Mineralogical Comparisons of Experimental Results Investigating the Biological Impacts on Rock Transport Processes

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This study investigates the influence of microbes on fluid transport in sedimentary and igneous host rock environments. It particularly focuses on granodiorite rock (Äspö; Sweden) and mudstone (Horonobe; Japan) that were utilised during laboratory-based column experiments. The results showed that biofilms form on both rock types in low nutrient conditions. Cryogenic scanning electron microscopy showed that the morphology of biofilaments varied from filamentous meshwork (in crushed granodiorite column experiments) to clusters of rod-like cells (fracture surfaces in mudstone). X-ray diffraction analysis of the fine fractions (<5 µm) revealed the formation of secondary clay mineral phases within the crushed Äspö granodiorite rock substrate only. The formation of secondary clay minerals appears to be enhanced when bacteria are present. All experiments showed biofilm formation, bacterial enhanced trapping of fines blocking off pore throats and/or secondary clay mineral formation. These observations illustrate the importance of bacteria on rock transport properties which will impact on the containment and migration of contaminants.

Introduction

The impact of bacteria on subsurface transport processes has been widely recognised[7,9,10,13,20,35] and has been linked to their ability to form biofilms. Biofilms are very common in the biosphere and geosphere and may be described as an agglomeration of microbial cells and their excreted organic and inorganic products that is attached to, or coats, mineral surfaces or other substrates[30]. Microbial activity in any environment is generally located on chemical or physical interfaces, usually within biofilms, and can result in changes in the physical properties of a geological environment, e.g. by altering porosity. In addition, microbial activity may also result in intracellular or extracellular mineral formation or degradation[5,11,21,23,32] by altering surrounding chemical conditions (e.g. redox conditions). This can result in a reduction of pore spaces and cause 'bioclogging', which can significantly reduce hydraulic conductivity of fractures flow paths and porous media[9,34,30].

Risk assessments for geological disposal of radioactive waste are based primarily on the precepts of contaminant transport. They mainly focus on understanding the movement of gas, water and solutes through engineered barriers and natural groundwater systems to assure long-term safety of containment sites. Microbial activity can have an impact on transport processes by changing the chemical and physical characteristics of the subsurface environment. Hence, the impact of microbes must be taken into account in the development of theoretical models on solute transport. This is illustrated by Coombs *et al.*[9] who showed that even the smallest amounts of biofilaments can significantly alter transport through porous media by 1.) forming bridges across pore throats (thereby reducing effective pore sizes), 2.) capturing fine mineral particles (e.g. contaminants that are being sorbed onto biofilm), 3.) or by the shearing-off and transport of larger sections of biofilm prior to lodging and potentially blocking off another flow path. In addition to promoting mineral precipitation along flow paths, biofilm growth can also reduce water inflow and thereby limit the transport of contaminants. This is very important when considering landfill and geological repositories for radioactive waste[36].

This paper focuses in particular on the development of an experimental protocol to assess the impact that microbial activity may have on transport processes in granitic and sedimentary environments. It provides a detailed analysis and comparison of key mineralogical-, petrographical and geochemical findings that were made during a number of different microbial transport studies undertaken over the past 10 years. These studies were carried out in collaboration with the Äspö Hard Rock Laboratory in Sweden[8,14,15,32] and the Horonobe Underground Research Laboratory (URL) in northern Japan[16]. This provides new insights on the influence that bacteria can have on transport properties within granodiorite or mudstone host rock environments due to the formation of biofilms.

Geological background

The two host rocks chosen were igneous granodiorite, from the Äspö Underground Research Laboratory in Sweden, and sedimentary diatomaceous mudstone supplied by the Japan Atomic Energy Agency (JAEA). Äspö granodiorite and Horonobe mudstone rock were chosen as materials, given that these rock types are analogous to the lithologies that are likely to

be considered for radioactive waste repositories.

The Äspö URL is located within the Precambrian Transcandinavian Granite Porphyry Belt that comprises predominantly porphyritic quartz monzogranodiorites and granodiorites. These granitic rocks have undergone extensive fracturing in parts and have been subjected to hydrothermal alteration adjacent to rock fractures[33]. A detailed account of the geology of the Äspö site is provided by Hama *et al.*[14]. The Äspö granodiorite samples were taken in immediate proximity to the *in situ* field-scale experiment (REX – redox experiment in detailed scale) which investigated groundwater redox-buffering capacity of the rock mass within the URL[27].

Horonobe is located in the north-western Hokkaido, Japan[16]. A detailed description of the geological setting of the URL is given by Milodowski *et al.*[24]. The mudstone cores used in the experiments described here were taken from boreholes within the Neogene sedimentary sequence of the Koetoi Formation.

Experimental methods

Flow-through column experiments were carried out on Äspö granodiorite and Horonobe mudstone with synthetic groundwater similar to the *in situ* groundwater composition of the host rock environment[14,16]. The experiments were conducted under aerobic, anaerobic and/or pressurized conditions, using a variety of microbe species. A detailed overview of the experimental setup and the parameters that were used is given in Table 1.

Methods applied to investigate the granodiorite

Four different approaches were used to investigate the granodiorite. However, the common features for all experiments were the use of:

1. crushed 125 to 250 µm Äspö granodiorite material, packed into
2. borosilicate glass columns, and
3. saline Äspö groundwater containing minimal nutrients (C, S, N, P).

