G21, had an identity higher than 98% with 16S rRNA sequences in the database, and are therefore probably identified on genus level. Acinetobacters are ubiquitous organisms that are present in soil, water and sewage and it has been estimated that *Acinetobacter* may constitute as much as 0.001% of the total aerobic population of soil and water (Tower, 1992). Finding *Acinetobacter*-like sequences (G21) in all boreholes investigated here, except the deep BAX01, is consequently in agreement with what is known about this group. The only other dominating clone which was matched to the database at an identity above 98% was G8, related to *Zoogloea*, and unlike G21, it was found only in BAX01. This genus is often found in environments enriched in organic nutrients (Dugan et al, 1992) and if *Zoogloea* should be expected at all in this investigation, borehole BAX01 would be the most the plausible one as it had the highest content of organic material detected in this investigation (14.2 mg l^{-1}).

Hydrological investigations and modelling have shown that there is a regional flow from the recharge area SW of the Bangombé reactor zone (Fig. 2.1) to the deep sandstone penetrated by BAX01. The water then discharges upwards towards the reactor (Gurban et al, 1995). Locally, the reactor environment is mainly influenced by a shallow-derived lateral groundwater flow with a minor deep discharging component. This means that some deep groundwater from BAX01 reaches the shallow boreholes, but there is certainly no recharge of groundwater from BAX02-07 to BAX01. There was an absolute dominance of members of the Proteobacteria beta group in BAX01 (Table 3.1). With one exception, this was the only major phylogenetic cluster found in BAX01. The other, more shallow boreholes, were populated by bacteria from 3 to 5 of the clusters of the phylogenetic tree (Fig. 3.4) including the Proteobacteria beta group. Some of the beta group members found in the BAX01 borehole were also found in the shallow boreholes. This implies that groundwater from BAX01 reaches and mixes with the groundwater in the shallow boreholes, but the shallow groundwater does not reach BAX01, as confirmed by hydrological modelling (Gurban et al, 1995). Details about the local flow around the reactor is more uncertain. Table 3.5 suggests that BAX02 and BAX04 have a hydraulic connection, as they share several common sequences and clone groups, but there is currently no direct hydrological evidence to support this suggestion. However, Tables 2.1 and 3.1 show that pH, conductivity, TOC and the total number of bacteria agree (with exception for the 1994 TOC values) with both BAX02 and BAX04, further supporting the possible existence of a hydraulic connection between these two boreholes.

4.3 COLLOIDS

Distribution coefficients of trace elements between the water and colloid phases (Kp) were estimated. As an example for uranium, an average of 200 pg·ml^{-1} of U was detected in the water; and 40 pg·ml^{-1} of U was detected in the colloid phase. The uranium concentration increased, especially in samples from inside or above reactor zones (i.e. BAX03 and BAX04) respectively.

$$K_p = \frac{[U]_{coll}}{[U]} \cdot \frac{1}{[coll]}$$

For uranium, a K_p value of $2 \cdot 10^6$ ml·g^{-1} was calculated considering [coll] = 100 ng·ml^{-1}. With this large K_p value, it is likely that uranium is not only sorbed (Degueldre, Ulrich & Silby, 1994) but also associated (embedded) with groundwater colloids. Its isotopic signature could then be used to assess transport behaviour of colloids.

In these Na-Mg-Ca-HCO_3 type waters of with a pH of 6-7 and a slightly negative Eh, the colloid concentration was rather low, about 20-100 ng·l^{-1}. The campaign in 1993 indicated a similar colloid concentration of 10-700 ng·l^{-1}. This low colloid concentration was due to the relatively high concentration of Ca, Mg and Na in the water which reduces the colloid (SiO_2, clay and iron oxide) concentration because the cations act as a colloid cement (aggregation sticking) in the aquifer. However, the presence of Fe(II) induces a large potential of artefact. Generation of iron hydroxide colloids by direct oxidation yields coprecipitation of elements with a large atomic number. Additional research is recommended at other sites or boreholes to study similarities between the selected system and a repository far-field.

