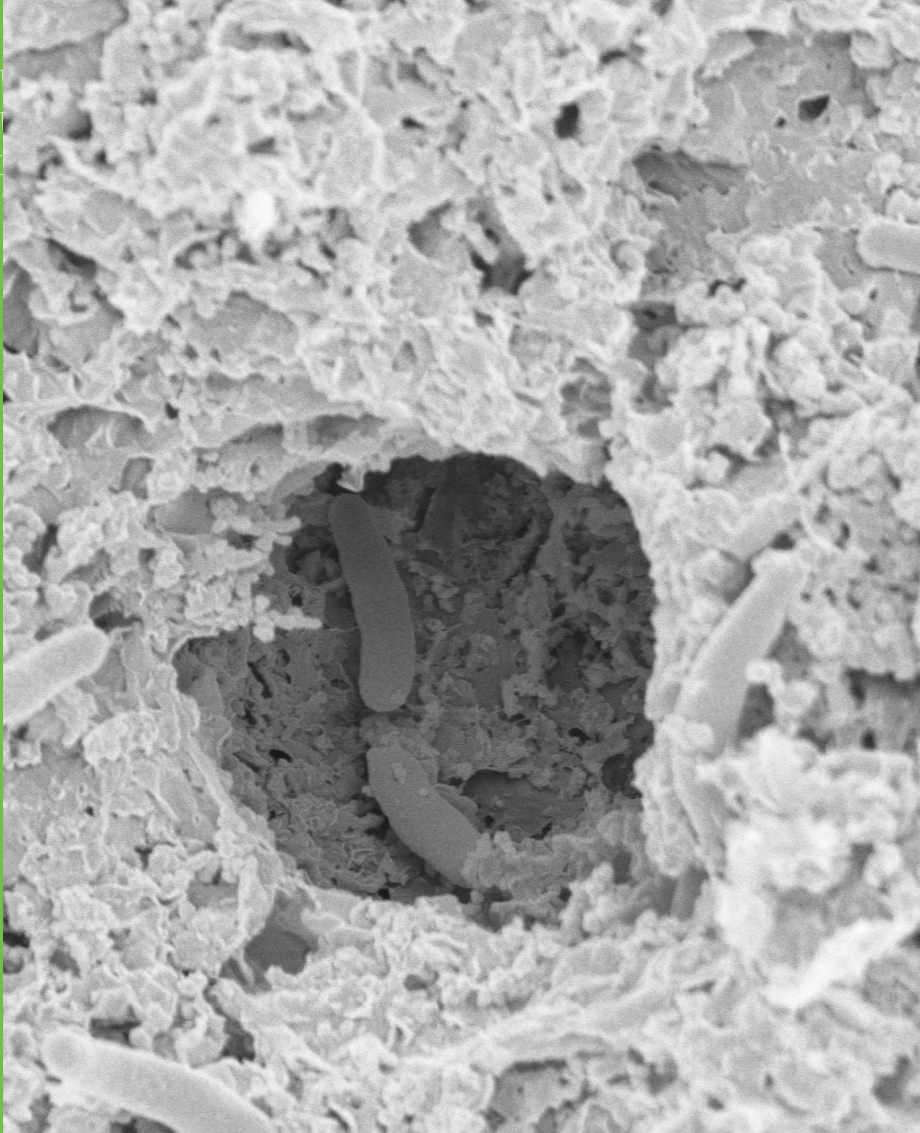


1101
0101
1010
0011



Microbially-induced corrosion of carbon steel in a geological repository environment

Pauliina Rajala



Microbially-induced corrosion of carbon steel in a geological repository environment

Pauliina Rajala

VTT Technical Research Centre of Finland Ltd

University of Helsinki

Faculty of Agriculture and Forestry

Department of Food and Environmental Sciences

Doctoral School in Environmental, Food and Biological Sciences

Doctoral Programme in Microbiology and Biotechnology

Thesis for the degree of Doctor of Science to be presented with due permission of the Faculty of Agriculture and Forestry, University of Helsinki for public examination and criticism in Auditorium 235, Info Centre Korona (Viikinkaari 11), on the July 5th 2017 at 12.00.



ISBN 978-951-38-8545-8 (Soft back ed.)

ISBN 978-951-38-8544-1 (URL: <http://www.vttresearch.com/impact/publications>)

VTT Science 155

ISSN-L 2242-119X

ISSN 2242-119X (Print)

ISSN 2242-1203 (Online)

<http://urn.fi/URN:ISBN:978-951-38-8544-1>

Copyright © VTT 2017

JULKAISIJA – UTGIVARE – PUBLISHER

Teknologian tutkimuskeskus VTT Oy

PL 1000 (Tekniikantie 4 A, Espoo)

02044 VTT

Puh. 020 722 111, faksi 020 722 7001

Teknologiska forskningscentralen VTT Ab

PB 1000 (Teknikvägen 4 A, Esbo)

FI-02044 VTT

Tfn +358 20 722 111, telefax +358 20 722 7001

VTT Technical Research Centre of Finland Ltd

P.O. Box 1000 (Tekniikantie 4 A, Espoo)

FI-02044 VTT, Finland

Tel. +358 20 722 111, fax +358 20 722 7001

Cover image: Image 1: SEM image of microorganisms embedded in corrosion product layer on surface of carbon steel, Mari Raulio. Image 2: Preparations for field experiments in TVO's VLJ-cave, Seppo Peltonen

Juvenes Print, Tampere 2017

Preface

This work was conducted at VTT Technical Research Centre of Finland Ltd and was funded by VTT and The Finnish Nuclear Waste Management Fund (projects REMIC and CORLINE). The scholarships, grants and awards from University of Helsinki (MBDP travel grant, Chancellor's travel grant), Fortum Foundation, Henrik and Ellen Tornberg Foundation, Roy G. Post Foundation, ECORD, DCO, DIMECC, The Finnish Concordia Fund and US Naval Research Laboratory, have enabled attending scientific conferences, workshops and courses as well as enabled the research visit to CSIRO, Australia.

I want to thank Professor Kaarina Sivonen, University of Helsinki, The Department of Food and Environmental Sciences, for her kind support and advice. The preliminary examiners Pierangela Christiani and Amelia-Elena Rotaru are thanked for their critical comments to improve this work. Michael Hardman is thanked for improving the language of this work.

I am deeply grateful to my supervisors Leena Carpén and Malin Bomberg for their friendship, mentoring, guidance, and trying to make time to read and comment my work. Timo Saario is thanked for critical comments on this work and general advice on life.

This work is overall a result of a joint effort. Thus, I would like to express my gratitude to all co-authors and collaborators in this study for their valuable contribution. I want to thank Mikko Vepsäläinen for inviting me to work at CSIRO and for his friendship and introduction to electrochemistry, Elina Huttunen-Saarivirta for bringing fresh ideas to our research and guidance with EIS, Mari Raulio and Irina Tsitko for performing high quality FE-SEM imaging, Jarkko Metsäjoki for SEM-EDS analysis, and Maija Raunio for providing stability diagrams. Taru Lehtikuusi and Mirva Pyrhönen are thanked for their skilled laboratory work, patience they have showed, and for questioning my ideas when they have made no sense. Marke, Tiina, Asta, Johanna, Seppo, Tuomo and Essi are equally acknowledged for their help in lab during this project and for their company and intriguing discussions during the breaks. Patrik Hautala is thanked for help with ICT problems and for never suggesting that the fault might be in the user. Tuire Haavisto and Liisa Heikinheimo from TVO are thanked for collaboration, enabling the field work for this research.

My family, are all thanked for their support and the help during my studies. I am grateful for my mother for setting the example for life-long studying. My brother has made my research visit and conference trips possible by accompanying me when I was not in shape to travel alone and has helped me in so many other ways. My father has been proud of my achievements and funded my brother's trips to accompany me. My sister has balanced my life asking me to art and music events. My precious partner, Antti, has always found a way to make me laugh, and has taught me that things just may get done also without stressing over them.

My fellow microbiologist Helena Wikman, Tiina Hyytiäinen and Sanna Laakso are thanked for long discussion on and off the topic to find fresh ideas in research and life in general. My dear friends @nakkiservo are thanked for putting life in perspective and providing all kinds of assistance and disturbance when needed, especially Vivi, Tuomas, Ensio, Johku and Jenna who have helped me finishing this project.

Espoo, May 2017
Pauliina Rajala

Academic dissertation

Supervisors

Docent Malin Bomberg, Senior Scientist
Material recycling and geotechnology
VTT Technical Research Centre of Finland Ltd, Finland

M.Sc. Leena Carpén, Principal Scientist
Materials performance
VTT Technical Research Centre of Finland Ltd, Finland

Custos

Professor Kaarina Sivonen
Department of Food and Environment Sciences
University of Helsinki, Finland

Preliminary examiners

Dr. Pierangela Cristiani
Sustainable Development and Energy Sources Department
Ricerca sul Sistema Energetico - RSE SpA, Italy

Assistant Professor Amelia-Elena Rotaru
Department of Biology
University of Southern Denmark, Denmark

Opponent

Dr. Anna Kaksonen
CSIRO Land and Water, Australia

Contents

Preface	3
Academic dissertation	5
List of publications	8
Author's contributions	9
List of abbreviations	10
1. Introduction	11
1.1 Repository for low and intermediate level radioactive waste	11
1.2 Corrosion	13
1.2.1 Forms of corrosion.....	15
1.2.2 General corrosion.....	15
1.2.3 Localized corrosion.....	15
1.2.4 Pitting corrosion.....	16
1.2.5 Crevice corrosion	16
1.2.6 Under deposit corrosion.....	16
1.2.7 Intergranular corrosion.....	16
1.2.8 Stress corrosion cracking.....	16
1.2.9 Selective leaching.....	17
1.2.10 Erosion corrosion	17
1.2.11 Hydrogen embrittlement.....	17
1.3 Microbially-induced corrosion	17
1.4 Biofilm formation.....	18
1.4.1 Microbial community and corrosion	20
1.4.2 Sulphate-reducing bacteria	21
1.4.3 Sulphur-oxidizing and -reducing microorganisms	22
1.4.4 Iron-reducing bacteria.....	23
1.4.5 Iron-oxidising microorganisms.....	24
1.4.6 Manganese-oxidising bacteria.....	25
1.4.7 Nitrate-reducing microorganisms.....	26
1.4.8 Methanogenic archaea	26
1.4.9 Acetogenic bacteria	27
1.4.10 Acid-producing bacteria	28

1.4.11 Fungi	29
1.4.12 Corrosion inhibition by microorganisms	29
1.5 Terrestrial deep biosphere	30
1.6 The deep geobiosphere at the Olkiluoto site	32
2. Aims of this thesis	33
3. Materials and methods.....	34
3.1 Experiment setup.....	35
3.2 Corrosion	37
3.3 Surface characterisation	40
3.4 Molecular biology.....	40
3.5 Bioinformatics.....	43
3.6 Statistics.....	43
4. Results and discussion.....	44
4.1 Groundwater	44
4.2 Corrosion	46
4.3 Biofilm-forming microbial community	53
4.4 Microbially-induced corrosion.....	62
4.5 Methodological considerations	64
5. Conclusions	66
6. Future outlook.....	68
References.....	69

Appendices

Publications I–IV

Abstract

Tiivistelmä

List of publications

This thesis is based on the following original publications, which are referred to in the text as I–IV. The publications are reproduced with kind permission from the publisher.

- I Rajala P, Carpén L, Vepsäläinen M, Raulio M, Sohlberg E, Bomberg M. Microbially induced corrosion of carbon steel in deep groundwater environment. *Frontiers in Microbiology*, 2015, 6:647.
- II Rajala P, Carpén L, Vepsäläinen M, Raulio M, Huttunen-Saarivirta E, Bomberg M. Influence of carbon sources and concrete on microbiologically influenced corrosion of carbon steel in subterranean groundwater environment. *CORROSION*, 2016, 72(12):1565-1579.
- III Rajala P, Bomberg M, Vepsäläinen M, Carpén L. Microbial fouling and corrosion of carbon steel in alkaline deep groundwater. *Biofouling*, 2017, 33(2):195-209.
- IV Rajala P, Carpén L, Raulio M. SRB and methanogens in corrosion of steel in anoxic groundwater. 19th International Corrosion Congress, Jeju, Korea, 2014, paper no. N-O-10.

Author's contributions

- I Pauliina Rajala wrote the paper as the corresponding author. She planned and performed the microbiological part of the experimental set-up, and was responsible for data analysis and interpretation.
- II Pauliina Rajala wrote the paper as corresponding author. She participated in designing and performing the experiments and fieldwork. She interpreted the results except for the electrochemical impedance spectroscopy.
- III Pauliina Rajala wrote the paper as corresponding author. She designed and performed the analysis, took part in the fieldwork, and interpreted the results.
- IV Pauliina Rajala wrote the paper as corresponding author. She conceived, designed and performed the microbiological experiments, interpreted the microbiological results and took part in the fieldwork.

List of abbreviations

AISI	American iron and steel institute
DGGE	Denaturing-gradient gel electrophoresis
DNA	Deoxyribonucleic acid
EDS	Energy-dispersive X-ray spectroscopy
EIS	Electrochemical impedance spectroscopy
EPS	Extracellular polymeric substances/exopolysaccharide
ILW	Intermediate-level radioactive waste
IOB	Iron-oxidizing bacteria
IRB	Iron-reducing bacteria
LLW	Low-level radioactive waste
LPR	Linear polarization resistance
MIC	Microbially-induced corrosion/Microbiologically-influenced corrosion
NPP	Nuclear power plant
NRB	Nitrate-reducing bacteria
OCP	Open circuit potential
OTU	Operational taxonomic unit
PCR	Polymerase chain reaction
rRNA	Ribosomal ribonucleic acid
SAE	Society of automotive engineers
SEM	Scanning electron microscope
SHE	Standard hydrogen electrode
SOB	Sulphur oxidizing bacteria
SRB	Sulphate reducing bacteria
TVO	Teollisuuden Voima Oyj
UNS	Unified numbering system
VLJ	Voimalaitosjäte/operation and decommissioning waste of power plant
VTT	VTT Technical Research Centre of Finland Ltd
XRD	X-ray diffraction

1. Introduction

1.1 Repository for low and intermediate level radioactive waste

Low and intermediate level radioactive waste (LLW and ILW) includes the radiation contaminated material generated during the operation, maintenance and decommissioning of nuclear power plants. This waste contains heterogeneous types of materials, both in the waste itself and as immobilization matrices of liquid waste (i.e., bitumen) and waste packaging. The waste also consists of different activity levels: LLW < 1MBq kg⁻¹; ILW 0.1–10 GBq kg⁻¹ ¹. The metallic waste comprises of pipes, valves, tools, etc., with the components being primarily made of carbon and stainless steel. This kind of waste can be decommissioned either in shallow repositories (short-lived waste), or in geological repositories ². On Olkiluoto island in western Finland, Teollisuuden Voima Oyj (TVO) has been disposing of operational waste from Olkiluoto nuclear power plant (NPP) into a final geological repository (VLJ-repository cave for LLW/ILW) since 1992 and is planning to place the decommissioning waste into the same repository after the reactors are closed down.

At the Olkiluoto NPP, LLW is deposited in their own rock silo inside a concrete liner and the ILW into a silo of steel-reinforced concrete (Nuclear Waste Management of the Olkiluoto and Loviisa Power plants, 2010). The repository silos are excavated into the crystalline bedrock of Olkiluoto island, 61°14'13"N 21°26'27"E (Figure 1). The silos extend from 60 to 95 m below ground level. The LLW silo has a capacity of about 5,000 m³, while the ILW silo is about 3,500 m³ (Nuclear Waste Management of the Olkiluoto and Loviisa Power plants, 2010). After the closedown of reactors the VLJ-repository cave will be further expanded to accommodate the decommissioning waste (Nuclear Waste Management of the Olkiluoto and Loviisa Power plants, 2010).The groundwater at the repository depth

¹www.stuk.fi/web/en/topics/nuclear-waste/disposal-of-low-and-intermediate-level-waste-in-finland

²www.iaea.org/OurWork/ST/NE/NEFW/Technical-Areas/WTS/geologicaldisposal-options.html

is anoxic and brackish, and presently flows into the silos at 39 L min⁻¹ (Nuclear Waste Management of the Olkiluoto and Loviisa Power plants, 2010).

The ILW silo currently contains mainly solid used resins mixed with bitumen in the barrels (Posiva, 2015). Barrels containing contaminated bitumen are encased in concrete boxes. After the closure of the disposal site, the facility will be flooded with concrete. The concrete generates an alkaline environment, which in turn is assumed to reduce the corrosion rate of metallic waste and containers. However, the concrete will degrade over time due to carbonization and other processes. Additionally, Small et al. (2008) showed that microbial processes could decrease the local pH as soon as four years after storage.

A durability of hundreds of years is required for a repository system containing LLW/ILW³. Understanding the changes occurring in the repository site over its intended lifespan is crucial to the design and maintenance of its long-term safety. In the safety assessment of radioactive waste repositories, the features, events and processes that affect the impact of radioactivity on the environment need to be considered (e.g. NEA, 2005). For the near-field assessment of a LLW/ILW repository, variables include microbial and chemical processes resulting from or affecting the waste degradation of repository materials that influence radionuclide speciation (Small et al., 2008). A particularly important factor to consider is the corrosion of metallic waste. In anoxic groundwater, the corrosion rate of steel is typically low unless microbial activity exists in the surrounding environment (King et al., 2014; Smart et al., 2013). The assessment of microbially-induced corrosion (MIC) of waste material in a deep bedrock environment is important when evaluating the long-term safety of the geological disposal of LLW/ILW. Microorganisms may facilitate processes (e.g., consume introduced oxygen, change redox conditions, produce aggressive metabolites) that affect the long-term stability of the repository (Pedersen, 1999). Corrosion and microbial activity related to corrosion are also partly responsible for gas generation in the repository environment that may cause overpressure and thus contribute to mobilisation of radioactive nuclides (Small et al., 2008).

Corrosion of metallic waste and associated microbial processes alter the environmental chemistry of the repository to such an extent that radionuclides may be released into groundwater and transferred to neighboring areas of the repository (Small et al., 2008). Sessile (i.e., biofilm) microbes may adsorb radionuclides and immobilize them, while planktonic microbes with adsorbed radionuclides have the potential to mobilize them (Anderson et al., 2011). Microorganisms can induce large changes in local redox potentials as well as directly transport radionuclides.

³ www.tvo.fi/

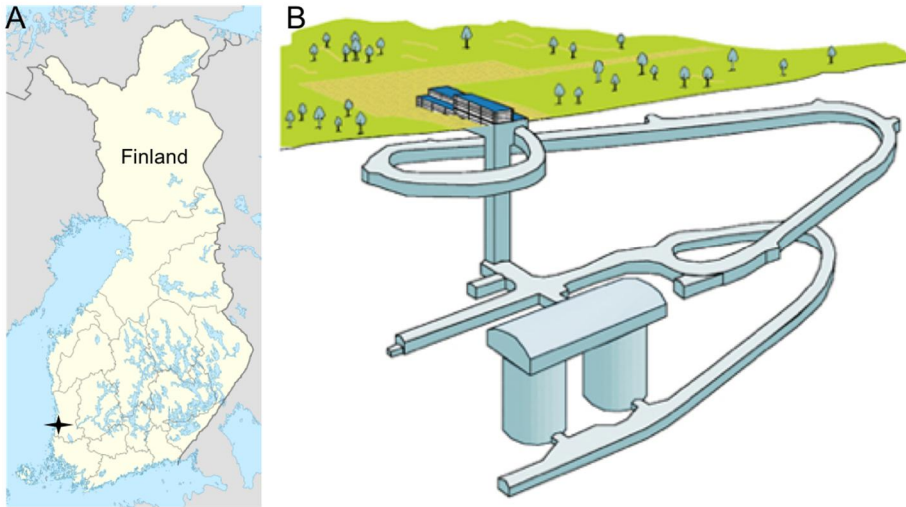


Figure 1. A) Location of Olkiluoto repository for LLW/ILW and B) schematic illustration of repository silos, figure: TVO.

1.2 Corrosion

Physicochemical interactions between a metallic material and its environment can lead to corrosion (Beech and Sunner, 2004). Electrochemical corrosion is a chemical reaction involving the transfer of electrons from zero-valent metal to an external electron acceptor, causing release of the metal ions into the surrounding medium and deterioration of the metal (Equation 1).



Electrons flow from anode to cathode and corrosion reactions take place, resulting in the dissolution of metal at the anode (Alasvand Zarasvand and Rai, 2014). In order for this reaction to proceed, a complementary reduction at the cathode takes place where electrons are transferred to an electron acceptor. As the electrons are removed from the cathode, it depolarises and allows the anodic oxidation to proceed. In oxic solutions, the cathodic reaction is the reduction of oxygen, whereas in anoxic solutions it is typically the evolution of hydrogen (Equation 2). The cathodic reaction is often the limiting part of the equation (Féron and Crusset, 2014). In aqueous media, electrochemical reactions are governed by physicochemical parameters (i.e., pH, redox potential, conductivity, etc.).

Reaction thermodynamics and kinetics determine susceptibility to and rate of corrosion. The tendency for a metal to act as either an electron donor (anode) or

electron acceptor (cathode) is described as its electrochemical potential. The thermodynamic property is an indication of how likely it is that corrosion will take place (Winston Revie and Uhlig, 2008). For corrosion to occur, differences in potential are needed. Electrons will flow from the more negative to positive potential, and the consequent oxidation will result in either film formation and/or anodic dissolution (i.e., corrosion) of the material with the more negative potential. The susceptibility to corrosion of the anodic metal in a given situation is dependent on the potential difference between the anode and the cathode. Moreover, metallic corrosion in aqueous solution is dependent not only on the metal potential but also the solution pH (Winston Revie and Uhlig, 2008). The thermodynamic prediction of corrosion can be visualised in potential-pH or Pourbaix diagram (Figure 2A). No corrosion occurs in the immunity region where the metallic form (here iron) is thermodynamically stable. Since the potential-pH diagrams are based on thermodynamic data, they provide no information concerning corrosion rate (Winston Revie and Uhlig, 2008). The rate of the anodic reaction (metal oxidation/dissolution) decreases gradually with time, because the oxidation products adhere to the surface forming a protective layer that functions as a diffusion barrier (Winston Revie and Uhlig, 2008). The stability of the layer depends on its chemistry and morphology and determines the overall susceptibility of the metal to corrosion.

Kinetics of electron transfer from the anode to the cathode are significant in establishing the corrosion rate, within thermodynamic limits (Hamilton, 2003). For the corrosion current to reach a sufficient magnitude, there must be a kinetic path available to facilitate the flow of electrons between the anode and the cathode. The rate of electron flow determines the rate of corrosion. The corrosion potential (E_{corr}) or the open circuit potential (OCP) is the potential of the metal component when the system is in equilibrium, i.e., the sum of anodic and the sum of cathodic currents are equal. The corrosion kinetic is usually described by the metal potential versus reaction current curves of both the anodic oxidation and the cathodic reduction (Figure 2B).

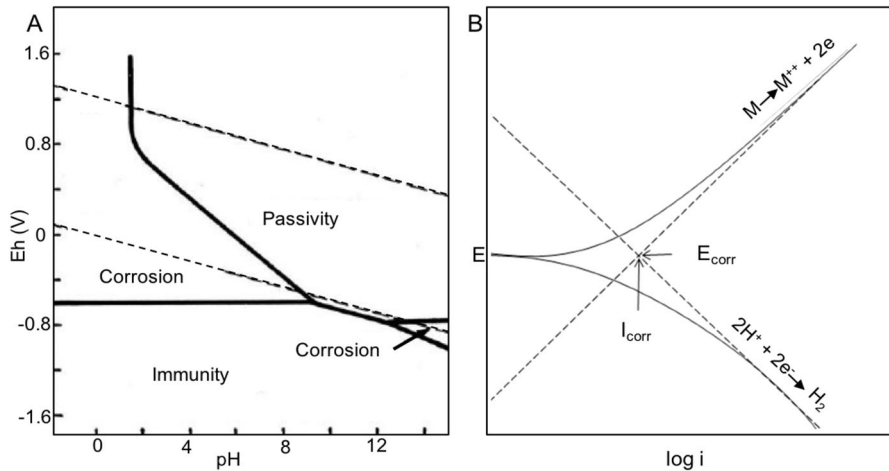


Figure 2. A) An example of potential-pH diagram of iron in pure water, dashed lines enclose the theoretical region of stability of the water. B) Conceptual potential-current curves of anodic and cathodic reactions for corrosion, i_{corr} presents the corrosion current, and E_{corr} is the corrosion potential.

1.2.1 Forms of corrosion

Corrosion is often categorized by the cause of the metal's chemical deterioration. The most commonly used categories for corrosion are: uniform or general corrosion, and localized corrosion including: pitting, galvanic corrosion, crevice corrosion, intergranular corrosion, stress corrosion cracking, selective leaching, under deposit corrosion, and erosion corrosion (Winston Revie and Uhlig, 2008).

1.2.2 General corrosion

General corrosion or uniform corrosion is the uniform loss of metal over an entire surface. General corrosion is relatively easy to detect and its effects are quite predictable (Winston Revie, 2011).

1.2.3 Localized corrosion

Localised corrosion affects small areas. The predominant forms of localized types of corrosion are pitting, crevice corrosion and under deposit corrosion (Winston Revie and Uhlig, 2008). Surface anomalies may increase the susceptibility of localised corrosion. In addition, aggressive substances in aqueous environment, for example chlorides or sulphate, may induce localised corrosion of carbon steel (Winston Revie, 2011).

1.2.4 Pitting corrosion

Pitting corrosion is a form of localized corrosion that leads to the creation of small holes or pits in the metal. Here, corrosion does not proceed uniformly but primarily occurs at distinct spots where deep pits are produced. The driving mechanism of pitting corrosion is the depassivation of a small area, which becomes anodic while an unknown but potentially vast area becomes cathodic (Winston Revie, 2011). Pit bottoms function as anodes in a small, localized corrosion cell, often aggravated by a large cathode-to-small anode area ratio (Winston Revie, 2011). Deep pits can develop with only a relatively small amount of metal loss and thus it can be missed in gravimetric analyses (Lynes, 2011).

