Table of Contents

List of Figures

List of Tables

List of Abbreviations

ATP	Adenosine triphosphate
DGGE	Denaturing gradient gel electrophoresis
DNA	Deoxyribonucleic acid
EAC	Equivalent active cells
EDTA	Ethylenediaminetetraacetic acid
GC-content	Guanine-cytosine content
HADES	High-Activity Disposal Experimental Site
HLW	High Level Waste
LILW-LL	Low-and intermediate level long-lived radioactive waste
MF23	Morpheus-23, Boom Clay borehole water
MIND	Mircobiology In Nuclear waste Disposal
$NaNO_3$	Sodium nitrate
Na_2SO_4	Sodium sulphate
ONDRAFT/NIRAS	Organisme National des Déchets Radioactifs et des Matières Fissiles / Nationale Instelling voor Radioactief Afval en Splijtstoffen
PCR	Polymerase chain reaction
PEX	Potassium Ethyl Xanthogenate

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Summary

Introduction: In Belgium, Boom Clay is a potential host rock for the geological disposal of radioactive waste. Part of this radioactive waste is immobilized in a bituminous matrix which typically contains also large amounts of soluble salts (e.g. sodium nitrate and sodium sulphate). Over time, water will infiltrate into the waste drums resulting in the solubilization and leaching of these salts and organic compounds from the waste. This could affect the redox conditions present in the Boom Clay and consequently decrease its reducing capacity. Sulphate is a known electron acceptor for microorganisms and most of the other leachates from bituminized waste are also biodegradable. In this study, we investigated whether the microbial population present in Boom Clay borehole water is able to reduce sulphate in the presence of relevant organic electron donors.

Material and Methods: Batch experiments were performed in anoxic septum bottles with Boom Clay borehole water as water environment and as inoculum. Sulphate was added as electron acceptor and acetate or formate were added as electron donors. Acetate and formate concentrations were measured spectrophotometrically, while the microbial population was followed up by intracellular ATP measurements and flow cytometry, PCR and DGGE.

Results: A significant decrease in formate was observed from day 2 onwards, while no decrease for acetate over the 84 days of testing was observed. Interestingly, without the addition of sulphate as electron acceptor, a significant decrease in formate was observed after 37 days. Microbial activity and cell count increased during the first week, but stayed constant onwards. There was variation in the bacteria population with time in all the samples, while the archaea population was present only at day 0, and not in all the samples of day 84.

Discussion and conclusions: Based on our results, microorganisms present in Boom Clay borehole water can probably reduce sulphate. Furthermore, formate is more favorable as electron donor compared to acetate in this process. Finally, we observed a change in the microbial population with days. However, it should be investigated if this can occur in relevant *in situ* conditions.

1 Introduction

The environment is made up of many different substances that could be harmful to life. Waste is one of such substances that can negatively affect living organisms if not well treated or contained in a sustainable way. Depending on the source, radioactive waste products can contain radiologically as well as chemically toxic substances that can be hazardous for a significant length of time, hence, requiring a specific management approach. Poor disposal of radioactive waste poses a threat to life, thus there is need for delicate precautions when designing a radioactive waste disposal system. This will ensure a safe environment for humans and living organisms in general, now and in the future, without imposing unacceptable burdens on the future generations. Worldwide, geological disposal is regarded as the best option for the disposal of radioactive waste. A combination of engineered and natural barriers between the waste and the environment should prevent the radionuclides from reaching the surface in such concentrations that could pose an unacceptable risk for humans and the environment. However, it could be detrimental if the natural or engineered barriers are compromised and therefore, next to the physical and chemical disturbances in the repository, the possible microbial interactions with the engineered barriers need to be investigated. This study will focus specifically on the Belgian situation for the disposal of low-and intermediate level long-lived radioactive waste (LILW-LL).

1.1 Sources and classification of radioactive wastes in Belgium

Radioactive waste in Belgium is managed by ONDRAF/NIRAS (the Belgian Agency for Radioactive Waste and Enriched Fissile Materials) that designs how radioactive waste is processed and treated or conditioned, and also how it is classified (1). In Belgium, there are three main sources of radioactive wastes which include: the exploitation of nuclear power reactors, the reprocessing of spent fuel, and also from the nuclear research performed at research institutes (2). Radioactive wastes are inevitably produced from the generation of electricity, industrial applications, research facilities and medical infrastructures. In Belgium, radioactive wastes are classified into three categories. Category A are disposed at the surface and is made up of short-lived Low intermediate level waste, category B is composed of LILW-LL, and category C is made up of high level waste (HLW) (3). These categories are further divided into twenty classes that are finally subdivided into sixty families. Each waste family forms the waste packages that are being stored at the disposal site.

1.2 Boom Clay

Boom Clay is regarded as a candidate host rock for the geological disposal of LILW-LL (4). This is because Boom Clay has a radionuclide retention capacity with very low water permeability, and a geochemical behaviour that renders the Boom clay formation to have a long time span delay migration of radionuclides in the biosphere (5). Also, Boom Clay has physico-chemical characteristics that limit the migration of contaminants ($NaNO_3$, heavy metals, organic molecules) that leached to the biosphere (6). It also has a self-sealing capacity which permits it to respond to mechanical damage (7). There also exists another type of clay known as the Ypresian clay which is considered as an alternative host formation in Belgium (8) .Boom Clay has been investigated in detail in the HADES (High-Activity Disposal Experimental Site) underground research laboratory in Belgium (6). Situated in the core of the Boom Clay, at a level of 230 meters below sea level, HADES provides unique access to Boom Clay. Boom Clay borehole water is made up of different chemicals with varying concentrations at a temperature of 16ºC as shown in table 1 (6).

