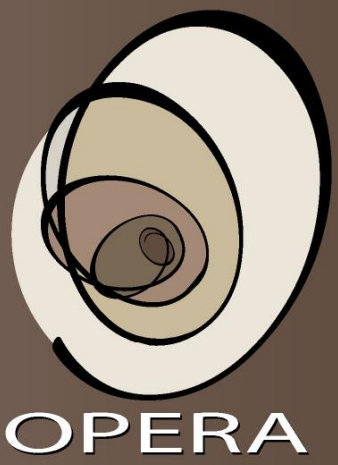

GePeTO

Geochemical Performance of the EBS: Translation and Orientation of existing Knowledge towards the Boom Clay in the Netherlands

OPERA Report of task 5.1.5
Microbial effects on the Engineered Barrier Systems (EBS) and Boom Clay

OPERA-PU-SCK515

Radioactive substances and ionizing radiation are used in medicine, industry, agriculture, research, education and electricity production. This generates radioactive waste. In the Netherlands, this waste is collected, treated and stored by COVRA (Centrale Organisatie Voor Radioactief Afval). After interim storage for a period of at least 100 years radioactive waste is intended for disposal. There is a world-wide scientific and technical consensus that geological disposal represents the safest long-term option for radioactive waste.
Geological disposal is emplacement of radioactive waste in deep underground formations. The goal of geological disposal is long-term isolation of radioactive waste from our living environment in order to avoid exposure of future generations to ionising radiation from the waste. OPERA (OnderzoeksProgramma Eindberging Radioactief Afval) is the Dutch research programme on geological disposal of radioactive waste.
Within OPERA, researchers of different organisations in different areas of expertise will cooperate on the initial, conditional Safety Cases for the host rocks Boom Clay and Zechstein rock salt. As the radioactive waste disposal process in the Netherlands is at an early, conceptual phase and the previous research programme has ended more than a decade ago, in OPERA a first preliminary or initial safety case will be developed to structure the research necessary for the eventual development of a repository in the Netherlands. The safety case is conditional since only the long-term safety of a generic repository will be assessed. OPERA is financed by the Dutch Ministry of Economic Affairs and the public limited liability company Electriciteits-Produktiemaatschappij Zuid-Nederland (EPZ) and coordinated by COVRA. Further details on OPERA and its outcomes can be accessed at www.covra.nl.

OPERA-PU-SCK515
Title: GePeTO
Geochemical Performance of the EBS: Translation
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OPERA Report of task 5.1.5 Microbial effects on the Engineered Barrier Systems (EBS) and Boom Clay
Authors: Katinka Wouters, Paul Janssen, Hugo Moors, Natalie Leys
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Keywords: microbiology, salinity, extremophiles, gas production and consumption, metal corrosion, cement degradation, deep subsurface clay, radioactive waste disposal

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"We know more about the movement of celestial bodies than about the soil underfoot."

Leonardo DaVinci, circa 1500

SUMMARY

Deep microbial ecosystems are nowadays assumed to be greater and more complex than previously expected. Microbes can be present in very old marine sediments below the sea floor in an abundancy that is comparable to the one-meter top layer of a forest soil. Indeed, micro-organisms exhibit a variety of traits which enable them to **persist in unfavourable, low energy conditions**. The metabolic potential of microorganisms goes all the way down the redox ladder, as they only need an electron acceptor and electron donor in order to remain active.

Within microbiology research of **clay layers** that are candidate for deep geological disposal, Boom Clay and Opalinus Clay have been described best so far, although information is still scarce. For both clay layers or their technical installations, presence and activity of microorganisms have been reported. In Boom Clay, both in Belgium and in the Netherlands, the major parameters determining microbial activity are *(i)* the estimated low availability of electron donors and acceptors, and *(ii)* the high consolidation which leads to small sizes of pores and pore throats.

Upon **excavation and waste disposal**, some opportunities could be created for microbial life, shifting this presumably low energy community towards a speculative state of heightened activity at first, due to oxygen penetration (a favourable electron acceptor), introduction of viable microbes, and availability of space and water. After **waste emplacement and closure**, rapid oxygen consumption and near extermination of the near-field microbial community is expected due to the nature of the engineered barrier system and the radioactive waste (e.g. high pH and radiation), and the sealing of any voids.

Nevertheless, many uncertainties remain concerning the survival and potential of microorganisms in disturbed or undisturbed Boom Clay, and in the engineered environment. Especially the microbial processes causing **deterioration of metals and cement** warrant some concern. To tackle part of these uncertainties, recommendations are made *(i)* to assess the microbial population and its boundary conditions for survival and activity in Dutch Boom Clay as such, and *(ii)* to simulate and monitor microbial processes underlying metal and cement corrosion for the Dutch scenario.

SAMENVATTING

Diepe microbiële ecosystemen worden verondersteld complexer te zijn dan eerder werd aangenomen. Micro-organismen hebben dan ook een verscheidenheid aan eigenschappen die hen toelaten te **overleven in ongunstige omgevingen** zoals een diepe geologische kleilaag.

In Boomse Klei, zowel in België als in Nederland, lijken de belangrijkste parameters die bepalen in welke mate microbiële activiteit aanwezig is: *(i)* de lage beschikbaarheid van energiebronnen voor microbieel leven en *(ii)* de hoge consolidatiedruk die leidt tot kleine poriën en verbindingen tussen poriën, en dus weinig ruimte voor microbiële cellen.

Excavatie van deze Boomse Klei kan in een eerste fase **kansen** bieden aan microbiële activiteit, doordat *(i)* zuurstof in het systeem penetreert, *(ii)* ruimte en water beschikbaar worden en *(iii)* levende micro-organismen aan het systeem worden toegevoegd. Na het aanbrengen van radioactief afval en het afsluiten van de bergingsinstallatie, wordt echter verwacht dat de microbiële activiteit fors zal **afnemen** doordat de aanwezige zuurstof snel zal worden opgebruikt, maar ook door de eigenschappen van de technische installatie en het afval zelf (hoge pH, hoge temperatuur, straling).

In het algemeen blijven er veel onzekerheden over omtrent de overlevingskansen en het potentieel van micro-organismen, in zowel onverstoorde klei, verstoorde klei als de technische bergingsinstallatie. Vooral de microbiële processen die **aantasting van metalen en cement** veroorzaken, zijn van belang in het kader van de veiligheid van de bergingsinstallatie. Er worden dan ook aanbevelingen geformuleerd om deze onzekerheden op te nemen in verder onderzoek.

1. Microbial life in the deep subsurface

Microbial life consists of microscopically small organisms (cells) that are invisible to the naked eye. Most micro-organisms replicate by doubling (binary fission), which renders their (uninhibited) growth exponential. Among microbial life in the deep subsurface are mainly Viruses, Bacteria, Archaea, Yeast, and Fungi. The smallest of Bacteria and Archaea (Prokaryotes) are approximately 0.2 µm in diameter, while Viruses can be even smaller, and Yeast and Fungi (Eukaryotes) can be up to 1 mm in size.

Geomicrobiology, the study of micro-organisms in the deep subsurface, has been a growing research field during the past two decades. It was recognized that a diverse and active microbial community can be present in a variety of geological niches and it is assumed that such deep microbial ecosystems are greater and more complex than previously expected (Pedersen, 2000, Fredrickson & Balkwill, 2006, Dong & Yu, 2007, Wolfaardt & Korber, 2012, Colwell & D'Hondt, 2013). Microbes can be present in very old marine sediments (16 – 111 My) as deep as 1.6 km below the sea floor in numbers of up to 10E+06 cells cm^{-3} (Roussel *et al.*, 2008), an abundancy that is comparable to the one-meter top layer of a forest soil, as described by Whitman *et al.* (1998). Calculations were made, although with considerable abstraction, to show that the total amount of biological carbon from subsurface life equals or even exceeds that of surface and marine life (Whitman *et al.*, 1998). In addition, the diversity of microbial communities in marine sediments (Fry *et al.*, 2008), and more in general the deep terrestrial biosphere (Wilkins *et al.*, 2014), as based on 16S rRNA or other target gene sequence data, is astonishingly high.

When isolated in a geological matrix, micro-organisms are subjected to a range of physical and chemical properties imposed by their direct environment, including space, temperature, water permeability, salinity and alkalinity, and the supply of energy and nutrients. Fig. 1.1 shows the interactions micro-organisms require with their environment in order to stay alive or to grow. **The prevalence of life forms of microscopic sizes in the deep subsurface is mainly constrained by lack of space and water and/or by lack of energy yielding compounds.** In subsurface clay environments, small pore sizes, low water availability and a lack of interconnection of niches typically inhibit microbial proliferation, either through limited space available for occupation or through inadequate diffusion of energy yielding compounds and growth factors.

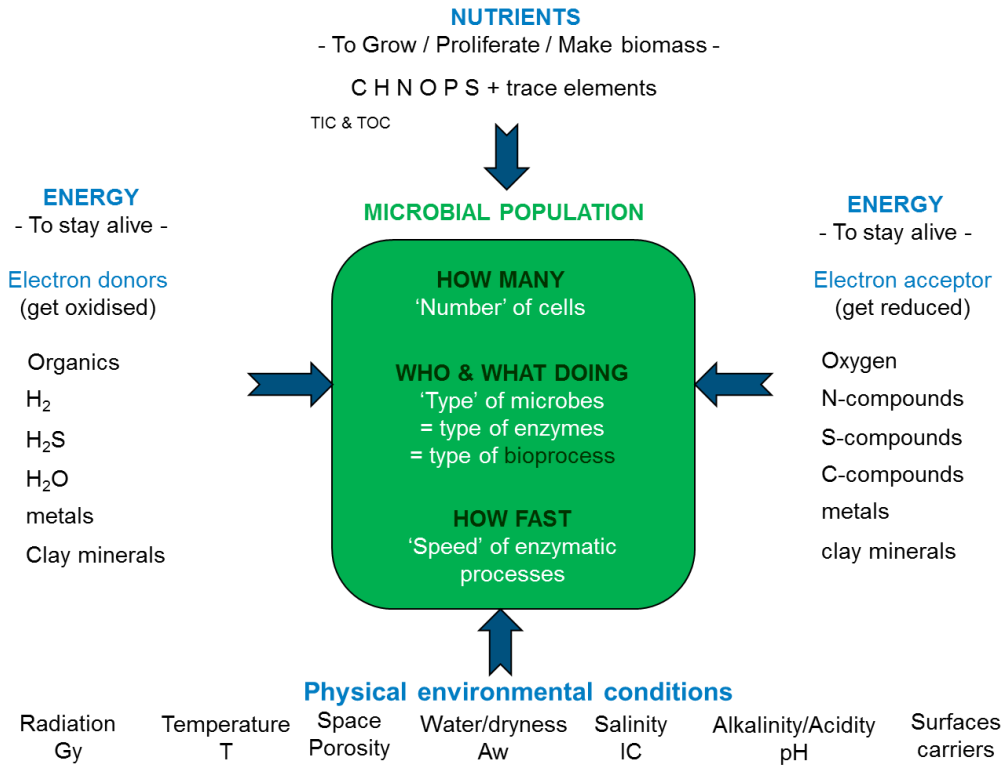

Fig. 1.1 Generalized overview of restricting and enhancing parameters for microbial life

1.1. Microbial growth, maintenance and survival

It is of importance to note the difference between microbial growth, maintenance and survival (Hoehler & Jørgensen, 2013).

Microbial growth is defined as reproduction, e.g. by binary fission. Typically, if one of the major factors shown in Fig. 1.1 is limiting (e.g. supply of carbon source), this one will be the controlling factor for growth of the microbial community.

Maintenance however, is recognized as a basic metabolism by which the cell is still active, but not reproducing. Indeed, the synthesis of new microbial cells is considered costly, with the synthesis of proteins in particular representing most of the costs. To enable cell maintenance in the deep subsurface, energy costs should be kept at a minimum.

Third, when a microbial cell is in *survival* mode, basically all energy is directed towards the preservation of cell integrity. Sporulation, by which a cell goes in an 'armoured' dormant state, is a form of cell survival, induced by harsh environmental properties (e.g. high temperature) and only present as a trait in a limited number of microbial genera, typically gram positive bacteria and fungi. Other, more energy efficient states of dormancy have been described throughout the microbial phylogenetic tree (Lennon & Jones, 2011). In these states, no energy is wasted, and all efforts are directed towards maintaining a membrane potential.

Archaea typically don't form spores, but seem to have a very efficient system of energy transduction. This might explain the relatively high abundance of Archaea (as opposed to Bacteria) that is observed in oligotrophic (low nutrient) subsurface environments (Lloyd *et al.*, 2013, Lloyd *et al.*, 2013). Since 30 years, Archaea have mostly been described as typical extremophiles, meaning they are capable of

dealing with environmental stressors like high temperature, extreme pH and high salinity (Huber *et al.*, 1987, Dennis & Shimmin, 1997, Huber *et al.*, 1997, Moser *et al.*, 2005, Mesbah & Wiegel, 2008). Like Bacteria, Archaea are competent in a number of metabolic pathways, of which some are unique to the Archaea (e.g. the methanogenesis pathway) (Swan & Valentine, 2009, Sato & Atomi, 2011). Some studies have elaborately studied Archaea in the deep subsurface in the context of radioactive waste disposal (Swanson *et al.*, 2012), but in general, Archaea are still largely unexplored or even neglected, evidenced by the focus on bacterial communities in the studies mentioned here.

1.2. Microbial energy

"Our understanding of microbial energy metabolism stems primarily from studying populations that are characterized by rapid growth, high metabolic rates and high cell densities – characteristics that do not apply to most micro-organisms in nature" (Hoehler & Jørgensen, 2013)

The current understanding of microbial life comes mostly of rapid growing populations with high metabolic rates in a laboratory environment. **The deep subsurface, including the Boom Clay layer in Belgium and The Netherlands, is considered a low energy environment.** Extensive reviews on microbial life under extreme energy limitation, are provided by Hoehler & Jørgensen (2013) and Lever *et al*. (2015), and the thermodynamic limitations to microbial reactions are described in detail by LaRowe *et al.* (2012).

Minimal levels of energy must be met in order to allow microbial growth, maintenance or survival. The energetic habitability of a geological niche can in this respect be defined as the balance between the biological demand for energy, and the corresponding potential for meeting that demand from the direct environment into a biological process (Hoehler, 2007). This energy demand is not only sensitive to chemical conditions, but also to physical conditions like temperature. In a subsurface environment, the flux of energy is generally low compared to the levels required for microbial growth. Therefore, **microbial metabolism in the deep subsurface is assumed to be primarily directed towards long term maintenance and survival**.

The **source of** this required **energy** is a recurring theme of study. It is accepted that a specific environment selects for the organisms capable of inhabiting it (Baas-Becking, 1934). A dark deep subsurface biotope will therefore logically not contain photosynthetic organisms that require light as energy source. Rather will deep subsurface microbial communities rely on a photosynthesis-independent, **chemical redox inequilibrium** (Fig. 1.2). Micro-organisms utilise the energy from exo-energetic redox reactions they catalyse, thereby producing ATP (Adenosine Tri Phosphate), which is the so-called motor of microbial life. These processes are typically of low energy (in contrast to high energy photosynthetic reactions) and define the equilibrium between micro-organisms and their direct geochemical environment.

In microbially catalysed reactions, the **redox couple** which yields the largest quantity of energy will be favoured first. As **electron donor**, primarily hydrogen gas (H_2) is considered the major fuel of deep subsurface microbial life (Pedersen, 2000, Dong & Yu, 2007). Organic compounds, if present and available to microbes, may also contribute to the redox equilibrium as electron donor. Apart from being a source of electrons, organic material is also degraded by microbes as a source of carbon to build into their biomass. Carbon sources, organic or inorganic, are therefore needed to enable microbial proliferation (growth).

If oxygen is present, it will be consumed first as **electron acceptor,** because it is thermodynamically most favourable, followed by oxidized nitrogen-, manganese-, ferric iron-, sulphur- and carbon-species, in that respective order, thereby increasingly reducing conditions (Fig. 1.2) and progressively releasing less energy. In the deep subsurface, **the predominant species to be reduced (electron acceptors) may be present in the mineral formation**, in which case the presumably low availability of the compounds and the rate of solute transport will limit the extent of the reaction (West *et al.*, 2008). The sources of energy available in Belgian and Dutch Boom Clay will be discussed in section 4.1.2.

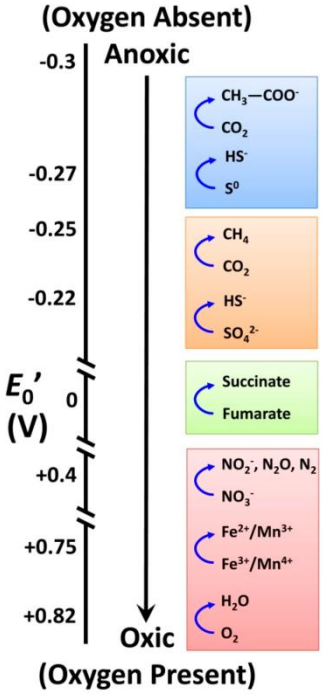

Fig. 1.2 Redox tower for electron acceptors relevant for microbial processes. Note that the reduction of Fe(III) to Fe(II) is only provided for low pH (lower E0' at neutral pH). Adapted from Madigan *et al.* (2011).

Recent studies indicate that strictly following this thermodynamic ladder is not requisite in a microbial community (Bethke *et al.*, 2011). For example, sulphur reducing microbes can be active while ferric iron is still present, and might even join in a mutual consortium with iron reducing microbes.

It can be assumed that open systems, with a dynamic range of physical and chemical influences, are potentially more complex, regarding the microbial biochemical processes that can occur. **Geological repositories however, are typically regarded as closed systems**.

As **deep subsurface geological layers are considered to be anoxic**, microbial life and activity -if present- is restricted to **anaerobic processes**. Among micro-organisms, three types of anaerobes exist. Those that are strict or obligate anaerobes can only survive in anoxic conditions. Exposure to oxygen will be lethal to these communities. The second group are the aerotolerant micro-organisms which logically can tolerate the presence of oxygen, but cannot use it as an electron acceptor to gain energy. The third group consists of facultative anaerobes (or facultative aerobes if you wish), which can survive and even thrive in both oxic and anoxic conditions. In the subsurface, all three groups can be present, although they may not all be active and growing.

