Flow cell systems for fieldwork

Three identical field systems with four flow cells each, a micropump (Micropump GAH series V21 J with PEEK impeller; Labinett, Göteborg, Sweden), two pressure meters (S-11, 40 Bar 4-20 G1/2; WIKA, Klingenberg am Main, Germany), a flow meter (Promag 50; Endress+Hauser Flowtech AG, Sollentuna, Sweden), and a 4-L expansion vessel (Pedersen, 2005) were installed in a container placed in a tunnel niche at a depth of 320 m in the ONKALO tunnel as shown in Supplementary Figure 2 and connected to the packer system in the ONK-PVA06 borehole. Each flow cell had a stainless steel shell (length 300 mm, diameter 65 mm) and was lined with polyvinyldifluoride (PVDF) plastic. Each flow cell had a 120-mm-long PVDF insert with a 22 × 32 mm opening that supported 110 g of crushed rock grains (2–4 mm in diameter) offering a rock surface area of approximately 3400 cm^2 per FCC for microbial adhesion and biofilm development, assuming spherical rock grains with an average diameter of 3 mm. The rock grains were heat sterilized (160°C for 5 h) and were obtained from the drill core of the ONK-PVA06 borehole at the approximate site of the intersected aquifer. Three flow stabilizers at each end of the insert ensured even distribution and a slow laminar flow of water through each flow cell (Pedersen, 1982). The flow cells were installed on 24 November 2010 and circulated with groundwater under the *in situ* pressure of 2.4 MPa for 110 d at a flow rate to and from the aquifer of 17–18 mL min^{-1}. The total volumes of groundwater circulated were 2597, 2840, and 2758 dm^3 in the three field systems, respectively, as registered by flow meters.

Configuration of growth experiments

The 12 FCs exposed to ONK-PVA06 groundwater for 110 d were transported under pressure from the ONKALO tunnel to the laboratory in Mölnlycke and installed, four by four, in three FCCs. These FCCs have been described in detail elsewhere (Pedersen, 2005; Hallbeck and Pedersen, 2008) and are shown in Supplementary Figure 3. The interior tubes and valves of the FCCs were sterilized with 20 mg L^{-1} chlorine dioxide, rinsed with sterile water, and filled with N_2 before FC installation. Each cabinet was temperature controlled at 16–18°C and a pressure of 2.4 MPa was maintained. A total of seven 4-L expansion vessels were filled with groundwater from ONK-PVA06, shipped pressurized with the FCs, and used to fill the FCCs with a total of 5000 mL of groundwater at the start of the experiments, as described previously (Pedersen, 2012a, b). Thereafter, gases were added as follows. Three Teflon-lined, 500-mL stainless steel cylinders (304L-HDF4-500-T; Swagelok, Göteborg, Sweden) were filled at RT (20°C) with air to a pressure of 220 KPa, methane to a pressure of 220 KPa, and 50% H_2 and 50% methane to a pressure of 440 KPa, respectively. The cylinders were filled with groundwater under pressure (2.4 MPa), resulting in a total circulating volume of 5500 mL per FCC. These gas additions then corresponded to final theoretical concentrations of 7.9 mM N_2 plus 2.1 mM O_2, 11 mM methane, and 11 mM methane plus 10 mM H_2, respectively (including 2.7 mM naturally occurring methane in the groundwater). These treatments are hereafter denoted O_2N_2, CH_4, and H_2:CH_4, respectively. The start date was 15 March 2011 (day 0) and the end date was 28 June 2011, resulting in an experimental time of 105 d. The flow rate was kept at 20 mL min^{-1}, corresponding to a flow of approximately 1 mm s^{-1} over the rock grains.