1.2.5 Crevice corrosion

Crevice corrosion occurs in crevices where microenvironments can develop due to reduced exchange with the immediate surroundings. The different environments result in corrosion because of differences in parameters such as pH, oxygen availability, or ionic concentration (Winston Revie, 2011). Crevices are formed when two surfaces are in close proximity to one another and may also occur under surface deposits.

1.2.6 Under deposit corrosion

Under deposit corrosion is a form of a localized corrosion that develops beneath or around deposits that form or aggregate on a metal surface (Winston Revie, 2011). This type of corrosion occurs where deposits create a localized concentration of a specific chemical (e.g., chloride or oxygen) that is notably different from the amount found in the environment generally (Almahamedh, 2015).

1.2.7 Intergranular corrosion

In the manufacturing of metal alloys, substances that weaken the corrosion resistance of metal can accumulate at grain boundaries. The causative agents may be enrichment of alloying substances or impurities. In such cases, metal corrosion results in a uniform attack at grain boundaries, since they are more reactive compared to the rest of the surface.

1.2.8 Stress corrosion cracking

Stress corrosion cracking (SCC), refers to the synergic action of an aggressive environment that causes SCC and the stress condition, which lead to the deterioration or loss of the mechanical properties of a metallic material (Biezma, 2001).

Tensile stresses can be caused for example by an external or internal load. For steel, some of the known SCC promoters such as sulfide, NaOH, H₂, CO₂, CO,

may be present in the repository. SCCs can be intergranular or transgranular, or a combination of the two (Winston Revie, 2011). Depending on the metal–environment combination and the stressing condition, the time to failure can vary from minutes to many years. Compared to the other corrosion mechanisms, the propagation rate of SCC is generally very high.

1.2.9 Selective leaching

Selective leaching refers to the removal of a less noble metal from an alloy via galvanic corrosion (Winston Revie, 2011). The most susceptible alloys are the ones containing metals with large distances between each other in the galvanic series (Winston Revie, 2011).

1.2.10 Erosion corrosion

Erosion corrosion is a degradation of the material surface due to mechanical action. The mechanism can be described as the mechanical erosion of the material, or the protective (or passive) oxide layer on its surface (Winston Revie, 2011). Enhanced corrosion is likely to occur when the corrosion rate of a material depends on the oxide layer.

1.2.11 Hydrogen embrittlement

Hydrogen embrittlement is the process by which metals such as steel become brittle and fracture due to the diffusion of hydrogen into the metal (Winston Revie, 2011). Hydrogen embrittlement may also be linked to the evolution of SCC. In many cases, the critical hydrogen concentration that can lead to failure of a sensitive material is low (Biezma, 2001).

1.3 Microbially-induced corrosion

The deterioration of metals or metal alloys due to microbial activity is termed microbially-induced corrosion, microbiologically-influenced corrosion or biocorrosion (MIC). The electrochemical nature of corrosion remains valid also for MIC. The participation of microorganisms in the process induces several effects, the most significant being local changes in the electrochemistry at the metal-solution interface under the microbial biofilm (Videla and Herrera, 2005).

MIC is not a one distinct type of corrosion but the term is used to designate corrosion resulting from the metabolic activity of microorganisms within biofilms at metal surfaces or in close proximity to the surface. MIC is a result of interactions between the metal surface, abiotic corrosion products, and microbial cells and their metabolites (Beech and Sunner, 2004). In most cases, MIC occurs as a localized corrosion that results in pitting, selective leaching, crevice corrosion, under deposit corrosion, or erosion corrosion (Little et al., 1992). Microorganisms may

also accelerate the rate of partial reaction (anodic or cathodic) and influence corrosion mechanisms in other ways. Different types of corrosion may occur simultaneously contributing to total corrosion rate at different magnitudes.

Microbiological processes are similarly subject to the laws of thermodynamics and kinetics as described above for the corrosion of metals. The capture and utilisation of energy from the surrounding environment involves electron flow from negative to more positive potentials, similar to the electron flow in corrosion (Hamilton, 2003). Microorganisms are commonly categorized by their primary energy source and electron donor (e.g., iron oxidisers, methanotrophs, etc.), or by the terminal electron acceptor that acts as the acceptor of the flow of electrons in the system (e.g., sulphate reducers, iron reducers, etc.). Several of these oxidation and reduction reactions are also related to MIC (Little and Wagner, 1996; Usher et al., 2014a). Microbial communities are open systems that require a constant supply of energy and building materials for cells, so in this aspect the metabolic reactions of living microorganisms differ from thermodynamics taking place in metal corrosion. Furthermore, microbial communities and their functions are in constant flux and thermodynamic equilibria are typically unstable (Hamilton, 2003).

Microorganisms identified on corroding surfaces encompass a wide range of species with a vast range of metabolic properties. Microbial metabolites that may induce the corrosion of iron and its alloys include organic and inorganic acids and volatile compounds such as hydrogen, hydrogen sulphide, carbon dioxide, or ammonia, and additionally microorganisms directly consume electrons from Fe^0 in steel (Beech and Sunner, 2004; Venzlaff et al., 2013). In addition, enzymes such as hydrogenases, a type of oxidoreductase, produced by microorganisms may induce corrosion (Landoulsi et al., 2008).

1.4 Biofilm formation

In natural aquatic environments, microorganisms are predominantly sessile and grow as multi-species communities attached to submerged surfaces (Flemming, 2009). Generally, the planktonic populations do not accurately reflect the type and number of microorganisms that form biofilms and induce corrosion on surfaces (Videla and Herrera, 2005). Biofilms are believed to represent the dominant lifestyle for microorganisms and nearly all microbes are able to form them (Flemming, 2002). In oligotrophic environments, the formation of biofilms is a survival strategy for microbial communities. In natural environments biofilms are often composed of a diverse community of microorganisms including bacteria, archaea, and eukaryotes such as fungi (Usher et al., 2014a). Biofilms are involved in the biogeochemical cycles of carbon, oxygen, hydrogen, nitrogen, sulphur, phosphorus and many metals (Table 1) (Ehrlich, 2002; Gadd, 2010). Biofilm formation induces corrosion through various mechanisms including the formation of differential concentration of cells, generation of corrosive substances, and alteration of anion ratios (Alasvand Zarasvand and Rai, 2014).

The development of a biofilm is facilitated by the production of gel-like matrices of extracellular polymeric substances (EPS) that consist of high molecular weight macromolecules such as polysaccharides, proteins, nucleic acids, metabolites, and other particulates from the surrounding environment (Flemming, 2009, 2016; Flemming and Wingender, 2010). EPS has diverse effects on surface processes that include immobilization of water, binding metal species (e.g., iron, manganese, chromium) and corrosion products, and altering ionic concentrations at the metal surface. Water immobilisation creates gradients of chemical species or pH. The metal binding properties of EPS are due to the negatively charged functional groups that are common on the protein or carbohydrate components of EPS (e.g., carboxyl, phosphate, sulphate, glycerate, pyruvate and succinate groups) (Beech and Sunner, 2004). Metal ions concentrated in the biofilm increase corrosion rates by providing an additional cathodic reaction, as well as influencing the pH and redox potential (E_h) of this microenvironment. EPS functional groups with different affinities for metal ions increase the metal concentration locally. The area immediately beneath EPS has a high affinity for the metal and acts as an anode while areas with low affinities act as cathodes. The presence of metal ions in different oxidation states in the biofilm matrix can result in substantial shifts in the standard reduction potentials. Metal ions bound to EPS can act as electron carriers and enable redox reaction pathways (i.e., a direct electron transfer) from the metal (e.g. iron) or biominerals (e.g. FeS) (Beech and Sunner, 2004). In the presence of a suitable electron acceptor, such redox pathways would lead to the depolarization of the cathode and thus increase the corrosion rate. In addition, biofilm can act as a good electrolyte and the conductivity inside it often is higher than in the adjacent environment, due to the accumulation of metabolically produced ions within the biofilm (Cristiani et al., 2013; Little et al., 1991). Diffusion is an important process to consider because it is the primary means by which solutes move toward or away from immobilized cells within the biofilm. These processes may increase the potential differences and consequently the corrosion current.

Microorganisms can also accelerate corrosion by breaking or destabilising the passive layer (usually an oxide layer) protecting the metal surface. The passive layer may be broken by microbially-produced metal chelating agents such as siderophores (Alasvand Zarasvand and Rai, 2014). Siderophores bind to metal cations that exist in the oxide film and promote iron oxide dissolution, thereby enabling corrosion. Apart from the siderophores, dissimilatory metal-reducing bacteria also may directly degrade passive films.

Minerals deposited on a metal surface as a result of microbial metabolism can also shift the corrosion potential in a positive or negative direction. For example, manganese dioxide is known to cause ennoblement of steels, whereas sulfides move the corrosion potential in a negative, more active direction (Little et al., 1998).

As well as EPS and microbial metabolic activity in biofilm, also the physical presence of microbial cells on surfaces alone might be enough to modify the electrochemical processes that occur on metal surfaces (Little et al., 1992).

Table 1. Microbial roles in elemental cycles involved in corrosion of steel, adapted from Gadd (2010).

Element	Suggested microbial roles in elemental cycles
C, H, O	assimilation, degradation and metabolism of organic and inorganic compounds biosynthesis of polymers, carbonate, oxalate formation, dissolution of carbonates, organometal degradation, metal biomethylation/demethylation, xenobiotic oxidation CO ₂ production, CO ₂ fixation, CO utilization methanotrophy, methanogenesis, hydrogen oxidation and production
N	decomposition of nitrogenous compounds assimilation and transformations of organic and inorganic N compounds, N ₂ fixation, nitrification and denitrification, ammonia and nitrite oxidation, anaerobic nitrification, production of N-containing metabolites and gases, e.g. N ₂ O, ammonia fermentation
P	dissolution of inorganic phosphates, decomposition of P-containing organic compounds, formation of insoluble P, release of organically bound P, assimilation and transformation of inorganic P spe- cies, oxidation of reduced forms of phosphate, production of diphosphates, phosphonates, phosphine
S	Degradation of S-containing organic compounds, organic-inorganic S transformations, uptake and assimilation of organic and inorganic S compounds, sulfidogenesis, S ⁰ accumulation and reduction, SO ₄ reduction and assimilation, oxidation of reduced S compounds, thiosulfate, tetrathionate, oxidation of H ₂ S to S ⁰ , reduction of S ⁰ to H ₂ S, dissolution of S-containing minerals in soils and rocks
Fe	Fe solubilization by siderophores, organic acids, metabolites etc., Fe(III) reduction to Fe(II), Fe(II) oxidation to Fe(III), Fe biomineralization, oxides, hydroxides, carbonates, sulfides, metal sorption to Fe oxides
Mn	Mn(II) oxidation and immobilization as Mn(IV) oxides, Mn(IV) reduction, indirect Mn(IV)O ₂ reduction by metabolites bioaccumulation of Mn oxides to surfaces and exopolymers, biosorption, accumulation, intracellular precipitation, Mn biomineralization oxides, carbonates, sulfides, oxalates, metal sorption to Mn oxides
Cr	Cr(VI) reduction, Cr(III) oxidation, accumulation of Cr oxyanions
Mg, Ca, Co, Ni, Zn	Biosorption and accumulation, bioprecipitation, carbonate, Co(III) reduction

1.4.1 Microbial community and corrosion

In natural environments, biofilm is a diverse community of different microbial species. Biofilm formation is crucial for the initiation of MIC. Although the roles of each species in a diverse and corrosive biofilm are not well known, similar communities are found in various environments where corrosion has caused the failure of steel structures (Duan et al., 2008; Nería-González et al., 2006; Vigneron et al., 2016, Papers I-IV). The main types of microorganisms associated with the corrosion of steel in terrestrial and aquatic habitats are sulphate-reducing bacteria (SRB), sulphur-oxidizing bacteria (SOB), iron-oxidizing bacteria (IOB), iron-reducing bacteria (IRB), microorganisms that can utilize zero-valent metals as an electron source, heterotrophic acid-producing bacteria and EPS-producing bacteria (Usher et al., 2014a; Vigneron et al., 2016). These organisms form complex consortia in naturally-occurring biofilms and are able to affect the corrosion process through

mechanisms that could not result from single species biofilm. Active members of a multiple-species biofilm generate metabolic by-products that can support the growth of other microbes. One example of a combined effect is with IRB and SRB that co-operate to increase the concentration of ferrous and sulphide ions and thereby sustain corrosion (Obuekwe et al., 1981).

For a complex ecosystem like a natural diverse biofilm, determining the ecology of each species and their potential effect on corrosion is challenging and the underlying mechanisms of MIC are complex and insufficiently understood (Lee and Newman, 2003). Furthermore, microbes from the deep biosphere, such as the repository site, generally have no cultivated relatives and their biochemistry and physiology are largely unknown (Hoehler and Jørgensen, 2013).

Some of the functional groups of microorganisms that are frequently linked to the corrosion of iron and its alloys in anoxic conditions are listed below along with their suggested mechanisms.

1.4.2 Sulphate-reducing bacteria

Sulphate-reducing bacteria (SRB) are anaerobic and use sulphate as a terminal electron acceptor and release hydrogen sulphide (H_2S) as a metabolic by-product (Barton and Hamilton, 2013). Although their name suggests they are composed entirely of bacteria, SRB also includes archaea in this large and diverse group of autotrophic and heterotrophic microbes that reduce sulphate (Barton and Hamilton, 2013). In addition to sulphate, SRB are capable of using a wide range of substrates such as inorganic sulphur compounds, including elemental sulphur, as a terminal electron acceptor.

SRB are the most thoroughly studied microbial group involved in MIC with respect to their corrosive activity in anoxic environments (e.g. Hamilton, 1985; Little et al., 1991). There are multiple processes through which SRB induce the corrosion of iron and its alloys. Under anoxic conditions, the production of an aggressive agent, H_2S , can lead to the precipitation of FeS on metal surfaces (Gadd, 2010). H_2S rapidly oxidises metallic iron, as per the net equation (3):



Sulphide produced by SRB may cause chemical corrosion by depolarization of the cathode via solid FeS (Figure 3). It is thought that, in the absence of oxygen, non-homogeneous films of sulphide products such as FeS serve as strong cathodes to accelerate the oxidation of metallic iron (Hamilton, 2003; Lee et al., 1995).

In addition to the corrosive properties of H_2S produced by SRB, their hydrogenase enzyme can remove the cathodic, abiotically produced, H_2 from the surfaces of iron and steel, and thus depolarise the system (Figure 3). Electron removal as a result of hydrogen utilization results in cathodic depolarization and forces more iron to be dissolved at the anode (Costello, 1974). However, recent research suggests that microbial consumption of H_2 on its own does not significantly increase

corrosion rates (Enning et al., 2012; Enning and Garrelfs, 2014; Venzlaff et al., 2013).

Instead, highly corrosion inducing SRB utilise electrons directly from iron for energy by oxidising metallic iron (Fe^0) to Fe(II) (Enning and Garrelfs, 2014; Venzlaff et al., 2013) (Figure 3). The mechanism by which corrosion rates are increased by direct electron uptake has not yet been confirmed and may vary according to species. Venzlaff et al. (2013) suggested that the consumption of electrons might enhance the cathodic reaction. The consequences of direct consumption of electrons from iron and its alloys are important, including higher rates of corrosion and the requirement for microorganisms to be attached to the steel or to a conductive film on the steel (Enning et al., 2012).

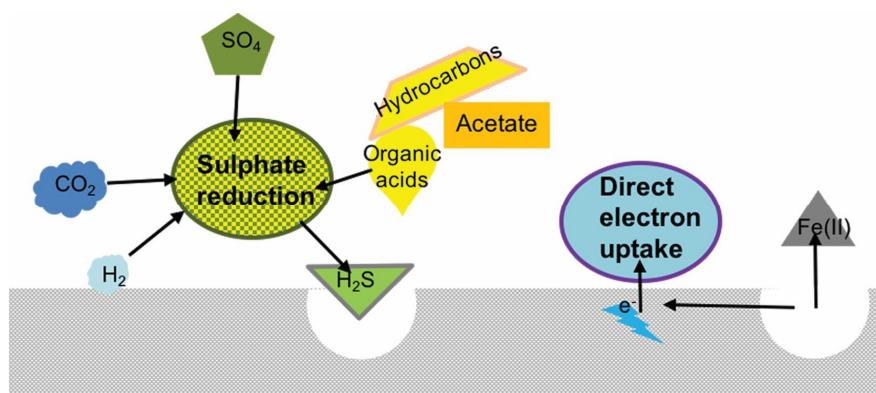


Figure 3. Examples of SRB processes influencing steel, generation of H_2S , consumption of H_2 causing depolarization, and direct electron up-take. Grey presenting the steel surface and holes possible pits.

1.4.3 Sulphur-oxidizing and -reducing microorganisms

Sulphur-oxidizing microorganisms oxidize sulphur, H_2S or sulphur containing compounds (for example FeS) to sulphate or sulphuric acid (Ehrlich, 2002). Sulphate may be further used by SRB. Sulphuric acid is corrosive to many metallic materials and increases acidity, hydrogen penetration to metal (Gadd, 2010; Little et al., 2000) (Figure 4).

Sulphur-reducing microorganisms reduce elemental sulphur to produce corrosive H_2S (Ehrlich, 2002). Sulphur reduction may be combined with methanogenesis or iron oxidation (Figure 4).

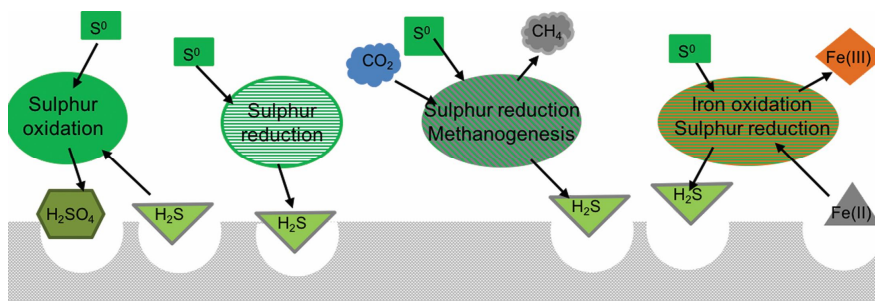


Figure 4. Examples of the mechanisms by which sulphur-oxidizing and -reducing microbes may influence the corrosion of carbon steel, production of sulphuric acid or hydrogen sulphide, and conversion of Fe(II) to Fe(III). Grey presenting the steel surface and holes possible pits.

1.4.4 Iron-reducing bacteria

Fe(III) serves as a terminal electron acceptor under anoxic conditions for lithotrophic and heterotrophic iron-reducing bacteria (IRB) (Ehrlich, 2002). The ferric iron, Fe(III), serves as the dominant or exclusive terminal electron acceptor in enzymatic ferric iron reduction during anaerobic respiration (Figure 5). Ferric iron reduction may also accompany fermentation, in which ferric iron serves as a supplementary, terminal electron acceptor (Figure 5). IRB can dissolve insoluble ferric oxide to extract the iron for iron dependent anaerobic respiration. The electron donors used by Fe(III) reducers include a wide range of organic compounds as well as H_2 or S^0 (Ehrlich, 2002; Schütz et al., 2015).

Proposed mechanisms of IRB-induced corrosion involve breaking or destabilising the passivating Fe(III) oxide (magnetite, Fe_3O_4 , in many cases) film from the metal surface through Fe(III) reduction (Herrera and Videla, 2009; Valencia-Cantero and Peña-Cabriales, 2014). IRB have been found in biofilms on corroding steel surfaces (Obuekwe et al., 1981).

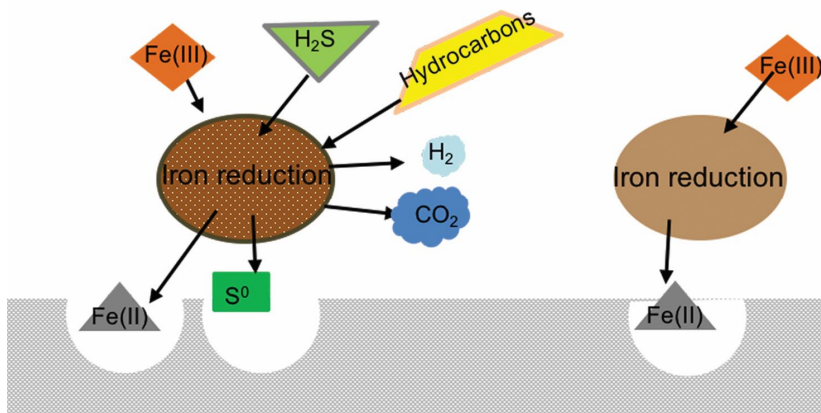


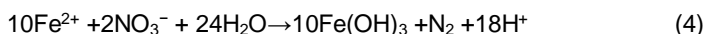
Figure 5. Examples of the mechanisms of iron-reducing bacteria induced steel corrosion, production of Fe(II) deposits, sulphur or hydrogen on the steel surfaces. Grey presenting the steel surface and holes possible pits.

1.4.5 Iron-oxidising microorganisms

Microorganisms that accumulate iron oxides on their surfaces are ubiquitous in nature (Ghiorse, 1984). Members of both archaea and bacteria exploit the favorable redox potential between the Fe(III)/Fe(II) couple and various electron donors or acceptors. In this way, Fe(II) is used as an electron donor to provide reducing equivalents for the assimilation of inorganic carbon by lithotrophic iron oxidizing microorganisms and derivation of energy.

The tendency of Fe(II) to spontaneously oxidise to Fe(III) and the low energy ratio available from Fe(II) oxidation make the niche for effective microbial iron oxidation rather narrow and the process requires an anoxic or highly acidic environment (Schädler et al., 2009; Straub et al., 2004). Microbes catalyse the oxidation of Fe(II) under pH-neutral anoxic conditions either with light as an energy source (phototrophs) or nitrate as an electron acceptor (Jiang et al., 2013; Schädler et al., 2009) (Figure 6). In the anoxic dark conditions of the repositories, the likely mechanisms for microbial iron oxidation would be dependent on nitrate (Equation 4) (Figure 6). The ability to oxidize ferrous iron with nitrate is widespread among Proteobacteria (Straub et al., 2004) but is also performed by certain archaea (e.g., *Ferroglobus placidus*) (Ehrlich, 2002).

Nitrate-dependent Fe(II) oxidation:



The end product of iron oxidation, Fe(III) has a poor solubility at a neutral pH. Fe(III) hydroxides and oxides are expected to precipitate rapidly after Fe(III) is

formed. Precipitate particles are positively charged causing them to bind either to negatively-charged cell surfaces or EPS compounds (i.e., carboxylic, phosphoryl and/or hydroxyl groups) (Schädler et al., 2009). Microbes must avoid situations where Fe(III) precipitates at cell surfaces since that would interfere with and possibly prevent the exchange of materials. Suggested mechanisms for Fe(III) solubilisation are complexation, creation of specific cellular pH microenvironments, modification of the cell surface charge, and the production of cellular exopolymers that act as precipitation templates (Schädler et al., 2009). Regardless of the mechanism, some bacteria deposit encrusted metal in the form of sheaths, stalks and amorphous masses (Ghiorse, 1984).

Microbially produced Fe(III) forms often ferric chloride (FeCl_3) that is aggressive corrosion inducing compound that produces very low pH locally. Microbial deposition of iron on surfaces may also induce underdeposit corrosion. Metal-oxidising bacteria may also remove electrons from steel via electron carriers or conductive pili (nanowires) that can exchange electrons directly with metals up to 10 mm away (Sherar et al., 2011).

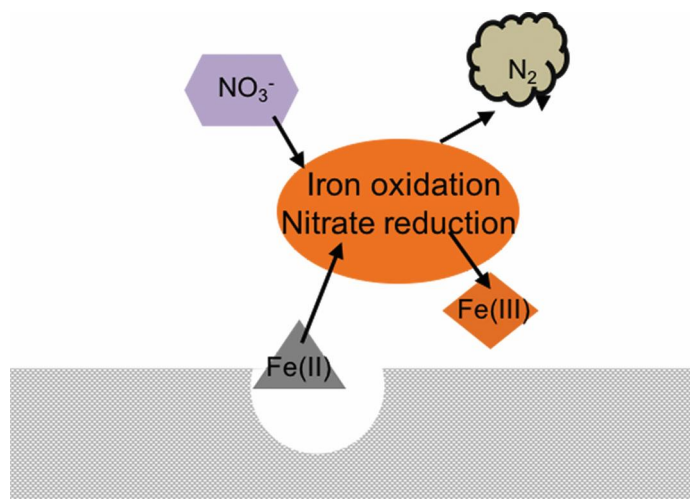


Figure 6. Possible interactive mechanisms of iron-oxidizing microorganisms with respect to steel and the corrosion-product layer, production of Fe(III) from Fe(II). Grey presenting the steel surface and holes possible pits.

1.4.6 Manganese-oxidising bacteria

Similar to anaerobic iron oxidation, manganese can be oxidized in anoxic conditions and often takes place coupled with nitrate reduction. Mn(II) oxidation is found to be widespread among the Alpha-, Beta- and Gammaproteobacteria, as well as Gram-positive bacteria (Hamilton, 2003). Manganese oxide deposition shifts the potential in the positive direction and may induce localized corrosion or pitting, especially on stainless steel surfaces (Little et al., 1998).

1.4.7 Nitrate-reducing microorganisms

The ability to respire nitrate has been described in taxonomically diverse microorganisms including members of the Alpha-, Beta-, Gamma- and Epsilon-proteobacteria, and also archaea (Ehrlich, 2002). Microorganisms that are capable of reducing nitrate are widespread (Ehrlich, 2002). As described above, nitrate-reducing bacteria (NRB) couple iron oxidation with nitrate reduction to induce corrosion (Figure 7). In addition, NRB are capable of inducing corrosion by directly utilizing electrons from metallic iron (Xu et al., 2013) (Figure 7). Some microorganisms use nitrate, nitrite, chlorate, or perchlorate as a terminal electron acceptor when oxidizing Fe(II). Fe(II) oxidation has been suggested to be a detoxification mechanism rather than an energy yielding pathway for NRB (Carlson et al., 2013).