Table 1**.** Average Boom Clay borehole water composition at a temperature of 16°C (6)

component	**Concentration**	**component**	**concentration**
HCO_3^-	880mg/l	SO_4^{2-}	2.2mg/l
Na^+	359mg/l	Ca^{2+}	2mg/l
Cl^-	26mg/l	Mg^{2+}	1.6mg/l
K^+	7.5mg/l	Br^-	0.6mg/l
Si	3.4mg/l	Fe	0.2mg/l
F^-	3mg/l	Al	0.0006mg/l

1.3 Composition of low-and intermediate level radioactive waste

The Belgian LILW-LL is made up of organic substances which contains about 90% bitumen (9) . Bitumen is a viscous liquid found naturally in crude oil after the distillation of petroleum. It exists in many forms, it is solid when cold and liquid at high temperature. It is used for road construction, and also as an encapsulation matrix for LILW-LL radioactive waste (10). Bitumen is made up of hydrocarbons and some quantities of heterocyclic compounds with functional groups containing oxygen, sulphur and nitrogen atoms, and metals (vanadium, iron, nickel) (11). There are mainly four chemical groups identified in bitumen with different proportions. i.e. aromatics (40-65% wt), resins (10-25%), asphaltenes (5-25%) and saturates (5-20%) make up the total percentage of

bitumen (12). In addition to bitumen, organic substances of LILW-LL are comprised of 2.5% cellulose (paper, cotton, wood...), 2% non-halogenated polymers (resin and polyethylene), 4% halogenated polymers. In addition, the LILW-LL could also contain a presumably low amount (percentage not yet clear) of citrate, EDTA, tartaric acid, oxalic acid and Tributyl phosphate (9). In Belgium, there are two types of bituminized waste: The soft and the hard bitumen. The soft bitumen is conditioned in a distilled matrix, while the hard bitumen also known as Eurobitum is conditioned in a matrix of blown bitumen and has a significant fraction of about 80% of the LILW-LL (9). Eurobitum waste is encapsulated in a bitumen matrix, comprising of 60.6% bitumen and 39.4% waste (13). The Eurobitum waste also contains some salts such as sodium nitrate (20-30% wt) and calcium sulphate (4-6% wt) (13).

1.4 The illustration of the waste disposal system

The LL-LILW system will be constructed in such a way that, bituminised waste is placed in carbon or steel drums, which are immobilized in mortar in a concrete container (fig. 1a) (4). These containers will then be stored in the geological disposal facility in the center of the Boom Clay layer (fig. 1b) (4) . The monoliths (massive stones) are placed in horizontal galleries lined with concrete wedge blocks.

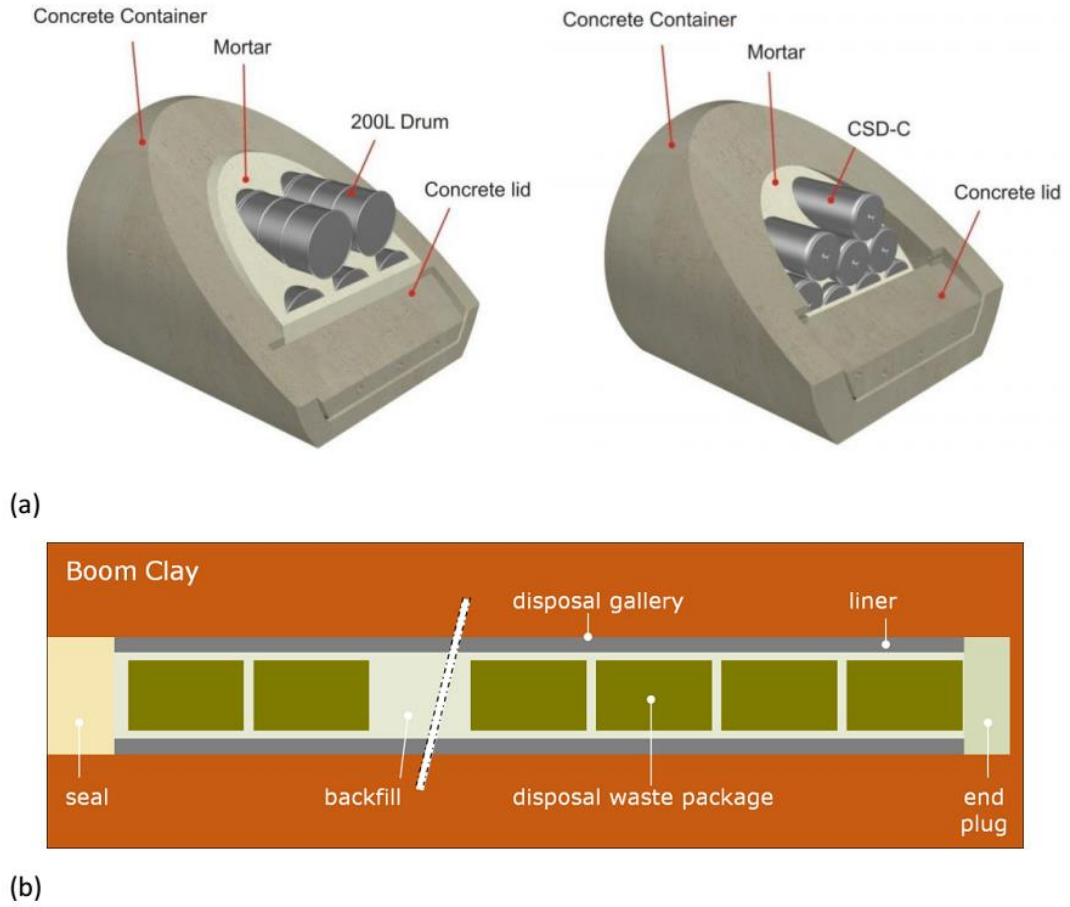

Figure 1: Schematic illustration of (a) cross sections through category B waste monoliths containing drums or canisters as primary waste packages and (b) configuration of waste package within a disposal gallery for the disposal concept in Boom Clay proposed in Belgium (4).

1.5 The activity of microbes in the repository

1.5.1 Microbial presence in the repository

The provenance of microorganisms in Boom Clay borehole water is not clear, nor whether they are indigenous or mostly introduced. However, Microorganisms can be present in geological disposal systems due to natural processes such as the formation of fissures and cracks, infiltration of foreign water or because of flooding, and also due to the excavation of the geological repository (14). Some studies have recently been performed, showing some presumptive reasons for microbial presence in the repository (15, 16). Microorganisms are speculated to have influences on waste form evolution *in situ*, multi-barrier integrity and ultimately radionuclide migration from the repository into the host-rock. It is therefore necessary to characterize and measure the effect of microbial activity on relevant processes affecting the safe disposal of radioactive waste.