1.3. Microbial resistance to physical and chemical constraints

Microbes are known to be highly versatile, with means to adapt to extreme conditions. Socalled extremophiles, whether Bacteria, Archaea, or other micro-organisms, can be divided according to their extremophile trait, *i.e.* their specific 'talent'. Relevant for geological disposal of nuclear waste are (hyper)thermophilic, acidophilic, alkaliphilic, halophilic, piezophilic and radioresistant micro-organisms, which thrive in conditions of respectively high temperature, high pH, high concentrations of salt, pressure and ionizing radiation (Table 1.1).

Table 1.1 Ranges of extremophilic traits in Bacteria and Archaea (changed after Wolfaardt & Korber, 2012)

Trait	Lower limit	Upper limit
Temperature	-20 °C	122 °C
pH	0	12
Salinity	n.r.	5 M NaCl
Radiation	n.r.	30 kGy
Pressure	n.r.	180 MPa

n.r.: not relevant

Extremophiles can be either facultative or obligate to their condition, meaning that for example high temperature is optional (facultative thermophile) or requisite (obligate thermophile) for survival. Different extremophilic traits can be present in one organism, demonstrated by the existence of e.g. halophilic alkalithermophiles (Mesbah & Wiegel, 2008).

1.3.1. (Hyper)thermophily

Micro-organisms that can be isolated from high temperature environments (both natural and man-made) are regarded as thermophiles if their favourable temperature for growth is > 45 °C. Hyperthermophiles even thrive at temperatures > 80 °C. As with all extremophiles, there is a presumed maximum tolerance of the extreme condition, defined by a specific record-holder. In case of thermophiles, the current record for highest growth temperature, 122 °C, goes to *Methanopyrus kandleri*. Scientists believe however, that the temperature limit for life can be extended still. The challenges high temperatures pose concern among others the protection of essential enzymes and proteins, and the high reaction rates needed to compete with high abiotic reaction rates at elevated temperatures (Amend & Shock, 2001).

1.3.2. Acidophily & Alkaliphily

Acidophiles are those organisms that can grow at low pH, usually below pH 2, and are known to occur within both Bacteria and Archaea. Most acidophiles have developed a mechanism to maintain their cytoplasm at neutral pH, so as to protect proteins against the acid environment. A limited number of acidophiles actually does have an acidified cytoplasm and therefore has developed ways to stabilize proteins at low pH (Menzel & Gottschalk, 1985, Ferris *et al.*, 1996, Kelch *et al.*, 2007). Acidophiles have mostly been described in anthropogenically influenced environments, like acid mine drainage sites, but also in natural chemical weathering sites (Baker & Banfield, 2003).

Alkaliphiles can thrive at a pH > 9. Life in environments of such alkaline pH and thus lacking in hydrogen ions, requires a mechanism to keep a neutral intracellular pH and a proton motive force across the cell membrane, in order to preserve proteins and enzymes and to produce ATP (adenosine triphosphate, a carrier of energy). Most studies focus on alkaliphiles that grow around pH 11, in

environments of natural high pH or as a results of anthropogenic activity (Mesbah & Wiegel, 2008, Yoshida, 2011, Alquier *et al.*, 2014, Watts *et al.*, 2015), but communities have been described that grow up to pH 13.2 (Roadcap *et al.*, 2006).

1.3.3. Halophily

Halophiles are adapted to thrive in an environment that is higher than 2M of inorganic salt (NaCl) (up to 5M) and have been described for both natural and anthropogenic environments (Dennis & Shimmin, 1997, Dong & Yu, 2007, Mesbah & Wiegel, 2008, Oren, 2010). A variety of (hyper)saline environments harbouring halophiles has been described, including anaerobic and alkaline environments (Ollivier B *et al.*, 1994). Again, it is the preservation of proteins and enzymes that is key to enable growth and survival of halophiles in their extreme environment. Generally, halophiles have developed a mechanism to maintain a low intracellular concentration of solute ions, by specific ion pumps in the membrane (Dennis & Shimmin, 1997). Halophiles can be fermentative, acetogenic, sulphate-reducing and methanogenic, like any other micro-organism (Ollivier B *et al.*, 1994).

1.3.4. Piezophily

When an organism is able to grow under high pressure, it is named a piezophile or barophile. Generally, such organisms have been described for the ocean floor, where pressures can exceed 40 MPa and even can go up to 120 MPa. Because of their specific habitat in the dark, piezophiles are often very sensitive to light and more specific to UV light. They seem to lack the mechanisms to repair their DNA upon exposure to photon radiation damage (Sharma *et al.*, 2002).

1.3.5. Radioresistance

Organisms that can survive high levels of ionizing radiation, are termed radioresistant. Radioresistance is defined by a certain LD10 or LD50 value, the dose rate which kills off 10 or 50 % of the individuals (microbial cells) in a population. In this respect, the LD100 is not the dose which kills off 100 % of the cells, but rather the dose that causes a 2 log reduction. Another term frequently used and less ambiguous, is the MIC. The MIC is the threshold dose which leaves no cultivable cells at all. Unlike the abovementioned extremophiles, radioresistance as such does not require optimal growth at a certain dose rate, but rather mere survival. Among microbes, MIC doses vary from 60 Gy (Escherichia Coli) to 15 kGy (*Deinococcus radiotolerans*) or even 30 kGy (*Thermococcus gammatolerans*) (Jolivet *et al.*, 2003, Cox & Battista, 2005).

1.4. Microbial ecosystems

When zooming out from the single cell level, interactions between microbial cells become apparent. It is generally assumed that microbial ecosystems are the sum of complex interactions between microbial individuals (intra-species interactions) and species (inter-species interactions).

In microbial **communities**, different metabolic traits may be interdependent. For example, sulphate reduction of one species might be depending on hydrogen produced by the second species, which in turn needs a third species to neutralize environmental pH by excretion of acids, and so on. Different species are also known to be involved in the sequential steps in one pathway, e.g. in the nitrogen cycle (Foshtomi *et al.*, 2015).

Microbial ecosystems can be divided in free swimming (planktonic) and attached (aggregates and biofilm states). Historically, microbial cells were thought to be mostly planktonic, but nowadays

microbial **aggregates and biofilms** are generally regarded as the most natural state for a microbial community (Stoodley *et al.*, 2002, Wuertz *et al.*, 2004). During biofilm development, adhesion of microbial cells to a surface will be followed by production of extracellular polymeric substances (EPS) in which the cells reside. This EPS matrix and its complex architecture of pores and channels enables thick biofilms to develop gradients of e.g. oxygen and pH, diffusion of nutrients, and protects the cells from harsh environments and attack by predators or antimicrobials (Stoodley *et al.*, 2002). The structure and protection of the EPS matrix gives biofilms cells a strong advantage over those in planktonic state (Wolfaardt & Korber, 2012). Indeed, biofilms have been reported in highly irradiated environments with dose rates of up to 100 Gy/h (Wolfram & Dirk, 1997). The close proximity of cells within a biofilm, which can consist of several species, also allows for genetic transfer of specific traits (e.g. substrate utilisation) among species. It should be noted however, that the production of such an extensive EPS matrix is costly for a microbial community, in terms of energy and carbon.

In undisturbed Boom Clay however, this extended biofilm state is not likely to be the dominant state of the microbial community. Due to spatial restriction (small pore size, section 4.1.1) within the consolidated clay matrix, and the low nutrient environment, only single cells or small clusters of cells are thought to be able to reside attached to the clay surface. In Belgian Boom Clay, flocs of biofilm were detected in borehole water samples, but they are presumably mainly detached from biofilms residing in the piezometer tubing or filters (Wouters *et al.*, 2013). **In the near field of a repository**, there is a potential of microbial colonization and subsequent biofilms development in fractures, areas of incomplete sealing and in fissures resulting from gas breakthrough or uneven drying (Wolfaardt & Korber, 2012). **Biofilm** development is considered of particular importance **on interface between materials** (Meleshyn, 2011), e.g. between the clay host rock and an engineered barrier, where sealing needs to occur. The oligotrophic (low nutrient) environment is unlikely to yield extensive biofilms, but can on the other hand trigger biofilm development as a survival mechanism.

With the emergence of new analytical techniques to study microbial communities as a whole, said communities are more often regarded from a holistic point of view, as entities with a macroscale behaviour (Comolli, 2014).

2. Microbial metabolism in the deep subsurface

2.1. Organic compounds, hydrogen gas and methane gas as electron donors

As mentioned in section 1.2, in order to sustain active microbial life, the presence of an electron donor and electron acceptor is essential as a means to provide energy for maintenance and survival. For biomass production (growth), a source of nutrients like carbon is required additionally. In this section, the focus will mainly lie on sources of energy for maintenance and survival, although some overlap of compounds used as both energy source and nutrient (e.g. organic compounds) can not be avoided.

The most straightforward microbial metabolism would be fuelled by any kind of organic carbon compound as both electron donor and carbon source, and oxygen as an electron acceptor (carbon oxidation and aerobic respiration). In the deep subsurface environments selected for nuclear waste disposal however, typically **little to no oxygen is available.** In anoxic conditions, organic carbon can still be used as electron donor, in combination with another compound as electron acceptor (Table 2.1).

Table 2.1 Overview of possible microbial respiration types catalysed by organic matter (OM), hydrogen or methane as electron donor.
Electron acceptors, their reaction products and (average) redox potentials (E0) of the redox reactions are provided for neutral pH. E0 for fermentation is not available (n.a.), as this varies largely with the organic compound used as electron acceptor.

e-donor	Respiration type (e-acceptor reaction)	e- acceptor	Reduced product	E0 (V)
OM, H_2	Aerobic respiration	O_2	H_2O	+0.816
OM, H_2, CH_4	Nitrate reduction	NO_3^-	N_2	+0.71
OM, H_2, CH_4	Iron reduction	Fe(III)	Fe(II)	+0.14
OM, H_2, CH_4	Sulphate reduction	SO_4^{2-}	HS^-	-0.22
OM, H_2	Methanogenesis	CO_2	CH_4	-0.26
OM, H_2	Acetogenesis	CO_2	acetate	-0.30
OM	Fermentation	OM	alcohols, organic acids, H_2, CO_2	n.a.

In some deep subsurface environments selected for nuclear waste disposal **natural organic matter** is present. In most of these cases, the organic carbon is removed from the biological carbon cycle upon burial and transformed into for example kerogen. In this state it is generally considered to be not readily available for microbial degradation and assimilation (use as C-source) nor as electron donor or acceptor (use as energy source), although some exceptions have been described (Petsch *et al.*, 2001, Frouz *et al.*, 2011). In this respect, the Boom Clay environment in Belgium has been described to harbour 1 to 5 wt% of organic carbon, comprised of a large kerogen fraction and a significant amount of dissolved organic matter (DOM) as well, the latter ranging from 50-150 mg C/L and consisting mostly of humic fractions (Bruggeman & De Craen, 2011). Dutch Boom Clay samples have shown to have a lower amount of total organic carbon than the Belgian ones, all below 0.7 wt% ((Behrends *et al.*, 2015) and Table 3.1). The nature and characteristics of Dutch Boom Clay organic matter have not been described so far.

Microbes using organic carbon, can be subdivided into two groups depending on their oxidative capability of the organics (Fig. 2.1). Some genera are able to completely oxidize an organic carbon source to CO_2, while others lack the mechanism for acetyl-Co-A oxidation and thus will oxidize an organic carbon source incompletely (Muyzer & Stams, 2008). In addition to using low molecular weight organic compounds like acetate, some microbes can also use more complex organics, like long aliphatic chains and aromatics.

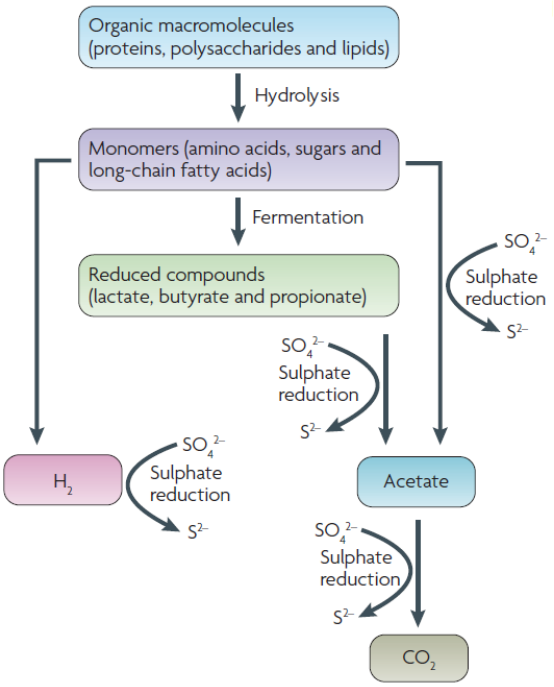

Fig. 2.1 Sequential pattern of microbial degradation of complex organic matter in anoxic environments by sulphate reducing microbes (Muyzer & Stams, 2008)

Generally, humic fractions are considered unavailable for microbial use as energy source as such (Scott *et al.*, 1998) . However, humic fractions, and especially their quinone moieties, which have been described for Belgian Boom Clay DOM (Bruggeman & De Craen, 2011), have been observed to serve as electron shuttles, accepting electrons and again donating them again (Lovley *et al.*, 1996). Indeed, humic-reducing micro-organisms have been described, which oxidize humic quinones into hydroquinones, which can in turn than be used for e.g. Fe(III) reduction (Scott *et al.*, 1998) as depicted in Fig. 2.2.

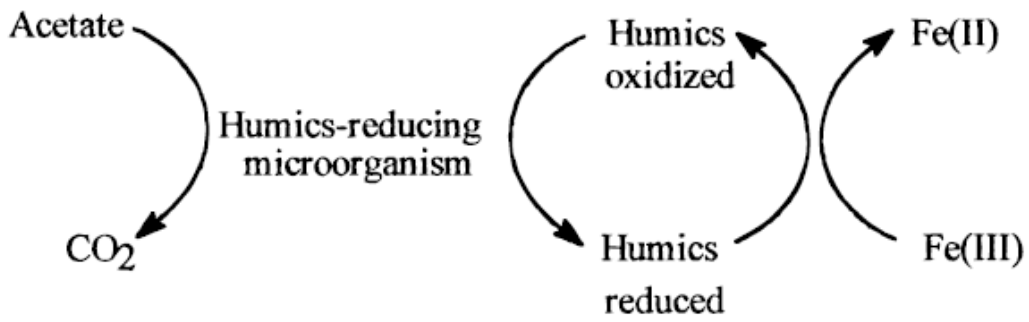

Fig. 2.2 Electron mediator role of humic substances for Fe(III) reduction, as proposed by (Scott *et al.*, 1998)

In the absence of organic carbon as energy source, **hydrogen gas** (H_2) is considered one of the most energetic substrates for microbial life, replacing OM as electron donor (Holloway & O'Day, 2000, Libert *et al.*, 2011). H_2 is expected to be readily available in nuclear waste repositories not only due to radiolysis of water and/or metal corrosion, but due to mineral H_2 formation by micro-organisms as well (Parkes *et al.*, 2011).

Potential electron acceptors for microbial activity, compatible with hydrogen oxidation (or bioavailable OM) as electron donor, in this case would be NO_3^- (nitrate reduction, section 2.2) (and downstream NO_2^- and NO), Fe(III) (iron reduction, section 2.3), SO_4^{2-} (sulphate reduction, section 2.4), or CO_2 (acetogenesis or methanogenesis, section 2.5), with varying optima regarding redox potential, as shown in Table 2.1. Equations 2-1 to 2-5 provide an overview of energy-yielding reactions using a variety of electron acceptors down the redox tower, and H_2 as electron donor.

$H_2 + NO_3^- \rightarrow NO_2^- + H_2O$ **Equation 2-1**	*(hydrogen oxidation and nitrate reduction)*
$H_2 + Fe(OH)_3 \rightarrow Fe^{2+} + 3H_2O$ **Equation 2-2**	*(hydrogen oxidation and iron reduction)*
$H_2 + SO_4^{2-} \rightarrow HS^- + H_2O$ **Equation 2-3**	*(hydrogen oxidation and sulphate reduction)*
$4H_2 + CO_2 \rightarrow CH_4 + 2H_2O$ **Equation 2-4**	*(hydrogen oxidation and methanogenesis)*
$4H_2 + 2CO_2 \rightarrow CH_3COOH + 2H_2O$ **Equation 2-5**	*(hydrogen oxidation and acetogenesis)*

As an alternative to hydrogen gas H_2, also **methane gas** CH_4 can in theory be used as electron donor, to be coupled to the microbial reduction of electron acceptors like nitrate NO_3^-, iron Fe(III), or sulphate SO_4^{2-} (Table 2.1) (Meleshyn, 2011).

$CH_4 + SO_4^{2-} \rightarrow HCO_3^- + HS^- + H2O$ **Equation 2-6**	*(methane oxidation and sulphate reduction)*

There is a double side-effect of these H_2 - or CH_4 - oxidation microbial reactions. First, all of the reactions shown in Fig. 2.6 (section 2.5) would result into a net decrease of the gas volume, even when for example H_2 gas is converted into N_2, due to the reaction stoichiometry (Libert *et al.*, 2011). Second, as CO_2 equilibrates with the liquid phase within the host rock, inorganic carbon would be available for autotrophic microbes as $CO_{2(aq)}$, H_2CO_3, HCO_3^- or CO_3^{2-}, with the species depending on the pH and the concentration of $CO_{2(aq)}$.

2.2. Nitrogen metabolism

Nitrogen (N) is the fourth most abundant element in the biosphere, after C, O and H, but unlike the other elements, it is only a minor constituent of the (sub)surface. N that is present in the deep subsurface, has been derived in earlier times from the atmosphere through biotic or abiotic N-fixation. Organic nitrogen is accumulated, because it becomes more resistant to biological degradation with aging. Globally, continental sediments are considered a sink of N (Wankel *et al.*,

2015). It is thus present in – relatively - large inert reservoirs (but still in much smaller reservoirs compared to those of C, O and H) and is processed in much smaller biological fluxes (Ward, 2012).

Transformation rates and nitrogen distribution among inorganic and organic pools, is largely controlled by micro-organisms (Ward, 2012), who use N-compounds as electron donor or electron acceptor. N occurs in nature in six of its eight possible oxidation states ranging from +5 to -3: NO_3^-, $NO_{2(g)}$, NO_2^-, $NO_{(g)}$, $N_2O_{(g)}$, $N_{2(g)}$ and NH_4^+. In this regard, a distinction is made between gaseous nitrogen (dinitrogen gases N_2 and N_2O) which is largely inaccessible to micro-organisms (with some exceptions) on one hand, and nitrogen compounds which contain no N-N bonds and which are considered biologically available on the other hand.