The first approach by Hama *et al.*[14] introduced a mixture of iron- and sulphate-reducing bacteria, i.e. *Shewanella putrefaciens* and *Desulfovibrio aespoeensis* respectively, in Äspö groundwater flowing through either columns or stirred tank reactors (CSTR) packed with crushed Äspö granodiorite. These types of bacteria were chosen as they are known to be indigenous to Äspö rock[25,26] and have been regarded as particularly important in Äspö groundwater chemistry[2]. The experiments were aimed to run over a period of 100 days under anaerobic conditions and a flow rate of 0.5 ml hr^{-1}. However, the columns became blocked soon after the introduction of bacteria and the experiments had to be terminated after 2 days[14].

The second approach by Tuck *et al.*[32] applied granodiorite packed column studies that were inoculated with a single or mixed population of chemolithotrophic bacteria under anaerobic and aerobic conditions. A similar experimental set-up to Hama *et al.*[14] was adapted but, a 'wild-type' mix of sulphate reducing bacteria was also introduced together with *Shewanella putrefaciens* and *Desulfovibrio aespoeensis*. This mixture of bacteria is commonly used in bioremediation experiments. As with previous work[14], the column experiments had to be terminated prematurely after 5 days due to blockage. Additional biotic and abiotic CSTR experiments investigated the influence of varying pH (ranging from between 1 and 10) on mineral dissolution aiming to free up' elements important for clay mineral formation[32].

The third approach by Coombs *et al.*[8] focussed in particular on the effect of biofilm formation on transport properties. A viable population of the bacterium *Pseudomonas aeruginosa* was introduced into a feed reservoir and any effects on fluid flow were monitored under aerobic conditions. The soil bacterium *P. aeruginosa* was chosen because it is robust and is known to live in a broad range of environmental conditions. It is also known for its capability to survive under anaerobic conditions as it is capable of using nitrate as respiratory acceptor in the absence of oxygen[22]. The level of groundwater in the header reservoirs was maintained using a peristaltic pump. This arrangement provided a fixed pressure head, and therefore changes in flow rate were proportional to permeability and calculated by mass balance over the course of the experiment. Investigations into the effect of varying the pH of simulated Äspö groundwater[14] between 7.2 and 5.2 on the test system were carried out in a 90 day aerobic experiment.

The fourth and most recent approach was based on that taken by Coombs *et al.*[8] but uses anaerobic column experiments in an automatically controlled environmental anaerobic chamber. The constant head reservoirs used by Coombs *et al.*[8] were replaced by syringe pumps to maintain a constant flow rate of 0.625 ml hr^{-1} and any changes in pressure were logged continuously using a data logging system. The experiment was terminated after 147 days. A full description of methodologies is given elsewhere[15].

Methods applied to investigate mudstone

For the mudstone investigations, two consecutive pressurized flow-through column experiments were carried out; one biotic and the other acting as a control. A fractured diatomaceous Horonobe mudstone core was used and positioned vertically in a Teflon sheath with caps fitted on both ends to allow fluid flow. The assembly was then placed into a pressure vessel and sterile synthetic Horonobe groundwater was then passed through the core. The synthetic groundwater had a composition similar to naturally occurring groundwater at Horonobe[16] (supplemented with 0.25 g l^{-1} sodium acetate to provide source of organic carbon to sustain bacterial growth).

After a 11 day period of system equilibration, the 'biotic' column was inoculated with approximately 10^5 ml^{-1} (suspended in 500 ml of synthetic groundwater) of the denitrifying soil bacteria *Pseudomonas denitrificans*. The choice of bacterium was based on research by Tochigi *et al.*[31] who found that this species appeared to have the greatest activity within Horonobe groundwater. These relatively short time pilot studies (39 days) were carried out under pressurised (1250 - 1260 kPa) fluid flow conditions with a flow rate of 0.3 ml hr^{-1} and any changes in fluid pressure were monitored using pressure transducers. A full description on the experimental methodologies is given in Harrison *et al.*[16].

Analytical methods

Prior to column experiments both Äspö granodiorite and

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Table 1 Overview of experimental conditions for different rock types