Previous research has shown that Boom Clay borehole water contains a wide community of metabolically active microorganisms (15), and also, microorganisms are known to be present in other possible geological disposals of nuclear waste (e.g. Bentonite, Opalinus Clay, Boom Clay) (16-18). Different types of bacteria have been identified in the Boom Clay borehole water, such as the Eubacterial genera; *Acinetobacter, Clostridium, Desulfotomaculum, Propionibacterium, Pseudoalteromonas and Pseudomonas*. In addition to these microbes, sulphate-reducing bacteria were also identified (16, 19). Sulphate-reducing bacteria are microorganisms that use sulphate as their terminal electron acceptor. Moreso, some extremophilic prokaryotes known as archaea (20) may be able to survive in the geological repository since the repository has harsh surviving conditions (anoxic, high salinity, high pH…) (21, 22). Microorganisms can as well be beneficial in the repository. For example, methane-forming bacteria and to a lesser extent sulphate-reducing bacteria aid in the reduction of hydrogen gas generated by the anaerobic corrosion of the steel drum (23). For microbes to be able to survive in the repository, microbial metabolic activities must occur. Microbial metabolic activity requires an electron acceptor and an electron donor. An electron donor is a reducing agent that causes the gain of electron by a molecule (atom or ion), while an electron acceptor is an oxidising agent that causes the loss of electron by a molecule.

1.5.2 Microbial degradation of bitumen

There is a possibility for microorganisms to degrade bitumen (24). However, the reaction kinetics and the extent of degradation is unknown (9). The rate of biodegradation of bitumen depends on whether they are exposed to aerobic or anaerobic conditions. It has been reported that the rate of microbial degradation of bitumen is greater in aerobic conditions than in anaerobic conditions (24). Also, microbial degradation of bitumen depends on the metabolic characteristics of the microbes, the chemical composition of the bitumen and some other chemical and physical parameters (25). Studies have shown that biofilm is formed during the degradation of bitumen (25, 26). A biofilm is formed when groups of microorganisms stick to each other and adhere on surfaces (27). In the case of bitumen biodegradation for example, microbes can adhere on the bituminised surface, causing the bitumen to become porous (25).

1.5.3 The possible influences of the salts that leaches from bitumen on microorganisms

Large amounts of relevant selection of electron acceptors such as sodium nitrate ($NaNO_3$) and to a certain extent sodium sulphate (Na_2SO_4) leaches out of the bituminised waste. In this study, some electron acceptors were selected based on their high concentrations that leaches out of the bituminised waste, thus were termed relevant selection of electron acceptors. It is anticipated

from calculations that the concentration of $NaNO_3$ within the monolith during the first approximate 300years will be above 1M (28). However, closer to the waste drums, the concentrations can be up to a few molars (28).

Since microorganisms can use nitrate as their terminal electron acceptor, the nitrogen cycle will be affected. Organisms can only utilize Nitrogen when converted from the inert form (N_2) to a more chemically available form such as ammonium(NH_4^+), nitrate (NO_3^-), or organic nitrogen (e.g. urea-$(NH_3)_2CO$). Hence, a proper recycling of Nitrogen in nature is necessary for its bioavailability to all kingdoms of life. Among the many electron acceptors for energy sources, nitrate is mostly preferred by microorganisms due to its relatively high standard redox potential (29). In summary, the nitrogen cycle consists of a chain of reactions namely; Nitrogen fixation, nitrification, denitrification, and ammonification (Fig. 2).

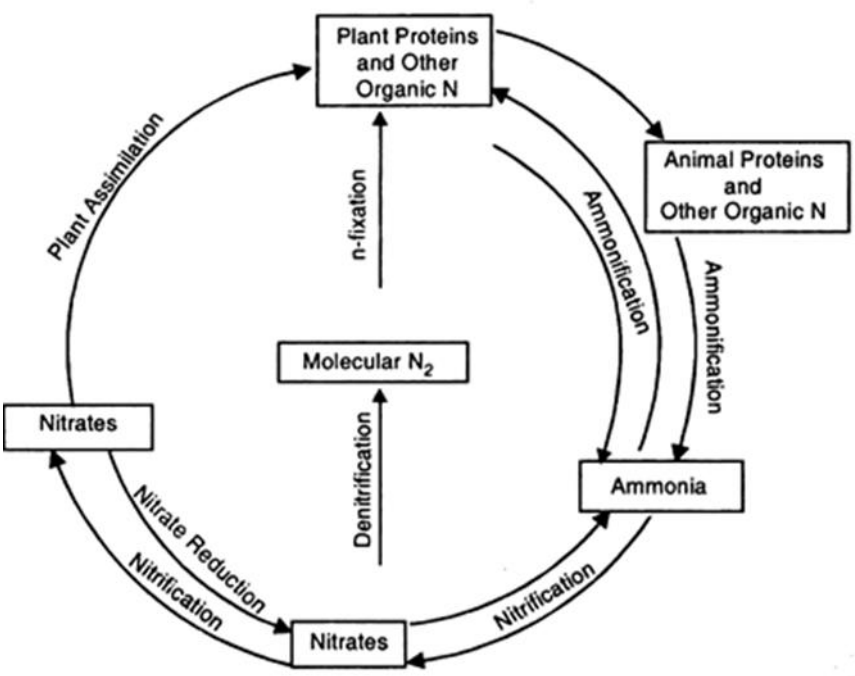

Figure 2 : The Nitrogen cycle in the ecosystem, showing the various steps involved from nitrification to ammonification. Adapted from Nikita D, www.biologydiscussion.com.

In brief, for Nitrogen fixation, certain bacteria particularly of the genus *Rhizobium,* convert Nitrogen to ammonium and these bacteria are often in symbiosis with certain plants. Whereas, Nitrogen uptake is the utilization of ammonium to organic Nitrogen and the reverse is true for ammonification as seen during decomposition. Nitrification is the conversion of ammonia to nitrate and this occurs only in the presence of Oxygen. For denitrification, oxidized forms of nitrate such as nitrite and nitrate are converted to dinitrogen and this process is mostly carried out by

denitrifying bacteria from where several intermediates are formed (nitric oxide, nitrous oxide and nitrous acid). Denitrification is assumed to be the main pathway for nitrate removal probably because it is the most studied (30). In anaerobic conditions, as in the geological repository, denitrification will mostly occur where bacteria will utilize nitrate contained in bitumen as a terminal substitute electron acceptor. Although nitrite is present only in small amounts in the repository, it is speculated that nitrate (NO_3^-) will be converted to nitrite (NO_2^-) as in the denitrification process in some years to come. This therefore, classifies nitrite as a relevant electron acceptor as well. As earlier mentioned, large amounts of nitrate will be released from the Eurobitum since Boom Clay borehole water will gradually infiltrate the Eurobitum. Microbes will therefore be influenced as a result of the induced osmotic pressure generated.