In the anoxic subsurface there are two major processes that dominate the N-cycle: Denitrification and anaerobic ammonium oxidation (left part of Fig. 2.3). During **denitrification**, N is used as electron acceptor, and a sequence of reduction processes takes place, ultimately transforming nitrate (NO_3^-) into N_2 gas (Equation 2-7).

$NO_3^- \rightarrow NO_2^- \rightarrow NO_{(g)} + N_2O_{(g)} \rightarrow N_{2\,(g)}$ *(denitrification)*
Equation 2-7

As mentioned above, N_2 gas is not available for most micro-organisms, except for those called N-fixators. Overall, N_2 gas is considered an end-product of denitrification. Most denitrifying microbes, of which nitrate reducing prokaryotes, (NRPs) and nitrite reducing prokaryotes (NiRPs) are best known, are versatile, facultative (an)aerobic and capable of using a wide range of organic compounds as electron donor and C-source (Ward, 2012). As mentioned in section 2.1 (Table 2.1), not only organic compounds, but also H_2 and CH_4 can act as an electron donor during denitrification. The best described genes involved in denitrification are *nirS* and *nar* genes, which can be specifically targeted for when assessing metabolic functions of a deep surface community.

Anaerobic ammonium oxidation (anammox) is a process by which ammonium is oxidized as electron donor, using nitrite as the terminal electron acceptor, thereby producing N_2 gas and water (Equation 2-8). This process requires the explosive hydrazine (N_2H_4) as an intermediate.

$NH_4^+ + NO_2^- \rightarrow N_2 + 2\,H_2O$ *(anaerobic ammonium oxidation)*
Equation 2-8

Anammox species thrive in anoxic environments in obligate syntrophy with other micro-organisms like denitrifiers, who supply both the necessary ammonium and nitrite. Ammonium is produced during growth of denitrifiers, by ammonification (i.e. degradation) of organic N-containing compounds (Fig. 2.3, bottom). In addition, they are obligate anaerobes and can only use CO_2 as a source of carbon.

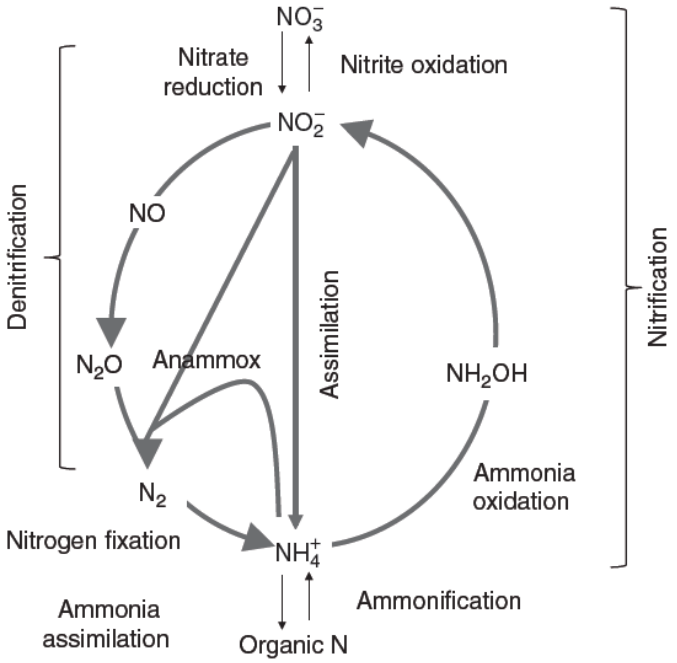

Fig. 2.3 Diagram of the biological nitrogen cycle showing the main inorganic forms in which nitrogen occurs in natural and anthropogenically influenced environments. On the left are the processes which can occur in anoxic environments (Ward, 2012)

Although both denitrification and anammox are typically anaerobic processes, in nature they often interact with aerobic nitrification for the supply of N-substrate, thus closing the circle. Such processes are well studied for aquatic environments (Ward, 2012). In the deep subsurface, however, in the absence of oxygen, N_2 gas is the most likely end product of the N-cycle.

2.3. Iron metabolism

In the subsurface, iron (Fe) is mainly present as Fe(III) or Fe(II), as part of the mineral fraction. Fe(III) oxides such as ferrihydrite, goethite, hematite and magnetite, and Fe(II)/Fe(III)-containing clays (smectites, illites) are ubiquitous in sediments. In addition, Fe(II)-sulfides, such as Mackinawite (FeS), greigite (Fe_3S_4) and pyrite (FeS_2) may occur in the subsurface as well. The presence of soluble Fe(III) is limited, and only possible at pH below 4.

It is generally accepted that microbial (bacterial) processes control iron speciation more than abiotic mechanisms in most environments, including the deep subsurface (Weber *et al.*, 2006). Archaea which can oxidize Fe(II) to Fe(III) under anoxic conditions (using e.g. sulphate as electron acceptor) have been described, but are not very common (Hafenbradl *et al.*, 1996). An oversight of the most common processes, both microbially and chemically mediated, controlling iron speciation on earth, is given in Fig. 2.4.

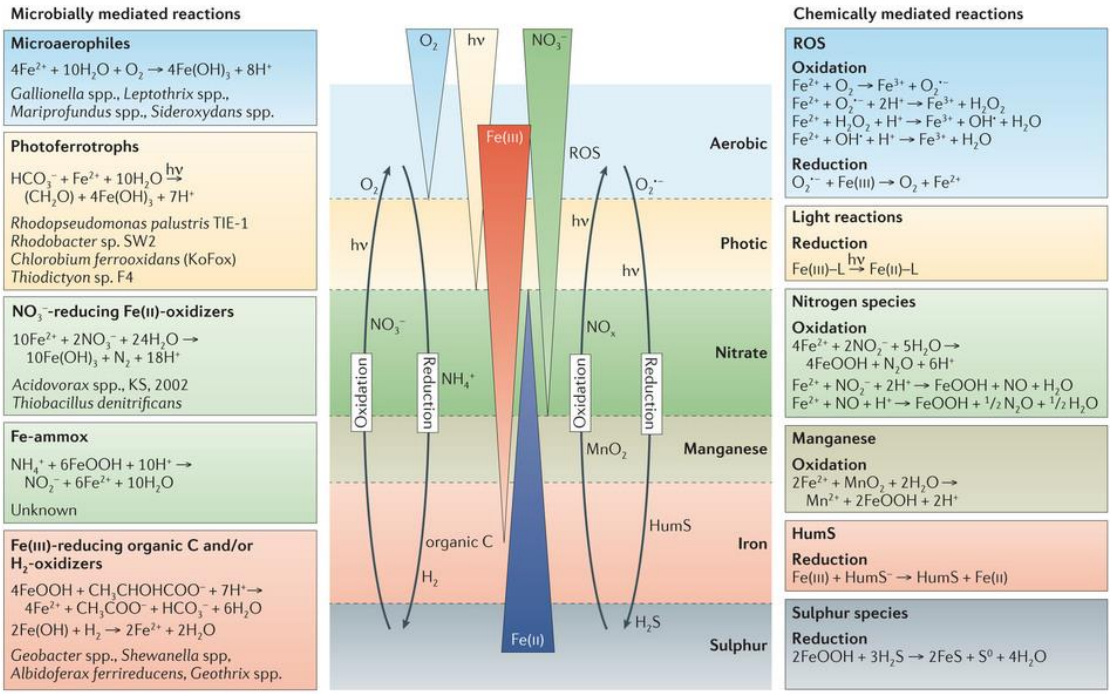

Fig. 2.4 Variety of microbially and chemically mediated reactions involving Fe(III) reduction. For microbially mediated reactions in the deep subsurface, mostly the reactions on the bottom left are relevant (Melton et al., 2014).

Among all metals that can be the terminal electron acceptor for microbial catalysed redox reactions in anoxic conditions as, Fe(III) is the most important one, given the abundance of Fe(III)oxides in the subsurface in general and of Fe(III)-bearing phyllosilicates in clay rich subsurface layers (Lovley, 2002). Despite the predominantly solid form of Fe(III), micro-organisms are capable of using it electron acceptor (dissimilatory iron reduction, equation 2-9), using a range of mechanisms, e.g. soluble electron shuttles and Fe(III) chelators. These micro-organisms are mostly known as dissimilatory iron reducing bacteria (DIRB), but Archaea with similar traits exist as well (Kashefi *et al.*, 2001). Specialized (and even thermophilic) bacteria have even been described that can reduce amorphous Fe(III)-oxyhydroxide to magnetic iron in deep subsurface basins (Liu *et al.*, 1997). A lot of DIRB are capable of reducing other metals and metalloids as well (Kashefi *et al.*, 2001). Some sulphate reducing micro-organisms and methanogens are also known to reduce Fe(III) as a side reaction, without the ability to use it as the sole source of energy (Lovley, 2002).

$2Fe(II)(OH) + H_2 \rightarrow 2Fe(III) + 2H_2O$ *(hydrogen oxidation and dissimilatory iron reduction)*

Equation 2-9

By reducing Fe(III), the more soluble Fe(II) is being formed, which has an impact on its mobility and that of anions like S^{2-} and other metals. Hence, the reductive dissolution of Fe(III) minerals by microbes could have a relatively large effect on Fe-transport and even on sediment genesis (Glasauer *et al.*, 2003). Some minerals are considered to be formed exclusively by microbial activity. As such, magnetite is considered to be a trustworthy signature of DIRB activity (Kashefi *et al.*, 2001). Which Fe-minerals are formed and the extent of the Fe-reducing capacity of the microbes, depends mostly on the availability of an electron donor and carbon source.

Electron donors can be either organic carbon, H_2 or CH_4 (Equation 2-3, section 2.1). A range of organic compounds can be oxidized to CO_2 by DIRB, including aromatics. DIRB generally don't compete with

fermentative micro-organisms, but they do tend to oxidize fermentation products, especially acetate (section 2.5). By this syntrophic relationship, fermentative and DIRB can together degrade relatively complex organic matter to CO_2 in anoxic environments (Lovley, 2002), if the initial substrate is bioavailable to the fermenters, but some DIRB are also capable of oxidizing the organic matter components directly.

2.4. Sulphur metabolism

In the subsurface, sulphur is mainly present as pyrite (FeS_2), gypsum ($CaSO_4$) or as dissolved sulphate (SO_4^{2-}). The sulphur cycle in the subsurface, is a combination of both chemical and biological reactions, and is highly coupled to the carbon and nitrogen cycle.

Micro-organisms play a key role in the transformation of sulphur, not only in the subsurface. Micro-organisms in general take up small amounts of sulphate as an essential nutrient, and reduce it to sulphide, which is then incorporated into amino-acids for proteins and enzymes. This is called *assimilatory* sulphate reduction.

In the presence of oxygen, chemolithotrophic sulphur bacteria are capable of oxidizing sulphide, thereby also gaining energy. These are therefore termed SOBs or sulphur oxidizing bacteria.

Under anaerobic conditions, microorganisms can reduce sulphate to sulphide as terminal electron acceptor (Beijerinck, 1895). When a micro-organism can obtain energy by reducing sulphate in relatively large amounts (not only as sulphate assimilation), it is recognized as a sulphate reducer. Micro-organisms showing such sulphate based anaerobic respiration, or *dissimilatory* sulphate reduction, are termed sulphate reducing prokaryotes, in short SRPs. Another term frequently used is SRBs, which refers to the Bacteria only (sulphate reducing bacteria). Over 220 species of SRPs have been described, of which SRBs comprise the largest group. These SRBs are found within the Deltaproteobacteria, Firmicutes, Nitrospirae, Thermodesulfobacteria and Thermodesulfobia. Within the Archaea, Archaeoglobus, Thermocladium and Caldivirga are the phyla known to harbour SRPs (Barton & Faugue, 2009).

The typical conversion equation of sulphate to sulphide reduction, using a carbon source as electron donor (Equation 2-10), is similar to the reaction with using H_2 or CH_4 as electron donor (section 2.1, Table 2.1 and Equation 2-6). Their use of a wide range of organic carbon sources causes SRPs to be major players in anaerobic carbon-cycling, especially in marine sediments (Jorgensen, 1982). In this reaction, eight electrons are transferred from the organic electron donor (in this case a low molecular weight compound, acetic acid), to the electron acceptor sulphate, ultimately producing sulphide.

$$CH_3COO^- + SO_4^{2-} \rightarrow 2\ HCO_3^- + HS^-$$ *(carbon oxidation and sulphate reduction)*

Equation 2-10

It has been shown that SRP can reduce other oxidized inorganic sulphur compounds such as sulphite, thiosulphate, thiosulphite, bisulphite and elemental sulphur, in a high range of pH, high pressure, temperature and salinity (Lie *et al.*, 1999). Some of these reduction reaction are depicted in Fig. 2.5.

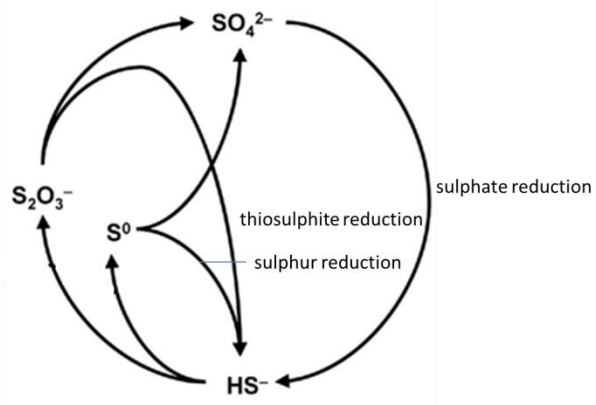

Fig. 2.5 Microbially mediated sulphur cycle, with reducing reactions indicated (after Pester *et al.* (2012).

Depending on the pH, the end product, sulphide will be present as H_2S (low pH), HS^- (neutral pH) or S_2^- (high pH). In the presence of metals, sulphide is also likely to react with the respective metal, producing highly insoluble metal sulphides (e.g. FeS).

All sulphate reducing reactions involve intermediates like sulphite and four major enzymatic complexes : hydrogenase, cytochrome C, adenylyl sulphate (APS) reductase and sulphite reductase (Almeida *et al.*, 2006). The two key genes that are typically targeted when assessing a microbial population for sulphate reduction are *apsA* end *dsrA*, respectively coding for adenine phosphosulphate reductase (aps-reductase) and dissimilatory sulphite reductase.

The presence of a SRP community, e.g. by detecting *apsA* or *dsrA* genes, does not necessarily mean that sulphate reduction in going on. SRPs typically can use many other electron acceptors besides sulphate. SRPs can grow fermentative (using organic compounds as electron acceptor) and acetogenic (using CO_2 as electron acceptor), which explain why they are ubiquitous, also in sediments and subsurface environments with low amounts of sulphate.

Depending on the presence of sulphate, SRPs can compete or grow syntrophically with methanogenic micro-organisms. In the presence of relatively high amounts of sulphate, SRPs will compete with methanogens for organic compounds (mainly CO_2 and acetate) or H_2 as the ultimate electron donor (Oremland & Polcin, 1982, Muyzer & Stams, 2008). On the other hand, in the absence (or low amounts of sulphate), SRPs will grow syntrophically with methanogens, by producing low molecular weight carbon sources (fermentative or acetogenic) for the methanogens.

2.5. Carbon reduction

Besides its most known role as source of C, carbon compounds can be used as sources of energy as well. In the deep subsurface at sufficiently low Eh (~-0.25-0.30), carbon compounds can be reduced. These processes in which organic compounds or CO_2 are used as electron acceptors are methanogenesis, acetogenesis and fermentation. In all these reactions, organic compounds or H_2 are used as electron donors (as described above for nitrate, sulphate and iron reduction).

Methanogenesis is a process that can only be mediated by microbes, and has so far only been described for Archaea (D'Hondt *et al.*, 2002, Thauer *et al.*, 2008). Methanogens are able to transform CO_2 and H_2 into CH_4 (methane) (Equation 2-11). Whenever methane is present in a system or environment, the presence and activity of microbes is confirmed, since methanogenesis can not

occur abiotically (Thauer *et al.*, 2008). Moreover, it is a reaction drastically decreasing the net gas volume in an environment, due to reaction stoichiometrics (Equation 2-11).

$CO_2 + 4H_2 \rightarrow CH_4 + 2H_2O$ *(hydrogen oxidation and methanogenesis)*
Equation 2-11

In many environments with E0 below -0.20 V, methanogenesis is commonly believed to be the dominant H_2-consuming process, because the energy yield from the conversion of CO_2 to H_2 to CH_4 is larger than that for the conversion to acetate (Ragsdale & Pierce, 2008). Recent findings however contest this (Lever, 2011).

During the latter process, **acetogenesis**, an organic molecule, acetate, is microbially constructed using CO_2 as building unit (Ragsdale & Pierce, 2008). Indeed, the so-called CO_2-reducing acetogens are typically obligately anaerobic microbes that produce acetate by reducing CO_2 and oxidizing H_2 (Equation 2-12). However, acetogens producing acetate from sugars like glucose exist as well. These are called fermentative acetogens (Equation 2-13).

$2CO_2 + 4H_2 \rightarrow CH_3COOH + 2H_2O$ *(hydrogen oxidation and CO_2-reducing acetogenesis)*
Equation 2-12

$C_6H_{12}O_6 + 3\ H_2O \rightarrow 2CH_3COOH + 4H_2 + 2CO_2$ *(fermentative acetogenesis)*
Equation 2-13

Fermentation is a metabolism by which energy is derived from oxidation of an organic compound, using other organic compounds as electron donor, thus excluding other, non-organic, electron acceptors. Different kinds of fermentation reactions are defined, mostly named after their end-product (e.g. ethanol fermentation, Equation 2-14).

$C_6H_{12}O_6 \rightarrow 2CH_3CH_2OH + CO_2$ *(ethanol fermentation)*
Equation 2-14

Most fermenters don't oxidize organic matter completely to carbon oxide, which makes fermentation a source reaction for other microbial metabolisms. Indeed, at redox conditions below those for nitrate reduction, micro-organisms are limited in the organic compounds they can use as electron donor. When using Fe(III) as an electron acceptor for example, mostly simple compounds like fatty acids can be oxidized. Therefore, in such environments, fermentative micro-organisms come in often, to first convert organic matter into smaller compounds, including H_2 and CO_2 (Lovley & Chapelle, 1995) (Equation 2-13).