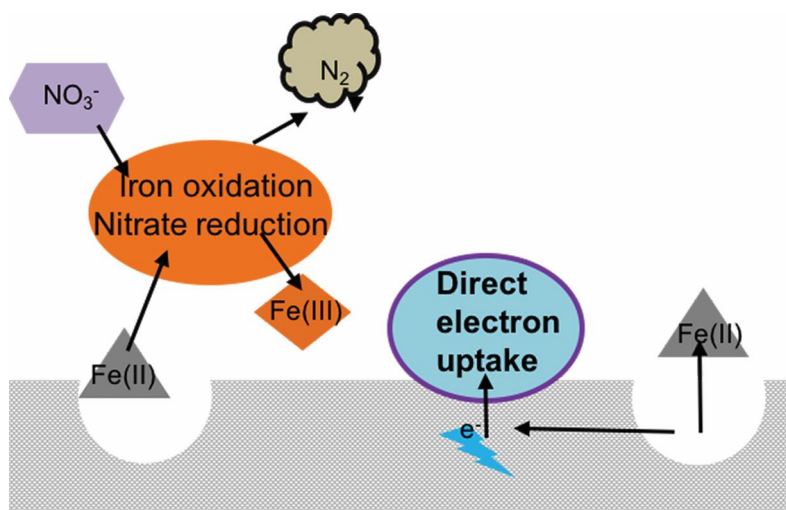


Figure 7. Possible interaction between nitrate reducing microorganisms, steel and the corrosion-product layer, production of Fe(III) and direct electron up-take. Grey presenting the steel surface and holes possible pits.

1.4.8 Methanogenic archaea

Methanogens belong exclusively to the domain Archaea, kingdom Euryarchaeota. They are obligate anaerobes that form methane as the major product of their energy metabolism (Whitman et al., 2006). Methanogenesis is the terminal step in the carbon flow in anoxic habitats and plays an important role in the anaerobic degradation of organic compounds (Ferry, 2010). Methanogenic archaea obtain their energy for growth from the conversion of a limited number of substrates to methane. The major substrates for methanogenesis are H₂, CO₂, formate, and acetate (Whitman et al., 2006). In addition, some other one-carbon compounds such as methanol, trimethylamine, and dimethylsulfide can serve as substrates for

certain methanogens (Whitman et al., 2006). About half of the methanogens are capable of autotrophic growth and they obtain all their organic carbon from the assimilation of CO_2 (Whitman et al., 2006). The proposed corrosive mechanisms of methanogens are the utilization of H_2 and direct electron uptake from Fe^0 , iron oxidation, sulphur reduction or iron oxidation coupled to methanogenesis (Boopathy and Daniels, 1991; Daniels et al., 1987; Deutzmann et al., 2015; Dinh et al., 2004; Lorowitz et al., 1992; Setter and Gaag, 1983) (Figure 8).

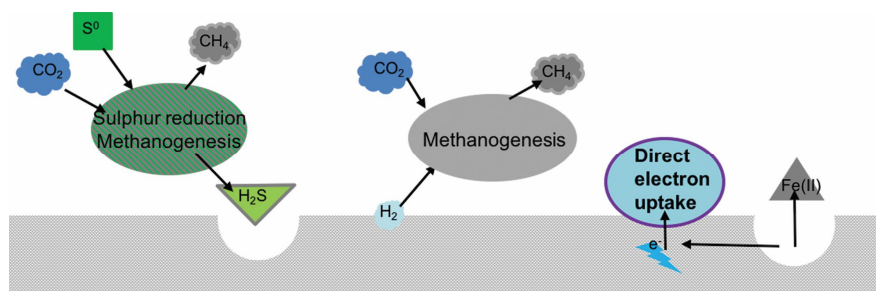


Figure 8. Methanogenesis coupled to sulphur reduction, autotrophic methanogenesis and direct electron uptake. Grey presenting the steel surface and holes possible pits.

1.4.9 Acetogenic bacteria

Acetogenic bacteria have the ability to conserve energy by producing acetate (CH_3COO^-) through reduction of carbon dioxide. Acetogens are a versatile metabolic group of bacteria. Homoacetogens could be the main group of bacteria responsible for acetate production in nutrient-poor environments. Homoacetogens are strictly anaerobic bacteria. Most homoacetogens are able to grow on very simple substrates such as carbon dioxide and hydrogen (Diekert and Wohlfarth, 1994).

Acetate is a common metabolic intermediate of microbes in anoxic environments. It provides a carbon source for a vast group of microorganisms and acetogens play an important role in producing acetate to support the heterotrophic community. Acetate can be further used by acetolastic methanogens, iron- and sulfate-reducing bacteria and other heterotrophic bacteria (Pedersen, 1997) (Figure 9). Acetate produced by acetogens may thus support corrosion indirectly by providing a carbon source to, for example, SRB (Mand et al., 2014). Acetogenic bacteria also consume the hydrogen released during corrosion and, although their importance to the induction of corrosion due to cathodic depolarisation is debated, these microbes are attracted to corrosion sites where they may become primary producers (Mand et al., 2014) (Figure 9).

Similar to SRB and methanogens, acetogenic bacteria use electrons from metallic iron and can thus induce corrosion directly (Kato et al., 2015) (Figure 9).

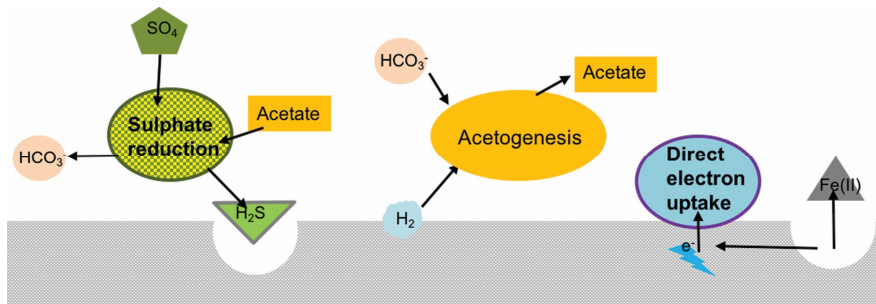


Figure 9. Possible interaction between acetogenic bacteria and carbon steel. Grey presenting the steel surface and holes possible pits.

1.4.10 Acid-producing bacteria

Hydrocarbons act as a carbon source and an electron donor for a wide variety of heterotrophic bacteria (AlAbbas et al., 2013). Most heterotrophic bacteria produce organic acids when fermenting organic substrates. Organic acids (i.e., acetic, oxalic, isocitric, citric, succinic, hydrobenzoic and coumaric acids) may force a shift in the tendency for corrosion to occur (Bento et al., 2005; Little and Ray, 2002a) (Figure 10). The impact of acidic metabolites is intensified when they are produced inside the biofilm matrix in close proximity to the metal surface (Gu, 2014). Organic acids can promote oxidation of a variety of metals by removing or preventing the formation of a protective passive layer. Microbes that produce acetic acid have been implicated as causal agents in the corrosion of metals in the same way as SOB that generate sulphuric acid (Gu, 2014). The presence of acetic acid is generally considered corrosive even at moderate concentrations (Mand et al., 2014).

Hydrogen is a side product of microbial processes such as fermentation (Figure 10). Microbially produced hydrogen may lead to embrittlement of steel (Biezma, 2001) by dissociating into atomic hydrogen and then being absorbed into the metal, by producing hydrogen ions via organic or mineral acids and being reduced to hydrogen atoms at cathodic sites, or by producing hydrogen sulphide which stimulates the absorption of atomic hydrogen onto metals by preventing its recombination into hydrogen molecules (Hamilton, 2003).

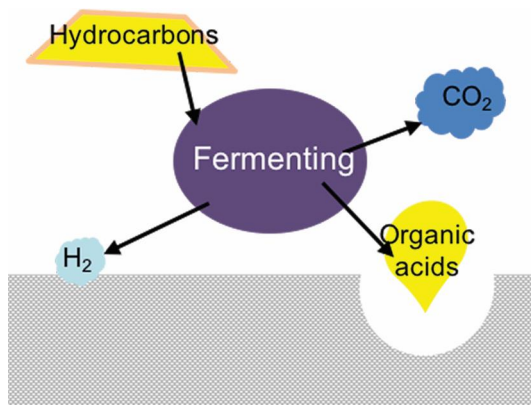


Figure 10. Production of organic acid and hydrogen via fermentation. Grey presenting the steel surface and holes possible pits.

1.4.11 Fungi

Fungi are extremely resistant to harsh conditions and are able to grow over a wide range of pH values (Das et al., 2009; Little and Ray, 2002a). The nature and diversity of fungi on the metallic structures remains poorly understood (Sette et al., 2010), but they are believed to play a role in certain forms of metal corrosion (Oliveira et al., 2011; Sette et al., 2010). Fungal colonies can produce oxalic, lactic, formic, acetic, citric, and propionic acid and directly cause corrosion of metal surfaces as well as lower the pH of an aqueous solution (Little and Ray, 2002a). Manganese oxidation and metal absorption of fungi affect the corrosion of steel in a similar manner to that described above for manganese-oxidising bacteria. In addition, fungi can reduce iron and sulphur (Das et al., 2009; Landoulsi et al., 2008). Fungi belonging to the Ascomycota phylum (e.g., *Fusarium* sp., *Penicillium* sp., *Hormoconis* sp. and *Aspergillus* sp.) have been linked to the corrosion of steel (Bento et al., 2005). Fungi also exert physical forces, unlike bacteria or archaea, and may penetrate hard substrates (Sterflinger, 2000).

1.4.12 Corrosion inhibition by microorganisms

In addition to the ability of microbes to induce and accelerate corrosion, they can alter the surface conditions of metal to reduce its tendency to corrode. The corrosive or corrosion-inhibiting behaviour of microorganisms vary considerably according to environmental variables (Alasvand Zarasvand and Rai, 2014). Mechanisms that inhibit corrosion under the biofilm are thought to be based on neutralizing the corrosive substances, the formation of a protective layer inhibiting the charge between cathodic and anodic sites (e.g., biofilm expolymers capable of binding metals), production of antimicrobial agents or competition with corrosive microbes, production of peptide corrosion inhibitors (i.e., siderophores), production of bio-

surfactants, and decreasing the hostility of the medium (e.g., by neutralizing acidity) (Alasvand Zarasvand and Rai, 2014; Little et al., 2007; Videla and Herrera, 2009). Successfully harnessing the microbial inhibition of corrosion would require a stable and predictable environment to ensure that the correct biofilm is formed and maintain (Little and Ray, 2002b). Most natural and industrial systems are too dynamic and cannot be precisely manipulated to take advantage of protective property of certain microbial biofilms (Little and Ray, 2002b).

1.5 Terrestrial deep biosphere

The biosphere is the part of Earth that is inhabited by living organisms. Microorganisms have been found in extreme environments where other organisms are not able to survive (Hoehler and Jørgensen, 2013; Rothschild and Mancinelli, 2001). Living microbes exist almost everywhere where there is liquid water, habitable space, suitable temperatures and electron donors and acceptors for energy and biomass production. A significant amount of the Earth's microorganisms exists in the oceans, soil, and in oceanic and terrestrial subsurface. It has been estimated that 2–19% of the total biomass exists in the fluid-filled pores and fractures of sediments and rocks below the Earth's surface in the terrestrial subsurface (McMahon and Parnell, 2014). Surprisingly large microbial populations (10^3 – 10^6 cells mL⁻¹) with considerable diversity have been found in sedimentary and igneous rocks at hundreds to thousands of metres deep in the terrestrial subsurface (Bomberg et al., 2014, 2015; Purkamo et al., 2016; Rajala et al., 2015; Rajala and Bomberg, 2017; Sass and Cypionka, 2004; Sohlberg et al., 2015; Wu et al., 2015). Besides inhabiting groundwater itself, microorganisms of the deep subsurface also live in microcolonies and biofilms on the surface of sediment particles or rock fractures (Pedersen et al., 2013).

The geology of the deep subsurface varies considerably, from porous soft sandstones to hard igneous rocks (Konhauser, 2007). Deep subterranean groundwater occurs in fractures or in pores between grains of igneous rocks and minerals. Deep subterranean groundwater has a much higher mineral and salt composition as a result of long-term interaction with the environment and can be characteristic for certain sites and depths (Ehrlich, 2002; Kotelnikova, 2002). In addition, the microbial community has been detected to affect the chemistry of fracture waters (Flynn et al., 2013).

The temperature, pH, pressure, salinity and lack of oxygen can all be limiting factors for life in the deep biosphere. The rate of microbial metabolism and growth in the deep subsurface has been reported to be several orders of magnitude slower than that in shallow soil (Hoehler and Jørgensen, 2013; Lovley and Chapelle, 1995). Due to the extremely low flux of energy and nutrients, estimates of generation time reach up to 1000 years (Jørgensen and D'Hondt, 2006). Thus, the microbial community in the deep subsurface is likely adapted to the low energy flux and relies on mechanisms that save energy (Hoehler and Jørgensen, 2013).

The aerobic microorganisms in surface ecosystems use oxygen as a terminal electron acceptor to degrade and metabolise organic substrates (Table 2). When oxygen has been consumed and cannot be replenished, the anaerobic microorganisms use other terminal electron acceptors such as nitrate, manganese, iron, sulphate and carbon dioxide instead of oxygen (Table 2) (Dose, 1989). Most subsurface environments may be characterized as electron donor limited rather than electron acceptor limited (Kotelnikova, 2002). The soluble redox couples are considered to be the most important because they are easily available for intracellular reactions (Jangir et al., 2016). However, it is also important to consider insoluble electron donors and acceptors in the form of redox active elements (e.g., S, Fe, and Mn) that are available in the subsurface geological environment in minerals associated with sediments and rocks (Jangir et al., 2016).

The anaerobic and facultative anaerobic microorganisms are the dominant inhabitants of the deep lithosphere. The energy to sustain the microbial community under anoxic conditions can be provided by three types of mechanisms: anoxygenic photosynthesis, anaerobic respiratory energy generation, and fermentative energy generation (Konhauser, 2007; Lovley and Chapelle, 1995). In deep subsurface environments, life is dependent upon chemical energy because light is not available for anoxygenic photosynthesis. Organic carbon, methane and reduced inorganic molecules including hydrogen, serve as possible energy sources in this environment (Table 2).

Deep terrestrial subsurface microbiology has been a target of interest in recent years due to the use of deep geological sites for long-term storage of nuclear waste or CO₂, mining activities, geothermal energy, and oil and hydrocarbon recovery and storage (e.g. Carpén et al., 2015a; Huttunen-Saarivirta et al., 2017; Morozova et al., 2011; Neria-González et al., 2006; Pedersen, 1999). Deep biosphere research in the Fennoscandian shield area has focused on the effects of microbial communities on nuclear waste storage. The safe disposal of nuclear waste in geological repositories requires that biogeochemical processes that could affect the storage environment and its contents are properly understood.

Table 2. Summary of microbial metabolic strategies.

	Substance	Definition
<i>Carbon source</i>	Inorganic (CO ₂)	Autotrophic
	Organic	Heterotrophic
<i>Energy source</i>	Light	Phototrophic
	Chemical	Chemotrophic
<i>Electron donor</i>	Inorganic	Lithotrophic
	Organic	Organotrophic
<i>Electron acceptor</i>	Oxygen	Aerobic
	NO ₂ ⁻ , NO ₃ ⁻ , SO ₄ ²⁻ , S ⁰	Anaerobic
	CO ₂ , Fe(III), Mn(IV), Fumarate	

1.6 The deep geobiosphere at the Olkiluoto site

The Olkiluoto deep geobiosphere has been a target of chemical, hydrological, geological, physical and biological research during recent decades following the construction of the ILW/LLW repository and planned construction of the final repository for spent nuclear fuel⁴.

The bedrock of Olkiluoto belongs to the Fennoscandian Shield and consists of old, Precambrian-age, highly deformed and metamorphosed migmatitic mica gneisses. More precisely, the bedrock consists of migmatitic gneiss (64% of the bedrock volume), pegmatitic granite (19%), gneiss (9%), tonalite-granodiorite-granite gneiss (8%), and the migmatitic gneiss (67%) that is veined and diatextitic gneiss (33%) (Kärki and Paulamäki, 2006). The groundwater in Olkiluoto is characteristically anoxic, with a salinity gradient increasing with depth from 0.1 g L⁻¹ at ground level to 100 g L⁻¹ at 900m depth (Vieno, 2000). The groundwater column is divided into four types according to increasing salinity and their distinctive anion contents: fresh/brackish HCO₃-type, brackish SO₄-type, brackish chloride-type, and saline groundwater (Pitkänen et al., 2003). Chloride typically occurs in all groundwater types, but the fraction of other anions is variable between groundwater types.

The microbiology of the Olkiluoto deep biosphere has been well characterized (Bomberg et al., 2015, 2016; Kutvonen et al., 2015; Nyysönen et al., 2012; Pedersen et al., 2008; Sohlberg et al., 2015). Much of this work has focused on deeper bedrock environments more relevant for the planned repository of spent nuclear fuel at 400–500 m depth. Microorganisms have been found in the Olkiluoto groundwater as deep as 900 m (Bomberg et al., 2015; Haveman and Pedersen, 1999; Kotelnikova and Pedersen, 1998; Nyysönen et al., 2012), and have been shown to contain methanogens, acetogens, SRB and IRB (Haveman and Pedersen, 1999, 2002). In addition, the fungal community of the groundwater has been surveyed (Sohlberg et al., 2015) although at deeper sites (296–798m) than the ILW/LLW repository. The biogas generated from the degradation of organic wastes in the LLW/ILW repository has been studied as well as the activity of the nitrogen-utilizing community at LLW/ILW repository depth (Kutvonen et al., 2015; Small et al., 2008). However, only a limited amount of research has focused on the MIC in the natural groundwater environment (Carpén et al., 2015a; Huttunen-Saarivirta et al., 2015, 2017; Rajala et al., 2014, Papers I-IV).

⁴ <http://www.posiva.fi/en/>

2. Aims of this thesis

The objective of this work was to focus on the MIC of carbon steel relevant to the conditions after closure of the LLW/ILW repository. This environment is anoxic, slightly saline, oligotrophic and cool with a stable year-round temperature of 10°C.

This thesis specifically concentrates on:

- The ability of deep groundwater microorganisms to induce corrosion of carbon steel in anoxic environments simulating the repository conditions.
- Microbial biofilm formation on carbon steel surface in deep groundwater.
- The effects of dynamic environmental conditions during the repository time-scale (the addition of carbon steel and concrete, temperature, availability of organic carbon sources) and their impact on corrosion and the microbial community.
- Compilation of biological, metallurgical and chemical knowledge to develop laboratory techniques for the investigation of MIC and its causal microbes in a groundwater environment corresponding to that of the LLW/ILW repository.

3. Materials and methods

The materials and methods used in this study are summarized in this chapter. More detailed information and references are presented in the original publications as well as in Table 3.

Table 3. Methods used in the study. Roman numerals refer to the original articles in which the methods were applied and fully described.

<i>Target</i>	<i>Method</i>	<i>Paper</i>
Composition of bacterial community	Denaturing gradient gel electrophoresis	I
	High-throughput sequencing	II–IV
	Quantitative PCR	I–IV
Composition of sulphate-reducing community	Denaturing gradient gel electrophoresis	I
	Quantitative PCR	I, II, IV
Composition of archaeal community	High-throughput sequencing	III
	Quantitative PCR (archaeal 16S rRNA gene, Methyl-coenzyme M reductase gene)	I–IV
Composition of fungal community	High-throughput sequencing	III
	Quantitative PCR	III
Corrosion behavior	Gravimetric analysis	I–IV
	Open circuit potential	II
	Linear polarization resistance	II
Surface characterization	Electrochemical impedance spectroscopy	II
	Energy-dispersive X-ray spectroscopy	I–IV
	X-ray diffraction	II–IV
	Scanning electron microscope	I–IV

3.1 Experiment setup

The immersion experiments (Papers I–III) were conducted using the natural groundwater sourced from TVO drillhole VLJ-KR-9 at Olkiluoto, Finland (Figure 11A, B).

Long-term *in situ* experiments were conducted in drillholes VLJ-KR-19 (5.75 years experiment time) and VLJ-KR-21 (14.8 years experiment time) that are located in close proximity to VLJ-KR-9 (Figure 11A). The drillholes are located 95 m below ground level and were drilled in 1995 (VLJ-KR-9) and 1998 (VLJ-KR-19, VLJ-KR-21).

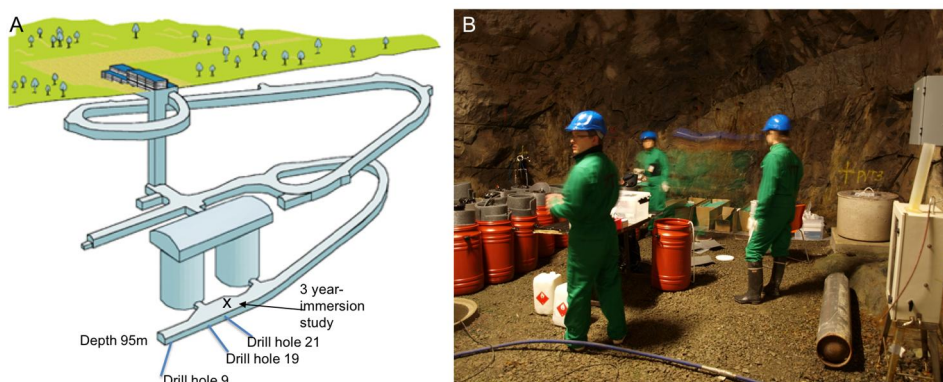


Figure 11. A) Illustration showing the location of repository silos (not to scale), and drillholes from which experimental water samples were obtained and experiments were conducted, B) preparations for three-year experiment (Paper III).

Carbon steel specimens (Papers I–IV) were prepared from cold-rolled thin sheet of 1 mm in thickness. The composition of the carbon steel corresponds with low carbon steel (AISI/SAE 1005/UNS G10050) (Table 4). Specimen surfaces for the microbiological and gravimetric studies were in as-received condition and did not receive any further treatment or finishing. However, specimens for electrochemical tests were ground to 600 grit finish; a surface roughness that corresponds to surface roughness, R_a , value of 110 nm.

Table 4. Composition (wt%) of the carbon steel AISI/SAE 1005 used in this study, Fe to balance.

C	Si	Mn	S	P	Cr	Ni	Mo	Cu	Al	W	V	Ti	Co	B
0.03	0.03	0.22	0.005	0.006	0.02	0.04	0.005	<0.003	0.054	<0.01	<0.003	0.001	0.01	0.0013

The concrete used in mesocosms (Papers II, III) was chosen on the basis of its correspondence to that used in repository structures (Table 5). Concrete particles ranging in size 1.6–8 mm were prepared by mechanical crushing and sieving.

Table 5. Composition of the concrete used in study.

Compound	kg m⁻³
Cement, type CEM I 42.5 R	395
Filler, particle size <0.1 cm	146
Particle size 0.1–0.6 cm	182
Particle size 0.5–1.2 cm	207
Particle size 1–2 cm	218
Particle size 2–3 cm	256
Particle size 3–5 cm	72
Particle size 5–10 cm	719
Water	177
Glenium (plasticiser)	2.96

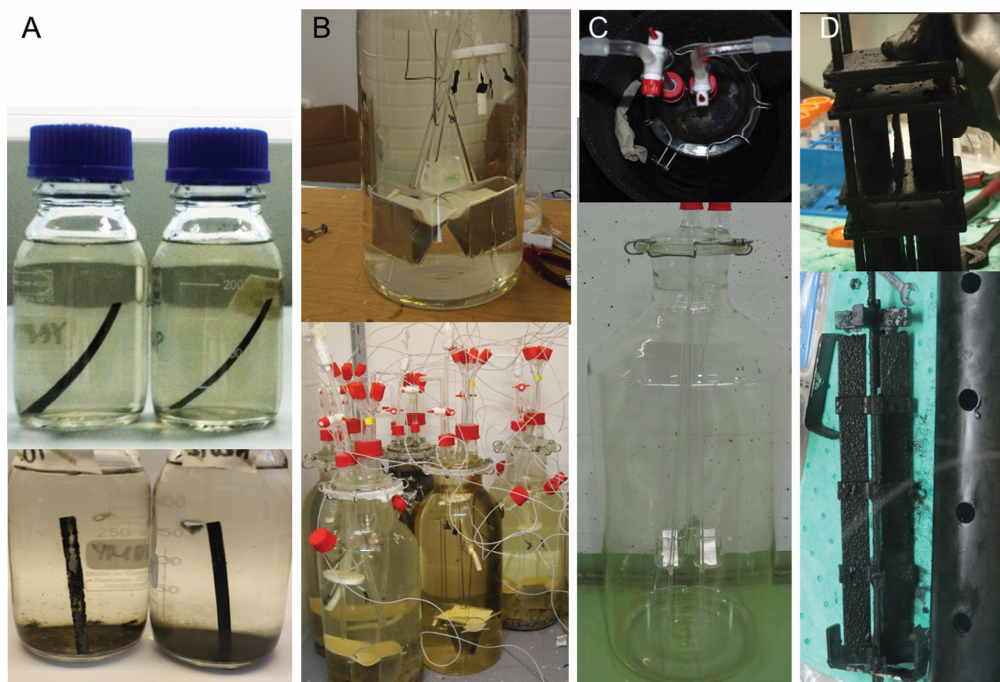


Figure 12. The mesocosm experiments. A) Immersion experiment, volume 0.25L, Paper I; B) Immersion experiment with electrochemical monitoring, volume 12L Paper II; C) Immersion experiment, volume 43L Paper III; D) The sample holders for long-term *in situ* experiments.

The mesocosms were made of borosilicate glass and were acid washed (5% HCl) and sterilized with 70% ethanol (Paper III) or by autoclaving (Papers I and II) prior to use. The experiment aiming to study the effect of the presence of carbon steel on microbial community and the effect of temperature on corrosion and microbial community was conducted in 250 mL glass mesocosms (Figure 12A) (Paper I). The study employing electrochemical monitoring to solve the roles of nutrient, concrete or biocide amendment on MIC was conducted in 12L glass mesocosms enabling the connections for electrochemical measurements (Figure 12B) (Paper II). The three-year survey of the effect of concrete on MIC was conducted in 43L glass mesocosms (Figure 12C) (Paper III).

In the *in situ* drillhole experiment the specimens were installed inside the drill-holes in specimen holders so that the groundwater had direct access to the specimens (Figure 12D) (Paper IV).