In addition, sulphur which occurs in two amino acids, cysteine and methionine is an essential element in living systems as it occurs in many vitamins and essential metabolites. Microbes can transform sulphur from its most oxidized form (sulphate or SO_4) to its most reduced state (sulphide or H_2S), (fig. 3) (31). Some unique groups of procaryotes and procaryotic processes are involved in the cycling of sulfur. For example, two distinct groups of procaryotes oxidize H_2S to S and S to SO_4. The first is the anoxygenic photosynthetic purple and green sulphur bacteria that oxidize H_2S as a source of electrons for cyclic photophosphorylation. The second group is the colorless sulfur bacteria and a group of Archeaea which oxidize H_2S and S as energy source. In both cases, these microbes can completely oxidise H_2S to SO_4. In a process called litho- or phototrophic sulfur oxidation. Such microbes are usually found around thermal vents and hot springs that are rich in H_2S (31, 32), and they may also be termed acidophiles, as they acidify their environment by producing sulphuric acid. In anaerobic environments SO_4 and S may be used obligatorily by sulphate-reducing bacteria producing H_2S. Bacteria (and plants) assimilate S as SO_4 and convert it to sulfide (SH) (33). Bacteria can remove the sulfide group from proteins as a source of S during decomposition which completes the sulfur cycle (32) (fig. 3).

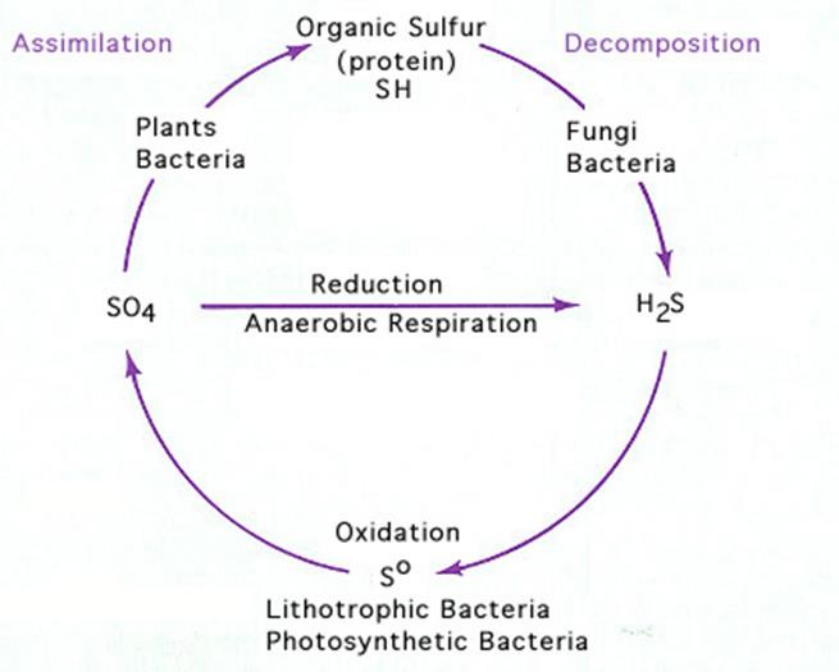

Figure 3: The sulfur cycle showing the major processes of assimilation, decomposition, oxidation and reduction (32)

In addition to the leaching out of sulphate from the bituminised waste, during construction and experimental coring of the gallery, pyrite (FeS_2) is being oxidized in Boom Clay, thus increasing the level of sulphate (34). As a result, sulphate reducing bacteria will in turn used sulphate as a terminal electron acceptor.

Another component of the engineered barrier that will also be used in the construction of the geological repositories are cementitious materials. Although cement is not the focus of this study, it has an essential role in pH variation (35), and is equally a concrete material commonly used for house constructions. The use of concrete leads to high alkaline conditions (pH > 13) which can be largely inhibitory for microbial activity (36). However, the pH will drop gradually to pH 10 in a long run (ca. 10^5 years) (35). In addition, several processes are expected to reduce the pH in the future. Moreover, the pH-lowering processes are expected to lead to niches were the growth of microorganisms are possible (37). For example, if the concrete is colonized by acidophilic microbes, they can produce acids which can lead to a decrease in pH and also promote the colonization by other microbes that can dissolve and disintegrate the concrete, and therefore the entire disposal system (38).

1.6 The Research Plan

This our study focused specifically on the Belgian situation. The goal of this study was to investigate whether the microbial population present in Boom Clay borehole water is able to

reduce sulphate in the presence of relevant organic electron donors. The derivatives of bitumen such as acetate and formate were selected as the relevant electron donors, while sulphate which leaches out of the bituminised waste was selected as the relevant electron acceptor.

To achieve our objective, before the start of the sulphate batch experiment, standard protocols to extract DNA were compared. In addition, an attempt was made to optimize a method to measure the concentration of oxalic acid. Afterwards, the sulphate batch experiment was performed. Boom Clay borehole water was inoculated with and without microbes in anaerobic conditions in septum bottles. In this same medium, bitumen derivatives (acetate and formate) and the electron acceptor (sulphate) were added. The concentration of formate and acetate were then analysed. This was followed by the analyses of the metabolic activities (intracellular ATP) and the microbial cell count. In addition, molecular analysis that was optimized, was further used to investigate the changes in the microbial community.

2 Material and Methods

2.1 Study site and sample collection

The research facility HADES of SCK.CEN (Mol, Belgium) accesses the Boom Clay which is located at a depth of 225m below sea level. Boom Clay borehole water was collected using a Piezometer (i.e. instrument for measuring the pressure of liquid or gas) named TD-11D (MORPHEUS, MF23) as described by De Crean *et al*., 2004 and Wouters *et al*., 2013 (fig. 4a) (6, 15). Another Boom Clay borehole water (TD-116E, previously known as SPRING116) was filtered using a 0.22-µm filter and, sterilized by Autoclaving at 121ºC. It was later filled into anoxic septum bottles, and placed in the anaerobic glove box overnight to render it anoxic. The sterile Boom Clay borehole water was also used as water environment to carry out the experiments (fig. 4b).

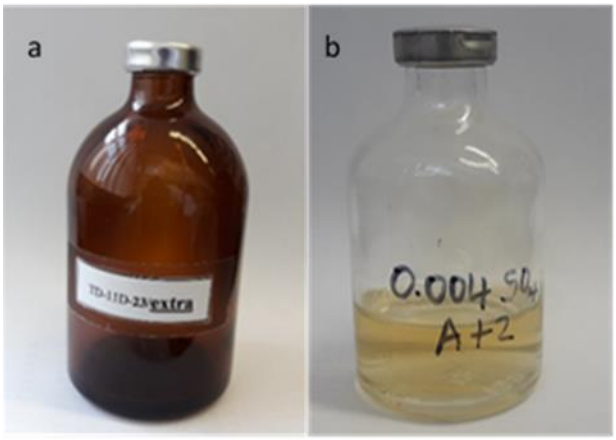

Figure 4: Boom Clay pore water sealed in septum bottles in anaerobic conditions (a) Boom Clay pore water known as MF23 containing the microbial population that was used as an inoculum during the experiment, (b) An example of a Boom Clay borehole water as water environment used in the sulphate batch experiment.

