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Sulphate Reducing Bacteria and Methanogenic Archaea Driving Corrosion of Steel in Deep Anoxic Ground Water

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During the operation, maintenance and decommissioning of nuclear power plant radioactive contaminated waste is produced. This waste is stored in an underground repository 60-100 meters below the surface. The metallic portion of this waste comprises mostly carbon and stainless steel. A long-term field exposure showed high corrosion rates, general corrosion up to 29 μm a^{-1} and localized corrosion even higher. High corrosion rate is possible if microbes produce corrosive products, or alter the local microenvironment to favor corrosion. The bacterial and archaeal composition of biofilm formed on the surface of carbon steel was studied using 16S rRNA gene targeting sequencing, followed by phylogenetic analyses of the microbial community. The functional potential of the microbial communities in biofilm was studied by functional gene targeting quantitative PCR. The corrosion rate was calculated from weight loss measurements and the deposits on the surfaces were analyzed with SEM/EDS and XRD. Our results demonstrate that microbial diversity on the surface of carbon steel and their functionality is vast. Our results suggest that in these nutrient poor conditions the role of methanogenic archaea in corrosive biofilm, in addition to sulphate reducing bacteria, could be greater than previously suspected.

Keywords: *MIC, Steel, SRB, Methanogenic archaea*

1. Introduction

During the operation, maintenance and decommissioning of nuclear power plant radioactive waste is produced. This waste is classified into low, intermediate and high level radioactive waste. In Finland the low and intermediate level radioactive waste is disposed of in the underground repositories excavated into the bedrock. The metallic waste consists mostly of carbon steel and stainless steel.

The corrosion of carbon steel is normally very slow in anoxic and alkaline environments, such as this repository environment. However, high corrosion rates have been detected in this environment leading to suspicion that microbes from ground water may be inducing the corrosion [1-5]. High corrosion rates are possible under alkaline and anoxic conditions if microbes are present and produce corrosive products or alter the local microenvironment to corrosive [1,2]. In natural aquatic environments, microorganisms are predominantly sessile and grow as multi-species communities attached to submerged surfaces [6]. In oligotrophic environments, the formation of biofilms is a survival strategy for microbial communities [6]. Biofilm formation is crucial for the initiation of MIC, under the biofilm microorganisms generate localized conditions that might be corrosion inducing even if the surrounding environment is not. Microbes are known to accelerate several types of corrosion such as general corrosion and localized corrosion like pitting and stress corrosion cracking. However, MIC is often reported as localized corrosion due to the heterogeneous, and often patchy, distribution of biofilms and their inorganic deposits [7].

To study the in situ corrosion rate and corrosion mechanisms of carbon steel at the underground nuclear waste repository for low and intermediate waste a long-term field exposure (6 to 15 years) was designed. The carbon steel coupons were exposed to natural groundwater at the repository environment and corrosion was studied using combination of gravimetric determination, chemistry, surface characterization and molecular biological methods.

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2. Experimental methods

2.1 Materials and sampling

Low alloy carbon steel from cold-rolled thin sheet was used as test material. The surface of the carbon steel was as received, and the coupon size was 25 × 75 × 1 mm (drill hole A) or 25 × 68 × 1 mm (drill hole B). Prior to the immersion into the drill holes, the length, width and thickness of the coupons were measured, and coupons were weighed with the accuracy of 0.1 mg. Coupons were cleaned with deionized water, 70% ethanol, and air-dried. Coupons in drill hole B were installed in June 1998 and coupons in drill hole A were installed in October 2007. Sampling was performed in October 2013, resulting experiment times of 5.9 a (drill hole A) and 14.8 a (drill hole B). The coupons were placed in sample holders so that the coupons were in direct contact with ground water filling the drill hole. Sample holders were transferred into anaerobic glove box immediately after they were lifted from drill holes. The steel coupons for corrosion studies (weight loss, microscopy, EDS, XRD) were immersed in 96% ethanol, air-dried and stored in glass desiccators. The coupons for FE-SEM were immersed in phosphate (0.1 M, pH 7.2) buffered 2.5% glutaraldehyde for fixation of presumed biofilm. After 16 h the coupons were rinsed with phosphate buffer three times. Dehydration was carried out with ethanol series, followed by final dehydration using hexamethyldisilazane. Samples for FE-SEM were coated with Au/Pd (10 nm) and examined with Hitachi S-4800 FESEM (Japan) operated at 1 to 5 kV. Coupons for microbiological analyses were frozen on dry ice and stored at – 80 °C until DNA extraction.

