

# Evidence for a Hydrogen-driven, Intra-terrestrial Biosphere in Deep Granitic Rock Aquifers

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## ABSTRACT

Transmission electron microscopy was used to detect bacterial microfossils buried in fracture surface calcites in a granitic rock aquifer from 207 m below ground. 16S rRNA sequencing demonstrated that bacteria in deep rock groundwater (400 m) were not contaminants from drilling operations. All groundwaters investigated contain hydrogen and methane, ranging from 0.1 to 70  $\mu\text{M}$  and 0.1 to 1000  $\mu\text{M}$ , respectively. Homoacetogens and acetoclastic and autotrophic methanogens have been cultured from many different deep sites down to 900 m at viable count numbers, constituting at an average 10 - 15 % of the total number of microorganisms. A new methanogenic species from deep rock aquifers, *Methanobacterium subterraneum*, was recently reported. *In vitro* methanogenic and acetogenic activities were shown for many of the sites investigated. The hydrogen driven deep biosphere theory includes chemoautotrophic production of acetate, methane and biomass with hydrogen produced by geological processes as the primary energy source. This biosphere is, therefore, independent of photoautotrophic organic carbon. The produced chemoautotrophic organic carbon can be used by heterotrophic microbes. Sulphate and iron reducing bacteria frequently occur in deep granitic groundwater, e.g. *Desulfovibrio aespoeensis* and *Shewanella putrefaciens*. Yeast and other fungi have been obtained from depth as 18S sequences and also as isolates. The existence of a deep hydrogen-driven, intra-terrestrial biosphere seems very probable.

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## Introduction

### *The hard rock environment*

The disposal of high level nuclear waste from the electrical power industry in Sweden is planned to be done in a repository approximately 500 m under ground. Other hazardous wastes, especially mercury, are also intended to be deposited deep underground. Most of the Swedish ground, except for the southern part and the Baltic Sea islands, consists of hard rocks such as granite or gneiss of the Fennoscandian Shield. The Swedish hazardous waste repositories will be placed in hard rocks of that kind. One of a series of different scientific questions regarding the isolation of non-destructible wastes underground concerns the effect from microorganisms on repository performance [18]. The Swedish research program on the influence of microorganisms on nuclear waste repository performance started in 1987 and is still in progress [20, 17]. This paper describes recent results obtained during that research endeavour.

### ***Microbial Biosystems: New Frontiers***

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The Fennoscandian Shield is 1.6 - 3.1 billion years old, and has been fractured by considerable forces, e.g. glaciations, during that time span. As result, the rock exhibits fractures at an overall average of 2 meters, with significant local variations. They range in width from less than a mm up to several meters thick faults with crushed rock. In common, they all may transport water. Intersecting large faults during tunnelling can cause inflows of several m<sup>3</sup> of groundwater per minute to the tunnel unless preventive measures are taken, demonstrating that parts of the rock contain much water and can have a very high hydraulic conductivity. The lifetime of a fracture is not eternal. Groundwater over-saturated with ions that may form precipitates, e.g. calcium carbonate, can eventually seal a fracture.

## **Methods**

### *Drilling for microorganisms in hard rock*

Drilling to access microbial ecosystems in hard rock is a forceful operation and the risk for microbial contamination of the aquifers during drilling must be minimised. Different techniques are available for hard rock drilling. The best method is termed triple tube drilling where the drill core is pushed into a protective liner that can be retrieved from the surface without lifting up the drill tube. A specially designed front washed drill bit should be used that does not flush the core itself. Drill water must be used to cool the drill bit and to transport drill debris out of the borehole. We use groundwater from adjacent boreholes as drill water, store it under nitrogen in clean tanks and add the fluorescent tracer dye uranine before use. This drill method gives drill cores with fractures that have been protected from flushing with drill water and the tracer will give an accurate estimation of how much drill water is left in the aquifers after drilling.