2.2 Comparing different DNA extraction methods

Before the start of the sulphate batch experiment, three different methods for DNA extraction were compared from which the best method was used. The samples (MF23 Boom Clay borehole water) were centrifuged to obtain the microbial pellet, followed by the use of the various procedures;

2.2.1 QIAGEN protocol for DNA extraction

To extract the microbial DNA population, QIAGEN protocol, QIAamp DNA Mini Kit 250 (QIAGEN GmbH, Germany) for DNA purification was used per the manufacturer instructions.

2.2.2 Customized Potassium Ethyl Xanthogenate DNA extraction method

This method is based on chemical lysis, whereby, Potassium Ethyl Xanthogenate (PEX) was used as the main lysing buffer. This PEX extraction method is a customized protocol designed at

SCK.CEN, and DNA was successfully extracted from microorganisms in Boom Clay borehole water as described by Wouters *et al*., 2013 (15). The microbial cell (pellet) were lysed by adding 900µl of autoclave lysing buffer containing potassium ethyl xanthogenate (0.062M), Tris-HCl (0.1M, pH 8.0), ammonium acetate (0.8M) sodium dodecyl sulphate (0.069M) and disodium-EDTA (0.2M). This was followed by incubating (70ºC) the mixture overnight. The day after, samples were centrifuged at 12000×g for 10 minutes. 750µl of the PEX-supernatant was transferred to 2ml Phase Lock Tubes (containing Phase Lock GelTM). 100µl of vitamin solution (1M thyamine hydrochloride and 1M pyridoxine hydrochloride) was then added in the PLG tube and incubated at room temperature for 1 hour. DNA was extracted by adding 750µl of phenol:chloroform:isoamyl alcohol (25:24:1), then centrifuged for 10 minutes at 12000×g to separate the phases. The aqueous phase containing DNA was transferred to a PLG tube (new), followed by the addition of 750µl of chloroform:isoamyl alcohol (24:1), it was then centrifuged for 10 minutes at 12000×g to separate phases again. The aqueous top phase containing the DNA was transferred to a new 1.5ml Eppendorf tube, 8M LiCl (750µl) and 2-propanol (750µl) were added, mixed (vortex) and incubated at room temperature for 1 hour (to allow precipitation of the DNA). Samples were then centrifuged for 30 minutes (at 4ºC) at 16000×g, followed by adding 1ml of 70% ethanol (to wash the pellet and eliminate 2-propanol) and incubated at room temperature for 30 minutes, then finally centrifuged for 30 minutes (at 4ºC) at 16000×g. The supernatant was discarded, DNA pellet was air dried and subsequently 500µl of nuclease free water was added to the DNA pellet.

The DNA was purified using the Amicon 30K filter cartridge (UFC5030BK) via some centrifugation steps. 500µl of the solution containing the DNA was centrifuge for 10 minutes at 14000×g and the filtrate was discarded. A double step of 450µl pure water (DNAse and RNAse free) was added in the Amicon filter and centrifuge for 10 minutes at 14,000×g. The Amicon filter was then placed upside down in a new Amicon collection tube and centrifuged for 2 minutes at 1000×g and a volume of 30-40µl containing DNA was obtained.

2.2.3 Customized Clay Lysis DNA extraction method

Another clay lysis DNA extraction customized method was used as described by Hurt Jr et *al*., 2014 (39) where they showed improved yield in DNA extracted from microorganisms in sub-surface clay environment. Summarily, at the initial step, the cell pellet was washed with phosphate buffered saline (PBS) and subsequently lysed with clay lysis solution. Clay lysis solution was made using 4M guanidine isothiocynate, 1M PBS (pH 7.2) and 10µl/ml 2-mercapthoethanol. This clay lysis solution was used to suspend the bacteria pellet in PBS, and then centrifuged at

10,000×g for 1 minute. An extraction buffer (6.4M urea, 1M NaCl, 10mM EDTA and 100mM MOPS at pH of 7) was diluted 10 times with water and then added to the sample and centrifuged at 4500×g for 10 minutes. Equal proportion of Chloroform (isoamyl alcohol) and Detergent (5% cetyltrimethylammonium bromide, and 5M potassium acetate at pH 5.2) were added, centrifuged at 7100×g for 20 minutes. Finally, Isopropyl alcohol was added to precipitate the DNA and then centrifuged in the Amicon 30k filter at 16,000×g for 20 minutes. This was followed by the DNA purification step using the amicon filter as described in the customized PEX method above.

2.3 Optimization for oxalic acid measurement

Before the beginning of the sulphate batch experiment, oxalic acid measurement was optimized using the following methods.

2.3.1 Oxalic acid colorimetric assay

To measure the concentration of oxalic acid, the oxalic acid colorimetric acid kit (Sigma-Aldrich, USA) was used. In this method, the concentration of oxalic acid is determined by coupled enzyme reaction that leads to the formation of a colorimetric product which is proportional to the concentration of oxalate present. In brief, 0, 2, 4, 8, and 10µl of 1mM oxalate standard solution were placed in a 96well plate, creating 0 (blank), 2, 4, 6, 8, and 10nmol/well standards. Oxalate assay buffer was added to each well to bring the volume to 50µl. Thereafter, 2µl of oxalate converter were added in each well and incubated for 1hour at 37ºC. Subsequently, 50µl of a master reaction mix (containing: 46µl oxalate development buffer, 2µl Oxalate enzyme mix and 2µl oxalate probe) was then finally added in each well and incubated in the dark for 1hour at 37ºC after which the absorbance of oxalic acid was immediately measured using a CLARlostar (BMG LABTECH GmbH, Germany) at a wave length of 450nm.