Rock type	Experimental system	Atmosphere	Bacteria culture	Flow conditions	References
Crushed granodiorite (Äspö), 125-250 µm packed into borosilicate columns	Flow-through column experiments (biotic and abiotic) circulated by Äspö groundwater. Planned duration of 100 days (blockage of biotic column after 2 days). Äspö groundwater, sampled within borehole KA2858A. Continuously stirred tank reactor (CSTR) experiments (biotic and abiotic). Duration of 100 days anaerobic and then further 100 days aerobic. Synthetic Äspö groundwater.	Anaerobic (flow-through columns) Anaerobic then aerobic (CSTR experiment)	*Shewanella putrefaciens and Desulfovibro aespoeensis* Column experiments: 10^7 bacteria ml^{-1} CTSR experiments: 10^5 bacteria ml^{-1}	Column and CSTR experiments: Flow rate of 0.5 ml hr^{-1} using a peristaltic pump	Hama *et al*., 2001. *Clay Minerals* **36**, 599-613.
	Flow-through column experiments (biotic and abiotic) circulated by synthetic Äspö groundwater. Blockage after 2-5 days. Continuously stirred tank reactor (CSTR) experiments (biotic and abiotic). Duration of 21 days, pH between 1 and 10. Synthetic Äspö groundwater.	Anaerobic and Aerobic (*S putrefaciens* only)	*Shewanella putrefaciens* and *Desulfovibro aespoeensis* and wild type sulphate reducing bacteria Column experiments: 10^7 bacteria ml^{-1} CSTR experiments: 10^5 bacteria ml^{-1}		Tuck *et al*., 2006. *J Geochem Explor*. **90**, 123-133
	Flow-through column experiments (2 biotic columns) circulated by synthetic Äspö groundwater. Changes of pH from 7.2 to 5.5. Experiment duration 90 days.	Aerobic	*Pseudomonas aeruginosa* (10^4 bacteria ml^{-1} in feed reservoir of 23 litre)	Fixed pressure head maintained with peristaltic pump (0.18 ml hr^{-1} to 270 ml hr^{-1})	Coombs *et al*., 2008. *Min Mag*. **72**(1), 393-397
	Flow-through column experiments (biotic and abiotic) circulated by synthetic Äspö groundwater. Experiment duration 147 days.	Anaerobic	*Pseudomonas aeruginosa* (one single 10 ml inoculation of 10^7 bacteria ml^{-1})	Syringe pumps (0.625 ml hr^{-1})	Harrison *et al*., 2009. *British Geological Survey* Report, OR/09/33
Intact mudstone cores (Horonobe) containing multiple fractures	Pressurised columns (biotic and abiotic) circulated by synthetic Horonobe groundwater (supplemented with 0.25g l^{-1} acetate). Experiment duration 39 days.	Aerobic	*Pseudomonas denitrificans,* (1.18 x 10^5 bacteria ml^{-1})	Constant fluid flow (0.3 ml hr^{-1}) under pressurised conditions	Harrison *et al*., 2011. *Min Mag*. **75**(4), 2449-2466

Horonobe mudstone were characterised by X-ray diffraction (XRD) and Backscattered Scanning Electron Microscopy (BSEM) techniques. In the subsequent experiments, reacted groundwater samples were taken at regular intervals and analysed for pH/alkalinity, selected microbial nutrients (C, S, N and P) and major and trace cations and anions by Inductively Coupled Plasma-Emission Spectrometry (ICP-ES) and Ion Chromatography (IC). If the timescale of the experiment allowed, the bacterial population within the reacted fluids was counted directly by staining with acridine orange and epifluorescence microscopy[17,35].

On completion of the experiments, the columns were sliced open. One half of the column was sprayed with acridine orange solution and the extent of biofilm growth imaged by Laser-Stimulated Scanning Fluorescence Imaging (LSSFI) using an Amersham Bioscience STORM860 laser fluorescence scanning system. Subsamples along the unstained length of the second half of the reacted columns were analysed using XRD and SEM techniques to document any mineralogical changes, and to confirm the formation of biofilms.

X-ray diffraction analysis

For bulk XRD analysis the dried samples were tema-milled and then micronised to a fine powder (<15 µm). Early experimental work[14,32] was carried out using a Phillips PW1700 Series diffractometer. During later studies this model was replaced by a PANalytical X'Pert Pro series diffractometer equipped with a X'Celerator detector. Data processing was performed using either Phillips X'Pert or PANalytical X'Pert HighScore Plus software packages that were linked to the latest version of the International Centre for Diffraction Data (ICDD) database. Both instruments were equipped with a cobalt-target tube and operated at 45 kV and 40 mV. Quantitative mineralogical data was accomplished by a least squares fitting process using the Rietveld refinement technique[29].

For clay mineral characterisation a nominal clay-size fine fraction (<5 µm) was extracted from the suspension of bulk material in deionised water, following settling times based on Stoke's Law. This was achieved for most of the experiments. However, Tuck *et al.*[32] noted that the high salinity of the fluid resulted in extensive flocculation of the suspension, which made separation of the fine fraction impossible. Here, a coarser mineral fraction of <61 µm had to be removed. The liberation of the particles was enhanced by ultrasonic treatment prior to gravity separation. The concentrated clay suspensions were then deposited onto either frosted glass slides or silicon wafers. The zero-background oriented mounts were then scanned from 2-32°2ϑ at 0.55°2ϑ/minute (2-40°2ϑ at 1°2ϑ /minute for the PANalytical X'Pert Pro series diffractometer) after air-drying, after ethylene glycol-solvation and after heating to 550°C for 2 hours.

Scanning Electron Microscopy

Samples of reacted Äspö granodiorite and mudstone were examined by a variety of SEM techniques using sample preparation methods that evolved over the time span of the experiments. Initial SEM observations were carried out on freeze-dried material and revealed collapsed microbial biofilamentous structures. Improved sample preparation techniques were established to maintain delicate biogenic structures that may have been affected by the precipitation of salt crystals produced from the saline fluids during the freeze-drying. These included rapid solvent replacement technique[28] aimed to remove the saline artificial porewater prior to SEM analysis. To produce a coherent sample capable of staying intact during SEM observation, the mineral grains were weakly diffusively impregnated with a polystyrene cement after drying. Prior to analysis, both starting materials and reacted core samples were deposited onto aluminium pin-type SEM stub mounts. SEM and BSEM analyses were performed using a LEO435VP variable pressure scanning electron microscope (VPSEM) equipped with a KE-Developments four-quadrant solid-state detector for BSEM and an Oxford Instruments INCA Energy 450 EDXA system to aid mineral identification. Both SEM and BSEM observations were recorded using 10-20 kV electron beam voltages.