3.2 Corrosion

Cumulative corrosion rate was calculated based on the weight loss of specimens (Papers I–IV); specimens were weighed prior to and after the experiment. The deposit formed on the specimen surface during the experiment was first cleaned mechanically with a brush and then chemically according to the standard ISO 8407/C3.1 (Standard Practice for Preparing, Cleaning, and Evaluating Corrosion Test Coupons, 2011). To determine mass loss of the base metal during the chemical cleaning, a replicate non-corroded control specimen received the same treatment as the test specimens. The average weight losses were determined and used to calculate the average corrosion rates ($\mu\text{m a}^{-1}$) (Equation 5). The chemically-cleaned specimens were also examined using stereomicroscopy to evaluate the nature of the corrosion.

$$\text{Corrosion rate} = \frac{K \times W}{A \times T \times D} \quad (5)$$

where,

K = constant (0.365×10^4)

W = mass loss (mg)

T = time of exposure (days)

A = area (cm^2)

D = density of carbon steel (g cm^{-3})

As corrosion is an electrochemical process, electrochemical methods can be used to monitor both the environment and the state of the metallic material. Studying the electrochemistry of corrosion relies on the monitoring of two parameters, the open circuit potential (a thermodynamic parameter that reveals the probability of corrosion) and the current density of the corrosion reaction (a kinetic parameter related to corrosion rate).

Electrochemical methods were employed to study the corrosion and development of surface phenomena continuously during the experiment (Paper II) (Figure

13). Specimens, with the exposed surface area of 1 cm × 1 cm were used as working electrodes. Open circuit potential (OCP) monitoring, linear polarization resistance (LPR) and electrochemical impedance spectroscopy (EIS) measurements were made during the experiment. Measurement data were collected with potentiostat Reference 600™ and DC105 and EIS300 software (Gamry Instruments, USA).

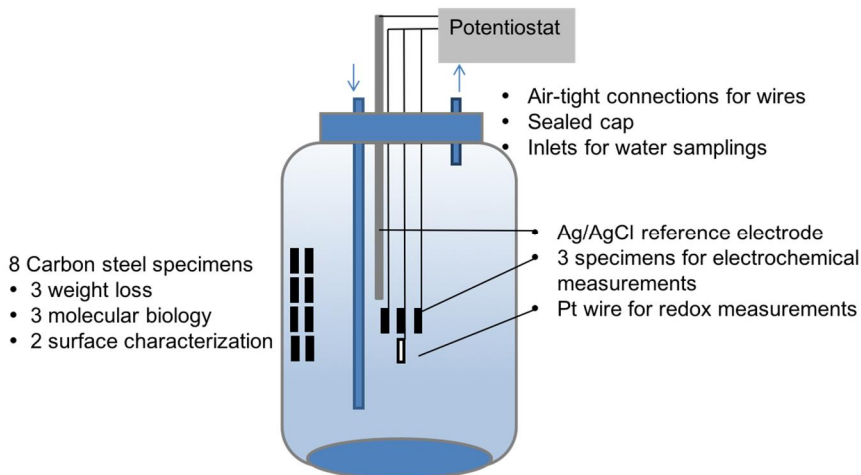


Figure 13. Schematic figure of experimental set-up enabling real-time electrochemical monitoring of corrosion rate, redox and OCP.

OCP

The OCP is the potential that a metal acquires in aqueous solution and is the potential at which the sum of the anodic and cathodic reaction currents is zero in the corresponding environment. OCP is sometimes referred to as corrosion potential (E_{corr}). This is the potential of the metal relative to the reference electrode when no external potential or current is being applied. Since the corrosion potential is determined by the specific chemistry of the system, it is a characteristic of the specific metal-solution system and can change over time if the test environment evolves. For example, biofilm growth on a metal surface can affect the cathodic or anodic processes inducing the shift in OCP. An Ag/AgCl (0.015 M KCl) reference electrode developed at VTT and a platinum wire as a counter electrode were used in electrochemistry experiments.

LPR

Polarization resistance methods allow real-time measurements of the corrosion rate without compromising the specimens. In the LPR technique, a potential (here

± 20 mV vs. E_{OCP}) is applied to a freely-corroding specimen, and the resulting current response is measured. The potential perturbation is usually applied stepwise, starting below and terminating above the free corrosion potential. The polarization resistance is the ratio of the applied potential and the resulting current response. Polarization resistance is inversely proportional to the corrosion current (Equation 6).

$$R_p = \frac{B}{i_{corr}} \quad (6)$$

$$, \text{where } B = \frac{\beta_a \beta_c}{2.3(\beta_a + \beta_c)}$$

The Tafel constants, β_a and β_c , were obtained experimentally from Tafel plots that were run prior to LPR measurement and over the potential range of ± 30 mV vs. E_{OCP} . Corrosion rate was calculated from the corrosion current according to Faraday's law. In LPR and Tafel measurements, another specimen was used as a reference electrode and a platinum wire as the counter electrode.

EIS

In EIS, the electrochemical reactions taking place at the surface of the metal are disturbed by applying a small AC signal and the impedance is measured as the result of the applied signal. Impedance measurements offer an index by which different surface reactions can be simultaneously measured (e.g., diffusion within a surface film, charge transfer reaction at the film-electrolyte interface and diffusion within the electrolyte, i.e., resistance of the electrolyte).

Electrical models (equivalent electrical circuits) can be used to explain the impedance results. EIS data are commonly analyzed by fitting the experimental data into an equivalent electrical circuit model. The circuit elements contain passive elements, such as resistors (R), capacitors (C) and inductors (L), with each pair of RC (i.e., time constant) being assigned to a different process that occurs in the system. Numerical values for the passive elements that present the system may be obtained by fitting appropriate equivalent electrical circuits to the experimental EIS data.

The EIS spectra were collected by applying alternating current potential with an amplitude of 10 mV (rms) in the frequency range from 100 kHz to 1 mHz.

Modelling the equivalent circuits

EIS data were analysed using the Echem Analyst software (Gamry Instruments, USA) by fitting appropriate electrical equivalent circuits and quantifying the numerical values for the circuit components.

3.3 Surface characterisation

At the time of sampling, specimens for the corrosion studies were quickly removed from the mesocosms under N₂ flow and immediately immersed in 96% ethanol, air-dried and placed in desiccators. In the laboratory, each metal specimen was photographed using a digital camera. Sample corrosion was evaluated by weight loss and the type of corrosion was verified under a stereomicroscope.

Corrosion products of selected specimens from each mesocosms were analyzed with an energy-dispersive x-ray spectrometer (EDS) coupled to a scanning electron microscope (SEM) and further with X-ray diffraction spectrometer (XRD).

Surface analysis and visual examination of deposits was performed by applying field-emission scanning electron microscopy (FE-SEM). To visualize the biofilm formed on the surface, selected samples were fixed in phosphate (0.1 M, pH 7.2) buffered with 2.5% glutaraldehyde for 2 h and dehydrated with an ethanol series followed by final drying in hexamethyldisilazane. The specimens were coated with Au/Pd (10–15 nm) prior to examination with a FE-SEM.

Prediction of surface products

The potential-pH diagrams, which correlate with redox and acidity conditions were generated using HSC Chemistry software version 8.2.0 (Outotec, Finland) to demonstrate the stability of corrosion products in aqueous media corresponding to that of the experiments.

3.4 Molecular biology

DNA based methods were used to determine the total bacterial (Papers I–III), archaeal (Papers II–III) and fungal (Paper III) community on the surface of carbon steel or in the groundwater. PCR primer details used in the molecular biological characterization and assessment of functionality of the microbial communities are presented in Table 6.

Quantitative PCR

Quantitative PCR (qPCR) was used to enumerate the copy numbers of several marker genes in the samples. The copy number of bacterial (Papers I–IV) or archaeal (Paper III) 16S rRNA genes was used as an approximation of bacterial and archaeal cell numbers and fungal 5.8S rRNA region targeting qPCR was used to estimate the number of fungi in the samples (Paper III). The abundance of sulphate reduction and methanogenesis marker genes were used as a proxy for potential of key anaerobic respiration processes (Paper II, IV).

Microbial community

Microbial community studies were conducted either by using PCR combined with denaturing gradient gel electrophoresis (DGGE) and Sanger sequencing of selected bands at Macrogen Inc. (Seoul, Korea) (Paper I) or high-throughput sequencing (HTP) methods of amplicon libraries (Papers II–III) using the GS-FLX-Titanium platform (454 Life Sciences, Roche, USA) at Macrogen Inc. (Seoul, Korea) (Papers II, IV) or the Ion Torrent PGM platform (Thermo Fisher Scientific, USA) at Bioser (Oulu, Finland) (Paper III).

Table 6. Primers used in molecular biological characterization of microbial community.

Gene		Assay	Primer name and sequence	Reference	Paper
Bacterial 16S rRNA		qPCR	P2 5'-ATTACCGCGGCTGCTGG-3' P1 5'-CCTACGGGAGGCAGCAG-3'	(Muyzer et al., 1993)	I-IV
Bacterial 16S rRNA		DGGE	P2 5'-ATTACCGCGGCTGCTGG-3' P3 5'-CGCCCGCCGCGCGCGGGCGGGGCGG GGGCACGGGGGCTACGGGAGGCAGCAG-3'	(Muyzer et al., 1993)	I
Bacterial 16S rRNA		454-sequencing	8f 5'-AGAGTTTGATCCTGGCTCAG-3' P2 5'-ATTACCGCGGCTGCTGG-3'	(Geets et al., 2006) (Muyzer et al., 1993)	II, IV
Bacterial 16S rRNA		IonTorrent sequencing	Bact_0341F 5'-CCTACGGGNGGCWGCAG-3' Bact_805R 5'-GACTACHVGGTATCTA ATCC-3'	(Herlemann et al., 2011)	III
Archaeal 16S rRNA		qPCR	A344F 5'-ACG GGG TGC AGC AGG CGC GA-3' A744R 5'-CCC GGG TAT CTA ATC C-3'	(Bano et al., 2004; Barns et al., 1994)	III
Archaeal 16S rRNA		454-sequencing (Nested PCR)	A109F ACKGCTCAGTAACACGT Arch915R GTGCTCCCCGCAATTCCT A344F 5'-ACG GGG TGC AGC AGG CGC GA-3' A744R 5'-CCC GGG TAT CTA ATC C-3'	(Grosskopf et al., 1998) (Stahl and Amann, 1991) (Bano et al., 2004; Barns et al., 1994)	IV
Archaeal 16S rRNA		IonTorrent sequencing	Arch349F 5'-GYGCASCAGKCGMGAAW-3' Arch-0787-a-A-20 5'-GGACTACVSGGGTATCTAAT-3'	(Klindworth et al., 2013)	III
Fungal 5.8S rRNA		qPCR	5.8F1 5'-AAC TTT CAA CAA CGG ATC TCT TGG-3' 5.8R1 5'-GCG TTC AAA GAC TCG ATG ATT CAC-3' 5.8P1 5'-CAT CGA TGA AGA ACG CAG CGA AAT GC-3'	(Haugland and Vespe, 2001)	III
Fungal internal transcribed spacer	(ITS)	IonTorrent sequencing	ITS1 5'-GCTGCGTTCTTCATCGATGC -3' ITS2 5'-CTTGGTCATTTAGAGGAAGTA -3'	(Gardes and Bruns, 1993)	III
Dissimilatory sulfite reductase gene, β -subunit	(<i>dsrB</i>)	qPCR	DSRp2060F 5'-CAACATCGTYCAYACCCAGGG -3' DRS4R 5'- GTGTAGCAGTTACCGCA -3'	(Geets et al., 2006) (Wagner et al., 1998)	I, II, IV
Dissimilatory sulfite reductase gene, β -subunit	(<i>dsrB</i>)	DGGE	2060F+GC 5'- GTGTAGCAGTTACCGCACGCCCGCCGCGCG CGCGGGCGGGGCGGGGGCACGGGGG-3' DRS4R 5'- GTGTAGCAGTTACCGCA -3'	(Wagner et al., 1998)	I
Methyl coenzyme M reductase, α -subunit	(<i>mcrA</i>)	qPCR	ME1 5'- GCMATGCARATHGGWATGTC-3' ME3 5'- GGTGGHGTMGWTTTCACACA-3'	(Hales et al., 1996)	I, II, IV

3.5 Bioinformatics

Sequence analyses

Sequences from DGGE were manually checked, edited, aligned and phylogenetic trees were constructed with the Geneious Pro software (Kearse et al., 2012) (Paper I). Amplicon libraries from HTP sequencing were analysed using mothur (Schloss et al., 2009) and QIIME software (Caporaso et al., 2010) (Papers II–IV). The setup for quality control is described in detail in Bomberg et al. (2015b). The bacterial and archaeal 16S rRNA gene sequences were compared against Silva reference alignment (Pruesse et al., 2007) and the taxonomy was assigned according to Ribosomal Database Project (RDP) (Wang et al., 2007) (Papers II–IV). Fungal sequences (Paper III) were aligned using the UNITE reference database (Kõljalg et al., 2013).

All sequences retrieved from DGGE and HTP sequencing libraries were deposited in the European Nucleotide Archive (ENA), Paper I: LN869402–LN869518, Paper II: ERS986872–ERS986876, Paper III: PRJEB18275, Paper IV: PRJEB19087.

Predicted metagenomes

Metagenomes of the biofilm-forming community (Paper III) were predicted based on 16S rRNA reads obtained from HTP-sequencing using the PICRUSt program (Langille et al., 2013). For the PICRUSt analysis, the taxonomy of the OTUs was reassigned using the Greengenes reference alignment, version gg_13_5 (DeSantis et al., 2006) with mothur software (Schloss et al., 2009). OTUs that could not be matched to a taxonomic reference were removed from the taxonomy data, which was subsequently uploaded to Galaxy pipeline (Afgan et al., 2016) for PICRUSt. Weighted nearest sequenced taxon indexes (NSTI) were calculated and metagenomes predicted from the normalized taxonomy data. Normalization was done by dividing the abundance of each organism by its predicted 16S rRNA gene copy number.

3.6 Statistics

Biological, chemical and electrochemical data were exposed to a principal components analysis (PCA) as applied in the PAST software package (Hammer et al., 2001) (Paper II).

The similarity of microbial communities between the different samples was tested by principal coordinates analysis (PCoA) using the Phyloseq package in R (McMurdie and Holmes, 2015; R Development Core Team, 2013) (Paper III).

4. Results and discussion

The ability of deep groundwater microorganisms to induce corrosion of carbon steel in anoxic mesocosms environments simulating the repository conditions or inside the drillholes at the repository site was examined (Papers I–IV). The study and identification of MIC required a multidisciplinary approach involving biological, metallurgical and chemical information (Little et al., 2006). The present study aims to combine this information to develop laboratory methods that allow us to study MIC in a meaningful way and to explore its behaviour in a groundwater environment corresponding to that of the LLW/ILW repository.

Dynamic environmental conditions and their impact on corrosion and the microbial community were simulated in several laboratory experiments. In Paper I, the effect of temperature on corrosion and the microbial community was evaluated, in addition to how the presence of carbon steel affects the microbial community. The influence of diverse organic carbon sources on corrosion and the microbial community was examined in Paper II and the effect of concrete on MIC was examined in Papers II and III. The long-term immersion experiments were conducted inside two drillholes (i.e., *in situ*) to study the corrosion process and the tendency for biofilm to form on carbon steel surfaces (Paper IV).

4.1 Groundwater

The hydrochemistry of the groundwater in drillhole VLJ-KR-9 was studied at the beginning of each laboratory experiment (Papers I–III) and twice each year during the three-year experiment (Paper III). The hydrochemistry of drillholes VLJ-KR-19 and 21 were studied at the time of specimen retrieval.

Our observations suggest that the chemical composition and microbial community inhabiting the groundwater at 100 m depth did not remain stable during the three-year survey period.

The conductivity, chloride and sulphate concentrations varied greatly among the three drillholes, being highest in VLJ-KR-21 (conductivity 6.18 mS cm⁻¹, chloride 1830 mg L⁻¹, sulphate 250 mg L⁻¹) and lowest in VLJ-KR-9 (conductivity 2.2 mS cm⁻¹, chloride 420 mg L⁻¹, sulphate 100 mg L⁻¹) (Carpén et al., 2013a, Papers I–IV). The amount of total organic carbon in groundwater varied between 5.28 and 14 mg L⁻¹, which is higher than detected in other subterranean sites of similar

depth in the Fennoscandian Shield (Haveman and Pedersen, 1999, Paper II-III). Compared to other groundwater data obtained from the Fennoscandian Shield, the groundwater in Olkiluoto VLJ cave drillholes has higher alkalinity (5–5.5 mmol L⁻¹, i.e., a higher buffering capacity) than, for example, the groundwater in the Outokumpu deep drillhole (0.5 mmol L⁻¹) (Kietäväinen et al., 2013).

In addition to changing chemical conditions, the microbial community and the groundwater in drillhole VLJ-KR-9 altered during the experimental period. During the three-year survey period, the bacterial community in the groundwater fluctuated in number (10⁴–10⁵ copies of 16S rRNA gene mL⁻¹) and in species composition (Figure 14) (Papers I, III). During the first year of the survey, the microbial community of drillhole VLJ-KR-9 consisted of Beta- and Epsilonproteobacteria in June and in October. Similar results were obtained at these time points despite different analytical techniques being applied (i.e., DGGE in Paper I and HTP sequencing in Paper III). In the following years, the bacterial community shifted to one dominated by members of Parcubacteria, Caldiserica, Nitrospirae, Dehalococcidia and Deltaproteobacteria.

The composition of the archaeal and fungal communities in groundwater was studied over three years (Paper III). Archaea were almost as numerous as bacteria (10⁴–10⁵ archaea mL⁻¹) but the community composition remained stable during the survey period (Paper III). It has previously been shown that at specific depths of the Olkiluoto site, the size of the archaeal community may even exceed that of the bacteria (Bomberg et al., 2016), suggesting that archaea might be the main player in the groundwater ecosystem at specific depths. The majority of archaea identified belonged to the order Halobacteriales (Figure 14). The number of fungi in the groundwater was low and the dominant orders shifted during the experimental period (Paper III) (Figure 14).

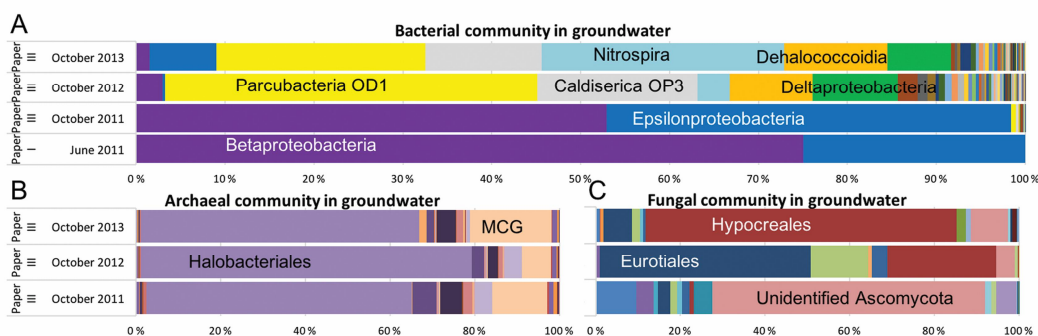


Figure 14. A) Bacterial, B) archaeal and C) fungal diversity in groundwater of drillhole VLJ-KR-9 during a three-year survey examined by DGGE+sequencing (Paper I) or HTP-sequencing of bacterial 16S rRNA gene (Papers II–IV) or archaeal 16S rRNA genes and fungal ITS gene (Paper III).

4.2 Corrosion

Corrosion rate

The average cumulative corrosion rates for carbon steel specimens were analysed based on their weight loss at the end of each experiment ranging from three months to fifteen years (Papers I–IV) (Table 7). The corrosion rates increased with longer exposure time up to $29 \mu\text{m a}^{-1}$ after six years of exposure (Paper IV). Longer exposure time (15 years) gave slightly lower corrosion rates due to complete deterioration of the specimens from which the corrosion rate was calculated (Paper IV). 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 (Winston Revie and Uhlig, 2008). 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 discussed below. Very low corrosion rates were observed in the experiments with concrete, indicating a corrosion inhibiting effect of this material (Papers II, III)

Table 7. Cumulative corrosion rates based on weight loss ($\mu\text{m a}^{-1}$).

<i>Duration</i>	<i>Corrosion rate ($\mu\text{m a}^{-1}$)</i>	<i>Temperature ($^{\circ}\text{C}$)</i>	<i>Additions</i>	<i>Paper</i>
3 months	1.4	24	-	I
	11.4	6	-	I
8 months	1.35	24	-	I
	-	6	-	I
1 year	4	10	-	II
	9.5	10	Glucose	II
	3.6	10	Methane	II
	6	10	Biocides	II
	0.004	10	Concrete	II
	0.13	10	Concrete	III
2 years	0.03	10	Concrete	III
3 years	0.03	10	Concrete	III
	18.6	10	-	III
6 years	29	10	-	IV
15 years	18.5	10	-	IV

The electrochemical methods (Paper II) were used to obtain a closer look into the behaviour of carbon steel during the experiment, whereas the weight loss analysis and surface characterization provide cumulative information at the end of

the experiment (Table 7, Figure 15). Measurements of linear polarization resistance (LPR) portray the instantaneous corrosion rate when a measurement was taken. In most cases, the trends in corrosion were similar to those revealed by the gravimetric technique. However, the absolute values detected by LPR were much higher than those obtained on the basis of the weight-loss measurements. According to LPR, corrosion was largely restrained in concrete environments as demonstrated also by gravimetric methods. High alkalinity is known to protect low-alloy steels from corrosion due to the formation of a passivating film (Winston Revie and Uhlig, 2008). In the absence of microbes, the corrosion of carbon steel in anoxic and slightly alkaline groundwater has been estimated to be $0.1\text{--}10 \mu\text{m a}^{-1}$ (He and Ahn, 2015; King et al., 2014; Smart et al., 2001). This agrees with our results in a concrete-based environment where a corrosion rate of less than $1 \mu\text{m a}^{-1}$ was observed in the weight loss data; the no-concrete reference environment saw corrosion rates 620-fold higher (Papers II–III).

The open circuit potential (OCP) of carbon steel remained stable even though the redox potential of the environment changed during incubation (Paper II). The redox potential of the aqueous environment was measured with a platinum wire immersed in each mesocosm and changes occurring at the platinum surface (e.g., formation of a deposit layer) could affect the measured redox potential. In methane amended environment the redox potential decreased sharply as the corrosion rate started to increase (Paper II), possibly due to microbial activity. A similar but less intense response was seen in the “natural” environment without any amendments (Paper II). The OCP in the concrete environment was higher than in no-concrete systems, and increased over the first four months of the experiment (Paper II). The oxygen trapped inside the concrete pores may have been released in the early stages of the experiment and have caused the increase in OCP (O_2 up to 4.5 mg L^{-1} ; Papers II–III). However, the measured oxygen concentration was significantly lower than would be for water exposed to the air (10.9 mg L^{-1}).

The water chemistry was determined after each experiment to observe any changes. In all mesocosms without concrete, the pH values increased during incubation with carbon steel from 7.5–7.8 up to 8.4 (Paper I–III). As expected, in the presence of concrete the pH increased up to a maximum value of 12. An increase in soluble ferrous iron (Fe[II]) and total iron in water was detected in all mesocosms without concrete, demonstrating dissolution of the carbon steel during the experiment. Total organic carbon, sulphur and sulphate concentrations decreased in the mesocosms during the incubation, indicating enrichment of these substances in a solid phase attached to surfaces. In addition, carbon dioxide concentrations decreased during the incubation in all mesocosms compared to the groundwater controls (Papers II–III).

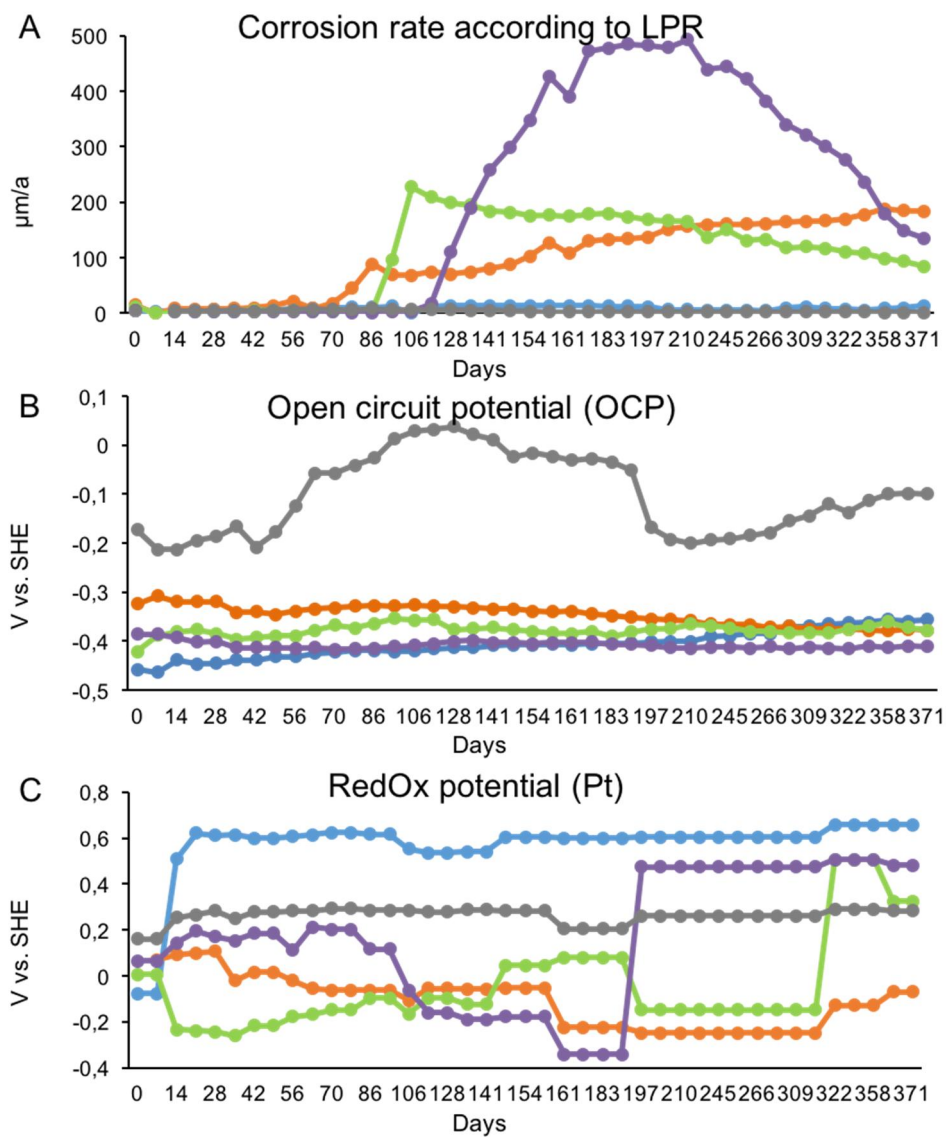


Figure 15. Summary of the electrochemical parameters. A) Corrosion rate (LPR), B) OCP and C) redox potential of platinum (Paper II)

Form of corrosion

The detected corrosion was mostly localized (e.g., pitting) and the rate of localized corrosion was considerably greater than that of general corrosion (Papers I–IV). Specimens were examined using a stereomicroscope after exposure and again after the deposit-layer was removed after the experiment to confirm the form of corrosion of the base metal. The corrosion detected in the presence of microorganisms was different from the biocide-amended groundwater where microbial activity was prevented from taking place (Paper II). In the presence of biocides, corrosion was general rather than localized. This was due to the corrosive nature of one of the biocides used (Paper II). MIC is often reported as localized corrosion due to the heterogeneous, and often patchy, distribution of biofilms and their inorganic deposits (Barton and Hamilton, 2013). The most dense and diverse microbial community was detected inside corrosion pits (Figure 16) (Paper I). Whether these microbes detected inside the pits are the ones causing pitting or lured there after pit initiation by released Fe(II), for example, remains unconfirmed. After longer exposure, the corrosion-product layer covered the whole surface and concealed the evolving pits and obscured any fine-grained observation of the microbial activity but microbes were detected throughout the corrosion-product layer.

