**Microbial cooperation in deep subsurface groundwaters of the Fennoscandian Shield**

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**Abstract (max *n* characters)**

Aquifers encompass a large proportion of microbial biomass while being an unsuitable environment for most other forms of life. With a low flux of chemical energy, deep biosphere communities are characterized by specialized microbial populations hypothesized to survive via a high degree of interactions. Studying these interactions provides insights into how these populations cope with the oligotrophic conditions. To do so, anaerobic enrichment cultures were inoculated using groundwater from Äspö Hard Rock Laboratory and enriched using a range of electron donors. The genomic potential of the populations in the cultures was assessed using metagenomics and combined with cryogenic electron microscopy to determine the morphology of the populations.

**Introduction**

* **Importance of aquifers as large reservoir of Earth’s freshwater**
  + **All three domains of life**
  + **Activity**
  + **Why do we study the microbes in these aquifers?**
* **Biogeochemical cycling plus examples**
  + **Briefly introduce some major cycles (S, N, C)**
  + **What is the contribution of the microbes to these major cycles?**
  + **Cryptic cycling / oligotrophy**
  + **The need for sequencing data because geochemistry alone does not detect the cycled components**
* **Adaptations to oligotrophy**
  + **Genome streamlining and variation in genome size.**
  + **What is the implication of a reduced genome?**
  + **Relation genome size and physical cell size.**
  + **Energetics of genome complexity.**
  + **CPR + DPANN**
  + **How do these microbes survive?**
* **Fennoscandian Shield**

Äspö Hard Rock Laboratory (HRL) is a 3.6 km long underground research facility, extending 460 m below sea level into 1.8 Ga years old Fennoscandian Shield granitoids. The underground tunnel network, operated by the Swedish Nuclear Fuel and Waste Management Company, is situated partly under land and partly under sea and its boreholes intersects aquifers that are primarily oligotrophic anoxic groundwaters. These aquifers are of different origin, such as those influenced by marine surface water, situated under land, or aquifers devoid of any hydrological connection to the surface. Baltic Sea water is drawn down in the bedrock through vertical to subvertical fractures, supplying connected subsurface aquifers with dissolved organic matter1 and thereby shaping the microbial communities2. The labile organic matter is rapidly consumed by microbes as a valuable energy source, leaving the refractory organic matter for specialized microbial populations1.

Previous studies have shown the microbial communities in this deep biosphere environment to be alive, active, and based on 16S rRNA gene amplicons abundant in the phyla Proteobacteria, Desulfobacterota, Patescibacteria, Chloroflexota, Spirochaetota, Omnitrophota, and Nanoarchaeota. Patescibacteria, being adapted to low-energy conditions and characterized by streamlined genomes, thrive in the more shallow groundwaters in terms of abundance and this phylum has been described together with the Nanoarchaeota as being dependent on neighboring populations for the supply of metabolites. Associative lifestyles as shown by Patescibacteria, Nanoarchaeota, and possibly also Omnitrophota make the aquifers in Äspö HRL a suitable environment for studying interactions among microbes.

* + **Connectivity of microbes and surface input of DOC**
  + **Summary of the microbial community (Depth and diversity paper)**
  + **Äspö Hard Rock Laboratory and Olkiluoto**
  + **What makes it a suitable site for studying interactions? (CPR + DPANN; presence and genomes)**
* **Study design:**

In this study, 96 anaerobic cultures were set up to enrich for uncharacterized clades. To do so, four pristine subsurface aquifers were sampled under anoxic conditions to inoculate either a medium either enriching for sulfate reducers or a medium favoring necrotrophs. Additionally, to investigate growth of smaller cells, larger cells were removed by filtering the inoculum using a pore size of 0.45 *µ*m. The microbial communities in the cultures were characterized using 16S rRNA gene amplicons, allowing to generate hypothesis on interactions and to select cultures for metagenomics based on composition. The reconstructed genomes were used to assess genomic potential and to investigate the relation between cell size and genome size.