Cryogenic Scanning Electron Microscopy (CryoSEM) was performed on a LEO 435VP variable pressure digital SEM fitted with an Oxford CT 1500 Instruments SEM cryogenic preparation and transfer system. This equipment allows the observation of any delicate biological matter in frozen in situ conditions, without the necessity of drying, which may cause collapse and disturbance of microstructures prior to analysis. Wet column residues were rapidly pre-frozen in a liquid nitrogen/solid nitrogen slush freezing mixture to limit the formation of coarse ice crystals that might cause microstructural deformation within the sample. The sample was then freeze-fractured to produce a fresh surface for observations, gold-coated in the SEM cryogenic preparation chamber and transferred via an airlock into the cryogenically-cooled SEM sample stage attachment maintained at -170°C. Once inside the SEM, the sample stage was slowly warmed to between -60 to -80°C to slowly ablate the frozen interstitial porewater under vacuum followed by rapid re-cooling to -170°C. This process was executed with extreme care to avoid any collapse of the delicate intergranular pore fabrics. Initial examination revealed the precipitation of salt crystals from the pore fluids as an artefact of freezing saline experimental fluids, which obscured the original texture of the samples. To prevent salt formation and thereby enabling observation of any microstructures that may be present in the samples, de-ionised sterile water was gently percolated through residual specimen to remove saline porewater precursory to cryogenic analyses. For higher resolution morphological cryoSEM observation of the pre- and post-experimental fracture surface of the Horonobe mudstone, subsamples were also examined using a FEI Company QUANTA 600 environmental scanning electron microscope (ESEM) equipped with a Gatan ALTO 2100 cryogenic sample preparation and transfer unit.

Results and discussion

Mineralogical and petrographical observations from the starting materials

Petrographic work by Hama *et al.*[14] revealed an Äspö granodiorite starting material that is hydrothermally altered, partially recrystallised and relatively homogeneous. The rock matrix was shown to comprise plagioclase and orthoclase feldspar, quartz, 'mica' (muscovite and biotite) and accessory epidote, magnetite, pyrite and calcite. Furthermore, XRD analysis revealed the presence of small amounts of chlorite which, based

on thin-section observations, originate from the chloritisation of biotite. SEM analysis showed the presence of fine mineral debris that had accumulated on grain surfaces, an inevitable side-product of the mechanical process of sample crushing[14]. These observations are in accordance with later column studies, where the starting material was re-analysed.

Detailed mineralogical and petrographical data of the mudstone cores from Horonobe is given in Harrison *et al.*[16]. In summary, XRD analysis showed that the mudstone cores are predominantly composed of quartz, feldspars (plagioclase and alkali feldspars) and clay minerals (illite, kaolinite, chlorite and smectite as identified from the <5µm fractions of the samples after glycolation and heating) with accessory amounts of pyrite. VPSEM analyses also revealed the presence of authigenic pyrite and magnesiosiderite crystals and abundant silt-sized fragments of diatoms and sand-grade cylindrical diatoms and sponge spicule fragments. The diatoms have delicate microporous skeletal frameworks that are entirely made of amorphous silica. Their abundant presence is also highlighted by the enhanced broad background between 20-35°2θ on XRD traces, indicating the presence of low crystalline and/or X-ray amorphous species. Detailed examination of the fracture surface by VPSEM revealed fine lineation structures forming an interconnected network of channels. Harrison *et al.*[16] argued that they probably represent slickenside features which had been formed by shear-movement along the fracture surface. Fine organic filaments, possibly fungal hyphal structures, were prevalent across the fracture surface but appeared to leave mineral surfaces unaffected (Figure 1). These observations by Harrison *et al.*[16] revealed that the core material was already microbially contaminated by fungi prior to experiments.

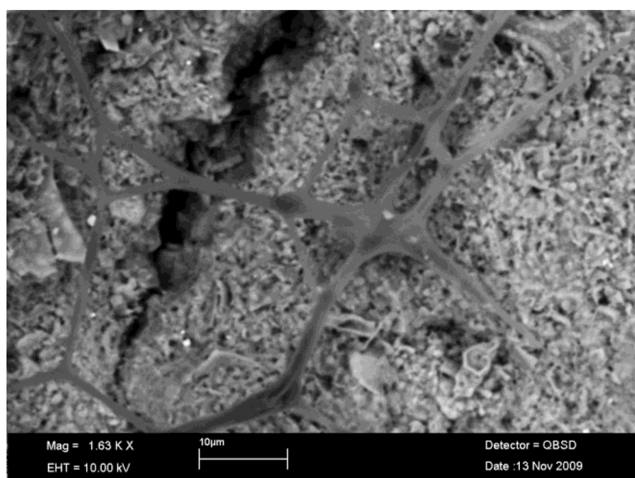

Figure 1 BSEM image showing detail of the fine organic filaments, possibly resembling fungal hyphal structures, coating the fracture plane (Horonobe mudstone, starting material).