Differences between the gravimetric measurements and the real-time corrosion rates deduced from LPR are likely due to localized corrosion that is too small to be measured accurately by weight loss but detectable with electrochemical monitoring. When the specimens from the methane-amended system were examined with a stereomicroscope, numerous small corrosion pits (\varnothing 5–10 μm) covered the whole surface and could influence the LPR measurement, where high corrosion rates were detected but only moderate corrosion was revealed by gravimetric analyses. Carpén et al. (2013b) studied the evolution of localized corrosion using real-time monitoring in an environment corresponding to that of Paper II and reported localized corrosion rates of up to 70 $\mu\text{m a}^{-1}$. Notably, localized corrosion was visually detected in the concrete-amended environment, whereas in abiotic experiments conducted in simulated groundwater this has not been previously reported (Smart et al., 2013). The scale and the passivation film formed due to concrete and high pH protected the carbon steel from general corrosion but local microenvironments formed under the microbial biofilm enabled pitting to take place.

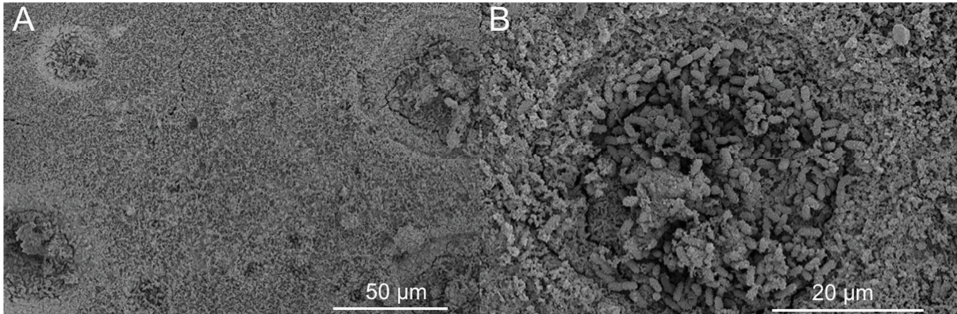


Figure 16. Microorganisms inside the corrosion pits on the surface of carbon steel.

Corrosion products

The corrosion product layer grew thicker and more uniform with longer exposure time (Papers I–IV). Several layers of corrosion products were detected on all surfaces, a thin and smooth inner layer and a porous outer layer (Figure 17). Fluid-filled tubercles inhabited by numerous microbial community were observed on specimens from both laboratory and long-term *in situ* experiments (Papers II, IV).

The corrosion of carbon steel in abiotic anoxic groundwater has been modelled and attempts made to predict the corrosion products but in the absence of microorganisms and in synthetic rather than natural groundwater (King et al., 2014; Smart et al., 2001, 2013). The type of corrosion products forming on carbon steel depends on temperature, pH and the concentration of Fe(II), chlorides, carbonates, oxygen and sulphate ions in the environment (Neff et al., 2005).

The potential-pH diagrams, which correlate with redox and acidity, can be used to demonstrate the stability of corrosion products (Figure 2A, Figure 18). Stability diagrams and the assumption of local equilibria can be used to construct trajectories consistent with principles of groundwater chemistry (McNeil and Little, 1999).

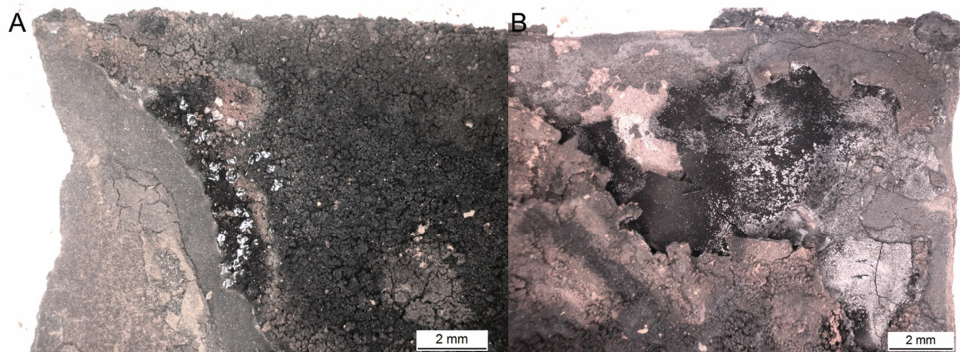


Figure 17. Stereomicrographs of A) a porous thick corrosion product and B) multiple corrosion product layers formed on carbon steel surfaces in natural groundwater during a three-year exposure.

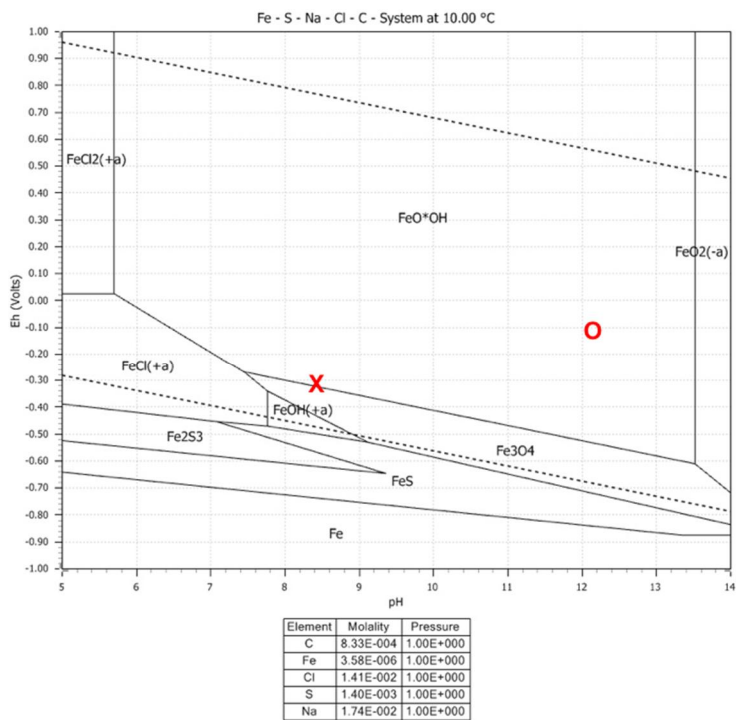


Figure 18. The potential-pH diagram portraying thermodynamically-favourable corrosion products. "X" pointing the likely conditions at the end of the experiments without concrete and "O" with concrete, dashed lines enclose the theoretical stability region of water.

Composition of the deposit layers was determined using EDS (Papers I–IV) and XRD (Papers II–IV). In the EDS analysis, Fe, C, O, S, Si, Ca were commonly detected on surface deposits. XRD spectra confirmed the presence of iron sulfide (FeS) and iron oxide (Fe₂O₃) on the surfaces of the carbon steel specimens in the absence of concrete after 1–3 years (Papers II–III). A more complex layer was detected after long-term exposure containing CaCO₃, FeCO₃, SiO₂ and iron sulphides possibly in the form of Fe₉S₈, and/or Fe₉S₁₁ (Paper IV). As stated above, the pH became more alkaline during the incubation; from 7.5–7.8 up to 8.4, and as high as 12 in the presence of concrete. The OCPs were between -350 mV (vs. SHE) and +500 mV (vs. SHE) (Paper II). With respect to thermodynamic favourability and potential-pH diagrams, the most stable surface deposits in the concrete-free mesocosms were magnetite (Fe₃O₄) and FeO*OH, and FeO*OH in mesocosm containing concrete. However, FeS was frequently observed in XRD, most likely produced by microbial activity. Similar corrosion products have been detected where MIC has been suspected (Kato, 2016; Usher et al., 2014b). Microbes in biofilms produce minerals through reactions that are not predicted by the thermodynamics of the medium (McNeil and Little, 1999). The decrease in sulphate of groundwater accompanied by slight pH increases supports the notion that some of the FeS formed may come from SRB metabolism. In the presence of sulphur species (either biogenic or abiotic), carbon steel first develops a film of FeS that later changes through several chemical and electrochemical pathways to more stable iron sulphides (Videla, 2000). Microbial metabolism and enzymatic reactions are constantly in flux, and therefore any equilibrium is likely brief and unstable (Usher et al., 2014a). Thus, corrosion products unlikely to form according to potential-pH diagrams can be detected due to the generation of local microenvironments created under microbial biofilms.

The conductive or semi-conductive corrosion products such as magnetite (Fe₃O₄) and ferrous sulphide (FeS) detected in this study are capable of transferring the electrons from corrosion product or carbon steel to microorganisms (Enning et al., 2012; Liu et al., 2015). In all cases, iron sulphides are characterized by strong cathodic effects on the hydrogen reduction reaction, causing an indirect increase in the corrosion rate (Videla and Herrera, 2005). Sulphur-containing deposits are frequently linked to the activity of sulphate- or thiosulphate-reducing bacteria on the carbon steel surface (Urios et al., 2014). Both abiotic and biotic iron sulphide films are related to the formation of tubercles on steel, as reported in Paper II. However, thin adherent layers of iron sulphides could also protect carbon steel from corrosion (Schütz et al., 2015).

In addition to sulphur-containing deposits, a greyish mineral crust mainly containing siderite (FeCO₃) is generally formed as a corrosion product under methanogenic, acetogenic and nitrate-reducing conditions (Kato, 2016). Siderite was confirmed in a deposit formed during long-term *in situ* exposure (Paper IV). However, siderite might also be easily transformed to iron oxyhydroxide. In EDS, the components of siderite were detected and the corrosion product resembled that reported for siderite after shorter exposure times (Papers II, III) (Figure 17).

In the presence of concrete, CaCO_3 and traces of iron oxide and iron hydroxide ($\beta\text{-FeOOH}$) were detected (Paper III). The scale formed in the concrete mesocosms clearly protected the carbon steel surface from corrosion and the corrosion rates decreased as the experiment continued and the deposit layer became increasingly uniform (Paper II, III). When examined using SEM, large ($100\text{--}200\ \mu\text{m}$) crystals were detected across the surface and few microbes were detected (Figure 19). The EIS measurements demonstrated that carbon steel and the formed CaCO_3 scale deposit in alkaline environments had higher corrosion resistance values than those in natural water systems (Paper II). It has been reported that the CaCO_3 scale on the steel is beneficial to reduce corrosion in aqueous systems (Lynes, 2011). Furthermore, the redox potential for hydrogen reduction decreases with increasing pH and the thermodynamic driving force for corrosion decreases. However, in contrast to earlier studies using a synthetic environment (Smart et al., 2001), local pitting was detected suggesting that microbial activity might be disrupting the passivating layer in certain locations (Paper III).

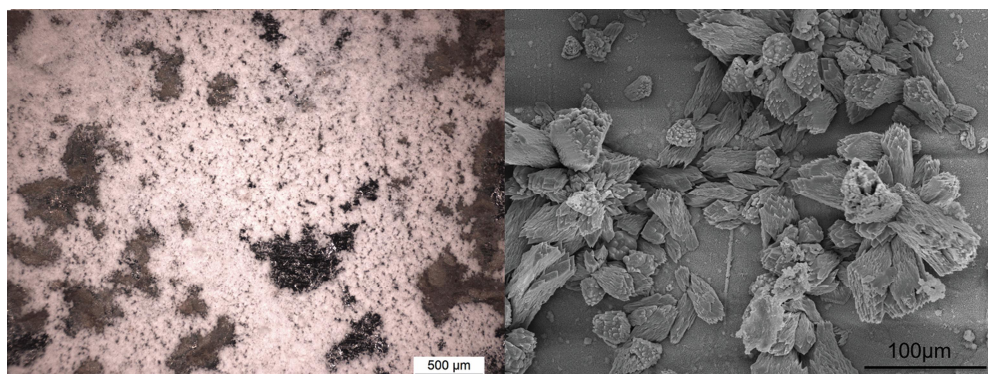


Figure 19. A) Stereomicrograph and B) SEM image of the deposit formed on carbon steel in the presence of concrete during a three-years exposure.

4.3 Biofilm-forming microbial community

Bacterial community

Microbial biofilms were studied with the aid of FE-SEM. An abundant and diverse biofilm was detected in the thick corrosion product layer deposited on carbon steel surfaces, as well as under the tubercles (Papers I–IV). The number of bacteria comprising the biofilm was estimated according to bacterial 16S rRNA gene copies. A similar trend was observed when comparing the results across experiments (Figure 20) in that the number of biofilm-forming bacteria increased during the first year (samples at three and eight months) after which it stabilized at 10^7 copies of 16S rRNA genes cm^{-2} of carbon steel (Papers I–IV). A higher temperature (RT) resulted in an increase in the number of biofilm-forming bacteria compared to

natural conditions (Paper I). The temperature increase had a greater influence on bacterial abundance in the biofilm than the availability of additional organic carbon sources (Papers I and II).

In the experiment where concrete was added to the mesocosms (Papers II and III), the number of biofilm-forming bacteria was stable at 10^4 16S rRNA gene copies cm^{-2} when studied after one, two or three years of immersion. Biofilm formation in the presence of concrete was studied during a one-year exposure (Paper II) and for three years with annual sampling (Paper III). Overall, concrete inhibited biofilm formation, and the number of biofilm-forming archaea and bacteria was 1000-fold lower than in the absence of concrete.

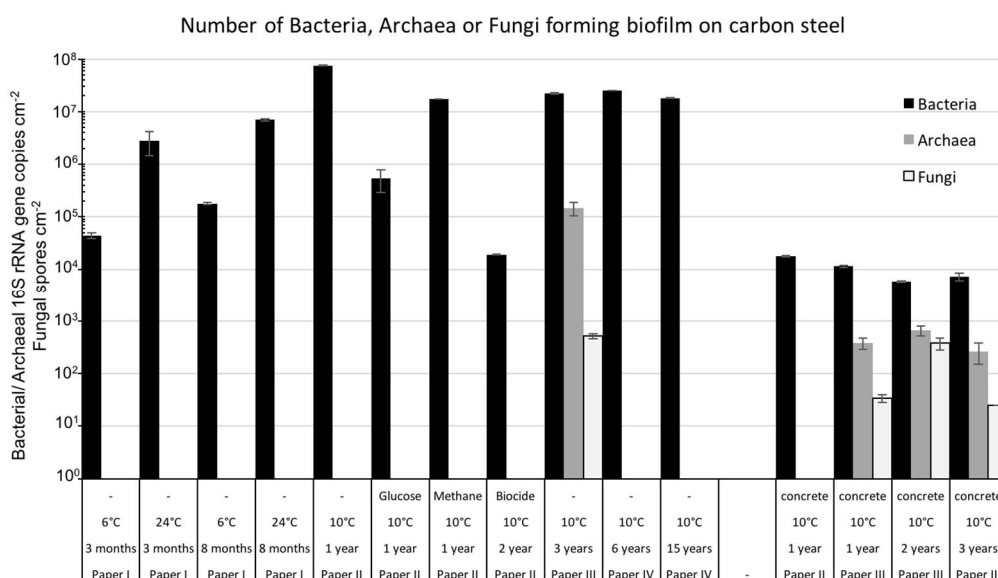


Figure 20. Number of bacterial (Papers I–IV) or archaeal 16S rRNA or fungal 5.8S rRNA (Paper III) gene copies on the surface of carbon steel (cm^{-2}) in various experiments and exposure times. Error bars represents standard deviation ($n=3$).

When the bacterial community was studied with DGGE and sequencing of the prominent bands (Paper I) or HTP-sequencing (Papers II–IV), changes in community structure were observed even though bacterial number remained constant. The availability of organic carbon sources affected the relative abundance of proteobacterial classes; the ratio between Alpha-, Beta-, and Deltaproteobacteria changed, so that Beta- and Deltaproteobacteria were favoured over Alphaproteobacteria (Paper II) (Figure 21). The temperature also had an effect on the species composition of biofilm-forming bacteria (Paper I) (Figure 21). The most dominant phylotypes detected in absence of concrete or biocides belonged to Proteobacteria. Betaproteobacteria or Alphaproteobacteria were the main classes forming biofilms in these conditions (Figure 21).

The bacterial biofilm formed in the presence of concrete after one, two, and three years of exposure consisted of Parcubacteria, Alphaproteobacteria (Rhodospirillales and Rhizobiales orders), and Gammaproteobacteria (Pseudomonadales, Moraxellaceae families) (Paper III). In addition to these, Clostridia, Bacilli and Deltaproteobacteria were detected after one, two and three years, respectively (Paper III). However, during one-year exposure in the electrochemical monitoring experiment (Paper II), the bacterial community differed completely from that described above in that the species were mostly spore-forming bacilli.

The bacterial communities in the corrosive biofilms did not resemble those detected more widely in the groundwater system, which suggests robustness in the mesocosm environment that effectively resisted the effects of new microbes being introduced when the groundwater was replenished.

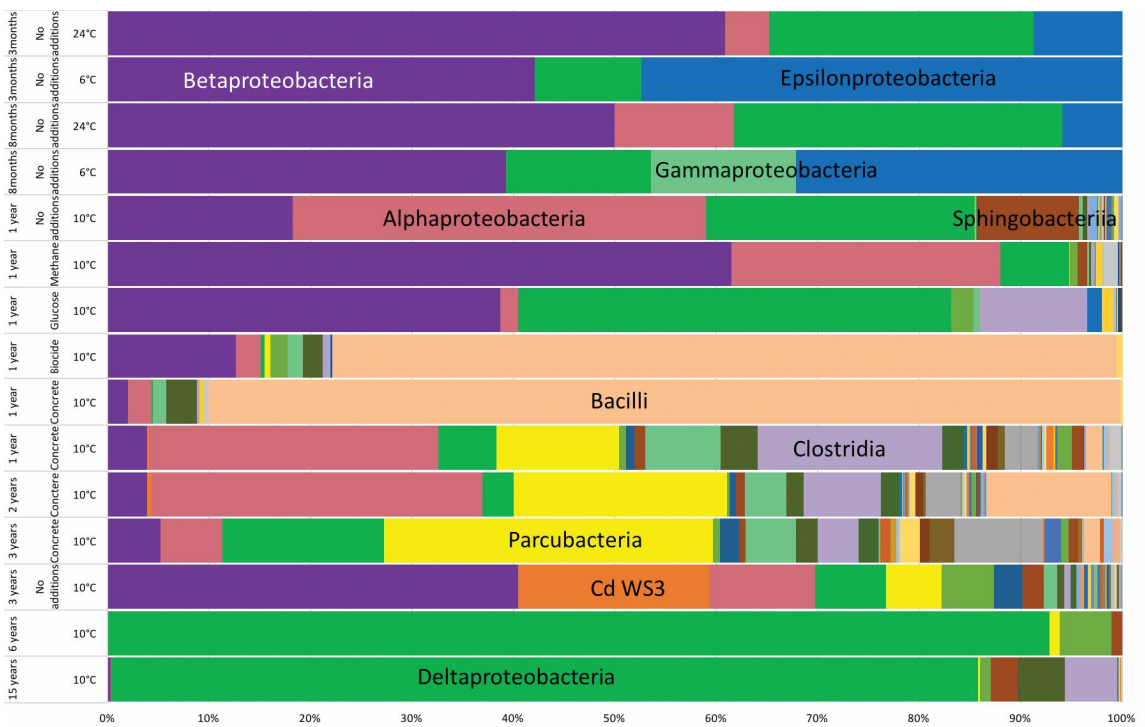


Figure 21. Relative abundance of biofilm-forming bacteria, data combined from two to three parallel samples.

Formation of biofilm and extracellular polymeric substances

The formation of biofilm is considered to be the result of complex processes involving adsorption of molecules to the surface, formation of the conditioning layer,

and initial attachment of microbial cells followed by their irreversible adhesion facilitated by the production of EPS, accumulation of organic and inorganic molecules and microbial cells to the surface (Flemming and Wingender, 2010). In SEM examinations, the evidence of EPS formation was seen on the steel surface (Papers I–IV). EPS has been shown to effectively bind metallic ions and metal accumulation has been demonstrated in many cases (Beech, 2004) that carries a charge to promote ionic and electrostatic binding of counterions including metals. Several classes of polymeric molecules participate in EPS–metal interactions by the formation of salt bridges with carboxyl groups on acidic polymers and by forming weak electrostatic bonds. EPS has a two-way relationship with the corrosion product; it binds metallic ions as described but the presence of metallic ions also increase the rate of EPS formation. When studying metal accumulation in EPS, it was noted that metals (especially molybdenum) had a positive effect on EPS production (Beech and Cheung, 1995).

Microbes in aqueous environments are often motile and move towards energy sources and away from unfavorable conditions (chemotaxis) (Dubiel et al., 2002). This behavior plays an important role in the formation of biofilms. Modeling of metagenomes based on 16S rRNA gene profiles from biofilm samples estimated complete pathways for motility including chemotaxis and the formation of flagella (Paper II). Furthermore, soluble Fe(II) or H₂ released from corroding steel may be important attractants for iron-oxidizing or hydrogen-utilizing microbes (e.g., SRB, methanogens), which in turn may increase the corrosion rate.

Sulphate-reducing bacteria

SRB are assumed to be the main microbial group causing MIC in anoxic environments (Hamilton, 1985; Lee et al., 1995) and, consequently, many studies have focused on this group. Results suggest that microbial sulphate reduction occurs in biofilms attached to steel surfaces (Papers I–II) (Figure 22), but it is significantly inhibited in the presence of concrete or a biocide (Papers II–III) (Figure 22). The detected SRB were affiliated with families Desulfobacteraceae, Desulfovibrionaceae, Desulfobulbaceae, and Thermodesulfobivrio (Papers I–IV). In relation to known mechanisms of corrosion, the potential for fully assimilatory and dissimilatory sulphate reduction and sulphate oxidation was predicted (Paper III). In an accurate simulation of their natural environment, SRB were associated with fast and localized corrosion (Carpén et al., 2015b). We found that the corrosion rate was enhanced beyond that measured by Carpen et al. (2015b) by providing additional organic carbon sources (Paper II), suggesting that MIC in groundwater environment relies on a heterogeneous community that includes taxa other than SRB (Figure 24).

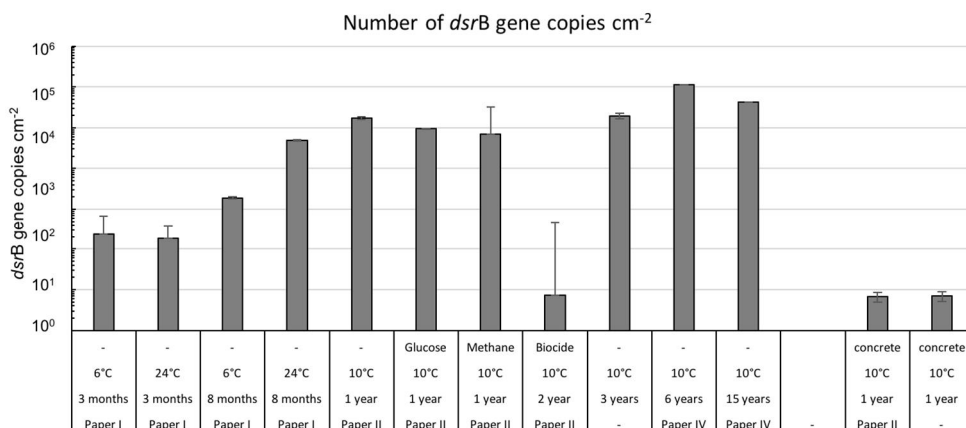


Figure 22. Number of *dsrB* gene copies on surface of carbon steel (cm⁻²), error bars presenting standard deviation (n=3) (Papers I,II,IV).

Sulphur reduction

Epsilonproteobacteria formed a large portion of the bacterial community in the groundwater but were also present in the biofilm. Epsilonproteobacteria such as *Sulfuricurvum* and *Sulfurovum* have been identified in brackish anoxic subsurface water (An et al., 2016). It has been suggested that Epsilonproteobacteria may be largely responsible for reduction of sulphur and generation of FeS scale and that they derive energy for growth by accelerating the reaction between metallic iron and elemental sulphur (An et al., 2016) (Figure 24).

Parcubacteria were detected in experiments containing concrete. These bacteria are thought to be ectosymbionts that attach to the external surfaces of other microbial cells (Nelson and Stegen, 2015). They occur in environments with elevated methane and ammonia concentrations (Peura et al., 2012) and are believed to reduce sulphur via a process linked to fermentation and thus might be contributing to MIC by producing H₂S (Nelson and Stegen, 2015) (Figure 24).