2.2 Molecular biological analyses

The biofilm was extracted from the surface of the steel coupons by bead beating coupons in 10 mL sterile phosphate buffered saline (PBS) and Tween®20 (1 µL Tween®20 1 mL^{-1} PBS) for 20 minutes at 150 rpm agitation and followed by ultra-sonication for 3 min. The biomass released from the steel coupons was subsequently collected on 0.22 µm -filtration units for subsequent DNA extraction. The DNA was subsequently extracted from filters using the PowerWater® DNA Isolation kit (MoBio Laboratories, Inc., CA, USA) in accordance with the manufacturer's protocol and the DNA was eluted in 100 µL elution buffer supplied by the manufacturer. Negative DNA extraction controls were included in the DNA extractions.

As a proxy for bacterial biomass, quantitative PCR (qPCR) was used to determine the amount of 16S rRNA gene copies in each sample. The amount of sulphate reducing microbes was determined on base of copies of β -subunit of dissimilatory sulphite reductase (*dsr*B) gene. The presence of methanogenic archaea was studied using qPCR targeting the α-subunit of methyl coenzyme-M reductase (*mcr*A) gene. qPCR was performed in 10 µL reaction volumes using the LightCycler® 480 qPCR machine and LightCycler® 480 Software 1.5.0 (Roche Applied Science, Germany). The reaction mixture contained 1 µL template, standard dilution or water, 1 × KAPA SYBR® FAST Universal qPCR Master Mix (KAPA Biosystems, MA, USA), 2.5 µM of both forward and reverse primer (P1 and P2 for 16S rRNA gene, DSRp2060F and DSR4R for *dsr*B gene, ME1 and ME3 for *mcr*A gene) and nuclease free water [8-11]. A ten-fold dilution series of plasmids containing the bacterial 16S rRNA gene, *dsr*B gene or *mcr*A gene ranging from 10^1 to 10^9 copies per reaction was used to estimate the concentration of corresponding gene copies in the samples and no template controls. The PCR program consisted of an initial 15 min incubation at 95 °C, followed by 45 cycles of denaturation at 95 °C for 10 s, annealing at 55 °C for 35 s and extension 72 °C for 30 s, and with final extension at 72 °C for 3 min. Sample fluorescence was measured at the end of each elongation phase. Subsequently, a melting curve was recorded, to test the specificity of the qPCR, with a program consisting of 10 s of denaturation at 95 °C, 1 min of annealing at 65 °C, and a melting and continuous measuring step rising gradually (20 °C s^{-1}) to 95 °C.

For sequencing with GS-FLX- Titanium platform (454 Life Sciences, Roche, USA) the bacterial 16S rRNA gene fragments were amplified with primers 8F and P2 equipped with adapter and barcode sequences at their 5' as described by Bomberg *et al* [12,13]. The archaeal 16S rRNA gene fragment (806 bp) from V3-V4 variable region was amplified with primers A109f [14] and Arch915R [15]. Second amplification was made using the primers ARC344f [16] and Ar774r [17], using the product from first reaction as a template. The amplification was performed in 50 µL reaction volumes, containing 2 µL template, 1 × Maxima HotStart buffer (Fermentas), dNTP 0.2 mM each (final concentration), $MgCl_2$ 4 mM (final concentration), 0.2 µM (final concentration) of both forward- and reverse primers, 1.5 U Maxima HotStart polymerase (Fermentas) and nuclease free H_2O. The amplification in all PCR reactions was carried out on a thermal PCR cycler using the following conditions: 95 °C initial denaturation for 4 min, followed by 40 cycles of 94 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min, with final extension step at 72 °C for 15 min. Amplicons were purified prior to sequencing. Amplicon libraries were sequenced by Macrogen Inc. (Seoul, Korea).