It can be argued that observed microbial life in deep groundwater is an artefact caused by the access operations [13] and not a true deep biosphere. We have approached this objection in several different ways. If microorganisms have been present deep underground over geological times, it should be possible to detect fossilised bacteria in fracture filling material. A successful, recent investigation of drill cores intersecting a granitic aquifer at 207 m depth demonstrated for the first time that this is the case [15]. Fossil bacteria were found partly organised in biofilms. This observation provides evidence for ancient life in deep granitic rock aquifers and suggests that the modern life found there is indigenous.

Proving that certain species of microorganisms found in drilled boreholes are indigenous and not introduced during drilling is extremely difficult. Instead, as was shown by Pedersen et al [16], the opposite situation was easier to investigate, i.e. testing if a known contaminating microbial population can colonise or not in deep granitic aquifers during drilling. A 600 m long tubing used for drill water supply constituted a source of bacterial contamination to the rest of the drilling equipment and the boreholes. The number of bacteria in the drill water could be determined, as could the numbers of bacteria in groundwater from the drilled boreholes. The amount of drillwater left in the aquifers after drilling was analysed using the uranine tracer method. This measurement enabled us to calculate the maximum number of microorganisms in the groundwater if the drill water alone was the source. The number of bacteria in the groundwater was 100 times larger than could be accounted for in the drillwater. This can only be explained if microorganisms were present in the aquifers before drilling. Molecular and culturing methods were also used and the results showed that, although large numbers of contaminating bacteria were

introduced from the drilling equipment into the boreholes during drilling, they did not become established in the aquifers at detectable levels. The reason for this is likely the inability of foreign microbes to adapt to the prevailing oligotrophic, reducing, anaerobic and low temperature environmental conditions in deep granitic aquifers. Earlier documentation of *in situ* activity of microbial populations in deep granitic rock environments suggests that the microbes present are active at low but significant levels [2, 10, 11, 12]. It can, therefore, be speculated that most of the microorganisms found in intersected aquifers were there before drilling.

## Results and Discussion

Two new species isolated from aquifers in deep fractured granitic rock were recently reported. *Desulfovibrio aespoeensis* is a mesophilic, salt tolerant sulphate reducing bacterium (SRB) that grows well at conditions typical for deep aquifers [7].

*Metanobacterium subterraneum* is an alkaliphilic, eurythermic and halotolerant methanogen that also was found to be well adapted to a life in hard rock aquifers [3]. It is the first example of a methanogen isolated from a deep granitic aquifer. Both these two species are adapted to life under the conditions prevailing where they were isolated from, and so provide additional evidence that they are indigenous.