4.4 HUMIC SUBSTANCES

Four of the five groundwater samples collected in Bangombé had fairly high concentrations of organic carbon. Only BAX04 had a TOC below 1.5 mg l^{-1} whereas the other samples had TOC values in the range of 4-14 mg l^{-1}, which is in contrast to groundwater from granitic bedrock where, in general, the concentration of organic carbon is a few mg l^{-1} (Karlsson et al, 1994). However, like to groundwater from granitic bedrock, the humic substances comprised only a minor fraction (0.5-3%) of the organic matter.

The molecular weight distribution was similar in BAX03 and BAX04 but differed from the distribution in BAX02 and BAX01. This could indicate that the organic matter originates from three different sources. However, this discussion is contradictory to the result from the 16S rRNA gene sequencing method which indicated similarities between BAX02 and BAX04. Since the 16S rRNA gene sequencing method is more specific than the determination of molecular weight distribution it is plausible that the composition of the bacterial community is a more reliable indication of the origin of the water.

The metal speciation study indicated that a large fraction of U in BAX03 (67%) and BAX04 (32%) was associated with the humic substances whereas in BAX01 and BAX02 approximately 10% of U formed humic complexes.

5 CONCLUSIONS

5.1 BACTERIA

- The 16S rRNA gene sequencing, of DNA extracted from the subterranean environment at the Bangombé site, showed that it was inhabited by a diversified microbiota. Each borehole was dominated by species that did not dominate any of the other boreholes; a result that probably reflects documented differences in the geochemical environment.

- Two of the boreholes, BAX02 and BAX04 contained many common 16S rRNA gene sequences in common and they also had similar bacterial counts of TOC, pH and equal conductivity, suggesting that these boreholes are hydrologically connected.

- The Bangombé natural analogue for a radioactive waste repository was inhabited by many different bacteria. This supports the idea that bacteria will also inhabit constructed repositories. The next step will be to gather information about the predominating species and their in situ activity. Culture media can now be directed towards these bacteria and the species of interest may be selected using nucleic acid probes and optical tweezers as described recently (Huber et al, 1995).

- The influence of bacterial activity on their environment must be studied further important parameters to study relating to radionuclide mobility, are redox effects, production of complexing agents and mobility of bacteria and uptake of radionuclides by bacteria (Pedersen and Karlsson, 1995; Pedersen, in press).

5.2 COLLOIDS

- The results from three analytical procedures SEM/EDS, ICP-MS and Single Particle Counting were consistent.

- Particle counts from BAX02 and BAX04 groundwaters with a single particle spectrometer correspond to SEM particle counts on membranes.

- Evaluation of mass concentration with density 2 (average for SiO_2 and clay) from both particle counting and SEM analysis was consistent and a concentration in the range of 20-60 $ng \cdot ml^{-1}$ (Table 3.10) is acceptable for a size range of 10-1000 nm. The obtained concentration from the elementary analysis of colloid cakes with oxides was smaller because Si is not

determinate. However, locally, one large particle (no preliminary separation at 1000 nm) may increase the mass concentration.

- Pareto coefficient b (<4) indicates that the mass concentration increases with colloid size. However, there must be an upper size cut-off dictated by sedimentation in the borehole during pumping.

- Trace element results show that transition metals and some heavy metals were associated with the colloid phase.

- Iodine, sulphur and selenium may also be associated with organic colloids.

- Sulphur and selenium were associated with transition (Cu, Zn, Fe, Ni, Pt etc.) and heavy metals (Pb) in the colloid phase. Because of an expected low Eh, sulphate results may be due to sulphide oxidation.

5.3 TOC AND HUMIC SUBSTANCES

- TOC varied in the range 4-14 mg l^{-1} in BAX01, BAX02 and BAX03 whereas in BAX04 TOC was <1.5 mg l^{-1}.

- Humic substances comprised only a minor fraction (<3%) of the TOC

- The humic substance from BAX02, BAX03 and BAX04 consisted of fractions with different molecular weights, whereas the humic matter from BAX01 had a more homogeneous character.

- Traces of LMW organic acids were detected, although they could not be identified nor verified.

- A large fraction, i.e. 8-67%, of the uranium was bound to the humic matter compared to the fractions of Ca and Fe (<0.4% and 0.02-10%, respectively). The largest fraction of U associated to humic substances was found in BAX03, i.e. in the reactor.