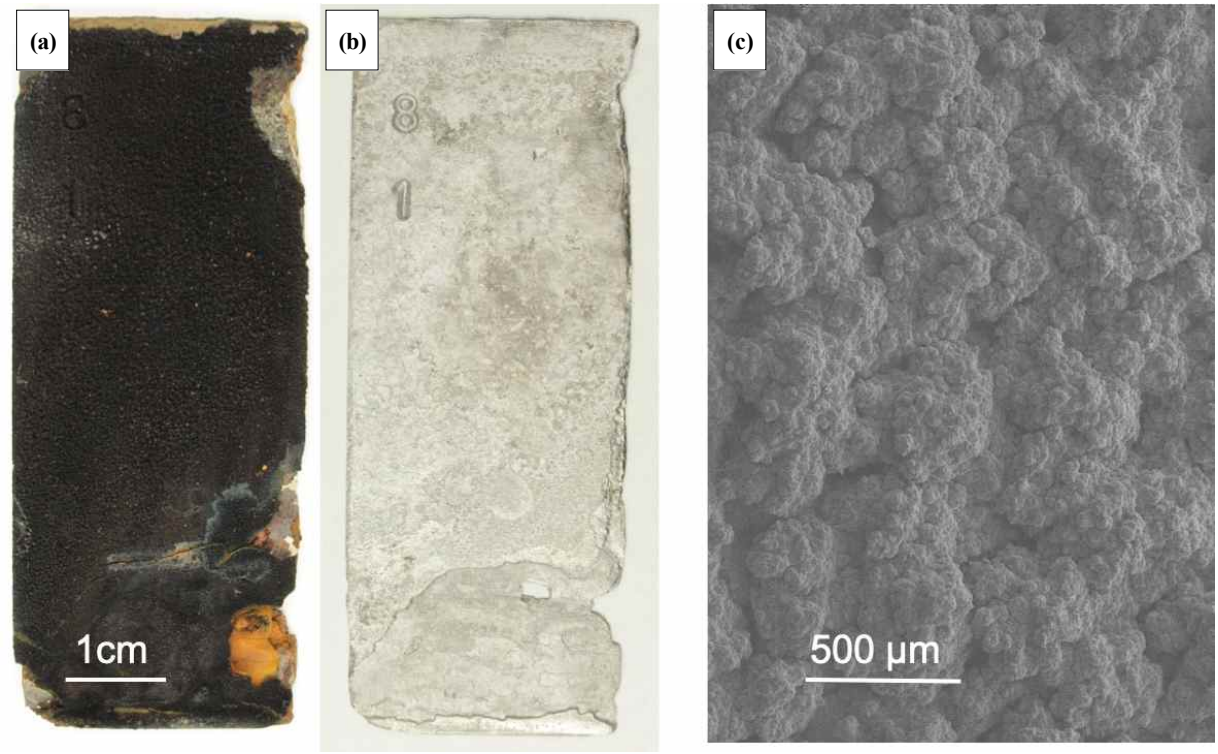

Fig. 1 (a) Carbon steel, 5.9 a, Drill hole A; (b) Carbon steel, 5.9 a, after chemical cleaning, Drill hole A; (c) Carbon steel, 5.9 a, surface imaging, Drill hole A.

The sequences obtained from the amplification libraries were analysed with the Mothur software v. 1.31.2 [18], aligning against Silva-reference database [19]. Due to the high number of similar sequences obtained, the sequences were grouped into operational taxonomic units (OTUs). Each OTU consisted of 16S rRNA sequences sharing over 97% of the nucleic acid sequence. One representative sequence was chosen from each sample for each OTU. Classification was made using Ribosomal Database Project- database [20].