2.3.2 Kinetic spectrophotometric measurement of oxalic acid

Oxalic acid measurement was optimized using a second method as described by Ensafi. A *et al*., 2001 (40). The principle is based on the absorbance of safranin at 530nm which is inversely proportional to the concentration of oxalic acid in a reaction oxidized by dichromate in the presence of diluted sulphuric acid. Sulphuric acid, safranin and dichromate were initially placed in a water bath at 60ºC before mixing them together. The reaction mixtures were prepared in 96 well plate and measured in a total volume of 225µl containing, 20µl diluted sulphuric (1M), 10µl safranin (8.4375×10^{-4}M), 25µl test sample (containing oxalic acid) and 145µl distilled water. This was followed by the automated addition of 25µl dichromate (0.1M), then the absorbance (530nm) was measured with the CLARlostar at the 0.5^{th} minute and at the 5^{th} minute. The absorbance

difference was calculated and the standard curve was plotted from which the concentration of oxalic acid was supposed to be calculated. In addition, the volume (15µl, 20µl, 25µl) of safranin was varied and was added to make up the same total volume of the reaction mixture (225µl), followed by the measurement of the oxalic acid. The temperature (25ºC, 37ºC, 60ºC and 70ºC) was also varied and tested as well.

2.4 Sample preparation for the assessment of microbial utilization of sulphate as an electron acceptor

A stock solution of 1M sodium acetate (SIGMA-ALDRICH, MO USA), 1M sodium formate (Merck KGaA, Darmstadt Germany) and 0.25M sodium sulphate (Merck KGaA, Darmstadt Germany) were initially prepared. The stock solutions were autoclaved and made anoxic by leaving them overnight in the anaerobic glove box. A 1/10 dilution of Boom Clay borehole water (MF23) was used for inoculating the samples. These solutions were used to prepare each condition of the samples in triplicates. A total volume of 20 ml sample each was prepared in triplicates in anoxic septum bottles that were sealed in the anaerobic glove box. The sulphate batch experiment was prepared for samples containing acetate or formate or no carbon source (table 2)

2.4.1 Sample preparation with sodium acetate

The samples were prepared in triplicate in anoxic septum bottles containing a total mixture of 0.015M sodium acetate, 0.004M sodium sulphate, MF23 (1/10 dilution), and water environment. Another triplicate sample was prepared containing a total mixture of 0.015M sodium acetate, no sodium sulphate (0.0M Na_2SO_4), MF23 (1/10 dilution) and water environment. In addition, control samples were prepared without any MF23 added. The first non-inoculated control sample was prepared in triplicate in a total mixture containing 0.015M sodium acetate, 0.004M sodium sulphate, and water environment. While the second non-inoculated control sample was prepared containing a total mixture of 0.015M sodium acetate and water environment only.

2.4.2 Samples preparation with sodium formate

The samples were prepared in similar conditions as in those containing sodium acetate. In these conditions, the samples were rather prepared with 0.015M sodium formate and not sodium acetate.

2.4.3 Samples preparation without acetate nor formate (no carbon source)

The samples were prepared in triplicate in anoxic septum bottles containing a total mixture of 0.004M sodium sulphate, MF23 (1/10 dilution), and water environment. Another triplicate sample was prepared containing only MF23 (1/10 dilution) and water environment. In addition, control

samples were prepared without any MF23 added. The first non-inoculated control sample was prepared in triplicate in a total mixture containing 0.004M sodium sulphate, and water environment. While the second non-inoculated control sample was prepared containing water environment only.

Table 2: Sample preparation for the sulphate batch experiments

Samples with acetate (In triplicate)	**Volume of sodium sulphate (ml)**	**Volume of sodium acetate (ml)**	**Volume of MF23 (ml)**	**Volume of water environment (ml)**
0.004SO4 A+	0.32	0.3	2	17.38
0.0SO4 A+	/	0.3	2	17.7
0.004SO4 A-	0.32	0.3	/	19.38
0.0SO4 A-	/	0.3	/	19.7
Samples with formate (In triplicate)	**Volume of sodium sulphate (ml)**	**Volume of sodium formate (ml)**	**Volume of MF23 (ml)**	**Volume of water environment (ml)**
0.004SO4 F+	0.32	0.3	2	17.38
0.0SO4 F+	/	0.3	2	17.7
0.004SO4 F-	0.32	0.3	/	19.38
0.0SO4 F-	/	0.3	/	19.7
Batch with no carbon source (In triplicate)	**Volume of sodium sulphate (ml)**	**No carbon source (ml)**	**Volume of MF23 (ml)**	**Volume of water environment (ml)**
0.004SO4 -+	0.32	/	2	17.68
0.0SO4 -+	/	/	2	18
0.004SO4 --	0.32	/	/	19.68
0.0SO4 --	/	/	/	20

A: Acetate, F: Formate, +: With MF, -: Without carbon source, 0.004SO4 and 0.0SO4: Concentration of sodium sulphate (M)

After sample preparation, the samples were incubated at 30°C. A 1ml syringe and a needle were used to collect sub-samples (500µl) at days 0, 2, 7, 18, 37, 66 and 84. The subsamples where stored at 4°C for DNA-extraction, while a fraction was stored at -20ºC for acetate and formate measurement.

2.5 Spectrophotometric measurement of acetic acid and formic acid

To obtain the concentration of the carbon sources present in the subsamples collected, their absorbance was measured spectrophotometrically using the microplate reader CLARIostar (BMG LABTECH GmbH, Germany). From the absorbance of the standard, standard curves were plotted from which the concentration of the carbon sources (acetate and formate) were calculated.

2.5.1 Acetic acid measurement

Acetic acid was measured from the subsamples containing sodium acetate with reagents from an Acetic Acid Kit (Megazyme, Co. Ireland). The measurement is based on the phosphorylation of

acetic acid in a series of reactions whereby the absorbance of nicotinamide-adenine dinucleotide (NADH) is being measured at a wavelength of 340nm. The absorbance of NADH is directly proportional to the concentration of acetic acid. From the kit, reagent 1 (R1) was prepared in a mixture containing the buffer and the enzyme, while reagent 2 (R2) was prepared in a mixture containing nicotinamide-adenine dinucleotide (NAD^+) with sterile water using requested volume by the manufacturer. 3µl of the sample and 200µl of reagent R1 were filled in 96well plates, followed by the automated addition of 20µl R2 in the mixture. The absorbance (340nm) was measured after 10 minutes of incubation at 25ºC and the acetic acid was then calculated from the standard curve obtained.