### *The hydrogen driven intra-terrestrial biosphere hypothesis*

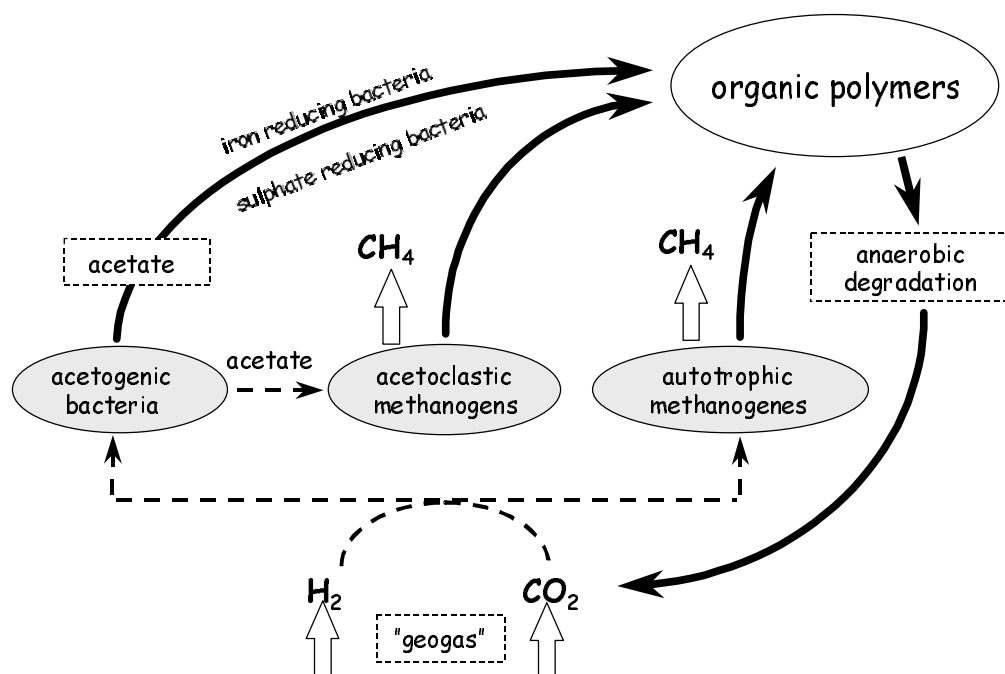
Groundwater at depths of 500 m can be very old and ages of 10,000 years are not uncommon. This poses a conceptual problem for the deep intra-terrestrial biosphere: What primary energy source is it using? Organic carbon from the photosynthetically driven surface ecosystem is consumed by the time it reaches such depths. Typical concentrations at depth are one mg dissolved organic carbon per litre groundwater or less [17]. Any energy source at this depth must be renewable. Throughout our work on deep granitic rock environments, results have indicated the presence of autotrophic microorganisms that utilise hydrogen as a source of energy. Therefore, a hypothesis of a hydrogen-driven biosphere in deep granitic aquifers has been suggested [14, 8]. The organismal basis for this biosphere is suggested to consist of acetogenic bacteria that have the capability of coupling hydrogen oxidation with carbon dioxide reduction to acetate (homoacetogens) and methanogens that yield methane from hydrogen and carbon dioxide (autotrophic methanogens) or from acetate produced by homoacetogens (acetoclastic methanogens) (Fig. 1). A similar hypothesis has recently been published for deep basaltic rock aquifers (Stevens and McKinley 1995) as have arguments against it [1]. One of the aims of our recent studies has, therefore, been to investigate the possibility of a hydrogen driven deep biosphere in deep granitic aquifers and we have focused on acetogenic bacteria and methanogens as the autotrophic base for such a biosphere.

Continuous support of hydrogen to the populations in the investigated granitic rock environment can be delivered from radiolysis of water. The Baltic Shield granites contain uranium that in its decay series among other species produces radium and radon. The radioactivity during this decay generate heat in the rock, e.g.  $7 \mu\text{Wm}^{-3}$  in Malingsbo granite [6] and explains a significant part of the heat flow from deep Swedish rock. Radium, and also thorium, migrate from the rock matrix to the aquifers where they tend to sorb on the aquifer surfaces. Additionally, radon gas will be generated and dissolved in the groundwater. The alpha radiation produced during decay of these, and other species as well [5], may be enough for a sustainable hydrogen production from radiolysis of water in deep

granitic aquifers to the intra-terrestrial biosphere. This possibility is presently being investigated in more detail.

### Distribution and activity of methanogens and homoacetogens in deep granitic aquifers

The Äspö Hard Rock Laboratory (HRL) is situated on the island of Äspö, adjacent to the Baltic coast of Sweden approximately 400 km south of Stockholm. The access tunnel to the HRL descends with a declination of 14 % from the Baltic shoreline for a distance of approximately 1700 m under the sea-floor, where it spirals down and terminates 460 m below the island of Äspö [19]. The total length of the tunnel is 3600 m. Distribution, numbers and physiological diversity of homoacetogens and methanogens in deep granitic rock aquifers at the Äspö HRL were investigated using a variety of methods [4]. The results revealed the existence of active chemoautolithotrophic microbial communities in the Äspö HRL deep granitic groundwater, which contains hydrogen and methane. Homoacetogens and acetotrophic methanogens dominated in the groundwater to a depth of 112 m, while autotrophic methanogens prevailed deeper, down to 446 m. The results from most probable number counts showed that viable methanogens and homoacetogens coexisted at all depths investigated, from 45 m to 446 m below sea level. *In vitro* radiotracer experiments demonstrated these microorganisms to form methane and acetate from CO<sub>2</sub> with hydrogen, and methane from [<sup>14</sup>C]-acetate at close to *in situ* temperature (17°C). The results suggest that autotrophic methanogens and homoacetogens initiate a deep subterranean food chain and that acetate-dependant methane formation is mediated by