All sequences are deposited in the European Nucleotide Archive (ENA) under accession number PRJEB19087.

2.3 Corrosion analyses

Corrosion was evaluated by weight loss of the coupons and the corrosion type was verified under a stereomicroscope. Carbon steel coupons were weighed, cleaned with a brush and chemically cleaned according to the ASTM standard G 1-90 [21]. To determine the mass loss of the base metal during the removal of the corrosion products, a replicate uncorroded control specimen was cleaned by the same procedure as the test specimens. The mass losses were determined and the average corrosion rates (µm a^{-1}) were calculated according to the ASTM standard G 1-90 (equation 1).

$$\text{Corrosion rate} = (K \times W) / (A \times T \times D) \text{ , where} \quad (1)$$

K = constant = 0.365×10^4
W = mass loss in mg
T = time in exposure in days
A = area in cm^2
D = density in g/cm^3

The corrosion products of selected coupons were analysed with an energy-dispersive x-ray spectrometry (EDS) coupled to scanning electron microscopy (SEM). Mineralogical composition of deposits was analysed by x-ray crystallography, using CuK_α-radiation with parameters 40 kV, 30 mA. The surface analysis and visual examination of deposits was performed by applying field-emission scanning electron microscopy (FE-SEM).

3. Results and Discussion

The water in both drill holes was alkaline and anoxic (Table 1). Concentration of dissolved salts and thus also conductivity measured from drill hole B was higher than that measured from drill hole A (530 mg L^{-1} and 1830 mg L^{-1} Cl,

Table 1 Water analyses

	pH	Cl (mg L^{-1})	SO_4 (mg L^{-1})	HCO_3 (mg L^{-1})	Ca (mg L^{-1})	Conductivity (mS cm^{-2})	Alkalinity (mmol L^{-1})	O_2 (ppb)
Drill hole A	7.9	530	128	311	71	2.37	5.07	< 10
Drill hole B	7.9	1830	250	214	250	6.18	4.59	< 10

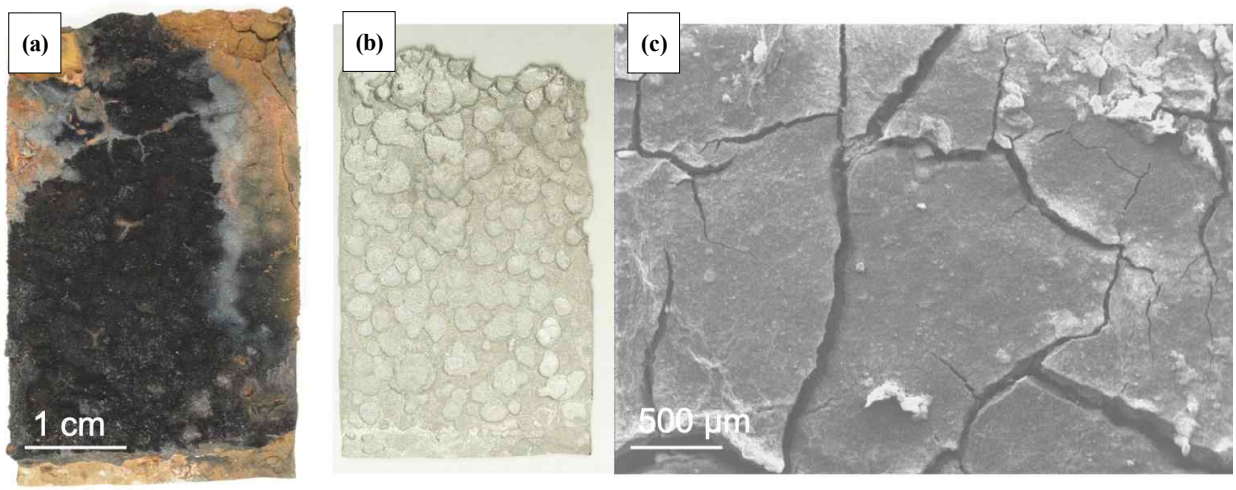

Fig. 2 (a) Carbon steel, 14.8 a, Drill hole B; (b) Carbon steel, 14.8 a, after chemical cleaning, Drill hole B; (c) Carbon steel, 14.8 a, surface imaging, Drill hole B.