Not only fermentation, but also microbial production of CH_4 and acetate by methanogens and acetogens can be considered as fuel reactions, since the metabolites can be used as energetically favourable compounds by other micro-organisms (Libert et al., 2011). Actually, acetate and CH_4 can be used as electron donor in some reactions shown in Fig. 2.6, thereby broadening the part of the microbial community that might be active in deep subsurface conditions. The possible microbial pathways involving H_2, CO_2, CH_4 and acetate can cause a complex network of interactions, as shown in Fig. 2.6.

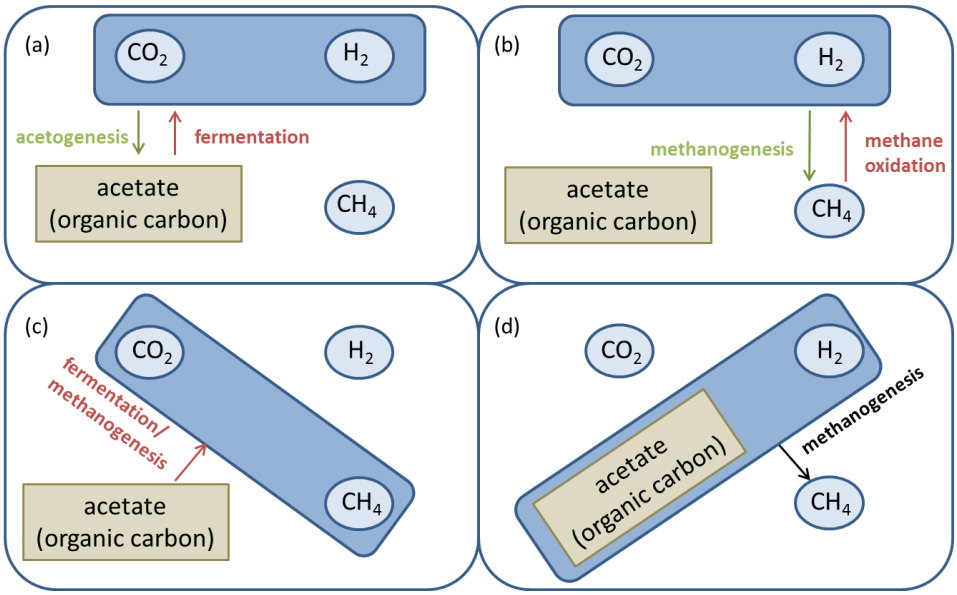

Fig. 2.6 Possible microbial pathways involving H_2, CO_2, CH_4 and acetate/organic matter. Gasses are indicated in circles while others are indicated in a square. Reactions in green, red and black result respectively in net gas consumption, production or conversion.

When considered as an energy source (electron acceptor) in these schemes (see also section 2.1), acetate can be replaced by other organic compounds as well, both simple and complex, if such would be present. The pathways displayed in (a), (b), (c) and (d) can be performed at the same time, by different groups of micro-organisms. However, depending on the dominance of a specific community of micro-organisms, different end products and a shift in the total net gas volume are expected. A shift in community composition towards fermentation of organic matter or direct methanogenesis of organic matter would produce CO_2 or CH_4 gas, and thus increase the net volume of gasses around the EBS, while a dominance of homoacetogens, reducing CO_2 to acetate, and autotrophic methanogens, producing CH_4 gas, while using hydrogen as electron donor instead of organic matter, would result in a net decrease of the gas volume.

3. Microbial processes in candidate clay layers for radioactive waste disposal

To date, in Europe, four types of clay rock with potential for high level long-lived radioactive waste (HLW) disposal in a deep geological repository (DGR) have been studied in detail. These are Opalinus Clay in Mont Teri, Switzerland, the Callovo-Oxfordian formation in Bure and the Toarcian argillite at Tournemire, both in France, and Boom Clay (Rupelian Clay) in Mol, Belgium. To investigate engineering-, scientific-, and safety-relevant issues associated with HLW deep subsurface storage, underground research laboratories and facilities (URLs and URFs) were established for each of these four clay types (reviewed by Birkholzer *et al.* (2012)). In recent years, a fifth European clay type, Boda Claystone, Mecsek Mountains, Hungary, is being scrutinized for its potential as a host rock for HLW storage (Lazar & Mathé, 2012). Non-clay deep geological formations, including granite, tuff, shale, limestone, and salt have also been considered as repositories for HLW and Spent Fuel (SF) (reviewed by Delay et al. (2014)). In total, 26 URLs/URFs were build worldwide (reviewed by Blechschmidt & Vomvoris (2012)).

Table 3.1 Indicative physical properties of five European clay types (ordered according to age), compiled from literature (De Craen *et al.* (2004); Birkholzer *et al.* (2012); Lazar and Mathé (2012); Delay *et al.* (2014); Vis and Verweij (2014); Behrends *et al.* (2015))

Rock clay type	Age (My)	Depth (m) (*)	Porosity (fraction)	Clay fraction (wt%)	Organic matter (wt%)	In situ temp. (°C)
Boom (Mol, BE)	30	200 - 300	0.39	23 - 59	1 – 5	16
Boom (NL)	30	ca. 500	0.40	41	< 0.7	27
Callovo-Oxfordian (Bure, FR)	155	420 - 520	0.11 - 0.17	55	1.1	?
Opalinus (Mont Teri, CH)	170	200 - 290	0.10 - 0.16	66	0.6	12 - 25
Toarcian (Tournemire, FR)	185	300 - 500	0.06 - 0.09	20 - 50	?	?
Boda (Mecsek, HU)	250	600 - 1000	1.5	35 - 50	< 0.1	?

(*) subsurface depth in the vicinity of the existing or planned URL/URF

The five European clay types currently under consideration for a deep geological repository much differ in age, characteristic minerals, organic matter content, water contents, *in situ* temperatures, etc. (Table 3.1). It has been considered that a microbial community potentially indigenous to the host clay rock may become part of a DGR environment. An indigenous community is not necessarily a community that is as ancient as the respective host rock deposit itself. Natural geological processes such as geological movement, landslides, the formation of cracks and fissures, infiltration of foreign water from aquifers or as a result of flooding, are just a few of the processes that can introduce microorganisms into rock formations, and be the source of a more recent indigenous community.

Evidently, the composition and metabolic activity of such indigenous microbial life depend on some of these intrinsic clay properties. Reversely, this microbial activity may change upon anthropogenic disturbances or upon the introduction of allochtoneous microbes and hence affect the integrity of any DGR over extended periods of time. A good knowledge of both native (if present) and introduced microbial life –regularly monitored during DGR construction – at prospective DGR sites is thus primordial.

Probably the best studied geological clays for microbial life are Boom Clay (Boivin-Jahns *et al.*, 1996, Aerts *et al.*, 2008, Wouters *et al.*, 2013) and Opalinus Clay (Mauclaire *et al.*, 2007, Stroes-Gascoyne *et al.*, 2007, Stroes-Gascoyne *et al.*, 2011). However, detailed microbial knowledge has been gathered for other argillaceous rocks (Urios *et al.*, 2012) as well, and also for a range of non-clay DGRs (Stroes-Gascoyne & West, 1997, Pedersen, 1999, Fredrickson *et al.*, 2004, Wang & Francis, 2005, Stroes-Gascoyne, 2010, Itavaara *et al.*, 2011, Colwell & D'Hondt, 2013).

3.1. Boom Clay

The first microbial study on Boom Clay was performed on 20 samples obtained within a 20-m long horizontally drilled core with diameter 10 cm at a gallery depth of 224 m (Boivin-Jahns *et al.*, 1996). In addition, three pore water samples were taken from piezometers extending from the gallery wall at the same depth of 224 m and extending 3, 7, and 15 m from the gallery wall. All samples were diluted and subcultivated on 13 different culture media chosen and used for bacterial counting as well as for bacterial activity measurements based on glucose usage. Microbial DNA extracted directly from solid samples and from pellets of centrifuged water samples served as template for bacterial 16S rRNA gene amplification (note: only universal eubacterial PCR primer pairs were used). Obtained amplicons were cloned and sequenced, and phylogenetic data were generated. All attempts to obtain scanning electron microscopy (SEM) images and other microscopy images failed. While, at aerobic conditions, high numbers of heterotrophic bacteria in the range of 10E+04 CFU mL^{-1} (colony forming units) could be observed for samples within a few cm of the gallery wall, this number dropped rapidly to a few viable counts per mL beyond 80 cm distance. Anaerobic bacteria were always poorly represented at or below the detection limit (10 cells per mL). Using a reference database of 16S rDNA sequences, rDNA amplicons that were 97.5% similar in sequence were considered as belonging to the same operational taxonomic unit (OTU). In total, 14 different OTUs were identified, of which 10 belonged to the phylum of Firmicutes while the other four classified to the phylum of Proteobacteria. The main eubacterial genera identified in Boom Clay were *Acinetobacter*, *Pseudoalteromonas*, *Clostridium*, *Desulfotomaculum, Pseudomonas*, and *Propionibacterium* while other OTUs were highly related to the genera *Azoarcus*, *Rhodocyclus*, and *Carnobacterium*.

In 2008, Aerts *et al.* investigated specifically the presence of sulphate reducing bacteria (SRB) in Boom Clay core samples taken from an eight meter long horizontally drilled hole at the Connecting Gallery of the HADES URF at Mol (depth 225 m). Since gallery construction and experimental coring cause the oxidation of pyrite (FeS_2) in Boom Clay, resulting in much higher sulphate levels in the excavation disturbed zone (EDZ) (Decraen *et al.*, 2004), and sulphate can be used as a terminal electron acceptor (TEA) by SRB, it is important to assess, from a corrosion perspective, the presence and activity of such SRBs. At gradual distance from the gallery wall, 13 clay sections were analysed i.e. eight within the first 2 meters and five in the following 6 meters. Unfortunately, multiple problems occurred when attempting to cultivate SRBs from these samples, and various DNA extraction methods failed. As a result, attention was redirected towards samples that were previously taken from MORPHEUS (TD-11D), a piezometer vertically extending 40 m below the HADES URF main gallery (S. Aerts, pers. comm.). The SRB counts for all MORPHEUS samples were estimated at approximately 11E+02 CFU mL^{-1} SRBs by the Most Probably Number (MPN) approach using three different SRB-specific growth media. DNAs extracted from the samples were used as a template for amplification and T-RFLP typing and/or sequencing of 16S rDNA as well as *apsA*- and

dsrA gene sequences. From these preliminary results it was found that most MORPHEUS (TD-11D) piezometer samples contained members of *Desulfobacter* and *Desulfotignum*, while some samples also contained SRB belonging to the genera *Desulfotomaculum* [also encountered in the Boivin-Jahns *et al.* (1996) study – see above], *Desulfovibrio*, *Desolfobotulus*, and *Desulfobulbus* (unpublished; poster presentation at the 2007 European Geosciences Union General Assembly, Vienna, Austria).

In a follow-up study by Aerts *et al*. (2009) the steel-clay interface at ring 5 of the main gallery of the HADES URF was investigated for the presence of SRB. This steel had been in contact with the Boom Clay for over 25 years (excavation period 1982-83). A 20 cm diameter hole was drilled through the steel into the Boom Clay and samples were taken at 53, 83, and 190 cm relative to the gallery lining. The numbers of SRB present in the three clay samples were estimated by decimal dilution series on two different media. Depending on the medium and sampling depth, between 20E+01 and 10E+05 CFU g^{-1} could be detected. The presence of SRB was also confirmed by positive amplification of *apsA* sequences. Besides the clay sampled at the steel-clay interface, SRB presence was also investigated in clay core samples. Here, SRB numbers were very low (4-40 CFU g^{-1}), agreeing with the low sulfate levels (<0.1 mM) in undisturbed Boom Clay. In this study, no sequence-based SRB identification was undertaken.

In 2011, Blanchart (2011) investigated the biodegradability of Boom Clay organic matter by sulphate reducing microorganisms. In this study, microbial activity in pristine clay (PC), oxidized clay (OC) and a sterilized control experiment (CE) were monitored during one year. As inoculum, borehole water from F20 (filter 20) of the above mentioned MORPHEUS piezometer (TD-11D) was taken. In part of the anaerobic reactors, lactate was added as an additional carbon source, in order to prime microbial activity towards co-metabolism of lactate and organic matter. Only in the set-up amended with lactate could microbial (SRP) activity be measured. However, organic analyses showed that these SRPs only used the amended lactate as electron donor, and not the Boom Clay organic matter. The hypothesis of sulphate reducing microbes affecting Boom Clay organic matter composition, in the presence of readily available sulphate, was therefore (preliminary) refuted. Other mechanisms by which Boom Clay organic matter can be used as either carbon or energy source are still conceivable though.

In 2013, Wouters *et al.* investigated the core bacterial community (CBC) of Boom Clay borehole water samples taken in 2011 from 10 filters of the above mentioned MORPHEUS piezometer (TD-11D). It should be noted that samples from this piezometer that was not installed in an aseptic way, must be considered to be contaminated with allochtoneous micro-organisms and moreover can be considered an *in situ* bioreactor where plenty of space and moisture is present to sustain a microbial community (as opposed to the highly consolidated clay rock). Nonetheless, the samples were scrutinized as a proxy for an excavation disturbed zone (EDZ), in which an introduction of microbial contaminants and voids is expected as well. A multidisciplinary approach was used, including scanning electron microscopy (SEM), extraction of DNA followed by 16S rDNA analysis via denaturing gradient gel electrophoresis (DGGE) and 16S rDNA-based metagenomics, metabolic activity analysis, and MPN cultivation. In addition, DNA extracted from each sample and corresponding enrichment cultures were subjected to PCR amplification using *apsA* and *nirS* gene-specific primers (as a proxy for sulphate and nitrite reduction, respectively). For each of the 10 samples, good-quality SEM images could be obtained (Fig. 3.1), providing for the first time visual evidence on the abundancy and diversity of microbes in Boom Clay borehole water.

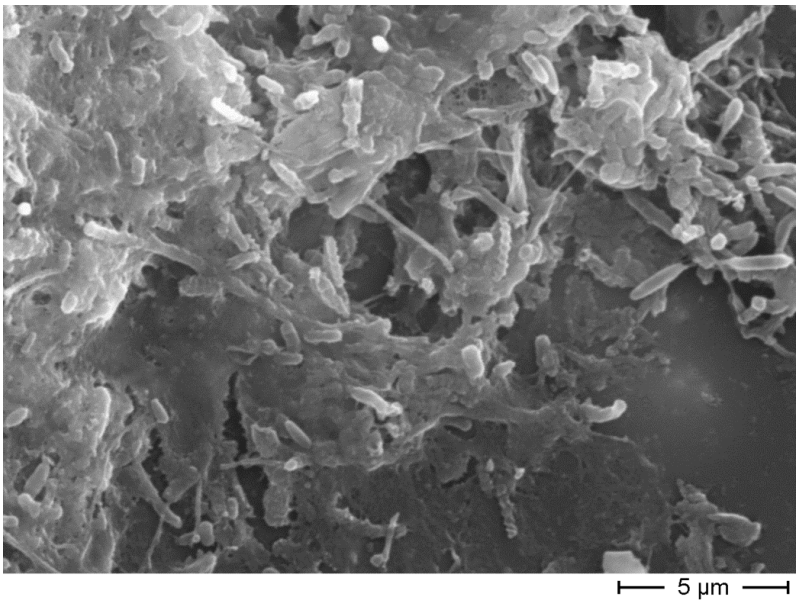

Fig. 3.1 Scanning Electron Microscopy vizualization of microbial cells from FD-11D borehole water concentrated on a 0.1 µm filter surface

Six samples with clearly distinct DGGE profile types were selected for further phylogenetic analyses via pyrosequencing of 16S rDNA amplicons, retaining roughly 10,000 sequences per sample, amounting to nearly 900 OTUs in total by which seven phyla were abundantly represented (>100 sequences per OTU): *Proteobacteria* (74%), *Actinobacteria* (7%), *Chlorobi* (9%), *Firmicutes* (7%), *Bacteroidetes* (3%), *Chloroflexi* (0.001%), and *Spirochaetes* (<0.001%) (percentual CBC abundancy in parentheses). Six of those phyla (i.e. excluding *Spirochaetes*) were present in all samples.

At genus level, the betaproteobacterial *Acidovorax* strongly dominated with 36% CBC abundancy. The genus *Acidovorax* currently holds at least 13 recognized species often found in soil and water habitats. It includes a number of phytopathogenic species as well as some opportunistic pathogens from clinical origin. Some species are known as degraders of xenobiotics and/or reducers of nitrate. Members of *Acidovorax* form gram-negative straight or slightly curved rods, are (mostly) aerobic and motile, and are metabolically versatile.

From these molecularly observed species, some were also cultivated, isolated and identified, meaning that the DNA was at least in part extracted from viable and cultivable micro-organisms and not merely from dead cell remnants.

In conclusion, Table 3.2 provides an overview of the findings of the above described publicly available data on microbiology studies on Belgian Boom Clay, indicating the samples used, the kind of micro-organisms and metabolisms addressed and the techniques used. It shows a variety of results, ranging for largely negative (cultivation) results to all positive outcomes. This contrast is most probably due to the nature of the samples (solid clay versus borehole water), the emergence of new techniques over time and/or the probable contamination of samples with allochtonous microorganisms.

Table 3.2 Overview of results from microbiology studies on Belgian Boom Clay.