**Fig. 1.** The deep hydrogen driven intra-terrestrial biosphere hypothesis, illustrated by its carbon cycle. At relevant temperature and water availability conditions, intra-terrestrial microorganisms theoretically are capable to perform a life cycle that is independent of sun-driven ecosystems. Hydrogen and carbon dioxide from the deep crust of earth or organic carbon from sedimentary deposits can be used as energy and carbon sources. Phosphorus is available in minerals like apatite and nitrogen for proteins, nucleic acids etc. can be obtained via nitrogen fixation; nitrogen is predominate in most ground waters [19].

homoacetogenesis. The finding of an active, deep, hydrogen based autotrophic biosphere adds a significant but previously overlooked reducing activity to deep granitic rock, in accordance with the deep hydrogen driven biosphere hypothesis as depicted in Fig. 1.

### **Eukarya in deep aquifers**

18S rRNA sequences closely related to yeast have occasionally appeared among sequenced clones from extracted and PCR-amplified environmental deep groundwater DNA [9, 22]. This triggered us to enrich for yeasts and other fungi in deep groundwater from Äspö HRL. Samples were taken from drilled boreholes and directly from fractures in the tunnel walls. Yeast and other fungi could be enriched from most of the sampled sites. The possibility for contamination during sampling is very low, but it is of course possible that boreholes were contaminated during drilling and installation of borehole equipment. That would, however, not apply to the sampled fractures. At the present stage, we do not know if the found yeast and other fungi are intrinsic and active in the deep aquifers or if they have been transported as spores with groundwater from the surface. Presence of indigenous eukaryotes would add evidence for an intra-terrestrial biosphere, as they would need to rely on a continuous autolithotrophic organic carbon production to be active.

### **Tunnel life**

The presence of active chemolithotrophic life in the Äspö HRL tunnel has been reported earlier [19, 17]. These bacteria utilise the energy from ferrous iron or sulphide oxidation with oxygen in the tunnel air; these reduced compounds may have been produced by sulphate- and iron-reducing bacteria in aquifers inside the rock. Recently, we have been investigating the potential for microbial oxygen reduction in backfilled nuclear waste repositories. Our investigations of the Äspö tunnel environment have demonstrated very viable populations of methanotrophs in tunnel water ponds and also in groundwater from boreholes. Significant amounts of methane are dissolved in most deep groundwater that reaches the tunnel through fractures and boreholes [4] and this methane is utilised as a source for energy and carbon by aerobic methanotrophs. This is of importance for the nuclear waste disposal industry because microorganisms will significantly contribute to the reduction of oxygen in a repository. Microbial oxygen reduction is beneficial because the copper canisters to be used for encapsulation of the waste are prone to corrosion in the presence of oxygen. Additionally, many dangerous radionuclides are less mobile in reducing environments compared to oxidised ones.

### **Conclusions**

Our results suggest that a deep intra-terrestrial granitic biosphere exists, driven by the energy available in hydrogen formed through radiolysis of water in the rock aquifers. Our planet apparently has two biospheres, the photosynthetically driven biosphere that exist at the surface, and the new, unexplored geologically driven intra-terrestrial biosphere (possibly radiation driven). Knowledge about this biosphere has just begun to emerge and is expanding the spatial borders for life from a thin layer on the surface of the planet Earth and in the seas to a several kilometre thick biosphere reaching deep below the ground surface and the sea floor [23]. If this theory holds, life may have been present and active deep down in Earth for a very long time and it cannot be excluded that the place for the origin of life was a deep subterranean igneous rock aquifer environment (probably hot with a high pressure) rather than a surface environment. Future research will aim at exploration

of the distribution, diversity, *in situ* activity and biogeochemistry of this intra-terrestrial biosphere.

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