6 ACKNOWLEDGEMENTS

This investigation was made possible by the Oklo-natural analogue project which is operated by Commissariat á l'Energie Atomique (CEA), France and supported by the EU. The authors would like to thank Dr John Smellie, Conterra AB, for suggesting the study and planning the campaign. We also thank John Smellie and Fred Karlsson, SKB AB, for their editorial and technical comments on the manuscript. Izabella Hallberg, GeoPoint AB, corrected the language. It has been a pleasure to co-operate with Paul-Louis Blanc and Didier Louvat at CEA.

7 REFERENCES

Amann, R.I., Ludwig, W. and Schleifer, K.-H., 1995. Phylogenetic identification and in situ detection of individual microbial cells without cultivation. *Microbiological Reviews*, **59**, 143-169.

Amann R, Springer N, Ludwig W, Görtz H-D, Schleifer K-H., 1991. Identification in situ and phylogeny of uncultured bacterial endosymbionts. *Nature*, **351**, 161-164.

Blanc, P.L. and Maravic, H.V., 1995. *4th joint Oklo working group CEC-CEA and final meeting of the "Oklo as a natural analogue" programme 1991-1995, Saclay, 22nd - 22nd June 1995.* pp 1-286. Institut de Protection et de Sûreté Nucléaire, Fontenay-aux-Roses.

Blanc P-L (Ed)., 1993. Oklo, Analogue Naturel de Stockage de Déchets Radioactifs. CEA-IPSN Rapport d'Avancement au 31 décembre 1992, SERGD 93/56 GEST.

Bodu, R., Bouzigues, H., Murin, N. and Pfiffelmann, J.P., 1972. Sur l'existence d'anomalies isotopiques rencontrées dans l'uranium du Gabon. *C R Acad Sci*, **275D**, 1731.

Boivin-Jahns, V., Bianchi, A., Ruimy, R., Garcin, J., Daumas, S. and Christen, R., 1995. Comparison of phenotypical and molecular methods for the identification of bacterial strains isolated from a deep subsurface environment. *Applied and Environmental Microbiology*, **61**, 3400-3406.

Brandberg F, Grundfelt B, Höglund L-O, Karlsson F, Skagius K and Smellie JAT., 1993. Studies of natural analogues and geological systems - Their importance to performance assessment. Nuclear Waste Commission of Finnish Power Companies, Rep. YJT-93-07, Helsinki; SKB Tech. Rep. (TR 93-05), Stockholm.

Braun-Howland EB, Danielsen SA, Nierzwicki-Bauer SA., 1992. Development of a rapid method for detecting bacterial cells in *Situ* using 16S rRNA-targeted probes. *Biotechniques*, **13**, 928-933.

Brosius J, Palmer ML, Kennedy PJ, Noller HF., 1978. Complete nucleotide sequence of a ribosomal RNA gene from *Escherichia coli. Proceedings of the National Academy of Science*, **75**, 4801-4805.

Brynhildsen L., 1991. Interactions between metals and bacteria-impotence for metal speciation. *Diss. Linköping Studies in Art and Science 70, Linköpings University.*

Choppin, G.R. and Allard, B., 1985. The complexes of actinides with naturally occurring organic compounds. In Keller, C., Freeman, A. and Landers, J. (Eds), Handbook of the Actinides, North-Holland Publ. Corp., Amsterdam, p.409-429.

Degueldre C., 1990. Grimsel colloid exercise: An international intercomparision exercise on the sampling and characterization of groundwater colloids. Report EUR 12660 EN, Commission of the European Communities, Brussels.

Degueldre C., 1994. Colloid properties in groundwaters from crystalline formation. NAGRA Technical Report NTB -92-06, Wettingen, Switzerland.

Degueldre C., 1995. Colloid properties in groundwaters from crystalline formations. PSI Bericht Nr 94-21, Villigen, Switzerland.

Degueldre C, Ulrich H-J, Silby H., 1994. Sorption of ^{241}Am onto montmorillonite, illite and hematite colloids. Radiochim. Acta **62**, 173-179.

Dugan PR, Stoner DL, Pickrum HM., 1992. The genus *Zoogloea*. In: Balows A, Truper HG, Dworkin M, Harder W, Schleifer K-Z (ed.) *The Prokaryotes*, pp 3137-3143. Springer-Verlag, New York.