2.37 and 6.18 mS cm^{-2}, drillholes A and B respectively) (Table 1).

The deposits on the surface of carbon steel coupons were mostly thick and black but some brownish deposits were also present (Figs 1 and 2). Several layers of corrosion products were detected on surfaces, a thin and smooth inner layer and a porous outer layer (Figs 1 and 2). Fluid-filled tubercles inhabited by numerous microbial community was observed on specimens (Fig. 3). According to the EDS analyses, the main components in black deposit, on coupons from both drill holes, were iron (Fe, average 53 w%), oxygen (O, average 21 w%) and sulphur (S, average 20 w%), also small amounts of calcium (Ca), carbon (C), magnesium (Mg), silicon (Si), aluminium (Al) and sodium (Na) could be detected. The brown deposit was found to contain iron (Fe, 50 w%) and oxygen (O, 40 w%) as main components. Results from the XRD-analyses (data not shown) suggested that deposits are mainly amorphic substances, but also small amounts of crystalline substances were detected. Main components were $CaCO_3$, $FeCO_3$, SiO_2 and metallic iron (Fe). Also, it is probable that iron sulphides were present, possibly in form of Fe_9S_8, and/or Fe_9S_{11}.

Average cumulative corrosion rate for coupons in drill hole A was 29 µm a^{-1} and 18.5 µm a^{-1} for coupons in drill hole B. Coupon in drill hole B had partly vanished during the experiment period (Fig. 2). Corrosion rate of carbon steel in drill hole B was earlier reported to be 10.1 - 12.4 µm a^{-1} after 11 years of exposure and in drill hole

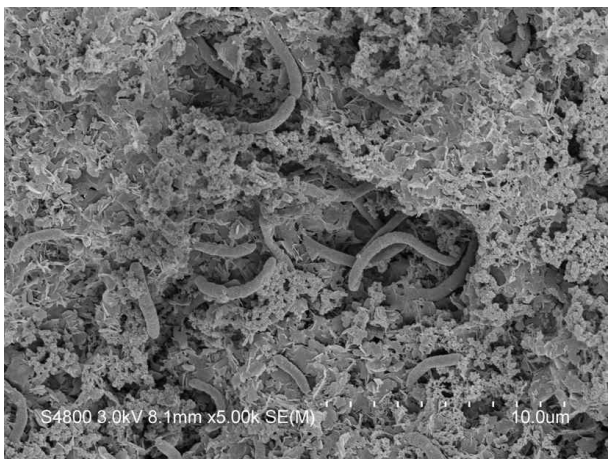

Fig. 3 Microbial biofilm embedded in corrosion product layers on carbon steel coupon from drill hole B.