Mineralogical and petrographical observations from the experimental residues

Scanning electron microscopy (SEM)

CryoSEM analyses revealed that the bacteria *Shewanella putrefaciens* and *Desulfovibro asponium* had formed substantial organic filamentous structures on granodiorite grain surfaces and within pore spaces during anaerobic column experiments[14]. Hama *et al.*[14] ascertained that these organic structures appeared to have trapped a significant amount of fine mineral debris (<2 µm) that originated from the rock crushing process during sample preparation. Trapping of these fine particles in pore throats resulted in blockage and/or restriction of intergranular porosity and may at least partly be responsible for the reduced permeability leading to early cessation of the experiment. On closer examination using SEM techniques Hama *et al.*[14] detected a secondary alumosilicate phase formed within these fines that further restricted pore spaces. It appears that the interaction of fine particles in the starting material with Äspö groundwater combined with microbial activity had resulted in secondary clay mineral formation. SEM images suggested that bacteria may aid liberation of fine particles from the grain surfaces. As the surfaces of bacterial cells are typically anionically charged, biofilms are predestined to attract other charged particles such as cations (e.g. $Fe^{2+/3+}$ or Si^{4+}) and as a result may act as nucleation points for the precipitation of secondary minerals[12]. In particular clay minerals, due to their high surface area and negative surface charge, are most reactive and will therefore play an important role within the microenvironments of rocks and soils. Similar observations were made during anaerobic column experiments by Tuck *et al.*[32] utilizing granodiorite, a mixture of *Shewanella putrefaciens*, *Desulfovibrio aespoeensis* and a wild type sulphate reducing bacteria. Interestingly, this secondary clay-type phase appeared to be less predominant in the aerobic column experiment. Aerobic column experiments by Coombs *et al.*[8] showed that the bacterium *P aeruginosa* had also formed biofilms and biofilaments on granodiorite grain surfaces (Figure 2).

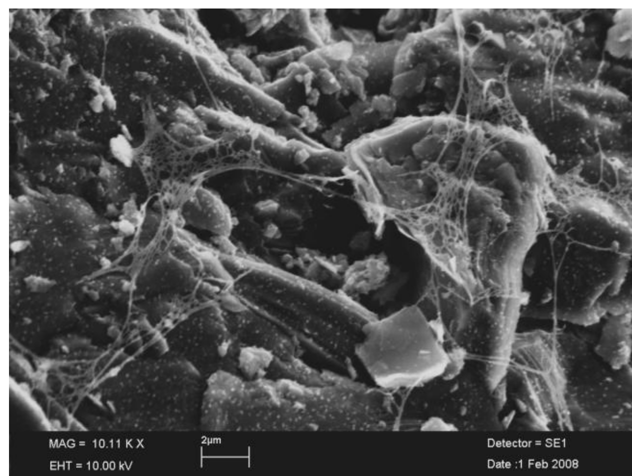

Figure 2 SEI photomicrograph showing a 'web-like- biofilm and biofilaments draped over the surface of a plagioclase grain (Äspö granodiorite, biotic experiment, aerobic).

Subsequent column experiments under anaerobic conditions[15] revealed that *P aeruginosa* was also capable of forming biofilamentous structures (Figures 3a and b) in the absence of oxygen under the given experimental conditions. Both aerobic and anaerobic experiments revealed a filamentous meshwork that consists of amorphous strands from 1-5µm thickness spanning intergranular pore spaces by anchoring themselves via pili structures onto granodiorite grain surfaces. These organic

structures appeared to be more developed in the aerobic column but were, in either case, particularly concentrated close to the column inlet. As in the previous short column studies[14], fine mineral debris appeared to be trapped within these strands, causing blockage of pore throats hence reducing permeability. However, the pressure and fluid flow through the anaerobic columns showed only limited changes throughout the experiments probably due to insufficient biofilm growth[15]. This was not unexpected, because the low nutrient levels in the synthetic Äspö groundwater limited the bacterium's ability to utilise nitrate as a respiratory electron acceptor. Detailed CryoSEM also revealed the formation of a non-aqueous, non-wetting organic liquid that appears in the form of 'oil droplets' within the biofilm. Coombs *et al.*[8] argued that this liquid was likely to originate from alginate that was produced by *P aeruginosa*. However, no secondary alumosilicate phase within the biofilaments was observed as during previous studies.