		Boivin-Jahns *et al.* (1996)	Aerts *et al.* (2008, 2009)	Blanchart (2011)	Wouters *et al.* (2013)
Samples	***Solid***	x	x	x	-

	Aqueous	x	-	x	x
Phylum		Bac, Mic	Bac, Mic	Mic	Bac, Mic
Metabolism	***General aerobic***	pos	-	-	pos
	General anaerobic	pos/neg	-	-	pos
	Sulphate reduction	neg	pos	neg	pos
	Nitrate reduction	neg	-	-	pos
	Anammox	neg	-	-	-
	Iron reduction	neg	-	-	-
	Methanogenesis	neg	-	-	-
Techniques	***Cultivation***	pos/neg	pos	neg	pos
	Visualisation	neg	-	-	pos
	Quantification	pos	-	-	pos
	Viability	pos	-	-	pos
	DNA-extraction	pos	pos	-	pos
	Identification	pos	pos	-	pos

x: sample used; -: sample/metabolism/technique not used or addressed; Bac: Bacteria, Mic: Microbes in general, pos: positive results; neg: negative results; pos/neg: both positive and negative results for different samples

3.2. Opalinus Clay

The Opalinus claystone is being studied at the international Mont Terri URL in Switzerland. From preliminary microbial investigations on Opalinus Clay borehole water by DAPI (4', 6-diamidino-2-phenylindole) staining, total cell counts varied from 60E+02 to 20E+05 cells mL^{-1} of which the percentage of active cells as measured by FISH varied from zero to 73% (Battaglia & Gaucher, 2004, Ishii, 2005, Mauclaire *et al.*, 2007). In addition, phospholipid fatty acid (PLFA) extracts from Opalinus Clay core samples (retaining on average 64 ng of PLFA per g of dry claystone) suggested the presence of 50E+05 cells per gram of clay (Mauclaire *et al.*, 2007). The obtained PLFA profiles clearly revealed lipid biomarkers specific for anaerobic Gram-negative bacteria and SRB, with indicative lipid profiles for *Desulfobulbus* and *Desulfovibrio* (Mauclaire *et al.*, 2007).

Stroes-Gascoyne *et al.* (2007) also studied the occurrence of presumably indigenous microbes, and their population size, community structure and metabolic activity, in undisturbed Opalinus Clay from the Mont Terri URL, Switzerland. Bore hole water samples were recovered from a 15 m long borehole drilled in the exploration gallery of the URL in early 2004 (known as the PP niche borehole, or BPP-1), and were analysed for microbes using photonic microscopy, molecular biology techniques (PFLA, Q-PCR, PCR-DGGE) and MPN cultivation and enrichment culturing. However, microbial cells could not be visualized with classic microscopy, acridine orange direct counting (AODC), and fluorescence in situ hybridization (FISH). All attempts to extract PCR-amplifiable DNA from the clay samples failed, and the vast majority of lipids detected by PFLA analysis were indicative for cell debris, not viable cells. Also, cultivation attempts were negative with the exception of one positive enrichment result for SRB and a few other partially successful enrichment cultures. The unperturbed Opalinus Clay environment thus rather seems biologically inactive (dormant), with little expectation for metabolic activity. Renewed efforts to directly extract DNA from the aforementioned samples remained unsuccessful (Poulain *et al.*, 2008) but PCR-amplifiable DNA could be obtained from a limited number of enrichment cultures and 16S rDNA sequence analysis could be performed. This eventually resulted in the isolation and characterization of seven strains, two of which could be

identified at genus level i.e. belonging to *Sphingomonas* and *Alicyclobacillus*. No further analysis on these strains was performed.

In stark contrast with the largely negative results obtained for "undisturbed" Opalinus core samples (Stroes-Gascoyne *et al.*, 2007, Poulain *et al.*, 2008), cultivation and molecular studies on clay and water samples taken from the *in situ* Porewater Chemistry (PC) experiment, carried out in the Opalinus Clay formation at the Mount Terri URL, indicate a diverse and active microbial community in PC water and adjacent clay (Stroes-Gascoyne *et al.*, 2011). Cell counts and quantitative culture results were 2-4 orders of magnitude higher (*i.e.,* up to 70E+07 cells mL^{-1}) in these samples as compared to undisturbed clay. MPN cultivation allowed the enumeration of various physiological groups of microorganisms: anaerobic heterotrophs, sulfate-reducing bacteria (SRB), nitrate-utilizing bacteria (NUB), nitrate-reducing bacteria (NRB), iron-reducing bacteria (IRB), anaerobic lithotrophs, and methanogens. DNA extracts from PC water, agar plate pure cultures, and enriched cultures were subjected to quantitative real-time PCR using universal primers for bacterial and archaeal 16S rDNA and amplicons were separated by DGGE, isolated from the electrophoresis matrix, re-amplified, and sequenced. In addition, SRB were quantified by targeting the *dsrA* gene, while methanogenic Archaea were quantified by targeting the methyl coenzyme M reductase gene *mcrA*. Using a 97% cut-off level of 16S rDNA sequence identity against database references, pore water included *Pseudomonas stutzeri*, *Bacillus licheniformis*, *Desulfosporosinus* sp., and *Hyphomonas*, while overcore samples included *Pseudomonas stutzeri*, three species of *Trichococcus*, *Nostocoida limicola*, *Caldanaerocella colombiensis*, *Geosporobacter subterrenus*, *Kocuria palustris*, and *Desulfosporosinus* sp. . The origin of these (mostly anaerobic) microbial species cannot be assessed with certainty. In spite of some precautions, species have most likely been introduced at the beginning of the PC experiment *i.e.* via contamination, while others might theoretically be indigenous to the Opalinus Clay stone and were only revived as a result of clay disturbances caused by drilling and excavation, providing dormant bacteria with water, space, and nutrients, including organics that might be used as electron acceptor. Especially given the contrast with earlier experiments in which no viable microbes could be detected, these results should be interpreted with some restraints.

In conclusion, Table 3.3 provides an overview of the findings of the above described publicly available data on microbiology studies on Opalinus Clay, indicating the samples used, the kind of micro-organisms and metabolisms addressed and the techniques used. As for Boom Clay, the table for Opalinus Clay shows again a variety of results, ranging for largely negative (cultivation) results to all positive outcomes. **Again, this contrast is most probably due to the nature of the samples (solid clay versus borehole water) and/or the likely contamination of samples with allochtonous microorganisms**. From these studies and those in Boom Clay, it thus seems fully warranted to take into account the potential consequences of stimulating and/or introducing microbial activity when designing and building large *in situ* experiments at clay-based URLs/URFs and ultimately a HLW repository in deep clay formations.

Table 3.3 Overview of results from microbiology studies on Opalinus Clay.

		Mauclaire *et al.* (2007)	Stroes-Gascoyne *et al.* (2007); Poulain *et al.* (2008)	Stroes-Gascoyne *et al.* (2011)
Samples	***Solid***	-	x	-
	Aqeuous	x	-	x

Phylum		Bac, Mic	Bac, Mic	Bac, Arch
Metabolism	***General aerobic***	-	pos/neg	-
	General anaerobic	pos	pos/neg	pos
	Sulphate reduction	pos	pos	pos
	Nitrate reduction	-	neg	pos
	Ammamox	-	neg	pos
	Iron reduction	-	neg	pos
	Methanogenesis	-	neg	pos
Techniques	***Cultivation***	-	pos/neg	-
	Visualisation	pos	neg	-
	Quantification	pos	neg	-
	Viability	pos	neg	-
	DNA-extraction	-	pos/neg	pos
	Identification	-	pos/neg	pos

x: sample used; -: sample/metabolism/technique not used or addressed; Bac: Bacteria, Mic: Microbes in general, Arch: Archaea, pos: positive results; neg: negative results; pos/neg: both positive and negative results for different samples

3.3. Toarcian argillite

The French Institute of Radioprotection and Nuclear Safety (IRSN) has been conducting a research program at the Tournemire experimental platform on the microbial diversity of Toarcian argillite (Urios *et al.*, 2012). At different locations of the Tournemire experimental platform samples were collected from the gallery wall (GW), the excavation disturbed zone (EDZ), the undisturbed zone (UZ), and from the faulted area (FA). Using 20 different growth media and three growth temperatures (20, 37, and 60 ^{o}C), a total of 112 isolates could be retrieved and studied: 34 from GW, 13 from EDZ, 36 from UZ, and 29 from FA, and of the 122 isolates, 59, 47 and 5 were obtained at 20, 37, and 60 ^{o}C, respectively. In addition, positive cultures were obtained in culture media designed for optimal growth of SRB and heterotrophic organisms, but no cultures were obtained for media that were designed for the optimal growth of nitrate reducing bacteria (NRB), iron reducing bacteria (IRB), homoacetogens, or methanogens. Based on 16S rDNA sequence identification, microbial diversity was the lowest in the UZ (3 genera) and the highest in the humid FA (14 genera). Some of the identifications could be interpreted as human-derived sample contamination (*Alcaligenes faecalis*, *Propionibacterium acnes*, *Staphylococcus hominis*, *Staphylococcus pasteuri*, *Corynebacterium appendicis*). However, these species are known to be ubiquitous and are not only of human origin. For instance, *P. acnes* and *S. hominis* strains have also been isolated from spacecraft assembly facilities, while *P. acnes* has also been found in deep granitic fracture groundwater. Moreover, not only were the contamination controls always negative, the diversities of the four different sample areas were so clearly different that procedural contamination could be ruled out. Most of the isolates characterized in this study may thus be assumed to be most likely indigenous to the geological formation. In 2013, a new bacterial species, *Pedobacter tournemirensis*, was isolated from subsurface water circulating in a fault inside of the Toarcian geological layer of Tournemire (Urios *et al.*, 2013). This species forms strictly aerobic, non-motile straight rods, with growth at 12–37 ^{o}C (optimum, 30 ^{o}C), at pH 6.0–9.0 (optimum pH 7.0) and at 0–2% NaCl (optimum, 0–1 %). The genus *Pedobacter* comprises over 35 species isolated from a range of different environments such as soils, compost, glaciers, freshwater, and activated sludge.

3.4. Callovo-Oxfordian (COx) clay

The claystone DGR for future HLW storage operated by the French Agence Nationale pour la Gestion des Déchets Radioactifs (ANDRA) at Bure consists of a network of 1 km-long interconnected galleries linked to the surface by two vertical shafts. Over the past 15 years, over 400 boreholes were drilled and many core samples were collected for physicochemical research and biodiversity studies. However, so far there are no public reports on microbial communities from these studies. In 2013, a new bacterial species, *Desulfosporosinus burensis*, was isolated from a porewater sample taken at 490 m depth (Mayeux *et al.*, 2013). This strictly anaerobic, spore-forming, sulfate-reducing species forms non-motile curved rods, with growth at 5-30 ^{o}C (optimum 25 ^{o}C) at pH 6-8 (optimum pH 7), and is tolerant to up to 15 g NaCl L^{-1}. It can use sulfate, thiosulfate or sulfite as TEA, but not elemental sulfur, fumarate, nitrate, nitrite, or Fe(III). Other species of the genus *Desulfosporosinus* have also been isolated from acid mine drainage sediments, freshwater sediments, permafrost, and gasoline-contaminated ground waters.

3.5. Conclusion

As evident from the sequence of studies performed over time, microbiology of geological clay layers for radioactive waste has only been addressed in recent time and faces quite some challenges. In some studies, only largely negative results could be obtained, or only very low amounts of cell counts were observed while in others, a seemingly thriving community could be detected, and new bacterial species were discovered. Ranges go from 4 cells towards 50E+05 cells per gram of clay and even 70E+07 cells per mL of pore/borehole water. **The nature of the samples** (pristine, seemingly undisturbed core samples versus water samples from non-sterile piezometers), **but also the rapidly progressing state of molecular technology might explain this stark contrast**. The seemingly contradicting results emphasize the **need for further exploration** of the deep clay biosphere, including microbial community evolution upon disturbance and introduction of new subcommunities, as is expected during the excavation phase.

In addition, studies have so far mostly targeted Bacteria (or microbes in general), while Archaea are expected to reside or even thrive in the deep biosphere as well. Methanogenesis for example, is a trait so far only detected in Archaea. Especially in extreme environments, Archaea can even grow dominant, as mentioned in section 1.1. Also, viruses, yeasts and fungi and have not been described for the above mentioned clay layers at all. Their contribution to the microbial community could be substantial, but can only be speculated for so far.

Large emphasis in previous studies was on the activity of SRBs, linking to microbially induced corrosion of the waste canisters (section 4.2.3). In most studies targeting SRBs, at least some were indeed detected. In more recent (still ongoing and unpublished) and future work, other traits of the microbial community are being/should be addressed as well, including impact on nitrate reduction, gas consumption and production, degradation of organic matter on long time scales, radionuclide transport and cement biodeterioration. The relevance of some of these microbial processes is described below, in section 4.

4. Microbial processes and repository safety

Geological formations that are selected for radioactive waste disposal are inevitably prone to disturbance by excavation and introduction of new materials. In this respect, **the excavation disturbed zone (EDZ) and the installation of the Engineered Barrier system (EBS) itself could provide space, moisture and additional energy and carbon sources to the microbial ecosystem**. In addition, excavation and installation will most likely **introduce a new subcommunity of microbes** to the habitat as well. Microbes might even inhabit some forms of low and intermediate level waste (L/ILW), which is in some countries also eligible for geological disposal. This review will however only focus on the disposal of High Level Waste (HLW), in which microbial processes affecting the waste itself are considered highly unlikely, due to high radiation, absence of water, lack of nutrients and high temperature (Wolfaardt & Korber, 2012). Therefore, microbial processes are only expected outside the HLW.

Characterization of the indigenous Boom Clay population - if present - seems mostly useful for the interpretation of geochemical data of candidate host rocks and to provide a prospect on geochemical equilibria over longer timespans. In much lesser extent is such indigenous population important for assessing the performance of the repository materials, since the latter is likely to come with their own population of micro-organisms (Wolfaardt & Korber, 2012). Therefore, when regarding microbial effects on DGR safety, characteristics and effects of microbial processes should be assessed in general terms, not limited to those processes already observed in the host rock.

The aspects of and bilateral interactions between microbial processes in a subsurface Boom Clay environment on one hand and components and characteristics of the EDZ and EBS on the other hand, will be the main subject of the next two chapters. In this respect, the performance- and safety-relevant processes and properties of a deep geological repository, which might be affected by microbial activity, will be highlighted.

4.1. Interactions between Boom Clay and microbial life

4.1.1. Physical environment

Space and water available for microbial life

In Belgium, the studied Boom Clay has an estimated **porosity** of 30-40% (Table 3.1) and **connecting pore throats smaller than 10-50 nm diameter** (Hemes *et al.*, 2015), while in The Netherlands pore sizes are suspected to be even smaller, due to higher depth and thus higher consolidation pressure of the clay layer (Vis & Verweij, 2014). Large, isolated pores (> 0.5 µm) have been detected in Belgian Boom Clay, however (Hemes *et al.*, 2015). With micro-organisms having diameters ranging from 0.2 µm to 2 µm (Kubitschek, 1990, Tortora *et al.*, 1998), Boom Clay pore throat diameters in Belgium are generally considered too small to enable any kind of microbial migration through the clay. Although it has been observed that microbes in a low energy environment drastically decrease in size and are morphologically flexible (Duda *et al.*, 2012, Kempes *et al.*, 2012), a microbial cell should have a certain minimal size in order to harbour all essential proteins and nucleic acids to maintain life (Benner, 1999). With a minimal reported volume of about 0.009 μm^3 (Luef *et al.*, 2015) and thus a diameter around 0.2 µm, such microbial cells are still regarded as too large to pass through the largest (undisturbed) subsurface Boom Clay pores throats (for given pore throats in the Belgian case). Microbes are therefore considered to be hindered in their mobility activity by **space restriction**.

However, mobility is not be requisite for microbial activity to occur. If **diffusion** enables water soluble electron acceptors, electron donors and carbon sources to move through wetted clay pores towards niches of microbes, microbial activity might still occur. However, when nutrient availability is restricted by low diffusion rates - e.g. due to low water availability and small pore throat sizes as in Boom Clay - and restricts the microbes to have direct contact to energy sources, **their metabolism is expected to be relatively slow** (Wolfaardt & Korber, 2012). A methodology to more precisely determine the impact of clay consolidation pressure and decreased porosity on the rates and boundaries of a given microbial process, will be discussed in section 7.1.3.

Microbes have been described that exhibit other means of reaching limiting, unreachable sources and sinks of electrons. The so-called cable bacteria are infamous for producing electron conductive cables, which can be as long as several centimetres (Larsen *et al.*, 2014). These microbes use such cables among others to connect two different niches to each other, one with a favourable electron acceptor, the other with an electron donor, thereby catalysing redox reactions on a centimetre scale. However, since these cables are made up from microbial cells, limitations of space restriction still apply.

Other microbes have been described to shuttle electrons through the mineral phase (personal communication prof. Templeton, University of Colorado, VS). In such events, microbes could exchanges electrons from a source towards an unreachable sink, or towards other microbial cells, across the mineral phase. The prevalence and extent of such electron conductive behaviour in the subsurface however, is debatable.

Temperature

Temperature as such has an impact on geochemical redox equilibria, yielding more energy at higher temperature. Indeed, most microbes are assumed to become more active with rising temperatures, since the reactions they catalyse will require less energy input. As such, the higher estimated temperature of Dutch Boom Clay at 500 meters depth (ca. 27 °C) as opposed to the temperature of Boom Clay at the Mol site (ca. 16 °C) (Table 3.1), would be more beneficial for microbial activity. Upon waste emplacement however, temperatures are expected to rise locally to a maximum of 72 °C in the Belgian case and 63 °C in the Dutch case (personal communication Eef Weetjens), which is both well above the limits for non-extreme microbial life (> 45 °C), thereby **restricting microbial life to thermophiles and/or hyperthermophiles** (section 1.3.1). Temperature restriction will be discussed further, among other restrictions, in section 4.2.2.

4.1.2. Chemical environment

Boom Clay in Belgium and the Netherlands seem to differ in their pore water composition, which is of importance when defining the boundary conditions for microbial life. Table 4.1 provides an overview of the most important geochemical characteristics of Boom Clay at different locations in The Netherlands and Belgium. It is important to note that the Dutch samples were analysed based upon their availability and not necessarily for their relevance for future geological disposal. Also, geochemical analyses after mechanical squeezing might not be the most representative for the real pore water solution (De Craen *et al.*, 2004).

Table 4.1 Chemical composition of solution retrieved by mechanical squeezing from Boom Clay core samples (from Behrends *et al.* (2015))

The reported uncertainties indicate the variation between duplicate measurements. Evaluation of the calibration with independent standards were in the range of ± 5%. Measured concentrations are compared to those reported for Boom Clay pore water in Mol (De Craen et al., 2004) and in Essen (De Craen et al., 2006). For comparison, also the seawater composition according to Appelo and Postma (2005) is listed.