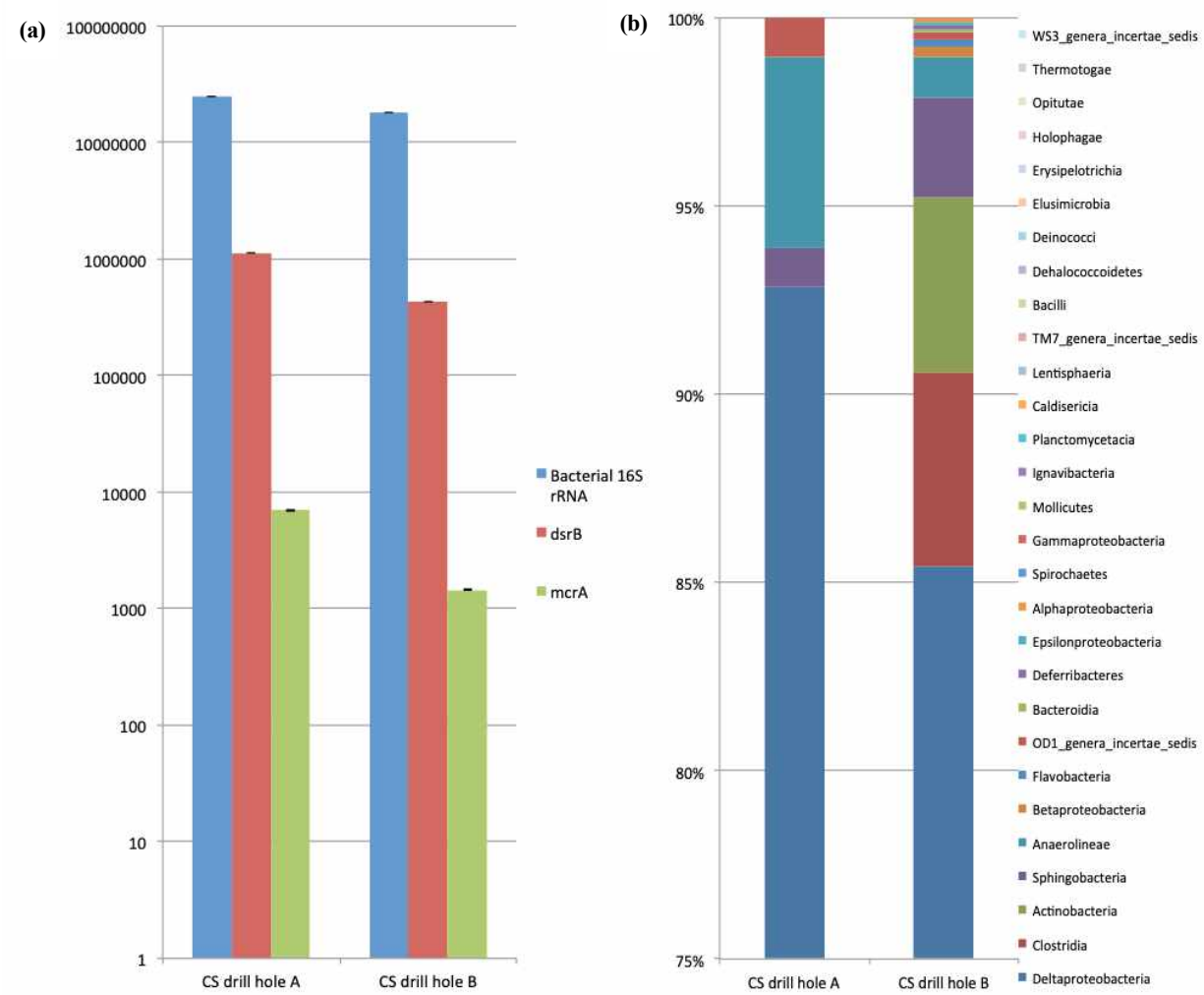

Fig. 4 (a) 16S rRNA, dsrB and mcrA gene copies detected from the biofilm formed on surface of carbon steel; (b) The diversity and abundance of bacterial classes detected on the surface of carbon steel months; All sequences of OTUs belonging to species within a phylum have been combined.

A after 3 years of exposure 29 μm a^{-1} [5].

Specimens were examined using a stereomicroscope after exposure and again after the deposit-layer was removed to confirm the form of corrosion of the base metal. The detected corrosion was mostly localized and the rate of localized corrosion was considerably greater than that of general corrosion. MIC is often reported as localized corrosion due to the heterogeneous, and often patchy, distribution of biofilms and their inorganic deposits [7]. Microbial biofilms were studied with the aid of FE-SEM. An abundant microbial community was detected embedded in the thick corrosion product layer deposited on carbon steel surfaces, as well as under the tubercles. The microbes appeared to be embedded in and attached to corrosion deposit layers forming intensive biofilm (Fig. 3). Long (2 to 7 μm in width) curved rod shaped cells were most abundant morphological form of microbes observed.