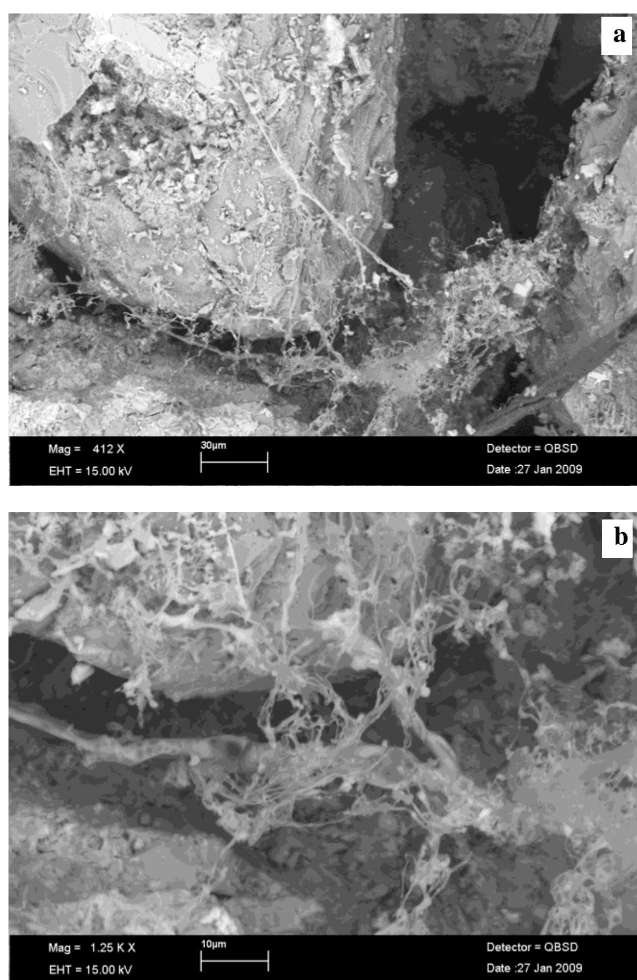

Figure 3 Image (a) is a BSEM image showing biofilaments. Mineral debris can be seen as bright particles trapped in the organic matter. Image (b) is a high magnification BSEM image, showing an intergranular granodiorite pore space, spanned by the amorphous meshwork of biofilaments revealed in image (a) (Äspö granodiorite, biotic experiment, anaerobic).

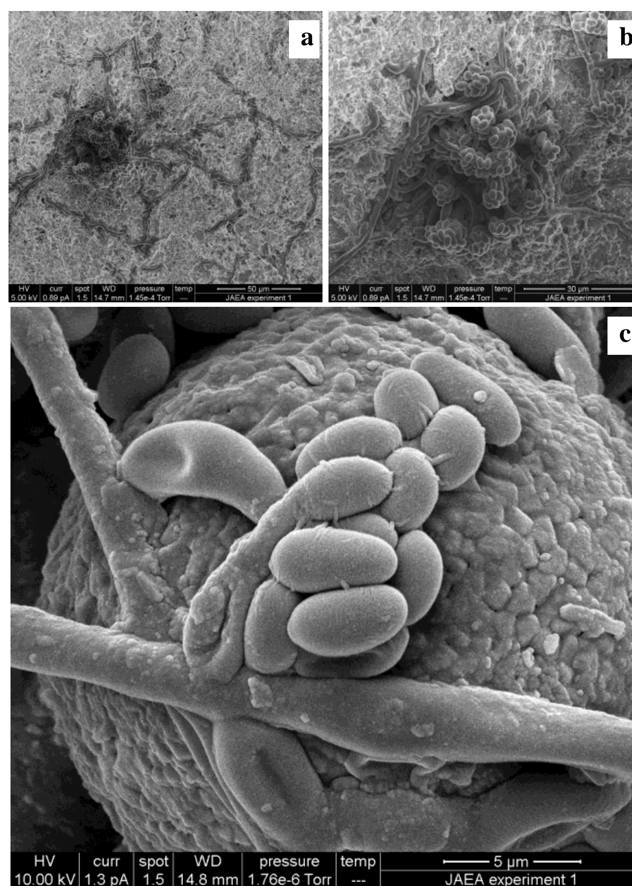

Figure 4 CryoSEM SEI images of the mudstone fracture surface showing (a) clusters of cellular structures associated with biofilaments where some biofilaments comprise strings of rod-like cells and (b) filamentous structures that are associated with isolated rod-like cells and clusters of cells ('bunches of grapes' aggregates). Image (c) shows detail of the clusters of rod-like cells associated with biofilaments on fresh framboidal pyrite. The pyrite appears to be unaffected by oxidation but the biofilaments can be seen to have etched into and are now embedded in the pyrite surface. (High-vacuum cryoSEM, biotic experiment)

CryoSEM analyses of the Horonobe mudstone residues, after exposure to *P denitrificans*, also revealed the presence of organic biofilaments that followed the open channel structures on the fracture surface (Figure 4a). Harrison *et al.*[16] suggested that this dendritic biofilm meshwork is made up of at least two different species of microorganism - fungal type structures similar to those observed in the starting material and rod-like cell structures exhibiting fine pili structures that probably represented bacterial cells. Harrison *et al.*[16] suggested that these pili structures possibly enabled the cells to attach to mineral surfaces and adjacent cells. The rod-like cells were also present in 'grape'-like clusters (Figure 4b) and were not observed in the starting material or in the abiotic residues. It appears that they are related to the introduction of *P denitrificans*. Data from flow pressure transducers revealed short but rapid changes in permeability of the biotic and control columns. However, they were more profound in the biotic column experiments. Harrison *et al.*[16] argued that they may be linked to the formation of biofilms

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and/or movement of fines within the column resulting in blockage of conductive pathways which were then later flushed as the hydraulic pressure locally increased. The fracture-surface mineralogy of the reacted biotic and abiotic residues was similar to the starting material, being dominated by biogenic silica derived from abundant fragments of siliceous diatom frustules and sponge spicules. The redox-sensitive minerals pyrite and siderite did not show any signs of oxidisation in both the biotic or control experiments. Interaction of biofilmaments with mineral surfaces resulted in some etching and dissolution of the underlying rock substrate (Figure 4c). The etching appeared to be non-specific with regard to surface mineralogy, affecting the silica-rich matrix, pyrite and siderite[16]. This was neither evident in the starting material nor in the control experiment. No secondary mineral formation was observed on the mudstone fracture surface.

X-ray diffraction analysis

X-ray diffraction analysis of the post experimental granodiorite and mudstone residues revealed that microbial-geochemical interaction with mineral surfaces did not appear to make any notable changes in bulk mineralogy[14,16,32]. Harrison *et al.*[16] noted a minor increase in chlorite content in reacted mudstone samples. However, because numerical changes were within the errors of the given quantification method, this increase could not be attributed to bacterial impact with certainty.