Iron reduction

Iron-reducing bacteria are met in association with SRB on carbon steel interfaces experiencing corrosion (Duan et al., 2008). IRB can influence corrosion by the release of H₂ or by interacting with the corrosion products or the passivating film formed earlier (Urios et al., 2014) (Figure 24). The IRB are usually attached to a solid substrate and therefore may be underrepresented in the aqueous phase (Williams et al., 2010). However, IRB have been identified at the anodic sites of microbial fuel cells (Daghio et al., 2015). The ability of IRB to function in alkaline environments, corresponding concrete environment, has been confirmed (Lee et al., 2014).

Iron oxidation

Betaproteobacteria was found to be the dominant group in almost all bacterial biofilms (Figure 21). These taxa are capable of diverse metabolic pathways and are found in various environments (Papers I–III). Betaproteobacteria have been detected in MIC instances (Li et al., 2010; Mand et al., 2014) and in several deep groundwater sites (Kutvonen et al., 2015; Moser et al., 2005). Betaproteobacterial sequences belonged to Fe(II)-oxidizing *Sideroxydans* species and sulfur-oxidizing *Sulfuricella* species similar to that which is believed to perform nitrate-dependent iron oxidation in anoxic environments (Blöthe and Roden, 2009) (Figure 24). The reddish deposit formed on the surface of corroded carbon steel is likely to be a result of IOB. Betaproteobacteria detected here, Rhodocyclaceae, can oxidise also iron-sulphur compounds (Fe_xS_x) (Rowe et al., 2015). In addition to *Sulfuricella* species, the capacity to oxidize elemental iron with nitrate has been reported for some species belonging to *Pseudomonas* (Gammaproteobacteria), *Pelobacteraceae* (Deltaproteobacteria), *Arcobacter* and *Sulfurimonas* (Epsilonproteobacteria) (Rowe et al., 2015), and may produce the extracellular structures detected in SEM analyses (Figure 23). Scanning-electron micrographs also revealed sheath and stalk-like structures typical of those produced by many iron- or manganese-depositing bacteria (Paper I and II) (Figure 23) (Fleming et al., 2014; Suzuki et al., 2015). Spiral stalk-like structures typical of iron-oxidizing Betaproteobacteria were also seen on carbon steel surfaces (Figure 23) (Fleming et al., 2014; Ghiorse, 1984). In addition, cell structures typical of nitrate-dependent IOB *Sphaerotilus natans* were commonly seen (Figure 23) and suggest this to be an active process (Park et al., 2014). Bacteria produce these structures for releasing poorly soluble compounds, such as Fe(III) oxides and to prevent fouling of the cell surface. Metal-encrusted sheaths and stalks may induce underdeposit corrosion (Ghiorse, 1984). Pathways for nitrate-dependent anoxic iron oxidation (Beller et al., 2013) were also predicted by PICRUST analysis of microbial community metabolic potential, supporting the SEM findings of extracellular structures characteristic of iron oxidizers (Paper III). The ability to oxidize ferrous iron in combination with nitrate reduction is widespread within Proteobacteria, although this process has been usually identified in neutral to slightly acidic environments (Straub et al., 2004). NRB, also detected in this study, can oxidize Fe(II) to poorly crystallized ferrihydrite (Fe_2O_3); an electron acceptor for Fe(III)-reducing bacteria (Straub et al., 2004).

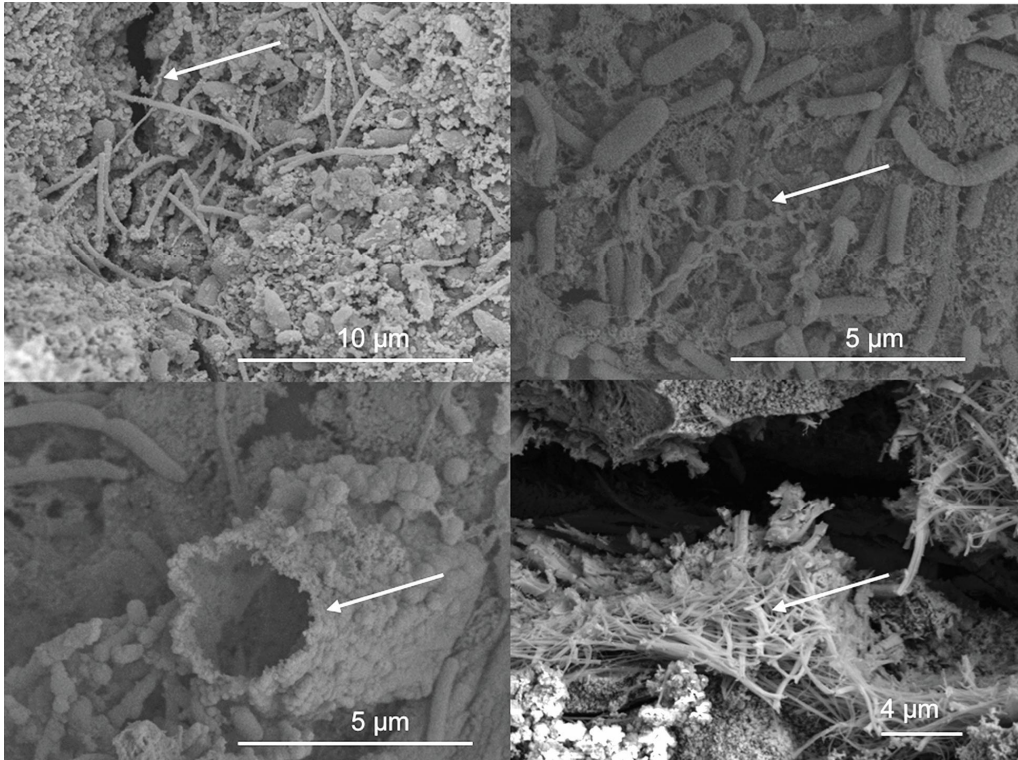


Figure 23. Typical structures formed by metal-depositing bacteria (arrows).

Nitrogen-fixing community

An abundant community of Alphaproteobacteria capable of fixing nitrogen was detected in the biofilm collected from carbon steel surfaces (Papers I–III), including Rhizobiales and Sphingomonadales (Papers II–III). Sphingomonadales have previously been detected in corrosive biofilms (Beale et al., 2012; Li et al., 2010) and are also common in various deep biosphere samples (Bomberg et al., 2015; Kietäväinen and Purkamo, 2015; Purkamo et al., 2015). All the mesocosms experiments were conducted under a N_2 atmosphere to ensure anoxic conditions (Papers I–III). It is possible that the microbial nitrogen cycle was activated due to the N_2 atmosphere since a high fraction of nitrogen pathways were predicted in metagenomic analyses but were not seen in the *in situ* experiment (Paper IV). Nitrogen-fixing bacteria produce ammonia or nitrate, which may in turn serve as a nutrient source for other microbes. In our mesocosm experiments, the concentration of ammonia increased over time from that recorded in the groundwater, supporting the prediction that nitrogen fixation and nitrate reduction pathways were active, at least *in vitro*. In addition, the potential for full dissimilatory and assimilatory nitrate reduction, denitrification, nitrogen fixation and nitrification were also

predicted (Paper III). Although their role is currently undefined, nitrogen-fixing bacteria have been detected and are believed to participate in the corrosion of steel (Usher et al., 2014b).

Acetogenic microorganisms

The mechanisms supporting this relatively numerous microbial community forming biofilms on the surfaces of carbon steel (Figure 20) were evaluated based on the known functionalities of the cultured relatives of detected bacteria. The autotrophic pathways were detected in the biofilm forming community. The potential for acetogenic carbon fixation was predicted as well as potential for reductive pentose phosphate cycle. Acetate produced by acetogens provide carbon source for vast microbial communities in environments where organic carbon is otherwise scarce. In addition, acetogens have been suggested to perform the direct electron uptake from metallic iron and might thus exacerbate the corrosion process of carbon steel (Mand et al., 2014).

Archaeal community

The number of archaea and methanogens were determined using archaeal 16S rRNA gene targeted (Paper III) and *mcrA* gene targeted (Papers II, IV) qPCR, respectively. Although methanogenic archaea in the original groundwater samples was below the detection limit of the assay, a diverse community of methanogens was detected on the surface of carbon steel (Papers II, IV). Methanogenic archaea could have been attracted to the surface by the H₂ released during corrosion, but also to oxidize iron compounds in combination with methanogenesis (Lorowitz et al., 1992).

Sequencing revealed a broader archaeal community in addition to the methanogenic archaea detected by qPCR (Figure 20), but this was still less diverse than the bacterial community (Paper III). The dominant archaeal groups belonged to the Halobacteriales (deep-sea hydrothermal vent euryarchaeota, DHVE) of the Euryarchaeota phylum and the Marine Benthic Group B (MBGB) of the Thaumarchaeota phylum. The majority of archaea belonged to uncultured groups often detected at deep-sea hydrothermal sites (DHVE and miscellaneous crenarchaeotal group, MCG) (Teske and Sørensen, 2008), but which have also been encountered in the Oikiluoto deep biosphere (Bomberg et al., 2015, 2016). DHVE archaea perform iron-, manganese- and sulphur-reduction and could thus be involved in corrosion of carbon steel through mechanisms discussed earlier (Davidova et al., 2012; Reysenbach et al., 2006) (Figure 24). DHVE archaea are believed to participate in a syntrophic relationship with Epsilonproteobacteria, a group which form a large portion of the groundwater bacterial community (Reysenbach et al., 2006) (Paper III). The potential role played by DHVE in corrosion could be overlooked as they are typically encountered in deep-sea hydrothermal vents where corrosion is not an issue.

Fungal community

Fungal sequences obtained from biofilm samples belonged mainly to the phylum Ascomycota, orders Hypocreales, Diaporthales, Eurothiales and unidentified Ascomycota (Paper III). Most aquatic fungi are known to belong to Ascomycetes and Chytridiomycetes (Grossart and Rojas-Jimenez, 2016) and Ascomycota is thought to be the dominant fungal phylum in the Olkiluoto deep biosphere (Sohlberg et al., 2015). Fungi are involved in many biogeochemical processes such as nitrogen- and sulfur-cycling and able to solubilize minerals, dissolve, and precipitate metal ions, degrade silicates and dissolve rock phosphates in oxygen-limited environments (Gadd, 2006; Sterflinger, 2000). The fungal biofilm was sparse compared to that of bacteria and archaea but was not affected by the presence of concrete (Figure 20). Fungi are known to tolerate environments with more extreme pH values than many prokaryotic microorganisms (Lugauskas et al., 2009) and have extremely resistant spores. Similar fungal communities including Hypocreales and Eurothiales have been associated with corrosive biofilms (Sette et al., 2010). Although the specific role(s) of these fungi in corrosion has not been confirmed (Bento et al., 2005; Sette et al., 2010), it is likely, that they produce organic acids (Figure 24). Some filamentous fungi and yeasts are able to oxidise sulfur and sulfur compounds and release sulfate, which might then support the SRB community (Sterflinger, 2000). In addition, fungi in aquatic environments are important components of the food web because they have a large repertoire of enzymes with which they are able to degrade a vast variety of organic materials, making bound nutrients available to the microbial community (Grossart and Rojas-Jimenez, 2016).

Direct electron uptake

In nutrient-poor conditions, such as deep anoxic groundwater seeps, certain microorganisms may use metallic iron (Fe^0) as their sole source of electrons (Jangir et al., 2016; Venzlaff et al., 2013). In this way, microbes can accelerate corrosion more aggressively than by producing corrosive agents (Venzlaff et al., 2013). The microbial groups known to induce corrosion through this mechanism were present in the biofilm (Paper III, IV) (Figure 24). Phyletically-diverse microorganisms including SRB (Proteobacteria), methanogens (Euryarchaeota), acetogens (Firmicutes) and NRB (Firmicutes and Bacteroidetes) stimulate iron corrosion via their direct electron uptake mechanism (Kato et al., 2015). In addition, Alphaproteobacteria (Comamonadaceae) and Betaproteobacteria (Rhodocyclaceae) have been demonstrated to perform direct electron uptake in the terrestrial subsurface environment (Jangir et al., 2016; Li et al., 2010). In this study, XRD results showed that the corrosion products on steel specimens exposed to groundwater in the drillholes included FeCO_3 , indicating the presence of SRB or methanogenic archaea that use metallic iron as their electron donor (Enning et al., 2012; Kato et al., 2015) (Papers III, IV).

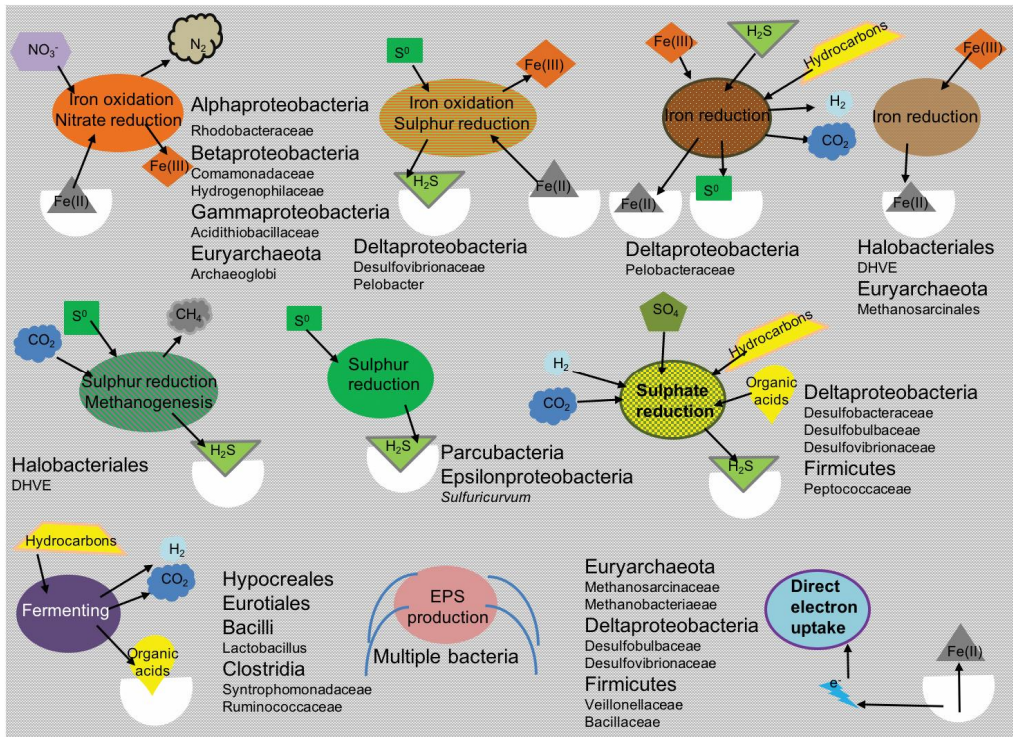


Figure 24. Summary of microbial groups detected in this study and likely mechanisms how they influence the corrosion of carbon steel.

4.4 Microbially-induced corrosion

MIC is a result of the interaction between the material itself, the surrounding environment, and the microorganisms (Féron and Crusset 2014). Both biological and inorganic processes occur on metal surfaces in natural environments within the same time period, but in the opposite direction at the metal-solution interface (Videla and Herrera, 2009). Corrosion and corrosion-product-accumulation occur from the metal surface towards the aqueous phase and biofilms form from the solution towards the metal surface. Microorganisms interact with the deposit layer but at the same time the abiotic deposit layer affects the local environment of the community forming the biofilm. As seen in this study, the microorganisms were embedded in the corrosion-product layer and appeared to have a close interaction with the deposit layer (Papers I–IV). Biofilms, once established, significantly modify the local environment and alter the electrochemical reactions in ways that may either promote (Beech and Sunner, 2004) or reduce corrosion rate (Videla and Herrera, 2009). Consequently, corrosion rate of the metal varies depending on the degree of this interaction and the new biologically-conditioned interface might influence the surface processes (Videla and Herrera, 2005). The rate of MIC in-

creased as a function of exposure time, in contrast to expectations based on a corrosive system where only the abiotic processes influence corrosion rate (Papers I–IV). In the present study, it was demonstrated that microorganisms in natural deep groundwater have a great affinity to form multi species biofilms on the surface of carbon steel and cause intense localized pitting even when gravimetric approaches suggest corrosion is minor (Papers I–IV).

The MIC in systems like deep groundwater seeps where sudden environmental changes are not expected are two-way systems; corrosion and the released compounds (e.g., H₂, Fe(III), etc.) interact with a resident microbial community, feeding and luring them onto the surface. The microbial community affects the base metal directly and cycles the elements of the corrosion products. As summarized above (Figure 24), the interactions between the biofilm community and carbon steel are various and complex (Papers I–IV).

Isolated from photosynthetic carbon as a nutrient source and abundant oxygen as an electron acceptor, subsurface microorganisms must rely on other nutrients for growth, cope with extreme environmental stressors, as well as extract energy for metabolic activity despite the scarcity of soluble electron donors and acceptors (Parnell and McMahon, 2016). It has been demonstrated that organic-carbon-starved microorganisms induce corrosion more aggressively than those maintained in more nutrient-rich environments (Xu and Gu, 2014). The incubation experiment with or without carbon steel demonstrated that the planktonic bacterial community of groundwater increased in abundance and diversity after the addition of carbon steel, especially the iron-oxidizing Betaproteobacteria and SRB (Paper I). Based on changes in the water chemistry, the increase in Fe(II) concentration could be the critical factor by providing an electron source for the iron-oxidizing community. Although the soluble electron acceptors and donors are traditionally thought to be crucial for the microbial community, Jangir et al. (2016) suggested that deep subsurface microorganisms may utilize insoluble electron donors and acceptors in the form of redox active elements (e.g., S, Fe, and Mn) in minerals and rocks. If the microbial community possesses mechanisms for utilizing insoluble electron acceptors and donors from minerals they could, in theory, also be capable of using carbon steel or the deposits formed on its surface as an electron source or sink in a similar manner.

The results (Papers I, III) demonstrate that the planktonic community does not reflect the biofilm forming community. Rather, the biofilm on carbon steel was formed by microbial groups present at abundances close to or below the limit of detection of the assay (Flemming, 2009; Videla and Herrera, 2005) (Papers I, III). This so called “rare biosphere” is suggested to possess an almost infinite bank of diversity in ecosystems (Aanderud et al., 2015; Sogin et al., 2006). In contrast, biofilms composed of archaea and fungi more-closely resembled the wider groundwater community in the three-year experiment (Paper III), although as experiments continued the archaeal community eventually became dominated by methanogens resembling *Methanobacterium* (Paper IV).

4.5 Methodological considerations

Molecular biological methods are powerful tools to rapidly detect and profile complex microbial communities, but these methods have their limitations that should be noted when comparing the results obtained by different methods. The DGGE is a well-established tool for comparing the qualitative structure of two or more communities (Paper I). However, the resolution of this method is not always sufficient to detect the rarer phylotypes. This is demonstrated in Paper I, where Deltaproteobacteria are a minor taxon in the 16S rRNA gene targeting analyses but a diverse community of SRB affiliating with Deltaproteobacteria is still detected when using a *dsrB* gene targeting assay. With respect to our three-year immersion experiment, some OTUs may have been missed in our previous work due to the primers and protocols used. For example, Peura et al. (2012) suggested that many bacterial 16S rRNA gene targeting primers do not anneal well to Parcubacteria templates, such as those used in Papers II and IV but are amplified successfully with others such as those used in Paper III. This is seen when comparing the results in Paper III to those in Rajala et al. (2014) where same samples were analysed using two different primer sets. The results were highly comparable with only difference being that with primers used in Rajala et al. (2014) the Parcubacteria were overlooked. In addition to possible bias created by primer choice, the thick corrosion-product layer may lower the efficiency of DNA extraction or inhibit PCR.

The analytical methods used in this study to detect the compounds in corrosion product are complementary, i.e., EDS provides the weight percent of elements while XRD gives the structure of crystalline minerals. However, in XRD the amorphous substances that may form a large fraction of corrosion products are overlooked.

The form of corrosion detected in this study was mainly localized (e.g., pitting), as is the case usually when microorganisms induce the corrosion. The gravimetric methods typically do not provide sufficient resolution to estimate the localized corrosion rate but do show the cumulative corrosion rate. Electrochemical methods used here to monitor corrosion in real-time (i.e., LPR and EIS) do not distinguish the localized corrosion rate sufficiently so that the whole corrosion phenomena could be resolved. A combination of methods is used to detect the nature of corrosion.

This study concentrated solely on the MIC of carbon steel and mapped the microbial community involved in the biofilm formed on the surface of carbon steel. It has been demonstrated that the biofilm can be affected by the type of steel introduced into otherwise identical environments (Rajala et al., 2014; Urios et al., 2014). As such, more research covering other steel materials relevant to the repository is also needed. In addition, as demonstrated in this study the microbial community responds to changes in environmental conditions in a way that affects corrosion. For example, the present results demonstrate that the biofilm-forming community differed on the basis of a moderate increase in the incubation temperature or changes in nutrition availability. Assuming the repository conditions will change over their lifespan, it is critically important to design experiments that will

provide the best understanding and predictive power concerning the nature of the most likely changes.

5. Conclusions

The objective of this work was to focus on the microbially-induced corrosion (MIC) of carbon steel. This work is relevant to the deep geological repository conditions after final closure of the LLW/ILW underground storage site. The results are important with respect to an improved understanding of the corrosion of carbon steel in geological repositories and are relevant to other industrial underground construction projects relying on the long-term integrity of metallic materials. The deep biosphere is largely unknown and its microbial diversity and activity are still poorly understood. These gaps in our knowledge present a great challenge in estimating the importance of MIC in these environments and its importance to the long-term storage of radioactive waste.

Here, MIC of carbon steel was studied in a groundwater environment under laboratory conditions and *in situ*, with experiments ranging from three months to 15 years.

- This work demonstrates that microbial biofilms and their metabolic activity play an important role in the corrosion of carbon steel in anoxic groundwater, and microorganisms in natural deep groundwater have a great affinity to form biofilms on the surface of carbon steel.
- Corrosion in the presence of microorganisms was mainly localized corrosion although it could not be detected by gravimetric analysis in all cases.
- The effect of changing environmental conditions was evaluated with respect to corrosion and microbial community structure. The biofilm is highly dependent on temperature, nutrient supply and nutrient quality.
- Deep groundwater microbes respond to the introduction of carbon steel by increasing in local abundance and diversity.
- The addition of organic carbon increased the rate of localized MIC.
- The presence of concrete evidently inhibits MIC. Properties of the corrosion product such as electrical resistance were different from those in the four other systems. The corrosion-inhibiting effects of concrete, i.e., increase of pH and calcium carbonate, improve the integrity, stability and long-term safety of the repository for carbon steel waste.

- One of the aims of this study was to develop methods that improve the realism and reliability of MIC experiments and to evaluate its importance in groundwater environments. Laboratory and *in situ* experiments corresponded well and proved useful for simulating different repository environments as well as allow real-time monitoring of corrosion and biofilm formation.

6. Future outlook

In spite of recent progress, the natural mechanisms involved in MIC remain still poorly understood. Predicting corrosive pathways in deep groundwater environments where the majority of native microbes are unavailable for laboratory culture and manipulation is even more challenging. As stated here, it is of crucial importance to conduct the experiments in a realistic environment. Thus, the future direction is to develop *in situ* measurement methods that combine real-time corrosion monitoring with microbial sampling over longer time periods. Localized corrosion is the predominant form of corrosion associated with MIC. Gravimetric methods in widespread use to measure corrosion in the field and laboratory are not always sensitive enough to detect MIC and the possible weaknesses it could be causing. Similarly, electrochemical methods are not always able to measure localised corrosion rates. Improved methods to distinguish localized and general corrosion are needed.

In addition, expanding our knowledge of the functionality of the microbial biofilm that forms naturally on surfaces in contact with groundwater is needed. This could be obtained by developing microsensor techniques or other microprobes targeting the metabolites produced locally within the biofilm and combined with mapping the local changes with electrochemistry, supplemented with molecularbiological techniques such as metagenomics and metabolomics to enlighten the full potential of microbial community.

Another way to obtain more insight into corrosive processes would be recording the products in relation to aggregates of particular microorganisms or their function. Corrosion-inducing mechanisms in deep groundwater (Figure 24) predict there is a potential for processes to release H_2 , CH_4 and N_2 as by-products of MIC. Measuring these gases, their relative abundance and isotopic composition could yield new insights into the importance of those processes in combination with corrosion product analyses.

It has been hypothesised that the direct uptake of electrons from metallic iron would provide microbes with a mechanism to induce corrosion and rapidly increase its rate. Microbial groups and corrosion products associated with direct electron uptake were detected in this study, and this hypothesis is plausible. However, the ability of deep groundwater microorganisms to perform direct electron uptake has not been experimentally confirmed. Further studies of direct electron transfer capability are needed.