According to quantitative PCR analysis (Fig. 4a) the number of bacteria (16S rRNA gene copies) in the biofilms on the surface of steel coupons from both drill holes was similar, 10^7 copies of bacterial 16S rRNA gene cm^{-2}. Also, the numbers of sulphate reducing bacteria and of methanogenic archaea were similar in the biofilms on the steel coupons from both drill holes, 10^6 copies of *dsr*B gene cm^{-2} and 10^3 copies of *mcr*A gene cm^{-2}, respectively. Bacterial 16S rRNA copies in native ground water are previously been reported to be in range of 10^4 - 10^5 copies mL^{-1} [4], *dsr*B gene copies 10^2 - 10^4 copies mL^{-1} [3]and *mcr*A gene copies were below detection limit of the assay [1]. Current result demonstrate that both methanogenic archaea and sulphate reducing bacteria are enriched on the carbon steel surface.

The microbial diversity in the biofilm was determined using high-throughput sequencing. Deltaproteobacteria were the dominant class in biofilm from both drill holes (Fig. 4b). Majority of the deltaproteobacteria resembled sulphate reducing *Desulfocapsa*- and *Desulfovibrio*-species. Also, sulphate reducing *Desulfosporosinus*-species belonging to clostridia class were detected in the biofilm, being more abundant on surface of coupon from drill hole B. Methanogens belonging Methanobacterium class formed the archaeal population in the biofilm on the steel coupons from both drill holes.

The observed increase in corrosion rate with increase in exposure time might be due to the formation of a microbial biofilm and its influence on corrosion [5]. In most cases where corrosion is dominated by abiotic reactions, the corrosion rate decreases gradually with time as corrosion products adhere to the surface and form a protective layer that functions as a diffusion barrier [23]. Corrosion under anaerobic environments is frequently linked to function of sulphate reducing bacteria. Sulphate reducing bacteria produce hydrogen sulphide, which is a corrosive agent. Methanogenic archaea are also suspected to cause corrosion of steels [24]. They are believed to consume the excess hydrogen and thus exhilarate the cathodic reaction. Recently an alternative reaction has been proposed by which microbes can induce corrosion of steels. Some sulphate reducing bacteria and methanogenic archaea are shown to use iron (Fe_0) as a source of electrons [25]. It has been demonstrated that in nutrient poor environments, like deep ground waters here, the microbes are more "aggressive" towards steels, since no organic electron donors are available [26]. Likely due to electron uptake from Fe_0 instead of organic electron donors. In the case of microbial electron uptake, the characteristic corrosion product is commonly $FeCO_3$ [27]. In this study data from XRD-analyses showed that the corrosion products on the steel coupons exposed to ground water in the drill holes were mainly $FeCO_3$ suggesting possibility of presence of sulphate reducing bacteria or methanogenic archaea that use metallic iron as their electron donor. This is in accordance with the sequencing results showing that archaeal population consisted solely of *Methanobacterium*-species in the biofilms formed on the steel coupons in the drill hole. Some *Methanobacterium*-species have been previously shown to grow metallic iron as their source of electrons [18]. The sequencing further showed that the majority of sulphate reducing bacteria found in the biofilms belonged to family Desulfobulbaceae. The reported Fe_0 utilizing sulphate reducing bacteria also belong to family Desulfobulbaceae [28].

4. Conclusions

The findings from long-term field experiment in repository cave in Olkiluoto, Finland, demonstrate that high corrosion rates in alkaline anaerobic ground water are caused by microbial metabolic functions. The findings demonstrate that the native occurring microbes in deep ground waters have great affinity to attach the surfaces, form biofilms and facilitate corrosion. Our results indicate that the microbial groups responsible for corrosion are both sulphate reducing bacteria and methanogenic archaea. These microbial groups are enriched on the surfaces of carbon steel despite being in minority in native groundwater. Our results also indicate that these microbial groups present in biofilm may use the steel direct as source of electrons and thus cause high corrosion rates.

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