The fine fractions (<2-5 µm) were analysed separately and revealed some interesting clay mineralogical changes in the granodiorite host rock during some of the biotic column experiments. Hama *et al.*[14] observed a newly formed mixed-layer clay phase, possibly a chlorite-smectite, shortly after the introduction of a mixture of sulphate-reducing and iron reducing bacteria to the granodiorite host rock type. This observation was confirmed by SEM-EDXA observations, where a secondary aluminosilicate phase had formed during the biotic experiments. This was not the case during control experiments and Hama *et al.*[14] argued that microbial activity must have stimulated the formation of this secondary clay phase, possibly by altering the chemistry within microenvironments. Interestingly, SEM data from CSTR experiments revealed that this neo-formed clay mineral phase appeared to form preferentially under anaerobic atmospheric conditions[14]. Tuck *et al.*[32] made similar observations, with the formation of a secondary mixed-layer clay mineral phase during biological column experiments. Tuck *et al.*[32] also demonstrated the dependency of clay mineral formation on pH, showing that a secondary smectitic clay had precipitated above pH 8, despite the absence of bacteria during CSTR experiments. However, secondary clay mineral phases appeared to develop predominantly during biotic experiments and Hama *et al.*[14] argued that intraporous reactions may be enhanced by the presence of bacteria. Clay mineral XRD traces of the granodiorite material that had been exposed to the bacterium *P aeruginosa* under anaerobic conditions[15] exhibited a small shift of the chlorite (001) d-spacing from 14.1Å to 15.5Å after glycolation (Figure 5). This swelling behaviour is indicative for the presence of smectite, possibly distributed sparsely within chlorite interlayers and may be described as a mixed-layer chlorite-smectite clay mineral, similar to previous observations made by Hama *et al.*[14] and Tuck *et al.*[32]. However, this was not supported by SEM observations because no secondary mineral phase was observed. Following glycolation of the aerobic biotic residues no obvious changes in peak positions were notable and hence no significant amounts of expanding clays appear to be present. In either case no control material was available for XRD analysis and therefore any mineralogical changes cannot be directly attributed to microbial activity.

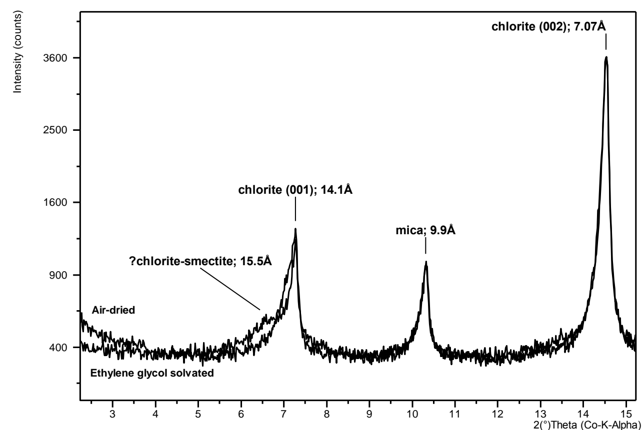

Figure 5 Air-dried and glycolated XRD traces of the granodiorite fines residues (<2 µm) that were prepared as oriented mounts (Äspö granodiorite, *P aeruginosa*, anaerobic).

In comparison, XRD analysis of the fine (<2 µm) fraction of the Horonobe mudstone residues did not reveal any changes in clay mineralogy that could be linked to microbial activity. It appears that the microenvironmental conditions did not support the formation of secondary clay phases. It is possible that the lack of fine material debris has limited the availability of necessary cations to form secondary clay minerals. Also, the pressurised conditions within the column may be unfavourable to secondary mineral precipitation.

Microbiological and geochemical observations from the outflow fluids and experimental residues

The columns containing Äspö granodiorite that had been exposed to *Shewanella putrefaciens* and/or *Desulfovibro asponium* and/or wild type sulphate reducing bacteria became blocked soon after the start of the experiments (2-5 days)[14,32]. Therefore no microbiological and only limited geochemical data from these experiments are available.

Bacterial counts taken from the outflow fluids of the columns packed with Äspö granodiorite *(P aeruginosa)*[8,15] and Horonobe mudstone (*P denitrificans*)[16] showed that bacteria were viable under aerobic[8], anaerobic[15] or pressurised conditions[16] for most part of the experiments. Overall, the results revealed that bacterial numbers were steadily increasing during the time these experiments were conducted. This indicates a bacterial population that can thrive under the given experimental conditions[8,15,16]. After equilibrium within these microenvironments is reached, a drop in bacterial numbers is to be expected (e.g. due to depletion of nutrient resources). This was evident in the outflow fluids of the granodiorite packed columns, where counts rates dropped significantly from *c.* 1800 hours after inoculation with *P*

aeruginosa under anaerobic[15] and aerobic[8] conditions.
Geochemical data showed, in general, little evidence for fluid-solid interactions using different type of rocks and groundwater. No significant changes in pH were recorded from any outflow fluid measurements[8,14,15,16,32]. Hama *et al.*[14] argued that although mineralogical changes may occur in microbially mediated microenvironments, they would be too small to be detected in the chemical analysis of the bulk fluid. Harrison *et al.*[16] also reasoned that the experiments using Horonobe mudstone material may not have been long enough to allow the observation of any changes in chemistry. However, short-time experiments by Hama *et al.*[14] using Äspö granodiorite did reveal limited dissolution of primary minerals, which is in agreement with SEM observations where etching of mineral surfaces was noted.