2.5.2 Formic acid measurement

Formic acid was measured from the subsamples containing sodium formate using a Formic Acid Kit (Megazyme, Co. Ireland). Two reagents from the kit, R1 and R2, were prepared (with sterile water) following the manufacturer instructions. R1 is made up of a buffer and NAD^+, and R2 contain an enzyme formate dehydrogenase. Before measuring the formic acid, the subsamples were diluted 10 times because the concentration was higher than the detection limit of the standard of the kit. 10µl of the diluted samples and 222µl of R1 were filled in 96 well plates, and the initial absorbance (340nm) were measured after 8 minutes of incubation at 37ºC. This was immediately followed by an automated addition of 20µl of R2. In the mixture, formic acid is being oxidized to form NADH. The absorbance of NADH was measured at a wavelength of 340nm at 37ºC for 12 minutes. From the absorbance of NADH, the concentration of formic acid was calculated.

2.6 Intracellular ATP measurement

Intracellular adenosine triphosphate (ATP) was analysed as an estimation of the microbial metabolic activity. The intracellular ATP was analysed at the days of subsample collection. An ATP Kit HS (Biothema Luminescent Assays, Sweden) was used, which contained ATP eliminating reagent that degrades extracellular ATP upon addition of 25µl together with 25µl of sample in a cuvette and incubated for 10 minutes. This was followed by the addition of 25µl Extractant B/S that aid in the release of intracellular ATP of the viable cells and the inactivation of the ATP Eliminating Reagent. ATP was then assayed with 160µl ATP reagent HS which contained luciferase and luciferin that produces light upon interaction with each other. The first intensity of the light (I_{smp}) was measured using the Lumitester C-100 (Kikkoman, Japan). The light intensity is proportional to the amount of ATP measured by the luminometer. An ATP standard (10µl) was immediately added in the cuvette and the second light intensity ($I_{smp+std}$) was measured. This

renders it possible to calculate the amount of ATP in unknown samples with the result express in pmol (10^{-12}mol). The luminescence was then calculated ($I_{smp}/(I_{smp+std}-I_{smp})$). The data obtained from the luminescence represented the units of equivalent active cells (EAC), which was further calculated from the amount of ATP per cell (2×10^{-18}) assume to be present as in most bacteria (41).

2.7 Flow cytometry

To count the microbial cells, flow cytometry was performed using a C6 Accuri flow cytometer (BD Biosciences, Erembodegem, Belgium). Flow cytometric analyses was performed at the same days of sample collection. Samples were diluted with mineral water that has been filtered with sterile 0.22µm-filter. The dilution was made in a total volume of 250µl each. This was followed by adding nucleic acid gel stain SYBR® Green I (Thermo Fisher Scientific, USA. 10,000×concentrate in 0.2µm-filter dimethyl sulfoxide) with a final concentration of 1×concentrate, and then incubated at 37ºC for 20 minutes. All samples were analysed within 1 hour of incubation. For the analysis of the C6 Accuri, 50µl of each of the 250µl samples was run using an FL1-H acquisition threshold of 1000. The number of event (event/µl) were plotted (FL1-H/FL3-H) and the background was eliminated by gating. The microbial cells (cells/ml) was calculated from the number of event (1000×number of event/µl).

2.8 Polymerase chain reaction

After DNA was extracted, it was amplified by PCR. The PCR was performed using primers for the microbial (bacteria and archaea) gene coding for 16S rRNA. The 16rDNA gene was used because it is specific for each microbial specie. For the bacteria, the forward primer 63F-GC (15, 42) having the sequence (CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGG GGGGCAGGCCTAACACATGCAAGTC*) with the GC-clamp (*) attached at the 5'-end and reversed primer 518R (ATTACCGCGGCTGCTGG) were used. For the archaea, the universal forward primer (CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGG GGGGCACGGGGGCGCAGCAGGCGCGA*) and universal reversed primer (GTGCTCCCCCGCCAATTCCT) were used (43). The PCR mixture was performed following the Thermoscientific product information for phusion high-fidelity DNA polymerase, and DreamTaq Green PCR master Mix (2X) for archaea and bacteria DNA respectively. Both methods were performed in a total reaction volume of 50µl each.

2.8.1 Polymerase chain reaction for bacteria

The 63F-GC forward primer and 518R reverse primer were used for the bacteria 16S rDNA. PCR was performed in stages using the following conditions on a thermocycler (GeneAmp® PCR System 9700, USA): Initial denaturing at 94ºC for 2 minutes followed by 30 cycles each of 95ºC ×30 seconds of denaturing, 56ºC × 30 seconds of annealing, and 72ºC × 1 minutes of elongation. The reaction was terminated after a 10 minutes' extension at 72ºC.

2.8.2 Polymerase chain reaction for archaea

The Universal Archaea GC forward primer and Universal Archaea reverse primer were used for archaea 16S rDNA. PCR was performed in stages for archaea presence in the samples using the following cycling conditions: Initial denaturing at 98ºC for 2 minutes followed by 15 cycles each of 98ºC × 30 seconds denaturing, 86ºC × 30 seconds of annealing (with a decreased in temperature by 1ºC after each cycle), and 72ºC × 1 minute of elongation. Another 25 cycles then followed the 15 cycles with a 98ºC × 30 seconds denaturing, 70ºC × 30 seconds of annealing, and 72ºC × 1 minute of elongation. The reaction was terminated after a 10 minutes' extension at 72ºC.

2.8.3 Agarose gel electrophoresis

The success of the PCR reactions was assessed by agarose gel electrophoresis using a Bio-Rad electrophoresis chamber (Bio-Rad, USA). A 1% (w/v) agarose gel in TBE (Tris-borate-EDTA) running buffer plus 5µl gel red (Biotium, USA. 10,000×concentrate) was used. 4µl of the archaea PCR was used along with the 6× loading dye (Thermoscientific, USA) to load the wells of the electrophoresis chamber. 4µl of the bacteria PCR samples were loaded in the wells of the electrophoresis chamber since the reaction mix already contained a loading dye. 2µl of 1kb plus Gene Ruler (Invitrogen, USA) was used as the ladder. Electrophoresis reactions were performed at 70V for 60 minutes. The PCR products were visualized using an ultraviolet spectrophotometer (Viber, Germany). From the PCR product of the samples obtained, a proportion of each was stored at 4ºC and further used for denaturing gradient gel electrophoresis.