	Seawater	Sample 104	Sample 101	Sample 103	BC in Mol (B)	BC in Essen (B)
Na [mM]	485	443** ± 3	237** ± 4	133.1 ± 0.1	15.6	56
Cl [mM]	566	546** ± 2	408** ± 5	394.0 ± 0.1	0.5	44.1
S [mM]	29	65** ± 2	57** ± 1	3.9 ± 0.1	0.02*	4.2*
K [mM]	11	15.95** ± 0.02	10.4 ± 0.2	3.7 ± 0.2	0.2	0.7
Ca [mM]	11	47.0 ± 0.1	60.4 ± 0.7	69.7 ± 0.1	0.04 – 0.2	0.9
Mg [mM]	55	51.7 ± 0.3	42.3 ± 0.5	58.4 ± 0.1	0.05 – 0.2	2
Fe [µM]		297 ± 1	746 ± 12	3260 ± 0.1	6 – 50	70
pH	7.5 – 8.4	3.05	3.17	6.7	8.3-8.6	8.3-8.6
Alkal, [meq/ l]	2.47			0.55		

* SO_4^{2-} concentration. ** concentrations exceeded the calibration range and might have an error > 5%.

When evaluating microbial studies performed on Belgian Boom Clay samples, extrapolations towards Dutch Boom Clay, on which no microbial studies have been published so far, should heed the differences in physical (Table 3.1) and chemical (Table 4.1) characteristics of Boom Clay at different locations.

Redox reactions : energy available for microbial life

As described in section 1.2, availability of an electron acceptor and electron donor are requisite to sustain microbial life. For growth (biomass production) to occur, other nutrients, such as a source of carbon, are also needed.

Based on the available electron acceptors, the biochemical processes which seems favoured in **Belgian Boom Clay**, from a thermodynamic point of view, would be **methanogenesis and acetogenesis**, i.e. using CO_2 as terminal electron acceptor. Energetically more favourable electron acceptors like dissolved sulphate, iron(III) or nitrate have long been depleted in Belgian Boom Clay, except for Fe(III) in the mineral phase. In the Netherlands however, more dissolved Fe(II) and sulphate seem still available (Behrends *et al.*, 2015) (Table 4.1). Although some oxidation and reduction processes during sample preservation and preparation should be taken into account, overestimating these concentrations, it is hypothesized that **iron and sulphate reduction would be the dominant microbial processes in Dutch Boom Clay**. Especially sulphate reduction can be coupled with methanogenesis, as described in section 2.4, if the local redox potential is low enough. In any case, the relevance of microbial activity for sulphate and sulphide concentrations both *in situ* and during laboratory analyses, remains to be investigated (Behrends *et al.*, 2015).

For these reactions to occur, a suitable electron donor is needed. This can be either organic matter, methane, or H_2 gas. Natural H_2 has not been described for undisturbed Boom Clay environments, neither in Belgium nor in The Netherlands. In undisturbed conditions, the **primary electron donor** would therefore be the **naturally occurring organic matter**. In Belgian Boom Clay, natural organic matter is present in a range of concentrations (1 to 5 wt%) and is largely composed of kerogen

(Bruggeman & De Craen, 2011) and humified DOM, which have been evaluated so far as largely unavailable as a source of energy in their current form.

In Dutch Boom Clay, organic matter concentrations are lower, below 0.7 wt% (Behrends *et al.*, 2015). Its bioavailability is yet to be determined, so whether it can act as electron donor during e.g. microbial iron or sulphate reduction or other metabolisms, could be a subject of future investigations.

In short, for Boom Clay conditions, both in Belgium and The Netherlands, a suitable electron donor should be found among the NOM to enable microbial processes. Given the low bioavailability of both the kerogen and the DOM, **the electron donor is considered to limiting factor of microbial activity in undisturbed Boom Clay**.

Besides being the fuel for microbial activity in general, **microbial redox reactions are able to influence mobility and transport of metals, and therefore of radionuclides (RN)**, throughout the clay. Microbes that are capable of reducing metals like iron are more likely to be able to reduce RN as well, thereby in some cases altering their mobility by precipitation or dissolution. In addition, RN could sorb on microbial cells and biofilms, or show different interactions with organic matter before and after biodegradation.

Chemical restrictions: salinity and alkalinity-acidity limiting life

Intrusion of sea water has provided Dutch Boom Clay with a higher **salinity** (ca. 0.5 M) (Behrends *et al.*, 2015) than its Belgian variant. It has yet to be determined whether this is a local or more widespread phenomenon (Behrends *et al.*, 2015). In any case, as indicated in section 1.3.3, extremophiles exist which can withstand a salinity above 2 M and up to 5 M of NaCl. These extreme values are well above the sea water salinity levels that are described for Dutch Boom Clay. Marine environments are full of microbial life of all kinds of metabolisms, both aerobic and anaerobic (Azam & Malfatti, 2007, Jorgensen & Boetius, 2007). The moderately high salinity of Dutch Boom Clay is therefore **not considered to inhibit microbial processes to much extent**. Moreover, since Boom Clay is a marine sediment, any indigenous microbial cells, are assumed to be adapted to sea water salinity.

Whereas the pH of Belgian Boom Clay is fairly neutral and stable between pH 8.3 and pH 8.6, the measured pH in Dutch Boom Clay pore water from squeezing experiments is much lower and ranges from pH 6.7 to as low as pH 3.05 (Behrends *et al.*, 2015). However, the low pH samples are not considered to reflect the in situ pore water due to experimental difficulties, causing the oxidation of dissolved Fe(II). The low values are indeed countered by estimations (in the same reference) based on dilution experiments, which indicate an in situ pH of around pH 7.3 – pH 8.2 in the Dutch Boom Clay.

If a low pH would occur due to oxidation of the clay environment *in situ* (e.g. by excavation), **it is expected to inhibit microbial processes to a certain extent**, especially when reaching a pH of 3. Certain acidophiles are described to thrive at pH 2 or lower (section 1.3.2), so microbial life is not entirely impossible. As sulphate reduction can acidify the (local) environment, depending on the electron donor used (Gallagher *et al.*, 2012), the catalysing microbes are expected to be able to withstand at least some decrease in pH. Before estimating the inhibiting potential of the Boom Clay pore water pH, the real *in situ* pH should first be confirmed.

4.1.3. Microbial impact on clay mineralogy

Boom Clay, both in Belgium and in The Netherlands, contains a fair amount of Fe-containing mineral phases like smectite. As discussed in section 2.3, microbes are able to reduce Fe(III) from such phases, even when solid (Weber *et al.*, 2006). The results and reversibility of this **microbial reduction process on clay minerals like smectite** are very similar to those of abiotic reduction, and depend on the extent of the reduction (Weber *et al.*, 2006, Meleshyn, 2011). When less than 1 mmol of Fe(III) is reduced per gram clay, the increase of negative charge of the smectite layers (by 1 mmol of charge per gram clay) only leads to a minor adjustment of the clay structure and is fully reversible. When about 2 mmol of Fe(II) is being produced, the process is still mostly reversible, but leads to a decrease in swelling pressure (~40 %), an increase of cation exchange capacity (~20-30%) and a decrease of specific surface area (~30-50%). When the reduction process exceeds 3 mmol Fe(II) production per gram clay, strong and largely irreversible changes occur, including a **decrease in cation exchange capacity and a decrease in swelling pressure, all negatively influencing the barrier function** of the clay in a DGR. Microbial Fe(III) reduction leading to more than 2 mmol Fe(II) production per gram clay have not been reported however (Meleshyn, 2011).

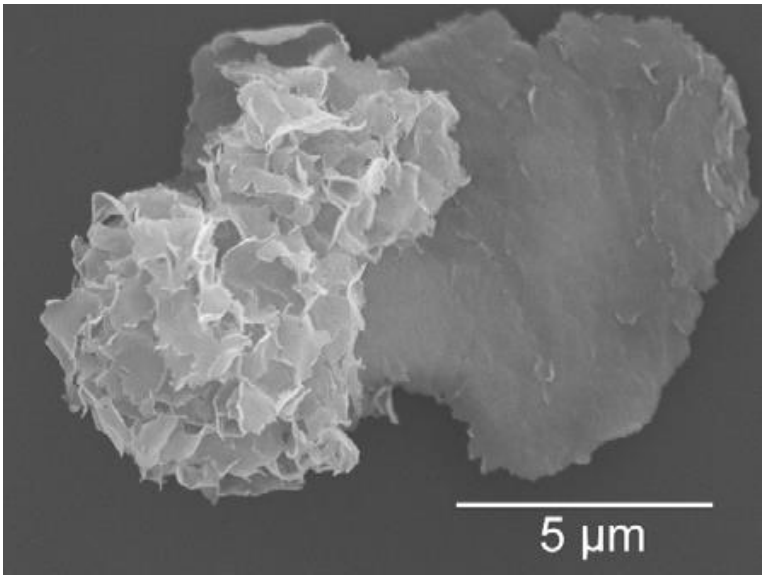

Fig. 4.1 Scanning Electron Microscopy visualization of microbiologically altered (left) versus unaltered (right) smectite (Dong et al., 2003)

Coupled to microbial reduction of Fe(III)-containing clay minerals, is the dissolution of structural Fe(II) from the mineral phase. In the presence of potassium, this results in the irreversible **conversion of smectite to illite** (illitization). This process is known to take place only at elevated temperatures in abiotic conditions, but has been described at temperatures as low as 25 °C in the presence of microbes (Kim *et al.*, 2004). This newly formed illite will precipitate directly in the pore space, thereby decreasing clay porosity and permeability. In extreme circumstances, this could lead to overpressure of the fluid and the accompanying (temporal) loss of clay plasticity and the development of fractures (Ortiz *et al.*, 2002, Meleshyn, 2011). In addition, illitization leads to **decrease in swelling pressure, cation exchange capacity and specific surface area and anion sorption capacity, again negatively influencing the barrier function of the clay** in a DGR. In this respect, it is important to note that the risk of abiotic illitization at high temperatures, has led to the

Belgian requirement of not storing radioactive waste at temperatures above 100 °C in Boom Clay (Baekelandt *et al.*, 2001).

Although Boom Clay is theoretically susceptible to microbial Fe(III) reduction at lower temperatures and detrimental consequences like illitization, **no direct evidence** has been reported **so far** on their occurrence in the deep subsurface (Meleshyn, 2011).

4.2. Interactions between microbial life and the Excavated Damaged Zone (EDZ) and the Engineered Barrier System (EBS).

The expected impact of the EDZ and the EBS on microbial activity will be described in short. First, enhancement and restriction of microbial activity by EBS and EDZ during excavation and operation and after closure will be discussed. After that, the main components of the HLW EBS with respect to microbial processes (metal canisters and cementitious materials), will be discussed. Finally, the dynamics of microbial gas production and consumption will be discussed as well, being of impact on EBS integrity.

4.2.1. Enhancement and restriction of microbial activity by EDZ/EBS, during excavation and operation

The two main factors that are considered to minimize microbial activity in undisturbed Boom Clay the most, are the small pore sizes and pore connectivity (space restriction) (section 4.1.1) on one hand, and the low energy availability (section 1.2) on the other hand. The relatively high salinity could account for a third main inhibitor, but can likely be considered to be of minor effect due to the well-spread natural adaptations of microbial life to seawater salinity. Upon disturbance of Boom Clay by excavation, inhibition by both low energy availability and space restriction is expected to decrease dramatically.

Decreased consolidation and increased water activity

First of all, excavation will temporarily provide microbial life with **increased space and water availability** to get access to energy and nutrient sources, in order to become active and mobile. Consolidation pressure will locally decrease and water activity will increase along the excavated clay surface. Microbes that have been confined for millions of years will gain the opportunity to become more active by the increased space and diffusivity. Surface colonization and even biofilm development can be expected, by both the indigenous and the introduced micro-organisms.

Upon waste emplacement and backfill, fast sealing of all voids is targeted, so this period of increased sapce will only last for a short period of time. Nevertheless, even this short period of surface and water colonization and biofilm development could drastically change the environmental settings for long term predictions and extrapolations. Especially the development of biofilms could onset corrosion, concrete attack or clay dissolution on very short time spans (weeks) that would not be expected in an abiotic setting (Meleshyn, 2011).

Oxygen, carbon and microbial intrusion

Secondly, upon excavation **oxygen** will be able to intrude the environment, and will provide microbes with a very efficient electron acceptor. Obviously, the strict anaerobic community will be inhibited or killed off by oxygen, but a net increase in activity is expected due to the high amount of energy that can be derived from oxygen reduction, and the **largely aerobic community** that is likely be introduced during excavation. Therefore, a dramatic shift in the microbial community composition

can be assumed due to oxygen intrusion followed by a gradual shift during oxygen depletion. In general, relatively active oxygen reducers are expected to colonize the environment, especially targeting surface and local aqueous environments. These subcommunities will speed up oxygen removal from the repository environment, after which a succession of facultative anaerobes is expected, amended with strict anaerobes when all oxygen is depleted.

Due to the oxidation of the clay environment, also the release of other **electron acceptors** like sulphate (from pyrite) can be expected as well, as already hypothesized by Behrends *et al.* (2015).

In addition, upon waste emplacement, the release of more bioavailable carbon sources from irradiated/oxidised (degraded) natural organic matter can be conjectured to further stimulate microbial activity by providing alternative **electron donors**. Indeed, the release of excess DOM and CO_2 upon thermal, oxidation or alkaline attack have been described for Belgian Boom Clay organic matter (Bruggeman & De Craen, 2011).

As such, **all requirements for microbial activity** – although still in relatively low amounts - could be met during operational and early closure phase.

4.2.2. Restriction of microbial activity by EDZ/EBS, after closure

The conditions that are expected in the near-field (primarily the EBS) after closure are generally assumed to significantly **reduce the number of surviving microorganisms,** whether indigenous or introduced (Stroes-Gascoyne, 2010, Wolfaardt & Korber, 2012). Undeniably, there will be extreme conditions near the surface of the nuclear waste containers, notably high radiation, high pH (and high temperature in most disposal scenarios), which will challenge microbial persistence.

Nevertheless, tolerance and even proliferation of certain types of microorganisms have been shown for all three conditions individually, as briefly described in section 1.3. Therefore, there is some likelihood of microbes surviving in the near-field and colonizing certain niches of the EBS or the EDZ, even after closure. This potential is however considered to be relatively low, given the combination of several extreme conditions, and the abrupt character of waste emplacement, the latter giving less opportunity to acclimation to the extreme conditions. Less microbes are expected to survive a combination of high heat, alkalinity and radiation, without any time to adapt or evolve (Wolfaardt & Korber, 2012).

Apart from these new extreme conditions imposed by the nuclear waste and EBS, micro-organisms residing in the deep subsurface already face challenges like low nutrient availability and small pore space. It remains unclear how such pre-adaption to harsh conditions will affect microbial survival rates. On one hand, such adaption in e.g. a dormant state could shield micro-organisms from further extreme conditions, enhancing survival, but on the other hand the long term persistence and low energy environment could weaken the cells, thereby further diminishing survival rates.

In conclusion, **the EDZ and EBS will impose both inhibition and enhancement of microbial activity**. Which mechanism will eventually prevail, can only be speculated and will most probably be determined by a succession in time of several inhibiting and enhancing factors.

4.2.3. EBS: Metal canisters

Steel and other iron surfaces can be prone to **microbial corrosion**, both in aerobic and anaerobic conditions. Given the anoxic nature of the subsurface and the limited oxygenated time expected after disposal closure, only anaerobic processes are taken into account for HLW canister corrosion. NRPs, DIRB, SRPs methanogens and acetogens, have been described to be involved in metal corrosion, but SRPs are considered to be most prominent (Enning & Garrelfs, 2014), and will thus be the focus of this paragraph. As discussed in section 3.1, presence of SRPs in Belgian Boom Clay samples (both clay and borehole samples) has been demonstrated.

In abiotic conditions, only slow chemical dissolution of Fe(II) from the metal surface takes place (Equation 4-1).

$$Fe(0) + 2H^{+} \rightarrow Fe(II) + H_2$$

Equation 4-1

At the metal surface, microbes like SRPs are able to facilitate electrochemical corrosion. Several decades ago, it was reported that the scavenging of H_2 by SRPs would change the electrochemical equilibrium, shifting it towards increased Fe(II) dissolution (Booth, 1964). Currently, this is more and more regarded as an outdated view (Enning & Garrelfs, 2014). Nowadays, SRP-induced corrosion of metal surfaces is considered the result of H_2S production, which is known to rapidly react with metallic Fe (or Cu, which is relevant in some disposal designs) (Equation 4-2).

$$Fe(0) + H_2S \rightarrow FeS + H_2$$

Equation 4-2

Other corrosive agents can also be produced by SRPs or by organisms in co-metabolism with SRPs. For example, the even more corrosive sulphuric acid can be produced from H_2S by sulphur oxidizing microbes, but only in aerobic conditions.

The rate of CMIC is most likely limited by the diffusion of aggressive species like H_2S/HS^- (produced in the near-field) towards the metal canister. Indeed, the direct attachment of microbes to the canister surface seems unlikely, due to space restrictions (in case of clay based backfill), high pH (in case of cementitious backfill or supercontainer concept), high radiation and/or high temperature at the canister surface. If, microbial colonization of the metal surface would occur, localized CMIC corrosion (called pitting corrosion) by SRPs might accelerate the process.

A common process that goes hand in hand with the production of FeS, is the development of a thin layer of FeS, which reduces, rather than accelerates Fe corrosion. This phenomenon is now agreed to be a controlling factor when it comes to metal corrosion (Enning & Garrelfs, 2014). It is however unclear how this passive layer would react to the relatively high concentrations of chloride that have been reported for Dutch Boom Clay (Behrends *et al.*, 2015).

In addition to such H_2S based chemical microbial influenced corrosion (CMIC), recently new mechanisms have been hypothesized by which microbes would be able to shuttle electrons directly from the metal surface, with the metal acting as a cathode (i.e. negatively charged electrode, providing electrons (energy) to the microbes), without the production of H_2. This phenomenon called

electrical microbially influenced corrosion MIC (EMIC) would cause higher rates of localised (pitting) corrosion than CMIC, but has only been observed in a limited number of SRP isolates (Enning *et al.*, 2012).

In conclusion, given the demonstrated presence of SRPs in Belgian Boom Clay, the presumed availability of SO_4^{2-} and the elevated Cl^- concentrations in Dutch Boom Clay (Behrends *et al.*, 2015), which could enhance pitting corrosion (Wang *et al.*, 2015), it is recommended to evaluate the likelihood of CMIC and EMIC effects on the canister surface, and to assess the maximum effect these processes would have on canister integrity. In this respect, it is also essential to further elucidate the amount of SO_4^{2-} available for SRPs and the amount of SO_4^{2-} that would be released from pyrite upon oxidation during the excavation phase (Behrends *et al.*, 2015).