Subsamples of the acridine orange stained granodiorite sample residues that had been exposed to *P aeruginosa* bacterium under anaerobic[15] conditions were taken from the centre and edge along the 15 cm column length and observed by epifluorescence microscopy. This showed that bacterial counts were greatest at the column inlet (0-3 cm) with the highest number in the centre of the column ($4.06x10^5$ bacteria g^{-1}). Under the microscope the bacteria appeared to be aggregated in groups rather than as individual cells. At 3-4 cm the groups of bacteria also appeared to be associated with organic material, possibly exopolysaccharids (EPS) from biofilm formation. In general there is a decrease of bacteria towards the end of the column. However, it appears that a high amount of bacteria ($2.18x10^5$ bacteria g^{-1}), predominantly non-viable, had accumulated at the end of the column causing some sort of plugging effect and were not carried through the outlet of the column. This is consistent with the fluorescence imaging of the acridine orange distribution within the intact column before sampling for petrographic and biological analysis showing that biofilm formation was largely limited to the first 4 cm distance along the column (Figure 6).

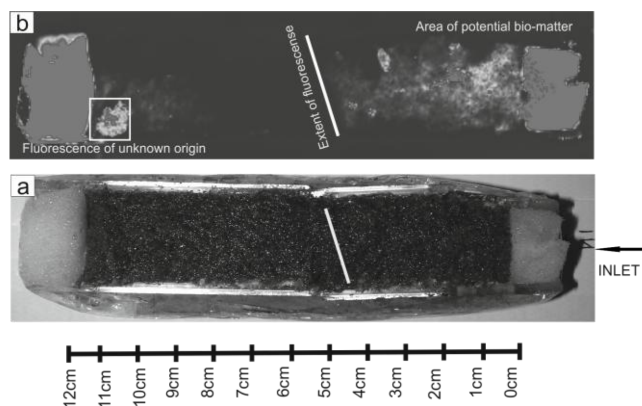

Figure 6 Visual images of the Acridine Orange stained column half obtained in (a) visible light and (b) blue excited fluorescence scan (Äspö granodiorite, biotic experiment, anaerobic)

In comparison, the aerobic column (25 cm length) revealed a much more profound biofilm development that is concentrated within 9 cm from column inlet[8]. No epifluorescence images of the Horonobe mudstone residues were taken.

Conclusions

In general, bacteria were able to form microbial biofilm on a mudstone or diorite rock substrate in the presence of bacteria and representative synthetic groundwater, despite the limited availability of nutrients. Imaging of these delicate structures was strongly dependent on sample preparation methods. It emerged that careful removal of any salt within the sample residues was essential to preserve any biofilamentous structures during freeze-drying. The biofilms exhibited a variety of shapes and sizes and developed as either web like structures or as clusters of elongated cell structures resembling organic filamentous structures. The experiments showed that the architecture of these biogenic structures appears to be linked to a variety of factors: the hydrodynamic environment, the availability of nutrients, the type of bacterium, the atmospheric conditions and the rock substrate. Selective types of bacteria appeared to flourish and form impressive filamentous biofilm structures. This was particularly apparent across granodiorite pore spaces containing the iron-reducing bacteria *Shewanella putrefaciens* resulting in early blockage after only 2 days[32].
The type of rock substrate has a significant impact on the volume of biofilm formed, depending on the availability of pore space and fracture porosity, consequently affecting the fluid flow pattern within the rock and nutrient supply. Biofilm formation within Äspö granodiorite rock material was enhanced because crushed material was used during the experiments increasing the surface area between the mineral grains. In the intact mudstone material from Horonobe, Japan, biofilm structures were predominantly along a rock fracture where fluid flow was focused.
Importantly, all experiments also showed that biofilm formation can have a dramatic impact on the hydraulic properties of a rock, causing blockage and/or a significant reduction in permeability. This appears to be a direct result of the constriction of pore throats and increased tortuosity of pore network flow paths and to a more or less extent the sorption of fine-grained particles onto biofilm. It also shows that microbes can enhance the liberation of fine particles from grain surfaces. At a larger scale, this could have significant implications for geological waste repositories by sorption of contaminants onto biofilm or uptake of contaminants onto mobile microbes. Similar observations were made by Brydie *et al.*[6] who found that the hydraulic conductivity, expressed by a change in effluent discharge flow rate, had significantly dropped after inoculation of bacteria within quartz sand packed columns due to biofilm formation. Furthermore, in the studies described here new mineral phases may precipitate within the rock pore spaces from porewater fluids and may contribute to this reduction in hydraulic conductivity. This was evident in some experiments, where a new mixed-layer clay mineral phase had formed as a result of interaction of fine diorite particles with groundwater. This secondary clay formation appeared to be enhanced by the presence of bacteria but appeared, however, to be absent during the experiments using intact mudstone material.
The results of these studies highlight the importance of considering the impact of microbes when assessing a potential host rocks for landfill and radioactive waste repositories. Currently the British Geological Survey is carrying out further column studies using sandstone material with a similar

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experimental set-up. This type of rock is of particular interest for radioactive waste and carbon capture & storage sites because of interconnected pore spaces that allow high storage capacity. However, more work is needed to fully evaluate the impact of microbial presence on transport processes underground to subsequently enhance transport models that are used as predictive tool for both safety of radioactive waste repositories and carbon capture and storage sites.

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