* + **Anaerobic enrichment cultures**
  + **Medium based on metagenomes**
  + **16S rRNA gene amplicons**
  + **Metagenomes**
  + **<Microscopy>**
  + **Hypothesis: Enrichment of populations passing 0.45 µm filter restricts cell division**

**Results and discussion**

**Aquifers used for enrichment**

Four aquifers, intersected by boreholes in Äspö HRL, were used as inoculum for anaerobic enrichment cultures. These aquifers have different retention times, mainly caused by a variable hydrological connectivity to surface waters as reflected in their isotope signatures (d18O and 87/86Sr). Compared to the other three groundwaters, SA1420 is characterized by a relatively high d18O ratio, more similar to the -6‰ value reported in the Baltic Sea, indicating infiltration of marine surface water. The meteoric aquifer was influenced by precipitation percolating through the soil to eventually reach this fracture after eight months to a year. This surface input is reflected in the relatively high DOC content, low chloride plus sulfate concentration and an intermediate d18O value. SA2600 has a very low d18O value of -12.2, revealing this fracture to be isolated from surface input, hence an estimated retention time over thousands of years3. In contrast to the meteoric aquifer, this fracture had a low DOC content yet high concentrations in chloride and sulfate ions of 389 and 6.56 mM, respectively. The saline groundwater was suggested to originate as a marine water that underwent changes in composition over geological time scales due to interactions with minerals on the fracture’ surface. KA3385, despite being the deepest aquifer, was described as having a mixed origin, showed by its d18O value and an intermediate chloride plus sulfate concentration.

The different hydrological and chemical properties of the aquifers are reflected in the composition and cell density of the microbial communities. For example, the Atribacterota were only scarcely present in the meteoric and marine aquifers while being the most abundant phylum in the saline fracture (Fig. X). Furthermore, the Patescibacteria were most abundant in the meteoric plus marine aquifer, where they together with Desulfobacterota, Omnitrophota, Chloroflexota, and Nitrospirota comprised 75% of the microbial community. These data support previous genome-based analysis4,5 that fermentation and sulfate/sulfur reduction are important processes in these groundwaters. Regarding cell density, microbial abundance decreased with depth and organic carbon content, as reviewed in McMahon et al.6, and were in the same order of magnitude as other aquifers in crystalline rocks at similar depths6. The meteoric groundwater was the most densely populated with 7.8 × 105 ± 1.5 × 105 cells ml-1 (mean ± standard deviation, *n* = 2, t0 in Fig. X), followed by the marine groundwater (6.7 × 104 ± 1.0 × 104 cells ml-1), the saline groundwater (1.9 × 104 ± 6.3 × 103) and the mixed groundwater (1.5 × 104 ± 2.5 × 103).

**Conclusion**

**Methods**

**Groundwater sampling**

* Properties of the groundwaters (hydrology, retention time, REE)

**Enrichment media and microscopy**

* SRB medium based on metagenomes
* Fluorescent microscopy

**Molecular work**

* Extraction of nucleic acids (discuss negative controls)
* Amplification (primers, negative controls)
* Sequencing 16S rRNA gene amplicons (MiSeq 2 x 300 bp)
* Library preparations + sequencing metagenomes (NovaSeq SP 2 x 150 bp)

**Bioinformatic analyses**

* Nf-core Ampliseq pipeline, GTDB-SBDI database (mention version)
* Nf-core MAG pipeline

**Statistics and reproducibility**

* Diversity estimates plus ordination method. Not a focus on statistics in manuscript.

**Data availability**

All sequencing data has been made publicly available at the European Nucleotide Archive under project reference PRJNAXXXX.

**Code availability**

**References**

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**Contributions:** M.D. and S.B. conceived the study; M.D., G.W., and S.T. designed the research; G.W. inoculated and maintained the cultures; G.W. performed the molecular work; G.W., S.T., and M.M. produced and/or analyzed the sequencing data; G.W. and M.D. drafted the manuscript with comments from all authors.