Sampling procedures

Complete sampling was performed six times, on days 3, 21, 42, 63, 84, and 105, and analysed as described below. A pilot sampling was performed on day 0 before the gas

additions to test the sampling procedures. On each sampling occasion, 20 mL of circulating water was drained and disposed of; thereafter, 5 mL of water was sampled and analysed for pH and 10 mL of water was collected in a sterile 15-mL polypropylene (PP) tube (Sarstedt, Landskrona, Sweden) for immediate analysis of ATP. Thereafter, 4 × 10 mL of water was collected via syringes in butyl-rubber-stoppered anaerobic glass tubes (no. 2048-00150; Bellco Glass,Vineland, NJ, USA) and 5 mL was collected for analysis of CHAB. Two 10-mL volumes of water were collected in polypropylene (PP) tubes, preserved with 0.02 µm of filtered, neutralized formaldehyde to a final concentration of 2.5%, and analysed for TNC and VLP, respectively. Thereafter, 8 mL was sampled for sulphate analysis, 9 mL for sulphide analysis, and two 5-mL volumes were sampled using a 0.2-µm filter (Sartorius Minisart hydrophilic syringe filter; Fisher Scientific, Göteborg, Sweden) and stored at –20°C until analysis of acetate and lactate, respectively. Then, 25 mL of water was sampled using a 0.2-µm filter (Minisart; Fisher Scientific) for immediate analysis of ferrous iron; thereafter, 100 mL of water was sampled and filtered for DNA extraction and qPCR analysis. This analysis was unfortunately not successful, probably due to the small biomass in 100 mL of groundwater combined with the low extraction efficiency of the extraction kit (DNeasy Blood & Tissue KitQiagen AB, Sollentuna, Sweden) as described elsewhere (Lloyd *et al.*, 2010). Finally, 10 mL of groundwater was collected for the analysis of E_h and 190 mL was collected for the analysis of gas on sampling occasions 1–3, while approximately 70 mL was sampled on each of the three remaining gas sampling occasions. In total, 452 mL of water was sampled on each of the first three sampling occasions and 332 mL was collected on each of the three last sampling occasions. After sampling the water, approximately 10 + 10 batches of rock grains were collected from two FCs in each FCC for subsequent analysis of the amount of attached ATP and for DNA analysis.

DNA extraction and concentration

DNA was extracted using the MO BIO Power Biofilm DNA isolation kit (catalogue no. 24000-50; Immuno Diagnostic Oy, Hameenlinna, Finland) from attached biomass on rock grains collected from the CH_4 and the H_2:CH_4 FCs on day 118. Four extractions from 10 grains each were performed according to the manufacturer's protocol and the extracted DNA was concentrated on a Microcon centrifugal filter (Ultracel, YM-100, 42424; Millipore, Billerica, MA, USA). The CH_4 FCs were re-sampled on day 120, and a total of 40 extractions of 10 grains each were concentrated to one sample. DNA concentrations were determined using Picogreen (Quant-iT™ PicoGreen® dsDNA reagent, catalogue no. P7589; Invitrogen, Carlsbad, CA, USA) and totals of 84, 450, and 87 ng of DNA were obtained from the CH_4 × 4, CH_4 × 40, and H_2:CH_4 × 4 extractions, respectively. The DNA was sent deep frozen (on CO_2 pellets) to Second Genome Inc. (San Bruno, CA, USA) for microarray analysis.

Analysis using the G3 PhyloChip assay

This analysis was performed by Second Genome Inc. as briefly outlined here (the methodology is fully described by Hazen *et al.*, 2010). The bacterial 16S rRNA genes were amplified using the degenerate forward primer 27F.1 5'-AGRGTTTGATCMTGGCTCAG-3'and the non-degenerate reverse primer 1492R.jgi 5'-GGTTACCTTGTTACGACTT-3'. For each sample, amplified products were concentrated using a solid-phase reversible immobilization method for purifying PCR products and quantified by means of electrophoresis using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). PhyloChip Control Mix was added to each amplified product. Thirty-five

cycles of bacterial 16S rRNA gene PCR amplification were performed. Labelled bacterial products were fragmented, biotin labelled, and hybridized to the PhyloChip Array, version G3. PhyloChip arrays were washed, stained, and scanned using a GeneArray scanner (Affymetrix, Santa Clara, CA, USA). Each scan was captured using standard Affymetrix software (GeneChip Microarray Analysis Suite). Hybridization values (i.e., the fluorescence intensity) for each taxon were calculated as trimmed averages, with maximum and minimum values removed before averaging. The data were evaluated using Second Genome's PhyCA-Stats analysis software package.

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