4.2.4. EBS: Cementitious materials

Concrete and other high performance cementitious materials will be prevalent when constructing a geological repository. It will likely be present as tunnel liners and floors, but can also be part of the waste form (supercontainer concept), backfill and/or plugs and seals. Because concrete has been used for decades in the building industry, its stability has been described over long time spans. However, it also has been shown that microbial deterioration can cause detrimental effects on concrete structures (Bertron, 2014).

Microbial deterioration of concrete is generally regarded to be caused by the biogenic production of chemically aggressive metabolites like acids and sulphur compounds (both organic and inorganic), solubilizing the concrete ($CaCO_3$), and has been known to initiate and intensify concrete degradation (Wei *et al.*, 2013). $CaCO_3$ dissolves readily, even in weak acids, following Equation 4-3.

$CaCO_3 + H^+ \rightarrow Ca^{2+} + HCO_3^-$	*(decarbonation)*
Equation 4-3	

In addition, neutralization of acids (Equation 4-4) and carbonation (Equation 4-5) would gradually reduce the pH of the concrete surface, followed by successive microbial attack. However, carbonation as such should reinforce the concrete structure.

$2CH_3COOH + Ca(OH)_2 \rightarrow Ca(CH_3COO)_2 + 2H_2O$	*(acid neutralization)*
Equation 4-4	
$Ca(OH)_2 + CO_2 \rightarrow CaCO_3 + H_2O$	*(carbonation)*
Equation 4-5	

Other microbial attack can be caused by biofilm development and hyphen protrusion by Fungi. Microbial attack can also indirectly lead to reprecipitation of ions that were dissolved from the cement matrix by the acids. A main mineral constituent of such precipitate would be gypsum, which drastically weakens the cement (Equation 4-6). The subsequent formation of the voluminous ettringite ($Ca_6Al_3(SO_4)_3(OH)_{12}$. $26H_2O$) from gypsum would lead to microcracking (Housewright *et al.*, 2004). Deterioration as such would imply **loss of alkalinity, erosion, increasing porosity, cracking or collapse** (Wei *et al.*, 2013, Bertron, 2014).

$Ca(OH)_2 + H_2SO_4 \rightarrow CaSO_4 + 2H_2O$	*(gypsum formation)*

Equation 4-6

It should be noted also that microbial activity in high pH concrete is considered to be largely inhibited. Also in the near field, the alkaline plume would restrict microbial activity to a large extent. Also, cement as such contains relatively low amounts of carbon sources to sustain microbial life, apart from organic plasticizers which have been shown to enable microbial activity (Stroes-Gascoyne & West, 1997). Yet, as shown in section 1.3.2, alkaliphiles do exist and might remain active in an environment relevant for nuclear waste disposal, up till pH 12 (Rizoulis *et al.*, 2012, Williamson *et al.*, 2013, Bertron, 2014, Bassil *et al.*, 2015). These alkaliphiles would be the first to colonize surfaces of and voids/pores within cementitious materials. It has been described that such early alkaliphilic colonizers can also exhibit a trait interfering with e.g. metal mobilization (Williamson *et al.*, 2013). In addition, subsequent lowering of local pH by these colonizers to pH 9 could pave the way for more general species to thrive and intensify deterioration (Wei *et al.*, 2013). This infers of course that the alkaliphiles are indeed producers of e.g. biogenic acids.

The type of biogenic acid produced will also influence degradation rate. Some acids (e.g. oxalate) will form insoluble precipitates (e.g. calciumoxalate) (Housewright *et al.*, 2004). As described for metal corrosion as well in section 4.2.3, a protective layer can hence be formed from cement degradation products, shielding the surface from further attack. However, there is a difference between a purely chemical attack and a biological attack. During a chemical attack, the impervious layer will indeed slow down cement corrosion. Microbes however would be in the transition zone between the corroded and the uncorroded layer, enabling the corrosion front to keep progressing. In addition, as mentioned in section 4.2.3, it is unclear how such protective layer would react to the presence of Cl^- in the Dutch Boom Clay environment.

Microbial activity might nevertheless have a **positive effect** on cement structures. Alkaliphilic microbes have been described to precipitate $CaCO_3$ or increase pH, thereby enhancing concrete repair (Housewright *et al.*, 2004). Indeed, such biomineralization mechanisms have been tested to produce so-called self-healing cement (Wang & De Belie, 2014).

In conclusion, **the activity of microbes inside cement structures of a DGR, and within the high pH plume in the near field remains disputable**. If present and active, microbial life is expected to locally lower pH and have detrimental or enforcing effects on concrete integrity, but of unknown extent so far.

4.2.5. Gas production and consumption

Within a DGR in a clay environment, the production of excess gas would imply a treat to repository integrity (cracking), to clay plasticity (overpressure) and to isolation of the waste in general (gas breakthrough). The study of mass balances has caused waste management organisations to rethink their design in order to minimize potential gas generation and migration (Meleshyn, 2011). In case of gas breakthrough, especially volatile radionuclides (^{14}C and ^{129}I) could be released or transported along with the carrier gas (Meleshyn, 2011).

The main gas of concern is **hydrogen gas**. H_2 is expected to be produced abiotically after HLW emplacement, through radiolysis of water and organic materials, and through abiotic corrosion of the metals in waste and waste canisters (Jacops *et al.*, 2013). This gas is expected to dissolve and be transported by diffusion. If however the rate of gas generation exceeds the capacity of diffusive transport, oversaturation occurs, followed by development of a free gas phase and build-up of gas pressure (Jacops *et al.*, 2013).

As H_2 is considered to be the most dominant source of energy in an environment low in carbon sources such as Boom Clay, **microbial consumption of H_2 as electron donor is to be expected**. In such cases, even when new, inorganic gasses are formed (e.g. during sulphate reduction), the gas mass balance turns negative. According to microbial reaction stoichiometrics, the volume of H_2 consumed is larger than the produced volume of inorganic gas (section 2.5) (Libert *et al.*, 2011).

When metabolisms combining H_2 with organic matter, **CO_2 and/or CH_4** are concerned and stoechiometrics turn more complex. As depicted earlier in Fig. 2.5 (section 2.5), a range of reactions is possible. Depending on the dominance of a specific community of micro-organisms, different end products and a shift in the total net gas volume are expected. For example, a shift in community composition towards direct methanogenesis of organic matter or fermentation would increase the net volume of gasses around the EBS, while a dominance of autotrophic methanogens and homoacetogens would result in a net decrease of the gas volume. Of course, the metabolisms increasing the net gas volume could in turn serve as fuel reactions for other metabolism like sulphate reduction. While such reaction would decrease the gas volume, aggressive species might be formed which imply other safety risks (section 4.2.3 and 4.2.4).

An assessment of the estimated impact of microbial activity on the global gas balance and gas reactivity in deep subsurface nuclear waste repositories is in this stage not yet possible. Microbial research should first of all focus on the overall complex network of microbial species and on their potential metabolic pathways – of which some would involve gas consumption and generation.

4.3. Conclusion

In undisturbed Belgian Boom Clay, and by extrapolation in undisturbed Dutch Boom Clay, both the physical and chemical environment are likely to cause low activity of micro-organisms and virtually no growth. Mostly **the lack of space, water and electron donors are considered to be limiting microbial activity**.

Upon excavation and during operation, microbial activity is expected to be **enhanced**, due to the **availability of space, water and oxygen, the release of alternative carbon sources and the introduction of allochtoneous micro-organisms**. Microbial processes induced during this limited time period might divert the geochemical environment from the one expected in abiotic conditions, thereby **changing the onset** for long-term geochemical modelling efforts.

After closure, microbial activity is expected to **decrease again** towards the conditions in undisturbed Boom Clay, **further restricted by radiation, high pH and high temperature** caused by the emplaced HLW. The local survival of microbial cells catalysing slow but detrimental processes can however not be ruled out and is especially of concern when regarding long time scales in a DGR.

Among these possibly detrimental processes are the illitization of the mineral phase by Fe(III) reduction, localised corrosion of metal canisters, cement deterioration and gas production. Concerning microbially catalysed **illitization**, no evidence has been reported so far of this process taking place in the deep subsurface. Therefore, this process can be considered to be of less concern for the safety of a DGR. Microbially induced localised **metal corrosion** and **cement deterioration** however, are well-described phenomena, of which the relevance in Belgian and Dutch Boom Clay remains unclear. Both processes are thus considered of significant concern in terms of DGR safety. When it comes to microbial **gas production**, which is also of concern for DGR safety, an insight in the prevailing microbial processes in general terms is needed first, to enable to start the unravelling of the gas mass balance, either in favour of net gas consumption or net gas production.

5. Identification of knowledge gaps on the microbiology aspects of Dutch Boom Clay

Although the extent of microbial life in the deep subsurface has gained a lot of attention in the past decades, relatively little is still known about microbial processes in geological clay layers as candidate host rocks for a DGR. Those best studied today are Boom Clay in Belgium, and Opalinus Clay in Switzerland (section 3.1 and 3.2). Only in recent years, molecular tools have become available to really probe the entire microbial community in an environmental sample, as has been done for some borehole water samples of Boom Clay in Belgium (Wouters *et al.*, 2013). Investigations on microbial communities presumably indigenous to undisturbed Boom Clay cores, boundary conditions for microbial life in Boom Clay and process-directed experiments (e.g. EBS degradation) are ongoing as part of the Belgian program and within a European project (*Microbiology In Nuclear waste Disposal (MIND)*[a]), but no results are publically available yet.

5.1. Microbial community composition

Because of the different pore water composition of Dutch Boom Clay and the expected higher consolidation, extrapolations concerning microbial community compositions from the Belgian to the Dutch Boom Clay remain speculative. Especially the higher salinity and chloride concentration of Dutch Boom Clay (compared to the Belgian Boom Clay) are expected to cause not only hydro-mechanical differences (Moors, 2005, Van Geet *et al.*, 2006), but also substantial differences between both indigenous communities (if such are present at all). Even though, the existence of a common core community can not be excluded.

It should be noted that within one vertical or horizontal transect of Boom Clay, different microbial communities can reside. So much has been hinted already by the different results obtained by different authors working on the same geological layer (section 3.1 and 3.2), although this discrepancy can also partly be caused by the evolution of microbiology techniques through time, and by contamination of samples due to unsterile sampling (section 3.5).

Overall, **studies on microbial communities in undisturbed Boom Clay samples are very scarce** up till now. One could argue that it is of little importance to understand the indigenous microbial population, since repository excavation and waste emplacement will come with an introduction of allochtonous microorganism and cause dramatic effects on survival rates of both subpopulations respectively. Nevertheless, the nature of the indigenous population could give insight in the currently prevailing biological processes and boundaries, and the allochtonous population seems problematic to predict, so it seems rational to first probe the existing population in Boom Clay for its metabolic potential and survival mechanisms (section 6).

5.2. Safety risks

Among the mechanisms described in section 4.2, and as concluded in section 4.3, **microbially induced localized metal corrosion** seems of specific concern for a DGR in Boom Clay. As described in section 3.1, an SRP community has been identified in Belgian Boom Clay, and can also be expected in the Dutch Boom Clay. With little knowledge on sulphate/sulphide dynamics in Dutch Boom Clay, and only hints of microbial sulphide production (Behrends *et al.*, 2015), no predictions can be made towards the risk of localized metal corrosion of microbial nature in a DGR scenario. The impact of

[a] *Euratom research and training programme 2014-2018 under Grant Agreement no. 66188*

specifically the chloride concentration on this process remains unclear. An **experimental program targeting microbial sulphide production and the influence of the latter on corrosion**, seems due (section 7.1).

Secondly, **microbially induced cement deterioration** iseven less understood and has not been described (publicly) for Boom Clay and HLW conditions at all. Cement, as part of the waste form or the DGR structure, should keep its integrity for a long period of time, similar to the metal canister. Passivation of the metal canister even relies on maintaining a high pH in the surrounding cementitious material. It seems therefore warranted to include a **study on microbial cement deterioration in future studies** (section 7.2).

As for **gas production and consumption**, microbial community dynamics are very complex. No microbial studies targeting microbial gas production in Boom Clay have been publicly reported so far. As can be derived from Fig. 2.5 (section 2.5), shifts in the microbial community can account for both a net increase and decrease in gas volume. As a first step towards defining the microbial gas mass balance, **a baseline characterization of the microbial community composition and its metabolic properties** should be a first step (section 6).

6. Recommendation 1 : Evaluation of the presumably indigenous microbial community in samples of Dutch Boom Clay

An assessment of the microbial community in (relatively) undisturbed Dutch Boom Clay samples (both clay and pore water) is recommended to provide a starting point for further experiments. Several tools for such microbial assessments have already been developed, tested and reported for Boom Clay. Mostly the work has been done on borehole water samples (Wouters *et al.*, 2013), but similar tests on clay samples are ongoing. Such analyses of microbial presence, viability and activity should be monitored during lab scale experiments, but should preferably include *in situ* experiments as well, combining both microbiological and geochemical analysis. A summary of some recommended analyses for water and/or clay samples is provided in Table 6.1.

Table 6.1 Selection of analyses for the evaluation of the microbial community in clay or clay water samples, indicating the expected outcome, the amount of sample and time needed for each analysis, and whether the analyses can be performed on solid (S) and/or aqueous (A) samples.

Sample Type	*Analysis*	*Technique*	*Outcome*	*Sample Volume (if liquid)*	*Time*
S, A	Visualization	Microscopy, SEM	Presence, morphology of microbial cells	500 - 1000 µL	1.5 days
A	Optical density	Spectrophotometry	OD_{600nm}	100 µL	5 min.
A	Cell count/viability	Flow cytometry, staining	Number and viability of microbial cells	500 µL	60 min
A	ATP	Luminometry	Microbial activity	50 µL	30 min
S, A	Cultivation	Media, MPN, Biolog	Microbial metabolism	5 - 10 mL	weeks
A	Spore count	Microscopy, staining	Number of sporulated (dormant) cells	500 µL	60 min
S, A	DNA-extraction	Customized protocol	Microbial metagenome	100-500 mL (conc.)	2 days
S, A	Molecular	PCR, AGE, DGGE	Microbial genes, community fingerprints	-	1.5 days
S, A	Sequencing	NGS	Microbial identities, community phylogeny	-	weeks

6.1. Sampling and storage

When **water samples** volumes are limited and should serve for both geochemical and microbial analyses, an aliquot of 1-2 mL should suffice for a quick microbial evaluation. More in depth analyses can be combined with geochemistry by filtration of a water sample over a (washed) 0.22 µm membrane filter, which preferably does not contaminate the filtrate with dissolved organic carbon (e.g. Supor® PES membranes). The filtrate can hence by used for geochemical analyses, while the microbial cells will be retained on the filter membrane.

Clay samples should be taken as sterile as possible. Preferably, this inside of clay cores should be used, sampled in a sterile and anoxic environment (e.g. glove box). It is advisable to include contamination controls, especially when sampling clay cores. For some analyses needing liquid samples, microbial cells will need to be extracted first from the clay environment (e.g. by adding a saline solution and/or sonicating), taking into account considerable bias due to cells attached to the clay surface.

Investigations are preferably performed on fresh samples, so as to not get biased results upon shifts in microbial community composition during sample storage. If **storage** is necessary, it should be aseptically, in the dark, at max. +4 °C, with an anaerobic, inert headspace such as argon. It is assumed this will keep most of the microbial cells intact and alive, but will slow their activity, due to lower temperature. If cells do not need to remain intact and alive (e.g. for molecular analyses), storage at -20 °C is also possible. Cells harvested from water samples by filtration, can be stored directly on the membrane at +4 °C or -20 °C, for later analyses.

6.2. Visualization of microbial cells

Scanning Electron Microscopy (SEM) is a high resolution microscopy technique that allows a visual confirmation of microbial presence, including details on cell sizes and shapes, and associations (e.g. cell aggregates, biofilms on surfaces, *etc).* When it is used on water samples, immediately after sampling, microbial cells from sample aliquots (0.5 - 1 mL depending on expected microbial biomass) should be collected and fixed on a 0.10 µm membrane filter (e.g. polycarbonate membranes). When it is performed on clay samples, subsamples of the clay can be placed on the membrane filter, followed by a fixation step. Following the fixation step, sample dehydration and drying will be required if SEM is to be performed under vacuum setting. After optional coating with gold or carbon particles (depending on the characteristics of the SEM), visual analyses can be performed.

6.3. Quantity of microbial cells

The optical density of a sample (OD_{600nm}) is a well-used and straightforward technique to provide a rough estimation of the (cell) density of a sample. It is mostly used during microbial cultivation, to keep track of the growth process. When using OD_{600nm} measurements on Boom Clay pore water samples, it should be kept in mind that interference due to colloids and clay particles could seriously bias the result. At least some form of filtration (e.g. filtration over 1.2 µm membranes) would be advisable.

Using flow cytometry (FC), the total number of microbial cells in a water sample can be determined with or without staining of the cells. By applying specific fluorescence staining of the cells (e.g. applying LIVE/DEAD® *Bac*Light™), a distinction can be made between live and dead cells, based on membrane integrity. Live cells with intact membranes will emit green fluorescence, while dead cells with compromised membranes will emit green and orange/red fluorescence. Some thoughtfulness is in place when applying such live/dead staining, as intermediate 'injured' forms have been described as well, and the distinction between live and dead is not as straightforward as one might assume. Regarding cell staining, other options can be explored as well, like specific staining of sporulated (dormant) cells.

To allow flow cytometry, samples should be in liquid and aliquots should be filtered over 1.2 µm membranes, to remove colloids and (cell) debris from the liquid in order to avoid clogging of the device and to exclude bias by environmental particles during the automated counting. For FC analyses, only a few 100 µL should suffice.