References

- Aanderud, Z. T., Jones, S. E., Fierer, N., and Lennon, J. T. (2015). Resuscitation of the rare biosphere contributes to pulses of ecosystem activity. *Front. Microbiol.* 6, 24. doi:10.3389/fmicb.2015.00024.
- Afgan, E., Baker, D., van den Beek, M., Blankenberg, D., Bouvier, D., Čech, M., et al. (2016). The Galaxy platform for accessible, reproducible and collaborative biomedical analyses: 2016 update. *Nucleic Acids Res.* 44, 1–8. doi:10.1093/nar/gkw343.
- AlAbbas, F. M., Williamson, C., Bhola, S. M., Spear, J. R., Olson, D. L., Mishra, B., et al. (2013). Influence of sulfate reducing bacterial biofilm on corrosion behavior of low-alloy, high-strength steel (API-5L X80). *Int. Biodeterior. Biodegradation* 78, 34–42. doi:10.1016/j.ibiod.2012.10.014.
- Alasvand Zarasvand, K., and Rai, V. R. (2014). Microorganisms: Induction and inhibition of corrosion in metals. *Int. Biodeterior. Biodegradation* 87, 66–74. doi:10.1016/j.ibiod.2013.10.023.
- Almahamedh, H. H. (2015). Under-Deposit Caustic Corrosion on Sodium Carbonate Pipeline. *Procedia Eng.* 114, 34–37. doi:10.1016/j.proeng.2015.08.026.
- An, D., Dong, X., An, A., Park, H. S., Strous, M., and Voordouw, G. (2016). Metagenomic Analysis Indicates Epsilonproteobacteria as a Potential Cause of Microbial Corrosion in Pipelines Injected with Bisulfite. *Front. Microbiol.* 7, 28. doi:10.3389/fmicb.2016.00028.
- Anderson, C., Johnsson, A., Moll, H., and Pedersen, K. (2011). Radionuclide Geomicrobiology of the Deep Biosphere. *Geomicrobiol. J.* 28, 540–561. doi:10.1080/01490451.2010.507644.
- Bano, N., Ruffin, S., Ransom, B., and Hollibaugh, J. T. (2004). Phylogenetic Composition of Arctic Ocean Archaeal Assemblages and Comparison with Antarctic Assemblages. *Appl. Environ. Microbiol.* 70, 781–789. doi:10.1128/AEM.70.2.781-789.2004.
- Barns, S. M., Fundyga, R. E., Jeffries, M. W., and Pace, N. R. (1994). Remarkable archaeal diversity detected in a Yellowstone National Park hot spring environment. *Proc. Natl. Acad. Sci.* 91, 1609–1613. doi:10.1073/pnas.91.5.1609.
- Barton, L. L., and Hamilton, W. A. eds. (2013). *Sulphate-reducing Bacteria, Environmental and Engineered Systems*. Cambridge University Press doi:10.1017/CBO9781107415324.004.
- Beale, D. J., Dunn, M. S., Morrison, P. D., Porter, N. A., and Marlow, D. R. (2012). Characterisation of bulk water samples from copper pipes undergoing

- microbially influenced corrosion by diagnostic metabolomic profiling. *Corros. Sci.* 55, 272–279. doi:10.1016/j.corsci.2011.10.026.
- Beech, I. B. (2004). Corrosion of technical materials in the presence of biofilms - Current understanding and state-of-the art methods of study. *Int. Biodeterior. Biodegrad.* 53, 177–183. doi:10.1016/S0964-8305(03)00092-1.
- Beech, I. B., and Cheung, C. W. S. (1995). Interactions of exopolymers produced by sulphate-reducing bacteria with metal ions. *Int. Biodeterior. Biodegrad.* 35, 59–72. doi:10.1016/0964-8305(95)00082-G.
- Beech, I. B., and Sunner, J. (2004). Biocorrosion: towards understanding interactions between biofilms and metals. *Curr. Opin. Biotechnol.* 15, 181–6. doi:10.1016/j.copbio.2004.05.001.
- Beller, H. R., Zhou, P., Legler, T. C., Chakicherla, A., Kane, S., Letain, T. E., et al. (2013). Genome-enabled studies of anaerobic, nitrate-dependent oxidation in the chemolithoautotrophic bacterium *Thiobacillus denitrificans*. *Front. Microbiol.* 4, 249. doi:10.3389/fmicb.2013.00249.
- Bento, F. M., Beech, I. B., Gaylarde, C. C., Englert, G. E., and Muller, I. L. (2005). Degradation and corrosive activities of fungi in a diesel-mild steel-aqueous system. *World J. Microbiol. Biotechnol.* 21, 135–142. doi:10.1007/s11274-004-3042-2.
- Biezma, M. V. (2001). The role of hydrogen in microbiologically influenced corrosion and stress corrosion cracking. *Int. J. Hydrogen Energy* 26, 515–520. doi:http://dx.doi.org/10.1016/S0360-3199(00)00091-4.
- Blöthe, M., and Roden, E. E. (2009). Composition and activity of an autotrophic Fe(II)-oxidizing, nitrate-reducing enrichment culture. *Appl. Environ. Microbiol.* 75, 6937–6940. doi:10.1128/AEM.01742-09.
- Bomberg, M., Lamminmäki, T., and Itävaara, M. (2016). Microbial communities and their predicted metabolic characteristics in deep fracture groundwaters of the crystalline bedrock at Olkiluoto, Finland. *Biogeosciences* 13, 6031–6047. doi:10.5194/bg-13-6031-2016.
- Bomberg, M., Nyyssönen, M., Nousiainen, A., Hultman, J., Paulin, L., Auvinen, P., et al. (2014). Evaluation of Molecular Techniques in Characterization of Deep Terrestrial Biosphere. *Open J. Ecol.* 4, 468–487. doi:10.4236/oje.2014.48040.
- Bomberg, M., Nyyssönen, M., Pitkänen, P., Lehtinen, A., and Itävaara, M. (2015). Active Microbial Communities Inhabit Sulphate-Methane Interphase in Deep Bedrock Fracture Fluids in Olkiluoto, Finland. *Biomed Res. Int.* 2015, 17. doi:10.1155/2015/979530.
- Boopathy, R., and Daniels, L. (1991). Effect of pH on Anaerobic Mild Steel Corrosion by Methanogenic Bacteria. *Appl. Envir. Microbiol.* 57, 2104–2108.

- Caporaso, J. G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F. D., Costello, E. K., et al. (2010). QIIME allows analysis of high-throughput community sequencing data. *Nat. Methods* 7, 335–336. doi:10.1038/nmeth.f.303.
- Carlson, H. K., Clark, I. C., Blazewicz, S. J., Iavarone, A. T., and Coates, J. D. (2013). Fe(II) oxidation is an innate capability of nitrate-reducing bacteria that involves abiotic and biotic reactions. *J. Bacteriol.* 195, 3260–3268. doi:10.1128/JB.00058-13.
- Carpén, L., Maukonen, J., and Salo, S. (2013a). Accelerated Corrosion of Carbon Steel and Zinc in Oxygen-Free Groundwater – Due to the Microbiological Activity? in *NACE - International Corrosion Conference Series*.
- Carpén, L., Rajala, P., and Bomberg, M. (2015a). Microbially Induced Corrosion in Deep Bedrock. *Adv. Mater. Res.* 1130, 75–78. doi:10.4028/www.scientific.net/AMR.1130.75.
- Carpén, L., Rajala, P., and Bomberg, M. (2015b). Real-Time Electrochemical Measurements Of Carbon Steel In Ground Water With Sulfate Reducing Bacteria Enrichment. in *Waste Management Symposia*, 15604.
- Carpén, L., Rajala, P., Vepsäläinen, M., and Bomberg, M. (2013b). Corrosion behaviour and biofilm formation on carbon steel and stainless steel in simulated repository environment. in *Eurocorr*, O-1589.
- Costello, J. A. (1974). Cathodic depolarization by sulphate-reducing bacteria. *S. Afr. J. Sci.* 70, 202–204.
- Cristiani, P., Carvalho, M. L., Guerrini, E., Daghighi, M., Santoro, C., and Li, B. (2013). Cathodic and anodic biofilms in Single Chamber Microbial Fuel Cells. *Bioelectrochemistry* 92, 6–13. doi:10.1016/j.bioelechem.2013.01.005.
- Daghighi, M., Gandolfi, I., Bestetti, G., Franzetti, A., Guerrini, E., and Cristiani, P. (2015). Anodic and cathodic microbial communities in single chamber microbial fuel cells. *N. Biotechnol.* 32, 79–84. doi:10.1016/j.nbt.2014.09.005.
- Daniels, L., Belay, N., Rajagopal, B. S., and Weimer, P. J. (1987). Bacterial methanogenesis and growth from CO₂ with elemental iron as the sole source of electrons. *Science*. 237, 509–511.
- Das, B. K., Roy, A., Koschorreck, M., Mandal, S. M., Wendt-Potthoff, K., and Bhattacharya, J. (2009). Occurrence and role of algae and fungi in acid mine drainage environment with special reference to metals and sulfate immobilization. *Water Res.* 43, 883–894. doi:10.1016/j.watres.2008.11.046.
- Davidova, I. A., Duncan, K. E., Perez-Ibarra, B. M., and Sufliya, J. M. (2012). Involvement of thermophilic archaea in the biocorrosion of oil pipelines. *Environ. Microbiol.* 14, 1762–1771. doi:10.1111/j.1462-2920.2012.02721.x.
- DeSantis, T. Z., Hugenholtz, P., Larsen, N., Rojas, M., Brodie, E. L., Keller, K., et

- al. (2006). Greengenes, a Chimera-Checked 16S rRNA Gene Database and Workbench Compatible with ARB. *Appl. Environ. Microbiol.* 72, 5069–5072. doi:10.1128/AEM.03006-05.
- Deutzmann, J. S., Sahin, M., and Spormann, A. M. (2015). Extracellular Enzymes Facilitate Electron Uptake in Biocorrosion and. *MBio* 6, 1–8. doi:10.1128/mBio.00496-15.Editor.
- Diekert, G., and Wohlfarth, G. (1994). Metabolism of homoacetogens. *Antonie Van Leeuwenhoek* 66, 209–221. doi:10.1007/BF00871640.
- Dinh, H. T., Kuever, J., Muszmann, M., Hassel, A. W., Stratmann, M., and Widdel, F. (2004). Iron corrosion by novel anaerobic microorganisms. *Nature* 427, 829–832.
- Dose, K. (1989). The bioenergetics of anaerobic bacteria: Evolutionary concepts. *Adv. Sp. Res.* 9, 93–100. doi:10.1016/0273-1177(89)90214-7.
- Duan, J., Wu, S., Zhang, X., Huang, G., Du, M., and Hou, B. (2008). Corrosion of carbon steel influenced by anaerobic biofilm in natural seawater. *Electrochim. Acta* 54, 22–28. doi:10.1016/j.electacta.2008.04.085.
- Dubiel, M., Hsu, C. H., Chien, C. C., Mansfeld, F., and Newman, D. K. (2002). Microbial respiration can protect steel from corrosion. *Appl. Environ. Microbiol.* 68, 1440–1445. doi:10.1128/AEM.68.3.1440-1445.2002.
- Ehrlich, H. L. (2002). *Geomicrobiology*. 3rd ed. New York, NY: Marcel Decker Inc. doi:10.1002/maco.19960470815.
- Enning, D., and Garrelfs, J. (2014). Corrosion of iron by sulfate-reducing bacteria: New views of an old problem. *Appl. Environ. Microbiol.* 80, 1226–1236. doi:10.1128/AEM.02848-13.
- Enning, D., Venzlaff, H., Garrelfs, J., Dinh, H. T., Meyer, V., Mayrhofer, K., et al. (2012). Marine sulfate-reducing bacteria cause serious corrosion of iron under electroconductive biogenic mineral crust. *Environ. Microbiol.* 14, 1772–1787. doi:10.1111/j.1462-2920.2012.02778.x.
- Féron, D., and Crusset, D. (2014). Microbial induced corrosion in French concept of nuclear waste underground disposal. *Corros. Eng. Sci. Technol.* 49, 540–547. doi:10.1179/1743278214Y.0000000193.
- Ferry, J. G. (2010). The chemical biology of methanogenesis. *Planet. Space Sci.* 58, 1775–1783. doi:10.1016/j.pss.2010.08.014.
- Fleming, E. J., Cetinić, I., Chan, C. S., Whitney King, D., and Emerson, D. (2014). Ecological succession among iron-oxidizing bacteria. *ISME J.* 8, 804–15. doi:10.1038/ismej.2013.197.
- Flemming, H. C. (2002). Biofouling in water systems - Cases, causes and countermeasures. *Appl. Microbiol. Biotechnol.* 59, 629–640.

doi:10.1007/s00253-002-1066-9.

- Flemming, H. C. (2009). "Why micro-organisms live in biofilms and the problem of biofouling," in *Marine and industrial biofouling*, eds. H. Flemming, P. Murthy, R. Venkatesan, and K. Cooksey (Berlin: Springer).
- Flemming, H. C. (2016). EPS—Then and Now. 1–18. doi:10.3390/microorganisms4040041.
- Flemming, H. C., and Wingender, J. (2010). The biofilm matrix. *Nat. Publ. Gr.* 8, 623–633. doi:10.1038/nrmicro2415.
- Flynn, T. M., Sanford, R. a, Ryu, H., Bethke, C. M., Levine, A. D., Ashbolt, N. J., et al. (2013). Functional microbial diversity explains groundwater chemistry in a pristine aquifer. *BMC Microbiol.* 13, 146. doi:10.1186/1471-2180-13-146.
- Gadd, G. M. ed. (2006). *Fungi in biogeochemical cycles*. 24th ed. Cambridge University Press.
- Gadd, G. M. (2010). Metals, minerals and microbes: Geomicrobiology and bioremediation. *Microbiology* 156, 609–643. doi:10.1099/mic.0.037143-0.
- Gardes, M., and Bruns, T. D. (1993). ITS primers with enhanced specificity for basidiomycetes - application to the identification of mycorrhizae and rusts. *Mol. Ecol.* 2, 113–118. doi:10.1111/j.1365-294X.1993.tb00005.x.
- Geets, J., Borremans, B., Diels, L., Springael, D., Vangronsveld, J., van der Lelie, D., et al. (2006). DsrB gene-based DGGE for community and diversity surveys of sulfate-reducing bacteria. *J. Microbiol. Methods* 66, 194–205. doi:10.1016/j.mimet.2005.11.002.
- Ghiorse, W. C. (1984). Biology of iron- and manganese-depositing Bacteria. *Annu. Rev. Microbiol.* 38, 515–550.
- Grossart, H. P., and Rojas-Jimenez, K. (2016). Aquatic fungi: Targeting the forgotten in microbial ecology. *Curr. Opin. Microbiol.* 31, 140–145. doi:10.1016/j.mib.2016.03.016.
- Grosskopf, R., Janssen, P. H., and Liesack, W. (1998). Diversity and structure of the methanogenic community in anoxic rice paddy soil microcosms as examined by cultivation and direct 16S rRNA gene sequence retrieval. *Appl. Environ. Microbiol.* 64, 960–9.
- Gu, T. (2014). Theoretical modeling of the possibility of acid producing bacteria causing fast pitting biocorrosion. *J. Microb. Biochem. Technol.* 6, 68–74. doi:10.4172/1948-5948.1000124.
- Hales, B., Edwards, C., Ritchie, D., Hall, G., Pickup, R., and Saunders, J. (1996). Isolation and identification of methanogen-specific DNA from blanket bog peat by PCR amplification and sequence analysis. *Appl. Envir. Microbiol.* 62, 668–675.

- Hamilton, W. (2003). Microbially influenced corrosion as a model system for the study of metal microbe interactions: a unifying electron transfer hypothesis. *Biofouling* 19, 65–76. doi:10.1080/0892701021000041078.
- Hamilton, W. A. (1985). Sulphate-reducing bacteria and anaerobic corrosion. *Annu. Rev. Microbiol.* 39, 195–217. doi:10.1146/annurev.mi.39.100185.001211.
- Hammer, Ø., Harper, D. A. ., and Ryan, P. D. (2001). PAST: Paleontological statistics software package for education and data analysis. *Palaeontol. Electron.* 4, 9.
- Haugland, R., and Vespe, S. J. (2001). Method of identifying and quantifying specific fungi and bacteria. CA2412993 A1.
- Haveman, S. A., and Pedersen, K. (1999). Distribution and Metabolic Diversity of Microorganisms in Deep Igneous Rock Aquifers of Finland. *Geomicrobiol. J.* 16, 277–294. doi:10.1080/014904599270541.
- Haveman, S. A., and Pedersen, K. (2002). Distribution of culturable microorganisms in Fennoscandian Shield groundwater. *FEMS Microbiol. Ecol.* 39, 129–37. doi:10.1111/j.1574-6941.2002.tb00914.x.
- He, X., and Ahn, T. (2015). Carbon steel corrosion in anoxic alkaline solution. in *NACE - International Corrosion Conference Series*.
- Herlemann, D. P., Labrenz, M., Jürgens, K., Bertilsson, S., Waniek, J. J., and Andersson, A. F. (2011). Transitions in bacterial communities along the 2000 km salinity gradient of the Baltic Sea. *ISME J.* 5, 1571–9. doi:10.1038/ismej.2011.41.
- Herrera, L. K., and Videla, H. A. (2009). Role of iron-reducing bacteria in corrosion and protection of carbon steel. *Int. Biodeterior. Biodegrad.* 63, 891–895. doi:10.1016/j.ibiod.2009.06.003.
- Hoehler, T. M., and Jørgensen, B. B. (2013). Microbial life under extreme energy limitation. *Nat. Rev. - Microbiol.* 11, 83–94. doi:10.1038/nrmicro2939.
- Huttunen-Saarivirta, E., Rajala, P., Bomberg, M., and Carpén, L. (2017). Corrosion of copper in oxygen-deficient groundwater with and without deep bedrock micro-organisms: Characterisation of microbial communities and surface processes. *Appl. Surf. Sci.* 396, 1044–1057. doi:10.1016/j.apsusc.2016.11.086.
- Huttunen-Saarivirta, E., Rajala, P., and Carpén, L. (2015). Corrosion behaviour of copper under biotic and abiotic conditions in anoxic ground water: Electrochemical study. *Electrochim. Acta* 203, 350–365. doi:10.1016/j.electacta.2016.01.098.
- Jangir, Y., French, S., Momper, L. M., Moser, D. P., Amend, J. P., and El-Naggar, M. Y. (2016). Isolation and Characterization of Electrochemically Active

- Subsurface Delftia and Azonexus Species. *Front. Microbiol.* 7, 756. doi:10.3389/fmicb.2016.00756.
- Jiang, S., Park, S., Yoon, Y., Lee, J.-H., Wu, W.-M., Phuoc Dan, N., et al. (2013). Methanogenesis Facilitated by Geobiochemical Iron Cycle in a Novel Syntrophic Methanogenic Microbial Community. *Environ. Sci. Technol.* 47, 10078–10084. doi:10.1021/es402412c.
- Jørgensen, B. B., and D'Hondt, S. (2006). A starving majority deep beneath the seafloor. *Science*. 314, 932–934. doi:10.1126/science.1133796.
- Kärki, A., and Paulamäki, S. (2006). *Petrology of Olkiluoto 2006-2*. Posiva Reports.
- Kato, S. (2016). Microbial extracellular electron transfer and its relevance to iron corrosion. *Microb. Biotechnol.* 9, 141–148. doi:10.1111/1751-7915.12340.
- Kato, S., Yumoto, I., and Kamagata, Y. (2015). Isolation of acetogenic bacteria that induce biocorrosion by utilizing metallic iron as the sole electron donor. *Appl. Environ. Microbiol.* 81, 67–73. doi:10.1128/AEM.02767-14.
- Kearse, M., Moir, R., Wilson, A., Stones-Havas, S., Cheung, M., Sturrock, S., et al. (2012). Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. *Bioinformatics* 28, 1647–1649.
- Kietäväinen, R., Ahonen, L., Kukkonen, I. T., Hendriksson, N., Nyssönen, M., and Itävaara, M. (2013). Characterisation and isotopic evolution of saline waters of the Outokumpu Deep Drill Hole, Finland - Implications for water origin and deep terrestrial biosphere. *Appl. Geochemistry* 32, 37–51. doi:10.1016/j.apgeochem.2012.10.013.
- Kietäväinen, R., and Purkamo, L. (2015). The origin, source, and cycling of methane in deep crystalline rock biosphere. *Front. Microbiol.* 6, 725. doi:10.3389/fmicb.2015.00725.
- King, F., Kolář, M., and Keech, P. G. (2014). Simulations of long-term anaerobic corrosion of carbon steel containers in Canadian deep geological repository. *Corros. Eng. Sci. Technol.* 49, 455–459. doi:10.1179/1743278214Y.0000000187.
- Klindworth, A., Pruesse, E., Schweer, T., Peplies, J., Quast, C., Horn, M., et al. (2013). Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. *Nucleic Acids Res.* 41, 1–11. doi:10.1093/nar/gks808.
- Köjljalg, U., Nilsson, R. H., Abarenkov, K., Tedersoo, L., Taylor, A. F. S., Bahram, M., et al. (2013). Towards a unified paradigm for sequence-based identification of fungi. *Mol. Ecol.* 22, 5271–5277. doi:10.1111/mec.12481.
- Konhauser, K. (2007). *Introduction to Geomicrobiology*. Oxford: Blackwell

Publishing Ltd.

- Kotelnikova, S. (2002). Microbial production and oxidation of methane in deep subsurface. *Earth-Science Rev.* 58, 367–395. doi:10.1016/S0012-8252(01)00082-4.
- Kotelnikova, S., and Pedersen, K. (1998). Distribution and activity of methanogens and homoacetogens in deep granitic aquifers at Aspo Hard Rock Laboratory, Sweden. *FEMS Microbiol. Ecol.* 26, 121–134. doi:10.1016/S0168-6496(98)00028-2.
- Kutvonen, H., Rajala, P., Carpén, L., and Bomberg, M. (2015). Nitrate and ammonia as nitrogen sources for deep subsurface microorganisms. *Front. Microbiol.* 6, 1079. doi:10.3389/fmicb.2015.01079.
- Landoulsi, J., El Kirat, K., Richard, C., Féron, D., and Pulvin, S. (2008). Enzymatic approach in microbial-influenced corrosion: A review based on stainless steels in natural waters. *Environ. Sci. Technol.* 42, 2233–2242. doi:10.1021/es071830g.
- Langille, M. G. I., Zaneveld, J., Caporaso, J. G., McDonald, D., Knights, D., Reyes, J. A., et al. (2013). Predictive functional profiling of microbial communities using 16S rRNA marker gene sequences. *Nat. Biotechnol.* 31, 814–821. doi:10.1038/nbt.2676.
- Lee, A., and Newman, D. (2003). Microbial iron respiration: Impacts on corrosion processes. *Appl. Microbiol. Biotechnol.* 62, 134–139. doi:10.1007/s00253-003-1314-7.
- Lee, S., Matsui, T., and Yoshikawa, H. (2014). Study of microbial corrosion behaviour of carbon steel both in alkaline liquid medium and liquid medium with anions. *Corros. Eng. Sci. Technol.* 49, 562–566. doi:10.1179/1743278214Y.0000000194.
- Lee, W., Lewandowski, Z., Nielsen, P. H., and Hamilton, W. A. (1995). Role of sulfate-reducing bacteria in corrosion of mild steel: a review. *Biofouling* 8, 165–194.
- Li, D., Li, Z., Yu, J., Cao, N., Liu, R., and Yang, M. (2010). Characterization of bacterial community structure in a drinking water distribution system during an occurrence of red water. *Appl. Environ. Microbiol.* 76, 7171–80. doi:10.1128/AEM.00832-10.
- Little, B. J., Lee, J. S., and Ray, R. I. (2006). Diagnosing microbiologically influenced corrosion: A state-of-the-art review. *Corrosion* 62, 1006–1017. doi:10.5006/1.3278228.
- Little, B. J., and Ray, R. I. (2002a). The Role of Fungi in Microbiologically Influenced Corrosion. in *15th International Corrosion Congress*.
- Little, B. J., Ray, R. I., and Pope, R. K. (2000). Relationship between corrosion

- and the biological sulfur cycle: A review. *Corrosion* 56, 433–443. doi:10.5006/1.3280548.
- Little, B., Lee, J., and Ray, R. (2007). A review of “green” strategies to prevent or mitigate microbiologically influenced corrosion. *Biofouling* 23, 87–97. doi:10.1080/08927010601151782.
- Little, B., and Ray, R. (2002b). A perspective on corrosion inhibition by biofilms. *Corrosion* 58, 424–428. doi:10.5006/1.3277632.
- Little, B., and Wagner, P. (1996). An overview of microbiologically influenced corrosion of metals and alloys used in the storage of nuclear wastes. *Can. J. Microbiol.* 42, 367–374.
- Little, B., Wagner, P., Hart, K., Ray, R., Lavoie, D., Neelson, K., et al. (1998). The role of biomineralization in microbiologically influenced corrosion. *Biodegradation* 9, 1–10. doi:10.1023/A:1008264313065.
- Little, B., Wagner, P., and Mansfeld, F. (1991). Microbiologically influenced corrosion of metals and alloys. *Int. Mater. Rev.* 36, 253–272. doi:10.1179/imr.1991.36.1.253.
- Little, B., Wagner, P., and Mansfeld, F. (1992). An overview of microbiologically influenced corrosion. *Electrochim. Acta* 37, 2185–2194. doi:10.1016/0013-4686(92)85110-7.
- Liu, F., Rotaru, A.-E., Shrestha, P. M., Malvankar, N. S., Nevin, K. P., and Lovley, D. R. (2015). Magnetite compensates for the lack of a pilin-associated c-type cytochrome in extracellular electron exchange. *Environ. Microbiol.* 17, 648–655. doi:10.1111/1462-2920.12485.
- Lorowitz, W. H., Nagle, D. P., and Tanner, R. S. (1992). Anaerobic oxidation of elemental metals coupled to methanogenesis by *Methanobacterium thermoautotrophicum*. *Environ. Sci. Technol.* 26, 1606–1610. doi:10.1021/es00036a034.
- Lovley, D. R., and Chapelle, F. H. (1995). Deep subsurface microbial processes. *Rev. Geophys.* 33, 365–381. doi:10.1029/95RG01305.
- Lynes, D. ed. (2011). *Internal Corrosion Control in Water Distribution Systems - Manual of Water Supply Practices, M58*. 1st Editio. American Water Works Association.
- Mand, J., Park, H. S., Jack, T. R., and Voordouw, G. (2014). The role of acetogens in microbially influenced corrosion of steel. *Front. Microbiol.* 5, 268. doi:10.3389/fmicb.2014.00268.
- McMahon, S., and Parnell, J. (2014). Weighing the deep continental biosphere. *FEMS Microbiol. Ecol.* 87, 113–120. doi:10.1111/1574-6941.12196.
- McMurdie, P. ., and Holmes, S. (2015). Shiny-phyloseq: Web Application for