Ekendahl S, Arlinger J, Ståhl F, Pedersen K., 1994. Characterization of attached bacterial populations in deep granitic groundwater from the Stripa research mine with 16S-rRNA gene sequencing technique and scanning electron microscopy. *Microbiology*, **140**, 1575-1583.

Farrelly V, Rainey FA, Stackebrandt E., 1995. Effect of genome size and *rrn* gene copy number on PCR amplification of 16S rRNA genes from a mixture of bacterial species. *Applied and Environmental Microbiology*, **61**, 2798-2801.

Felsenstein J., 1989. PHYLIP - phylogeny inference package. *Cladistics*, **5**, 164-166.

Fox GE, Wisotzkey JD, Jurtshuk JR P., 1992. How close is close: 16S rRNA sequence identity may not be sufficient to guarantee species identity. *International Journal of Systematic Bacteriology*, **42**, 166-170.

Gauthier-Lafaye, F (Ed)., 1994. Bangombé Reactor: Petrography, mineralogy and geochemistry of the solid phases. Prel. Inter. Rep. Univ. Strasbourg.

Gauthier-Lafaye, F. and Weber, F., 1989. Natural fission reactors of Oklo. *Economic Geol.* **84**, 2286-2295.

Giovannoni SJ, Britschgi TB, Moyer CL, Field KG., 1990. Genetic diversity in Sargasso sea bacterioplankton. *Nature*, **345**, 60-63.

Gurban, I., Ledoux, E., Made, B., Salignac, A.-L. and Winberg, A., 1995. Hydrology and modelling of flow and solute transport in the Okelobondo and Bangombé deposits. In: *4th joint Oklo working group CEC-CEA and final meeting of the "Oklo as a natural analogue" programme 1991-1995, Saclay, 22nd - 22rd june 1995.* pp 1-286. editors: **Blanc, P.L. and Maravic, H.V.** Institut de Protection et de Sûreté Nucléaire, Fontenay-aux-Roses.

Hallbeck, L. and Pedersen, K., 1990. Culture parameters regulating stalk formation and growth rate of *Gallionella ferruginea*. *Journal of General Microbiology*, **136,** 1675-1680.

Hobbie JE, Daley RJ, Jasper S., 1977. Use of nuclepore filter for counting bacteria by fluorescence microscopy. *Applied and Environmental Microbiology*, **33**, 1225-1228.

Huber R, Burggraf S, Mayer T, Barns SM, Rossnagel P, Stetter KO., 1995. Isolation of a hyperthermophilic archaeum predicted by in *situ* RNA analysis. *Nature*, **376**, 57-58.

Karlsson, F. Allard, B. Hummel, W Sargent, F.P. Vieno, T. Voinis, S. Merceron, T. Yoshikawa, H., 1994. Natural organic substances in granitic groundwater and their implications for nuclear waste disposal. Presentation at the "Workshop on binding models concerning natural organic substances in performance assessment" organised jointly by OECD/NEA and PSI and hold in Bad Zurzach, Switzerland 14-16 Sept. 1994 (To be published by OECD/NEA).

Laaksoharju M, Vuorinen U, Snellman M, Allard B, Pettersson C, Helenius J and Hinkkanen H., 1994. Colloids or artefacts? A TVO/SKB co-operation project in Olkiluoto, Finland. Report YJT-94-01, Nuclear Waste Commission of Finnish Power Companies, Helsinki.

Ledin, A., 1993. Colloidal carrier substances - properties and impact on trace metal distribution in natural waters. Diss. Linköpings Studies in Art and Science 91. Linköping University.

Louvat D., 1994. The Oklo Natural Analogue Project. Proceedings of the 6th CECNAWG Meeting, September 12-16, 1994, Sante Fe, New Mexico.

Malcolm, R., 1991. Factors to be considered in the isolation and characterization of aquatic humic substances. In: Allard, B., Borén, H. and Grimvall, A. (Eds). Humic Substances in the Aquatic and Terrestrial Environment, **pp 9-36.** Springer Verlag.

Marmur JA., 1961. A procedure for the isolation of deoxyribonucleic acid from microorganisms. *Molecular Biology*, **3**, 208-218.