6.4. Viability of microbial cells

By luminescence assay of intracellular adenosine triphosphate (ATP), the overall metabolic activity of the microbial community in a water sample can rapidly be estimated. In comparison with SEM and FC results, ATP analysis will allow to estimate on average which proportion of the (live) population is actually metabolically active. Due to suspected changes in microbial activity upon sampling, this analysis should be done on fresh samples, preferably the first to be performed. Preceding ATP analysis, optical density of the samples (OD_{600nm}) will routinely be checked, to provide a rough estimation of the (cell) density of the sample. This will allow estimating the need for sample dilution prior to ATP analysis, in order to stay within the reliable range of the luminescence assay. Preference should be given to ATP assays which include an internal ATP standard. This will allow to calculate ATP more reliably in samples which interfere with the typical luminescence output of the analysis.

6.5. Metabolic potential of microbial cells

6.5.1. Cultivation and isolation

Cultivation of a microbial subcommunity targets enrichment by using a growth medium that includes the electron donors, electron acceptors, and nutrients for proliferation (such as carbon, nitrogen and phosphorus sources) it needs. Non-selective media can be used, which target a broad range of generalist microorganisms. On the other hand, specific media have been developed as well, as for example to cultivate sulphate reducing microbes from subsurface samples (e.g. medium 63, DSMZ, GmbH, Germany). A growth medium can also be constructed which mimics the environment the sample originated from.

When estimations of the amount of microbial cells for a specific metabolic trait are desired, the Most Probably Number (MPN) technique can be applied. MPN is based on a series of dilutions in the specific medium, which allows to count back from the last positive dilution towards the initial amount of cells present in the sample. Incubation temperatures can be chosen for either *in situ* relevance or for growth enhancement. Cultivation of anaerobic microbes generally seems to take longer compared to aerobes (weeks compared to days), and some incubation periods can even take up to months.

A variation on the cultivation effort to probe for the metabolic potential in a fast and efficient way, which is widely used for environmental samples (Wolfaardt & Korber, 2012), could be the use of the Biolog system. This systems targets metabolic groups that are able to use certain sole carbon sources, including anaerobic assays (Biolog AN Microplate™). The use of this system for DGR microbial analyses has not been reported so far.

When cultivation efforts end up with an individual microbial strain, this strain is considered to be isolated from the sample. Such isolate can then be further characterized for its metabolic potential and boundary conditions for survival.

It should be noted however that the enrichment of a subcommunity or the isolation of a specific strain from a sample may not reflect its *in situ* activity. From Boom Clay borehole water samples for example, a range of oxygen reducing bacteria could be isolated (Wouters *et al.*, 2013). But this metabolism (oxygen reduction) is considered not to be active in the anaerobic environment, where they probably rely on their facultative anaerobic metabolic pathways to survive *in situ*. In addition, it is generally assumed that only a small fraction of microbial species is actually cultivable. More than 99 % is believed to remain undetected when using cultivation techniques only.

6.6. Diversity and Identity of microbial cells – DNA analysis

While the tests described in sections 6.2 to 6.4 are highly valuable in providing a rapid, more general screening on the presence and quantity of microbial cells in the sample, they hold only little explanatory value. DNA-extraction followed by basic molecular analyses could in that case offer more detailed insight in the microbial community and microbe identity. The molecular analyses as described below aim at providing a detailed, yet cost efficient, screening of the genetic content of the microbial community and can be applied on microbial cells retained on a filter or directly on clay samples.

DNA-based molecular analysis, will help to answer questions on the community composition and microbe identity and its correlation with geochemistry, especially when microbial presence, viability and/or activity will change coinciding with drastic changes in geochemical parameters. It is expected to yield information on shifts in the dominant metabolic properties and composition of the microbial community, based on molecular fingerprinting techniques (Fig. 6.1, for water samples; a similar pathway can be followed for solid clay samples as well). To allow a screening of fingerprints in time, a more detailed, taxonomic analysis of the first set of samples (time zero) by Next Generation Sequencing (NGS) would be highly valuable in order to link the fingerprints to an actual microbial phylogeny.

In addition, the outcome of the molecular analyses will allow to compare the microbial community in the Dutch Boom Clay with the one that was previously defined in Belgian Boom Clay samples using the same techniques (Wouters *et al.*, 2013), and with those that are currently being investigated in other borehole water samples and in clay samples.

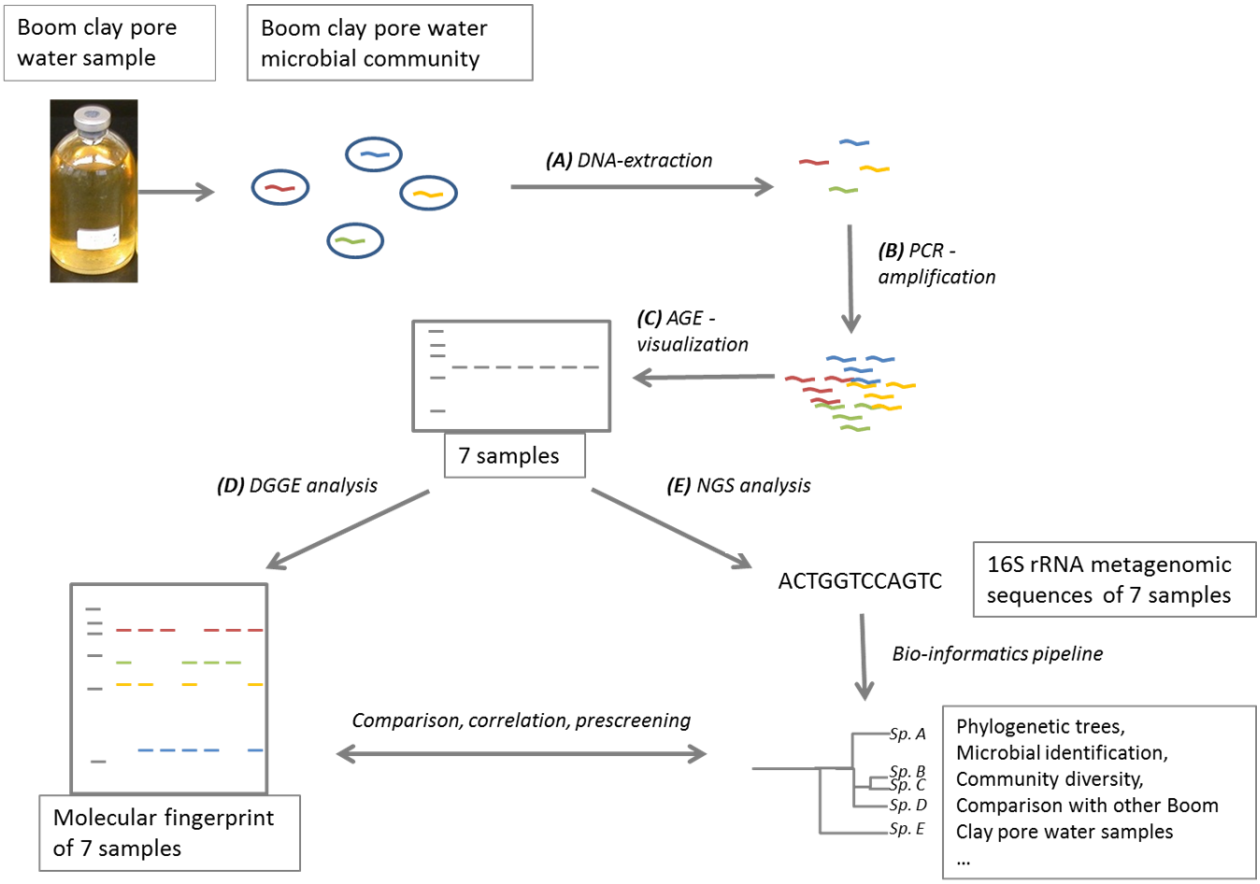

Fig. 6.1 Proposed workflow of molecular analyses on Boom Clay pore water samples (e.g. for 7 samples). A similar workflow can be used for solid clay samples, starting from a few grams of clay.

6.6.1. DNA extraction

DNA can readily be extracted from the total microbial community that was retained from the water samples on the 0.22 µm membrane filters or from clay samples, yielding the so-called microbial metagenome (Fig. 6.1 (A)). Especially for clay samples, a customized protocol for DNA extraction should be used. Generally described DNA-extraction techniques and kits tend to provide low yields

on Boom Clay samples due to *(i)* sorption of the negatively charged DNA to clay surfaces and *(ii)* interference with the natural organic matter. After extraction, DNA can be purified using size cut-off filtration, rendering it suitable for downstream analysis (PCR, AGE, DGGE, NGS) and storage at -20 °C or -80 °C.

These downstream analyses, however, are only possible if a sufficient amount of DNA is yielded. It can not be excluded that the number of microbial cells and therefore of DNA present in the samples will be low, but workable yields have been extracted from Boom Clay borehole water before, using a customized technique (Wouters *et al.*, 2013). Yields from clay samples are expected to be lower though.

A specialized DNA-extraction protocol for clay samples was developed at SCK•CEN (Moors et al., personal communication), and has been successfully used for the analysis of Boom Clay and Opalinus Clay communities.

6.6.2. Polymerase Chain Reaction (PCR) and Agarose Gel Electrophoresis (AGE)

By nucleic acid polymerisation reactions, specific parts of the microbial (meta)genomes can be multiplied a thousand fold, allowing more accurate downstream analysis of appropriate phylogenetic and metabolic genes (Fig. 6.1 (B)). Genes that can be targeted are universal housekeeping genes that will allow phylogenetic analyses (for identification), or functional genes that will indicate the presence of certain metabolic pathways (e.g. sulphate reduction). Bacteria, Archaea and Fungi can be probed for separately using a series of PCR primers.

The presence of a certain gene in the metagenome of a sample can be assessed by introducing the obtained PCR products in an agarose gel and subsequently applying an electric field, which will move the negatively charged DNA through the agarose matrix. This way, PCR fragments of different length can be visualized and checked for their presence in all samples (Fig. 6.1 (C)). After PCR-AGE, samples can be further analyzed by DGGE or NGS .

6.6.3. Denaturing Gradient Gel Electrophoresis (DGGE)

DGGE can be used to determine the molecular fingerprint of a community based on the nucleic acid composition (gene sequence)of the PCR products (Fig. 6.1 (D)). As DNA is built up of bonds between oligonucleotides A, T, C and G, which have different denaturing properties, the ATCG-composition of the PCR-fragment will determine the migration of the different pieces of DNA through a denaturing gel matrix when applying an electric field. Since PCR fragments resulting from a same gene (having the same length) can have different compositions within different microbial species, this technique allows a basic comparison of microbial community compositions for a certain gene (phylogenetic and/or metabolic). This comparison can especially be useful when comparing communities between samples, both within and between time points. A DGGE analysis as such merely provides a fingerprint and does not provide identification of micro-organisms, but it can be followed by cutting out the markers from the DGGE gel, purification and subsequent sequencing of the fragment for further identification.

6.6.4. Next Generation Sequencing (NGS)

The composition (gene sequence) of phylogenetic markers in the metagenome (16S rRNA genes) of a sample can be assessed by NGS (Fig. 6.1 (E)) and subsequent bio-informatic analysis. A customized workflow has already been optimized for Boom Clay samples (Wouters *et al.*, 2013). The aimed output of such analysis is among others a microbial phylogenetic tree, indications of species abundance, species diversity, indications of dominant versus rare species, etc. Such full view of the community can then be linked to the DGGE fingerprint of the community.

7. Recommendation 2 : Assessment of the contribution of the microbial community to specific safety risks

As concluded from the literature study (section 4.3), the microbial interference with the **highest risk** perception seems to be **microbial sulphide production**, related to **metal corrosion** in a chloride rich environment. On the other hand, the concept that is yet **least well understood** in Boom Clay conditions, is the **microbial deterioration of cementitious materials** in contact with Boom Clay and pore water. Therefore, microbiologically directed experiments targeting both sulphide production and cement deterioration will be discussed in section 7.1 and 7.2 respectively. The experimental programs recommended in each section is estimated to take up about 18 to 24 months to finish and can for the majority of the analyses (with exception of the consolidation and irradiation experiments) be performed in a standard microbiology lab that is equipped for anaerobic cultivation and handling.

7.1. Microbial sulphide production

7.1.1. Presence and potential

Following the basic screening described in section 6, first of all the absence or presence of a SRP community in the Dutch Boom Clay should be confirmed or refuted. This can be done by either or both a cultivation based approach (enriching SRPs in specific medium) or a molecular approach (targeting phylogenetic genes like the 16s RNA gene and metabolic specific genes like *dsr* and *aps*). If the presence of SRPs is confirmed, more in depth analyses of the sulphate reduction and corrosion potential can be performed.

7.1.2. Kinetics, rates and phylogeny

In an optimally performing repository, sulphide produced from sulphate by SRPs in the near- and far-field is not expected to reach the waste canisters because of slow diffusion in clay, backfill and concrete. However, in the case of a failing barrier, sulphide will have a larger probability to reach the canisters and generate localized (pitting) CMIC corrosion. It is therefore advisable to investigate the requirements needed for sulphide production in the near- and far-field.

First, the use of electron donors by the present SRPs should be studied. This can be done by amending SRP batch cultures with different amounts of organic carbon, H_2, or CH_4, and subsequent monitoring of rates of microbial growth by a combination of basic analyses, as described in section 6 and by following up sulphide production.

Secondly, the availability of these electron donors and of sulphate as electron acceptor in undisturbed Boom Clay and in the EDZ/EBS should be assessed, in order to extrapolate the growth and sulphide production rates from the batch experiments to rates expected *in situ*. These analyses would be part of an in depth geochemical assessment of Boom Clay water and solid samples, or the result of modelling of e.g. H_2 production by radiolysis.

Finally, the competition of the iron(III) reduction process (by DIRB) with sulphate reduction (by SRP), and thus sulphide production, should be assessed. If Fe(II) is produced in the sample, precipitation of FeS could mitigate sulphide migration. Cocultures of SRPs and DIRBs can be set-

up for this purpose, amended with different amounts of electron donors, Fe(III) and sulphate sources, followed up by the techniques described in section 6.

7.1.3. Boundary conditions

Since the experiments described in section 7.1.2 should be done in semi-optimal conditions for microbial growth, yielding maximum activity rates, the boundary conditions for all reactions, or at least the rate-limiting steps, should be assessed in a second phase. These boundary conditions in Dutch Boom Clay and disposal conditions are expected to be space restriction, salinity, chloride concentration, alkalinity, heat and radiation. The effect of most of these boundary conditions can be assessed in monitored batch experiments, which are amended with varying concentrations of salts and hydroxides, or incubated in the presence of a heat or radiation source.

As for the assessment of space restriction, oedometers can be used to study the behavior of a clay sample exposed to a sequence of varying consolidation pressures. The evolution of the position and motion of an oedometer piston indicates whether the clay sample in an oedometer cell is in geomechanical equilibrium with the imposed consolidation pressure. By equipping the cell of an oedometer with a series of water lines, it is also possible to simultaneously mimic groundwater flow through the studied clay sample, or to feed the present microbial community with a constant nutrient flow, keeping all but the consolidation conditions optimal. Such set-up can be used to study the effect of consolidation on space/transport restriction for activity/metabolism, mobility, and proliferation of microorganisms. In addition, the experimental design allows a simultaneous study of the availability of a energy source, electron acceptor and essential components (e.g. C-source) as a function of increasing consolidation pressure which is correlated with pore size reduction. In the case of sulphate reduction, metal surfaces could be placed inside the clay cores, enabling a check for actual pitting corrosion after dismantling of the set-up.

7.2. Microbial cement deterioration

As indicated in section 4.2.4, the integrity of cementitious materials should be ensured over a long period of time. Microbial activity, although expected to be inhibited by high pH, might affect the performance of cement on the long term, mostly by either *(i)* the production of biogenic acids, thereby lowering pH and/or enhancing calcium leaching (a detrimental effect), *(ii)* the enhancement of carbonation, thereby clogging the cement pores (a desirable effect) or *(iii)* minor processes, like biologically induced sulphate release, triggering the production of the voluminous ettringite (also detrimental).

7.2.1. Kinetics, rates and phylogeny

It is therefore recommended to estimate the dominant metabolic processes of microbial communities indigenous to Boom Clay or communities known to thrive at high pH, with respect to their impact on cement, being either deterioration (pH lowering, calcium leaching) or improvement (carbonation) of its barrier function. In a lab scale batch set-up, cementitious reference materials can be subjected to incubation in the presence or absence of Boom Clay (water or solid) or a microbial inoculum that is accustomed to high pH. Microbial activity, biofilm development and overall shifts in microbial communities can be monitored as described in

section 6, while cement integrity could primarily be monitored by electron microscopy and chemical analysis of the liquid phase.

7.2.2. Boundary conditions

Similarly to the experimental plan proposed in section 7.1.3, a range of parameters can be tested in batch experiments, to estimate the boundary conditions of the processes *(i)* relevant to the cementitious environment (neutral versus high pH, different bicarbonate concentrations, addition of cement plasticizers, which can also be used as carbon source by the microbes) or *(ii)* relevant to the total Boom Clay/EDZ/EBS environment (space restriction, salinity and chloride concentration, heat, radiation, *etc.*,).

8. Concluding remarks

The overall potential for microbial activity in undisturbed Dutch Boom Clay is considered low, mostly due to its highly consolidated and low energy nature. Upon excavation and waste disposal, both opportunities and inhibitory factors arise, shifting this presumably dormant community towards a speculative state of heightened activity at first, due to increased space and water availability, oxygen penetration, and introduction of viable microbes. After waste emplacement and closure, rapid oxygen consumption and near extermination of the near-field microbial community is expected due to the nature of the EBS and radioactive waste (e.g. high pH and radiation), and the sealing of any voids.

Nevertheless, even this short period of presumed microbial activity could drastically change the environmental settings for long term predictions and extrapolations. Especially the colonization of clay and EBS surfaces could onset clay dissolution and deterioration of metals and concrete on very short time spans. Once colonization of a surface is established and damage has been done, it seems reasonable to assume that the ongoing processes would prevail longer than assumed, although at lower rates, or that otherwise unexpected abiotic processes could be triggered. Overall, many uncertainties remain concerning the survival and potential of microorganisms in a Boom Clay, EDZ and EBS environment.

To tackle some of these uncertainties, it is recommended *(i)* to assess the microbial population and its boundary conditions for survival and activity in Dutch Boom Clay as such, and *(ii)* to simulate and monitor microbial processes underlying metal and cement corrosion for the Dutch scenario. Apart from such microbiologically directed programs, basic microbial analyses are recommended to be added to experimental set-ups with an abiotic focus, in order to either ensure sterility or to facilitate interpretation of (unexpected) geochemical data.

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