- Interactive Microbiome Analysis with Provenance Tracking. *Bioinformatics* 31, 282–283.
- McNeil, M. B., and Little, B. J. (1999). The Use of Mineralogical Data in Interpretation of Long-Term Microbiological Corrosion Processes : Sulfiding Reactions. *J. Am. Inst. Conserv.* 38, 186–199.
- Morozova, D., Alawi, M., Shaheed, M., Martin, K., Kock, D., and Würdemann, H. (2011). The influence of microbial activity on rock fluid interaction: Baseline characterization of deep biosphere for enhanced gas recovery in the Altmark natural gas reservoir. *Energy Procedia* 4, 4633–4640. doi:10.1016/j.egypro.2011.02.423.
- Moser, D. P., Gihring, T., Fredrickson, J. K., Brockman, F. J., Balkwill, D., Dollhopf, M. E., et al. (2005). Desulfotomaculum spp. and Methanobacterium spp. Dominate 4-5 km Deep Fault. *Appl. Environ. Microbiol.* 71, 8773–8783. doi:10.1128/AEM.71.12.8773.
- Muyzer, G., Waal, E. C. De, and Uitterlinden, A. G. (1993). Profiling of Complex Microbial Populations by Denaturing Gradient Gel Electrophoresis Analysis of Polymerase Chain Reaction-Amplified Genes Coding for 16S rRNA. *Appl. Environ. Microbiol.* 59, 695–700.
- NEA (2005). Achieving the Goals of the Decommissioning Safety Case - A Status Report Prepared on Behalf of the WPDD by its Task Group on the Decommissioning Safety Case. doi:ISBN 92-64-01068-8.
- Neff, D., Dillmann, P., Bellot-Gurlet, L., and Beranger, G. (2005). Corrosion of iron archaeological artefacts in soil: Characterisation of the corrosion system. *Corros. Sci.* 47, 515–535. doi:10.1016/j.corsci.2004.05.029.
- Nelson, W. C., and Stegen, J. C. (2015). The reduced genomes of Parcubacteria (OD1) contain signatures of a symbiotic lifestyle. *Front. Microbiol.* 6, 713. doi:10.3389/fmicb.2015.00713.
- Neria-González, I., Wang, E. T., Ramírez, F., Romero, J. M., and Hernández-Rodríguez, C. (2006). Characterization of bacterial community associated to biofilms of corroded oil pipelines from the southeast of Mexico. *Anaerobe* 12, 122–33. doi:10.1016/j.anaerobe.2006.02.001.
- Nuclear Waste Management of the Olkiluoto and Loviisa Power plants (2010).
- Nyysönen, M., Bomberg, M., Kapanen, A., Nousiainen, A., Pitkänen, P., and Itävaara, M. (2012). Methanogenic and Sulphate-Reducing Microbial Communities in Deep Groundwater of Crystalline Rock Fractures in Olkiluoto, Finland. *Geomicrobiol. J.* 29, 863–878. doi:10.1080/01490451.2011.635759.
- Obuekwe, C. O., Westlake, D. W. S., Cook, F. D., and William Costerton, J. (1981). Surface Changes in Mild Steel Coupons from the Action of

- Corrosion-Causing Bacteria. *Appl. Environ. Microbiol.* 41, 766–774.
- Oliveira, V. M., Lopes-Oliveira, P. F., Passarini, M. R. Z., Menezes, C. B. a, Oliveira, W. R. C., Rocha, A. J., et al. (2011). Molecular analysis of microbial diversity in corrosion samples from energy transmission towers. *Biofouling* 27, 435–447. doi:10.1080/08927014.2011.581751.
- Park, S., Kim, D. H., Lee, J. H., and Hur, H. G. (2014). Sphaerotilus natans encrusted with nanoball-shaped Fe(III) oxide minerals formed by nitrate-reducing mixotrophic Fe(II) oxidation. *FEMS Microbiol. Ecol.* 90, 68–77. doi:10.1111/1574-6941.12372.
- Pedersen, K. (1997). Microbial life in deep granitic rock. *FEMS Microbiol. Rev.* 20, 399–414. doi:10.1016/S0168-6445(97)00022-3.
- Pedersen, K. (1999). Subterranean microorganisms and radioactive waste disposal in Sweden. *Eng. Geol.* 52, 163–176. doi:10.1016/S0013-7952(99)00004-6.
- Pedersen, K., Arlinger, J., Bengtsson, A., Edlund, J., Eriksson, L., Hallbeck, L., et al. (2013). Sulphate Reduction Experiment: SURE-1. *POSIVA Work. Rep.* 57.
- Pedersen, K., Arlinger, J., Eriksson, S., Hallbeck, A., Hallbeck, L., and Johansson, J. (2008). Numbers, biomass and cultivable diversity of microbial populations relate to depth and borehole-specific conditions in groundwater from depths of 4-450 m in Olkiluoto, Finland. *ISME J.* 2, 760–775. doi:10.1038/ismej.2008.43.
- Peura, S., Eiler, A., Bertilsson, S., Nykänen, H., Tirola, M., and Jones, R. I. (2012). Distinct and diverse anaerobic bacterial communities in boreal lakes dominated by candidate division OD1. *ISME J.* 6, 1640–1652. doi:10.1038/ismej.2012.21.
- Pitkänen, P., Partamies, S., and Luukkonen, S. (2003). Hydrogeochemical interpretation of baseline groundwater conditions at the Olkiluoto site. *POSIVA Work. Rep.* 7.
- Posiva (2015). Olkiluodon ja Loviisan voimalaitosten ydinjätehuolto - Yhteenveto vuoden 2015 toiminnasta.
- Pruesse, E., Quast, C., Knittel, K., Fuchs, B. M., Ludwig, W., Peplies, J., et al. (2007). SILVA: a comprehensive online resource for quality checked and aligned ribosomal RNA sequence data compatible with ARB. *Nucleic Acids Res.* 35, 7188–96. doi:10.1093/nar/gkm864.
- Purkamo, L., Bomberg, M., Kietäväinen, R., Salavirta, H., Nyysönen, M., Nuppenen-Puputti, M., et al. (2015). The keystone species of Precambrian deep bedrock biosphere belong to Burkholderiales and Clostridiales. *Biogeosciences Discuss.* 12, 18103–18150. doi:10.5194/bgd-12-18103-

2015.

- Purkamo, L., Bomberg, M., Kietäväinen, R., Salavirta, H., Nyssönen, M., Nuppenen-Puputti, M., et al. (2016). Microbial co-occurrence patterns in deep Precambrian bedrock fracture fluids. *Biogeosciences* 13, 3091–3108. doi:10.5194/bg-13-3091-2016.
- R Development Core Team (2013). R: A language and environment for statistical computing. Available at: <http://www.r-project.org/>.
- Rajala, P., and Bomberg, M. (2017). Reactivation of Deep Subsurface Microbial Community in Response to Methane or Methanol Amendment. *Front. Microbiol.* 8, 1–13. doi:10.3389/fmicb.2017.00431.
- Rajala, P., Bomberg, M., Kietäväinen, R., Kukkonen, I., Ahonen, L., Nyssönen, M., et al. (2015). Rapid Reactivation of Deep Subsurface Microbes in the Presence of C-1 Compounds. *Microorganisms* 3, 17–33. doi:10.3390/microorganisms3010017.
- Rajala, P., Carpén, L., Vepsäläinen, M., Bomberg, M., and Raulio, M. (2014). Microbially Induced Corrosion of Carbon Steel and Stainless Steel in Alkaline Ground Water – Composition and Metabolic Functionality of Biofilm. in *Waste Management Symposia*, 14391.
- Reysenbach, A.-L., Liu, Y., Banta, A. B., Beveridge, T. J., Kirshtein, J. D., Schouten, S., et al. (2006). A ubiquitous thermoacidophilic archaeon from deep-sea hydrothermal vents. *Nature* 442, 444–447. doi:10.1038/nature04921.
- Rothschild, L. J., and Mancinelli, R. L. (2001). Life in extreme environments. *Nature* 409, 1092–101. doi:10.1038/35059215.
- Rowe, A. R., Chellamuthu, P., Lam, B., Okamoto, A., and Nealson, K. H. (2015). Marine sediments microbes capable of electrode oxidation as a surrogate for lithotrophic insoluble substrate metabolism. *Front. Microbiol.* 6, 784. doi:10.3389/fmicb.2014.00784.
- Sass, H., and Cypionka, H. (2004). Isolation of sulfate-reducing bacteria from the terrestrial deep subsurface and description of *Desulfovibrio cavernae* sp. nov. *Syst. Appl. Microbiol.* 27, 541–8. doi:10.1078/0723202041748181.
- Schädler, S., Burkhardt, C., Hegler, F., Straub, K. L., Miot, J., Benzerara, K., et al. (2009). Formation of Cell-Iron-Mineral Aggregates by Phototrophic and Nitrate-Reducing Anaerobic Fe(II)-Oxidizing Bacteria. *Geomicrobiol. J.* 26, 93–103. doi:10.1080/01490450802660573.
- Schloss, P. D., Westcott, S. L., Ryabin, T., Hall, J. R., Hartmann, M., Hollister, E. B., et al. (2009). Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Appl. Environ. Microbiol.* 75, 7537–41.

doi:10.1128/AEM.01541-09.

- Schütz, M. K., Schlegel, M. L., Libert, M., and Bildstein, O. (2015). Impact of Iron-Reducing Bacteria on the Corrosion Rate of Carbon Steel under Simulated Geological Disposal Conditions. *Environ. Sci. Technol.* 49, 7483–7490. doi:10.1021/acs.est.5b00693.
- Sette, L. D., Passarini, M. R. Z., Rodrigues, A., Leal, R. R., Simioni, K. C. M., Nobre, F. S., et al. (2010). Fungal diversity associated with Brazilian energy transmission towers. *Fungal Divers.* 44, 53–63. doi:10.1007/s13225-010-0048-y.
- Setter, K. O., and Gaag, G. (1983). Reduction of molecular sulphur by methanogenic bacteria. *Nature* 305, 309–311. doi:10.1038/305309a0.
- Sherar, B. W. A., Power, I. M., Keech, P. G., Mitlin, S., Southam, G., and Shoesmith, D. W. (2011). Characterizing the effect of carbon steel exposure in sulfide containing solutions to microbially induced corrosion. *Corros. Sci.* 53, 955–960. doi:10.1016/j.corsci.2010.11.027.
- Small, J., Nykyri, M., Helin, M., Hovi, U., Sarlin, T., and Itävaara, M. (2008). Experimental and modelling investigations of the biogeochemistry of gas production from low and intermediate level radioactive waste. *Appl. Geochemistry* 23, 1383–1418. doi:10.1016/j.apgeochem.2007.11.020.
- Smart, N., Blackwood, D., and Werme, L. (2001). The anaerobic corrosion of carbon steel and cast iron in artificial groundwaters. *Swedish Nucl. Fuel Waste Manag. Tech. Rep.*, 45.
- Smart, N. R., Rance, A. P., Fennell, P. A. H., and Kursten, B. (2013). The anaerobic corrosion of carbon steel in alkaline media – Phase 2 results. *EPJ Web Conf.* 56. doi:10.1051/epjconf/20135606003.
- Sogin, M. L., Morrison, H. G., Huber, J. A., Welch, D. M., Huse, S. M., Neal, P. R., et al. (2006). Microbial diversity in the deep sea and the underexplored rare biosphere. *Proc. Natl. Acad. Sci.* 103, 12115–12120.
- Sohlberg, E., Bomberg, M., Miettinen, H., Nyssönen, M., Salavirta, H., Vikman, M., et al. (2015). Revealing the unexplored fungal communities in deep groundwater of crystalline bedrock fracture zones in Olkiluoto, Finland. *Front. Microbiol.* 6, 573. doi:10.3389/fmicb.2015.00573.
- Stahl, D. A., and Amann, R. (1991). “Development and application of nucleic acid probes,” in *Nucleic Acid Techniques in Bacterial Systematics*, eds. E. Stackebrandt and M. Goodfellow (Chichester, England: John Wiley & Sons Ltd), 205–248.
- Standard Practice for Preparing, Cleaning, and Evaluating Corrosion Test Coupons (2011). G 1-90.
- Sterflinger, K. (2000). Fungi as Geologic Agents. *Geomicrobiol. J.* 17, 97–124.

doi:10.1080/01490450050023791.

- Straub, K. L., Schönhuber, W. a., Buchholz-Cleven, B. E. E., and Schink, B. (2004). Diversity of Ferrous Iron-Oxidizing, Nitrate-Reducing Bacteria and their Involvement in Oxygen-Independent Iron Cycling. *Geomicrobiol. J.* 21, 371–378. doi:10.1080/01490450490485854.
- Suzuki, T., Kunoh, T., Nakatsuka, D., Hashimoto, H., Tamura, K., Kunoh, H., et al. (2015). Use of Iron Powder to Obtain High Yields of *Leptothrix* Sheaths in Culture. *Minerals* 5, 335–345. doi:10.3390/min5020335.
- Teske, A., and Sørensen, K. B. (2008). Uncultured archaea in deep marine subsurface sediments: have we caught them all? *ISME J.* 2, 3–18. doi:10.1038/ismej.2007.90.
- Urios, L., Maillet, a., Dauzères, a., Flachet, M., and Magot, M. (2014). Tournemire argillite/carbon steel evolution under bacterial influence after 10 years of in situ interaction. *Corros. Eng. Sci. Technol.* 49, 554–561. doi:10.1179/1743278214Y.0000000220.
- Usher, K. M., Kaksonen, A. H., Cole, I., and Marney, D. (2014a). Critical review: Microbially influenced corrosion of buried carbon steel pipes. *Int. Biodeterior. Biodegradation* 93, 84–106. doi:10.1016/j.ibiod.2014.05.007.
- Usher, K. M., Kaksonen, A. H., and MacLeod, I. D. (2014b). Marine rust tubercles harbour iron corroding archaea and sulphate reducing bacteria. *Corros. Sci.* 83, 189–197. doi:10.1016/j.corsci.2014.02.014.
- Valencia-Cantero, E., and Peña-Cabrales, J. J. (2014). Effects of iron-reducing bacteria on carbon steel corrosion induced by thermophilic sulfate-reducing consortia. *J. Microbiol. Biotechnol.* 24, 280–286. doi:10.4014/jmb.1310.10002.
- Venzlaff, H., Enning, D., Srinivasan, J., Mayrhofer, K. J. J., Hassel, A. W., Widdel, F., et al. (2013). Accelerated cathodic reaction in microbial corrosion of iron due to direct electron uptake by sulfate-reducing bacteria. *Corros. Sci.* 66, 88–96. doi:10.1016/j.corsci.2012.09.006.
- Videla, H. A. (2000). An overview of mechanisms by which sulphate-reducing bacteria influence corrosion of steel in marine environments. *Biofouling* 15, 37–47. doi:10.1080/08927010009386296.
- Videla, H. A., and Herrera, L. K. (2005). Microbiologically influenced corrosion: looking to the future. *Int. Microbiol.* 8, 169–180. doi:im2305026 [pii].
- Videla, H. A., and Herrera, L. K. (2009). Understanding microbial inhibition of corrosion. A comprehensive overview. *Int. Biodeterior. Biodegradation* 63, 896–900. doi:10.1016/j.ibiod.2009.02.002.
- Vieno, T. (2000). Groundwater salinity at Olkiluoto and its effects an a spent fuel repository 2000-11, Posiva.

- Vigneron, A., Alsop, E. B., Chambers, B., Lomans, B. P., Head, I. M., and Tsesmetzis, N. (2016). Complementary microorganisms in highly corrosive biofilms from an offshore oil production facility. *Appl. Environ. Microbiol.* 82, 2545–2554. doi:10.1128/AEM.03842-15.
- Wagner, M., Roger, A. J., Flax, J. L., Gregory, A., Stahl, D. A., Wagner, M., et al. (1998). Phylogeny of Dissimilatory Sulfite Reductases Supports an Early Origin of Sulfate Respiration. *J. Bacteriol.* 180, 2975–2982.
- Wang, Q., Garrity, G. M., Tiedje, J. M., and Cole, J. R. (2007). Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Appl. Environ. Microbiol.* 73, 5261–7. doi:10.1128/AEM.00062-07.
- Whitman, W. B., Bowen, T. L., and Boone, D. R. (2006). “The Methanogenic Bacteria,” in *The Prokaryotes: Volume 3: Archaea. Bacteria: Firmicutes, Actinomycetes*, eds. M. Dworkin, S. Falkow, E. Rosenberg, K.-H. Schleifer, and E. Stackebrandt (New York, NY: Springer New York), 165–207. doi:10.1007/0-387-30743-5_9.
- Williams, K. H., Nevin, K. P., Franks, a, Englert, a, Long, P. E., and Lovley, D. R. (2010). Electrode-based approach for monitoring *in situ* microbial activity during subsurface bioremediation. *Environ. Sci. Technol.* 44, 47–54. doi:10.1021/es9017464.
- Winston Revie, R. ed. (2011). *Uhlig’s corrosion handbook*. 3rd Editio. Wiley.
- Winston Revie, R., and Uhlig, H. H. (2008). *Corrosion and Corrosion Control: An introduction to corrosion science and engineering*. 4th Editio. WILEY-VCH Verlag GmbH doi:10.1179/000705972798323134.
- Wu, X., Holmfeldt, K., Hubalek, V., Lundin, D., Åström, M., Bertilsson, S., et al. (2015). Microbial metagenomes from three aquifers in the Fennoscandian shield terrestrial deep biosphere reveal metabolic partitioning among populations. *ISME J.*, 1–12. doi:10.1038/ismej.2015.185.
- Xu, D., and Gu, T. (2014). Carbon source starvation triggered more aggressive corrosion against carbon steel by the *Desulfovibrio vulgaris* biofilm. *Int. Biodeterior. Biodegrad.* 91. doi:10.1016/j.ibiod.2014.03.014.
- Xu, D., Li, Y., Song, F., and Gu, T. (2013). Laboratory investigation of microbiologically influenced corrosion of C1018 carbon steel by nitrate reducing bacterium *Bacillus licheniformis*. *Corros. Sci.* 77. doi:10.1016/j.corsci.2013.07.044.

Title	Microbially-induced corrosion of carbon steel in a geological repository environment
Author(s)	Pauliina Rajala
Abstract	<p>Low- and intermediate-level radioactive waste (LLW/ILW) is produced during the operation and decommission of nuclear power plants. At the Olkiluoto power plant, LLW/ILW is disposed of in an underground repository excavated into the bedrock 60–100 m below sea level. The metallic portion of this waste is typically made of carbon steel and stainless steels.</p> <p>In anoxic conditions, such as the groundwater at the Olkiluoto repository site, carbon steel corrosion rate is very slow unless the groundwater is highly acidic or microbial activity is high, altering local conditions to corrosion inducing direction. Microorganisms are able to accelerate general corrosion as well as induce localized corrosion forms and stress corrosion cracking as conditions under the biofilm can differ markedly from those in the adjacent environment. Critically, corrosion of metallic waste can release radioactive nuclides into the groundwater and threaten the long-term integrity of the storage site.</p> <p>The objective of this research was to determine the importance of microbially-induced corrosion (MIC) of carbon steel placed in deep geological repository containing LLW/ILW. The structure and function of microbial communities in the deep biosphere are still poorly understood but could have important consequences for the long-term storage of radioactive waste in underground repositories.</p> <p>MIC of carbon steel in anoxic groundwater was studied in the laboratory and in situ in experiments with exposure time ranging from 3 months to 15 years. MIC was examined using gravimetric and electrochemical techniques complemented by molecular biology and surface characterization methods.</p> <p>It was shown that conditions beneath the microbial biofilm accelerated corrosion rate of carbon steel, especially localized corrosion, and that microbial activity in deep groundwater is enhanced by the presence of carbon steel. Naturally-occurring microorganisms in deep groundwater environments have a great affinity for the surface of carbon steel and rapidly form a biofilm. Phylum proteobacteria, beta- or deltaproteobacteria depending on the experiment, were in the majority in the biofilm forming bacterial community. Archaeal biofilm was formed by phylas Euryarchaeota (DHVE) and Thaumarcheota (MBGB). However, corrosion was inhibited in concrete-encased environments, due to high alkalinity and calcium carbonate concentration in the environment. In many cases, LLW/ILW repositories contain concrete materials, which according to the present results hinders the corrosion at least in the beginning of repository time scale.</p>
ISBN, ISSN, URN	ISBN 978-951-38-8545-8 (Soft back ed.) ISBN 978-951-38-8544-1 (URL: http://www.vttresearch.com/impact/publications) ISSN-L 2242-119X ISSN 2242-119X (Print) ISSN 2242-1203 (Online) http://urn.fi/URN:ISBN:978-951-38-8544-1
Date	July 2017
Language	English, Finnish abstract
Pages	83 p. + app. 54 p.
Name of the project	
Commissioned by	
Keywords	Microbially-induced corrosion, corrosion, deep biosphere, groundwater, geological repository, biofilm
Publisher	VTT Technical Research Centre of Finland Ltd P.O. Box 1000, FI-02044 VTT, Finland, Tel. 020 722 111

Nimeke	Hiiliteräksen mikrobiologinen korroosio geologisessa loppusijoitusympäristössä
Tekijä(t)	Pauliina Rajala
Tiivistelmä	<p>Ydinvoimalaitoksen käytön ja purkamisen yhteydessä syntyy radioaktiivisesti kontaminoitunutta materiaalia, joka luokitellaan aktiivisuustason perusteella pääosin matala- ja keskiaktiiviseksi radioaktiiviseksi jätteeksi. Olkiluodon ydinvoimalassa tämä jäte sijoitetaan maanlaiseen loppusijoitustilaan 60-100 m maanpinnan alapuolelle. Metallinen matala- ja keskiaktiivinen jäte on pääosin hiiliterästä ja ruostumattomia teräksiä.</p> <p>Hapettomissa olosuhteissa, kuten pohjavesi Olkiluodon loppusijoitustilassa, hiiliteräksen korroosionopeus on hyvin matala, ellei pohjavesi ole hyvin hapanta tai mikrobiologinen toiminta ole aktiivista, muuttaen paikallisia olosuhteita korroosiota kiihdyttäväksi. Mikrobit voivat kiihdyttää teräksen yleistä korroosiota sekä edesauttaa paikallisen ja jännityskorroosion syntymistä luomalla biofilmin alle korroosiolle altistavat olosuhteet, jotka voivat merkittävästi erota ympäröivistä olosuhteista.</p> <p>Metallisen radioaktiivisen jätteen korroosio voi vaikuttaa radionuklidien kulkeutumiseen sekä vaikuttaa loppusijoituksen pitkäaikaisturvallisuuteen. Tämän tutkimuksen tavoite oli kartoittaa hiiliteräksen mikrobiologisen korroosion riskejä loppusijoitusolosuhteissa. Mikrobiyhteisön koostumus ja toiminnallisuus syvässä kallioperässä on yhä heikosti tunnettua, mutta sillä saattaa olla vaikutusta vaikuttaa matala- ja keskiaktiivisen jätteen loppusijoituksen pitkäaikaisturvallisuuteen.</p> <p>Mikrobiologista korroosiota hapettomassa pohjavedessä on tutkittu sekä laboratorio- että in situ -olosuhteissa koeaikojen ollessa kolmesta kuukaudesta viiteentoista vuoteen. Mikrobiologista korroosiota tutkittiin sekä painohäviöön perustuvilla että sähkökemiallisilla menetelmillä yhdistettynä molekyylibiologiisiin menetelmiin ja pinnan karakterisointiin.</p> <p>Tulokset osoittavat, että mikrobien muodostaman biofilmin alla syntyy olosuhteet, joissa hiiliteräksen korroosio, erityisesti paikallinen korroosio, merkittävästi kiihtyi. Tulokset myös osoittivat, että pohjaveden mikrobiyhteisö hyötyi hiiliteräksestä.</p> <p>Pohjaveden luonnollinen mikrobiyhteisö hakeutui hiiliteräksen pinnoille muodostaen nopeasti biofilmiä. Proteobacteria pääjaksoon kuuluvat bakteerit, beta- tai deltaproteobacteria riippuen koeolosuhteista, olivat suurimmassa osassa biofilmin bakteeriyhteisöä. Biofilmin arkeoniyhteisön muodostivat pääjaksojen Euryarchaeota (DHVE) ja Thaumarcheota (MBGB) arkeonit. Koesarjoissa, jotka suoritettiin betonin läsnäollessa, korroosionopeus oli huomattavasti matalampi, johtuen korkeasta pH:sta ja kalsiumkarbonaatti-pitoisuudesta. Matala- ja keskiaktiivisen jätteen loppusijoitusolosuhteissa käytetään betonirakenteita, jotka näiden tulosten pohjalta voivat hidastaa korroosiota ainakin loppusijoituksen alkuvaiheessa.</p>
ISBN, ISSN, URN	ISBN 978-951-38-8545-8 (nid.) ISBN 978-951-38-8544-1 (URL: http://www.vtt.fi/julkaisut) ISSN-L 2242-119X ISSN 2242-119X (Painettu) ISSN 2242-1203 (Verkkojulkaisu) http://urn.fi/URN:ISBN:978-951-38-8544-1
Julkaisuaika	Heinäkuu 2017
Kieli	Englanti, suomenkielinen tiivistelmä
Sivumäärä	83 s. + liitt. 54 s.
Projektin nimi	
Rahoittajat	
Avainsanat	Mikrobiologinen korroosio, Korroosio, Syväbiosfääri, Pohjavesi, Geologinen loppusijoitus, Biofilmi
Julkaisija	Teknologian tutkimuskeskus VTT Oy PL 1000, 02044 VTT, puh. 020 722 111

Microbially-induced corrosion of carbon steel in a geological repository environment

Microbially-induced corrosion in deep bedrock environment is important when evaluating the long-term safety of the disposal of low and intermediate level radioactive waste. The metallic waste consists largely of steels. In Olkiluoto, this waste has been disposed of in an underground repository excavated into the bedrock 60–100 m below sea level. In oxygen-free groundwater, the corrosion of metals is expected to be low. Microorganisms however, may accelerate several types of corrosion. The activity of microorganisms attached to the surfaces and the properties of formed biofilms are essential factors when considering the possibility of microbially-induced corrosion. Under the biofilm the conditions may differ remarkably from the surrounding solution and thus induce circumstances where the corrosion is locally increased.

The objective of this work was to focus on the microbially-induced corrosion of carbon steel. This work is relevant to the deep geological repository conditions after final closure of the low and intermediate level radioactive waste underground storage site. This work demonstrates that microbial biofilms and their metabolic activity play an important role in the corrosion of carbon steel in anoxic groundwater, and microorganisms in natural deep groundwater have a great affinity to form biofilms on the surface of carbon steel. However, the presence of concrete, a barrier material used in disposal system, evidently inhibits microbially-induced corrosion improving the integrity, stability and long-term safety of the repository for carbon steel waste.

ISBN 978-951-38-8545-8 (Soft back ed.)
ISBN 978-951-38-8544-1 (URL: <http://www.vttresearch.com/impact/publications>)
ISSN-L 2242-119X
ISSN 2242-119X (Print)
ISSN 2242-1203 (Online)
<http://urn.fi/URN:ISBN:978-951-38-8544-1>