McCarthy J, Degueldre C., 1993. Sampling and characterization of groundwater colloids for studying their role in subsurface transport of contaminants. In: J. Buffle and H. Van Leeuwen (Eds) Environmental Particles, Vol II. Lewis Publishers, Chelsea MI. Chapter 6, pp. 247-315.

McCarthy J, Zachara J., 1989. Subsurface transport of contaminants. Environ. Sci. Technol., **23**, 496-502.

Miekeley N, Coutinho de Jesus H, Porto da Silviera C, Degueldre C., 1992. Chemical and physical characterization of suspended particles and colloids in waters from the Osamu Utsumi mine and Morro do Ferro analogue study sites, Poços de Caldas, Brazil. J. Geochem. Exploration **43**, 409-437.

Miller W, Alexander R, Chapman N, McKinley I, Smellie J., 1994. Natural analogue studies in the geological disposal of radioactive waste. *Studies in Environmental Science 57*, Elsevier, London.

Neidhardt FC, Ingraham JL, Schaechter M., 1990. *Physiology of the bacterial cell.* Sinauer Associates, Inc., Sunderland.

Niemelä, S., 1983. Statistical evaluation of results from quantitative microbiological examinations. Nordic committee on food analysis. Report no 1, 2nd edn, ISSN 0281-5303.

Pedersen, K. (1996) Investigations of subterranean bacteria in deep crystalline bedrock and their importance for the disposal of nuclear waste. Canadian Journal of Microbiology, (in press).

Pedersen, K., 1993a. The deep subterranean biosphere. *Earth-Science Reviews*, **34,** 243-260.

Pedersen, K., 1993b. Bacterial processes in nuclear waste disposal. *Microbiology Europe*, **1,** 18-24.

Pedersen, K. and Ekendahl, S., 1990. Distribution and activity of bacteria in deep granitic groundwaters of southeastern Sweden. *Microbial Ecology*, **20,** 37-52.

Pedersen, K. and Karlsson, F., 1995. Investigations of subterranean bacteria - Their importance for performance assessment of radioactive waste disposal. *SKB Technical Report 95-10.* Swedish Nuclear Fuel and Waste Co., Stockholm. pp 1-222.

Pedersen K, Ekendahl S., 1990. Distribution and activity of bacteria in deep granitic groundwaters of southeastern Sweden. *Microbial Ecology*, **20**, 37-52.

Pettersson, C., 1992. Properties of humic substances from groundwater and surface waters. Diss. Linköping Studies in Arts and Science 79. Linköping University.

Pettersson, C., Allard, B., Ephraim, J., Vilks, P., 1994. Humic substances in groundwater from the Cigarr Lake area. In: Cramer, J.J. and Smellie, J.A.T. Final report of the AECL/SKB Cigarr Lake Analog Study. AECL-10851, COG-93-147, SKB-TR 94-04. AECL Research, Manitoba, Canada.

Pettersson, C., Ephraim, J., Allard, B. and Borén, H., 1990. Characterization of humic substances from deep groundwaters in granitic bedrock in Sweden. SKB TR 90-29. Swedish Nuclear Fuel and Waste Management Co, Stockholm.

Risatti JB, Capman WC, Stahl DA., 1994. Community structure of a microbial mat: The phylogenitic dimension.(sulfate-reducing / 16S rRNA/oligonucleotide probes). *Proceedings of the National Academy of Science, USA*, **91**, 10173-10177.

Smellie JAT, Winberg A and Karlsson F., 1993. Swedish activities in the Oklo Natural Analogue Project. Proceedings of the 2nd. CEC-CEA Oklo Working Group Meeting, Brussels, April 6-7, 1992. Report EUR 14877.

Smellie JAT and Winberg A., 1992. Background and status of SKB participation in the Oklo Project (August 1990 - December 1991), and future recommendations. In: Oklo, Analogue Naturel de Stockage de Déchets Radioactifs. (Eds. A-M, Chapuis and P-L, Blanc). CEA-IPSN Rapport d'Avancement 1991, SERGD 92/25, Paris.

Smith P, Degueldre C., 1993. Colloid facilitated transport of radionuclides through fractured media. J. Contam. Hydrol. **13,** 143-166.