**Competing interests:** The authors declare no competing interests

**Figure legends**

**Fig. 1. Overview of the sampling site plus chemistry of the groundwaters**

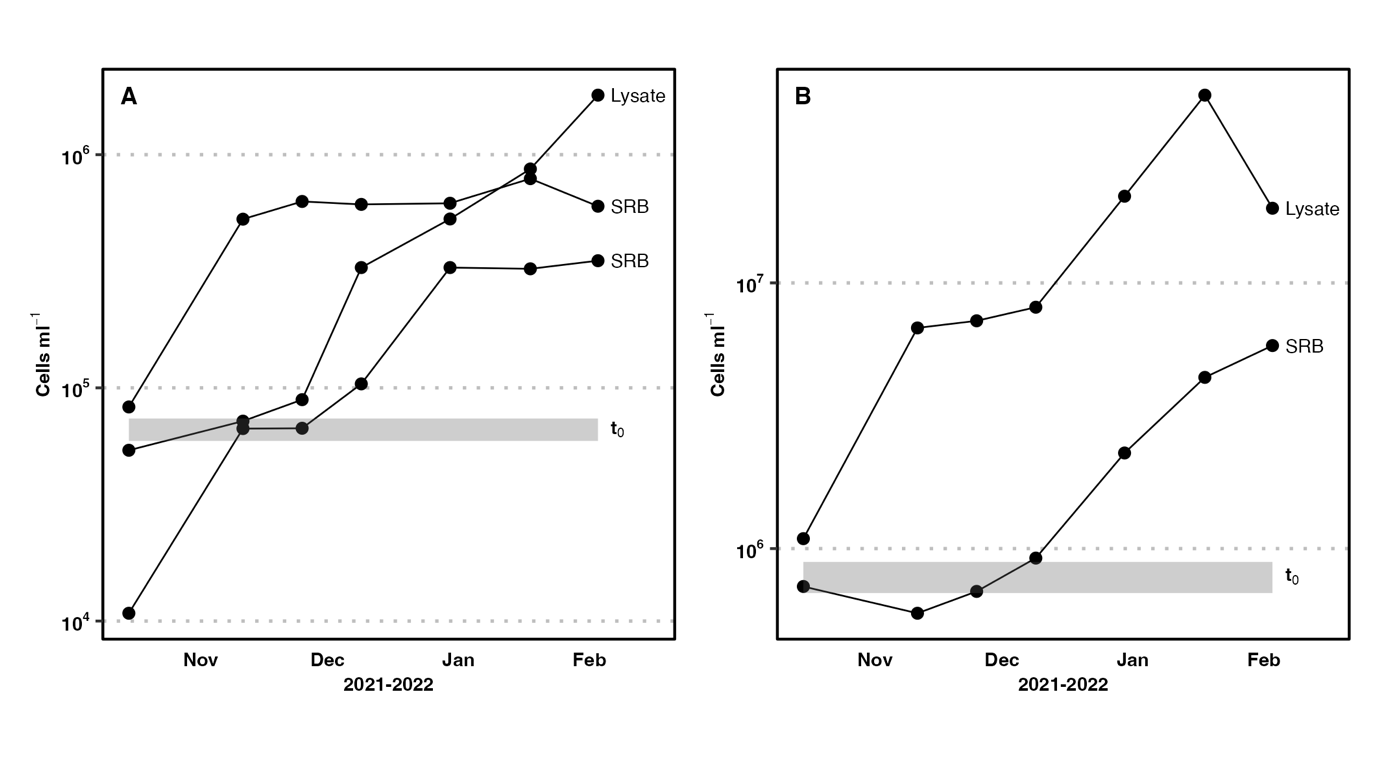
**Fig. 2. Growth and community diversity**

**Fig. 3. Composition of selected enrichment cultures**

|  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  | **Origin\*** | **Depth (m)** | **d18O (**‰) | **DOC (mM)** | **87/86Sr** | **SO42- (mM)** | **NH4+ (mM)** | **Cl- (mM)** | **pH** |
| **KR0015** | meteoric | 69.00 | -10.5 | 1.28 | 0.716 | 0.628 | 0.0179 | 18.7 | 7.6 |
| **SA1420** | marine | 200.6 | -7.45 | 0.549 | 0.716 | 3.27 | 0.106 | 83.5 | 7.5 |
| **KA3385** | mixed | 448.4 | -11.1 | 0.0916 | 0.719 | 4.34 | NA | 228 | 7.5 |
| **SA2600** | saline | 345.0 | -12.2 | 0.0832 | 0.719 | 6.56 | 2.41× 10-3 | 389 | 7.5 |

**\* According to Osterholz et al.**1

**Molar mass carbon 12.011, sulfate 96.06, ammonium 18.039, and chloride 35.45 g mol-1**

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