Stackebrandt E, Goebel BM., 1994. Taxonomic Note: A place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. *International Journal of Systematic Bacteriology*, **44**, 846-849.

Toulhoat P, Gallien J-P, Louvat D, Moulin V, l'Henoret P, Ledoux E, Gurban I, Smellie JAT and Winberg A., 1994. Present-time migration around the Oklo natural reactor. Evidence of uranium isotopic anomalies in groundwaters close to reaction zones. Migration '93, Charleston, S. Carolina (1993). 383-390.

Tower KJ., 1992. The genus *Acinetobacter*. In: Balows A, Truper HG, Dworkin M, Harder W, Schleifer K-Z (ed.) *The Prokaryotes*, pp 3137-3143. Springer-Verlag, New York.

Wallace DM., 1987. Large and small-scale phenol extractions. *Methods in Enzymology*, **152**, 33-41.

Ward DM, Bateson MM, Weller R, Ruff-Roberts AL., 1992. Ribosomal RNA analysis of microorganisms as they occur in nature. *Advances in Microbial Ecology*, **12**, 219-286.

Ward DM, Weller R, Bateson MM., 1990. 16S rRNA sequences reveal numerous uncultured microorganisms in a natural community. *Nature*, **345**, 63-65.

Winberg A and Smellie JAT., 1993. Oklo Natural Analogue Study: Preliminary reporting of CEA/EdM/SKB activities at Bangombé performed during September-December, 1992. In: Oklo, Analogue Naturel de Stockage de Déchets Radioactifs. (Ed. P-L, Blanc). CEA-IPSN Rapport d'Avancement au 31 décembre 1992, SERGD 93/56 GEST, Paris.

Woese C., 1987. Bacterial evolution. *Microbiological Reviews*, **51**, 221-271.

List of SKB reports

Annual Reports

1977-78
TR 121
KBS Technical Reports 1 – 120
Summaries
Stockholm, May 1979

1979
TR 79-28
The KBS Annual Report 1979
KBS Technical Reports 79-01 – 79-27
Summaries
Stockholm, March 1980

1980
TR 80-26
The KBS Annual Report 1980
KBS Technical Reports 80-01 – 80-25
Summaries
Stockholm, March 1981

1981
TR 81-17
The KBS Annual Report 1981
KBS Technical Reports 81-01 – 81-16
Summaries
Stockholm, April 1982

1982
TR 82-28
The KBS Annual Report 1982
KBS Technical Reports 82-01 – 82-27
Summaries
Stockholm, July 1983

1983
TR 83-77
The KBS Annual Report 1983
KBS Technical Reports 83-01 – 83-76
Summaries
Stockholm, June 1984

1984
TR 85-01
Annual Research and Development Report 1984
Including Summaries of Technical Reports Issued during 1984. (Technical Reports 84-01 – 84-19)
Stockholm, June 1985

1985
TR 85-20
Annual Research and Development Report 1985
Including Summaries of Technical Reports Issued during 1985. (Technical Reports 85-01 – 85-19)
Stockholm, May 1986

1986
TR 86-31
SKB Annual Report 1986
Including Summaries of Technical Reports Issued during 1986
Stockholm, May 1987

1987
TR 87-33
SKB Annual Report 1987
Including Summaries of Technical Reports Issued during 1987
Stockholm, May 1988

1988
TR 88-32
SKB Annual Report 1988
Including Summaries of Technical Reports Issued during 1988
Stockholm, May 1989

1989
TR 89-40
SKB Annual Report 1989
Including Summaries of Technical Reports Issued during 1989
Stockholm, May 1990

1990
TR 90-46
SKB Annual Report 1990
Including Summaries of Technical Reports Issued during 1990
Stockholm, May 1991

1991
TR 91-64
SKB Annual Report 1991
Including Summaries of Technical Reports Issued during 1991
Stockholm, April 1992

1992
TR 92-46
SKB Annual Report 1992
Including Summaries of Technical Reports Issued during 1992
Stockholm, May 1993

1993
TR 93-34
SKB Annual Report 1993
Including Summaries of Technical Reports Issued during 1993
Stockholm, May 1994

1994
TR 94-33
SKB Annual Report 1994
Including Summaries of Technical Reports Issued during 1994.
Stockholm, May 1995