2.9 Denaturing gradient gel electrophoresis

Denaturing gradient gel electrophoresis (DGGE) was performed from the PCR samples using an INGENY phorU system (INGENY International, The Netherlands). This technique separate the DNA strand and aligned the 16S rDNA according to their differences in GC content and distribution on the gel indicated by bands which represent the microbial population present in the sample. In brief, The DGGE gel was prepared and placed in between glass plates with a comb. Denaturing solutions were prepared in two different tubes containing the low (35%) and high

(65%) percentage appropriate for the fragment. The denaturing solution were made up of acrylamide/bis-acrylamide, urea, formamide, and diluted with water to a total of 250ml. 26ml of each of the denaturing solutions were immediately mixed with 16µl N,N,N',N'-Tetramethylethylenediamine (TEMED) and 160µl of fresh ammonium peroxydedipersulphate (10%w/v APS). The mixture was then poured to fill up the comb limit, starting with the high concentration (65%) followed by the low concentration (35%) for polymerization. Afterwards, sterile water was added to fill up the gel and left for 3hours. The water was then replaced with a mixture containing 10ml 0% denaturant, 100µl APS and 10µl TEMED, and left for 1 hour. The gel was then placed in a water bath at 60ºC, and the PCR samples were loaded along with the DGGE leader. 10µl bacteria PCR sample (because samples already contained the dye) was loaded while 5-10µl archaea PCR sample plus 4µl 6× loading dye was used. The INGENY phorU system was connected and the gels were run at 200V for 10 minutes, this was followed by changing the volt (120V) and run for 17 hours in Tris-Acetate-EDTA (TAE) – buffer at 60ºC. After 17 hours, the gel was stained with GelRed in a bath containing TAE for 10 minutes. Afterwards, the gel was placed in another bath (with water) for 10 min, then the gel was immediately visualized using an ultraviolet spectrophotometer.

2.10 Statistical analysis

Microsoft excel 2016 was used to analyze the results. All experimental conditions had three replicates (n=3). The student's T-test was used to evaluate the results and was considered significant when $p<0.05$.

3 Results

3.1 Optimization

3.1.1 Oxalic acid measurement

Oxalic acid was measured using different methods, but a good standard curve was not obtained from the different methods. Although the spectrometric measurement of oxalic acid had a straight line only within a short range (between 0.625mM-2.5mM safranin). Therefore, the oxalic acid could not be calculated from the absorbance.

3.1.2 Comparing different DNA extraction methods

The Qiagen DNA extraction method and two other customized methods (PEX and chemical lysis) were compared (fig. 5). The extracted DNA on Nano drop had an estimated concentration between 5-100ng/µl (Due to the nature of Boom Clay, there was a lot of variation when repeatedly measured, probably because Boom Clay borehole water still contained particles, chemicals and RNA from the Boom Clay). As a result, the extracted DNA by the different methods, were later validated base on their PCR (fig. 5a, b) and DGGE (fig. 5c, d) analysis. The PCR was performed for the analysis archaea (fig 5a, c) and bacteria (fig 5b, d). Qiagen method was used to extract DNA from non-Boom Clay borehole water sample that were used as the positive control bacteria (*Cupriavidus metallidurans* CH34) and the positive control archaea (*Haloferax volcanii* DSM 3757). MF23 Boom Clay borehole water sample was used along with the positive control bacteria (band B and C) and the positive control archaea (band E) as shown in figure 3. From the PCR band patterns, Qiagen method for DNA extraction (band A) and PEX extraction method (band B) had bands with the same size (650bp) as in the control archaea (band E), (fig. 5a). The PCR band patterns for both the Qiagen and PEX DNA extraction method had band sizes of 500bp (fig. 5b). From the PCR analysis, DGGE was further performed for the successful DNA extraction methods which are represented by the observed bands present with DGGE analysis (fig 5c, d). The chemical lysis (band D) customized DNA extraction methods had no bands present on the PCR analysis. While both the Qiagen method (band A) and the PEX extraction method (band B) had bands in the same alignment (approximately same size) as those of their controls.

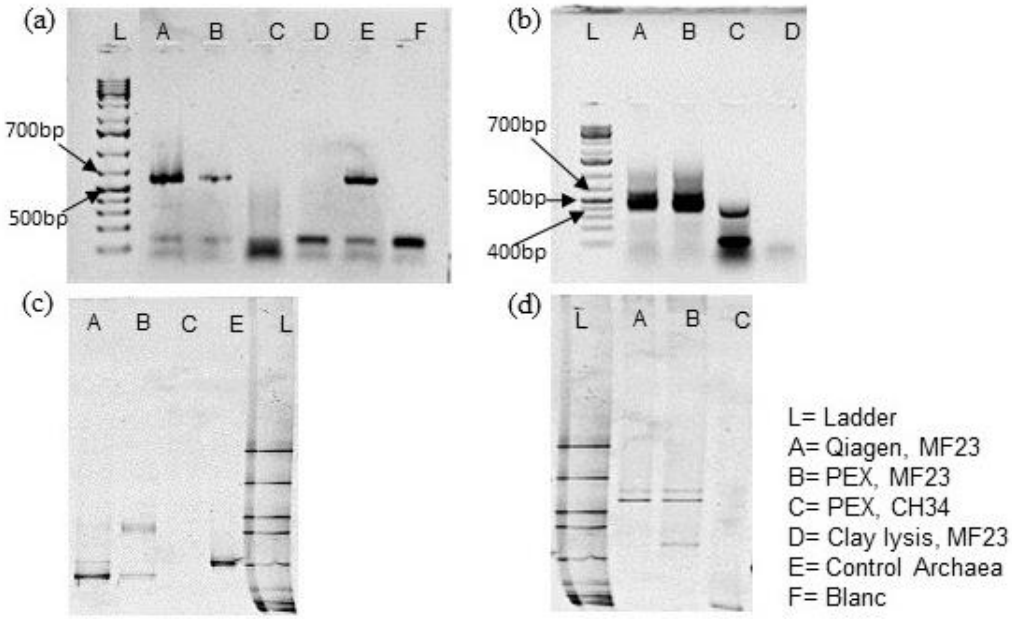

Figure 5: Optimization of a suitable Boom Clay pore water DNA extraction method representations on a PCR (a, b) and DGGE (c, d); The presence of archaea is indicated after DNA extraction by PCR and DGGE (a, c), likewise those of bacteria (b, d). The DNA extraction methods are indicated by the labeled bands with the size of bacteria (500bp) and archaea (650bp).

3.2 Microbial population analysis for the sulphate batch experiment

3.2.1 Flow cytometric Analysis

The microbial populations in the samples were analyzed at different time points of sample by flow cytometry. It was observed that the microbial cells significantly increased at day 2 compared to day 0, but after that, it remains constant with time in all the samples with and without Na_2SO_4 (fig. 6). The 0.004M Na_2SO_4 and 0.0M Na_2SO_4 without any addition of MF23 were used as controls in the samples and the microbial population was below the detection limit of the flow cytometer and were assumed to be sterile.

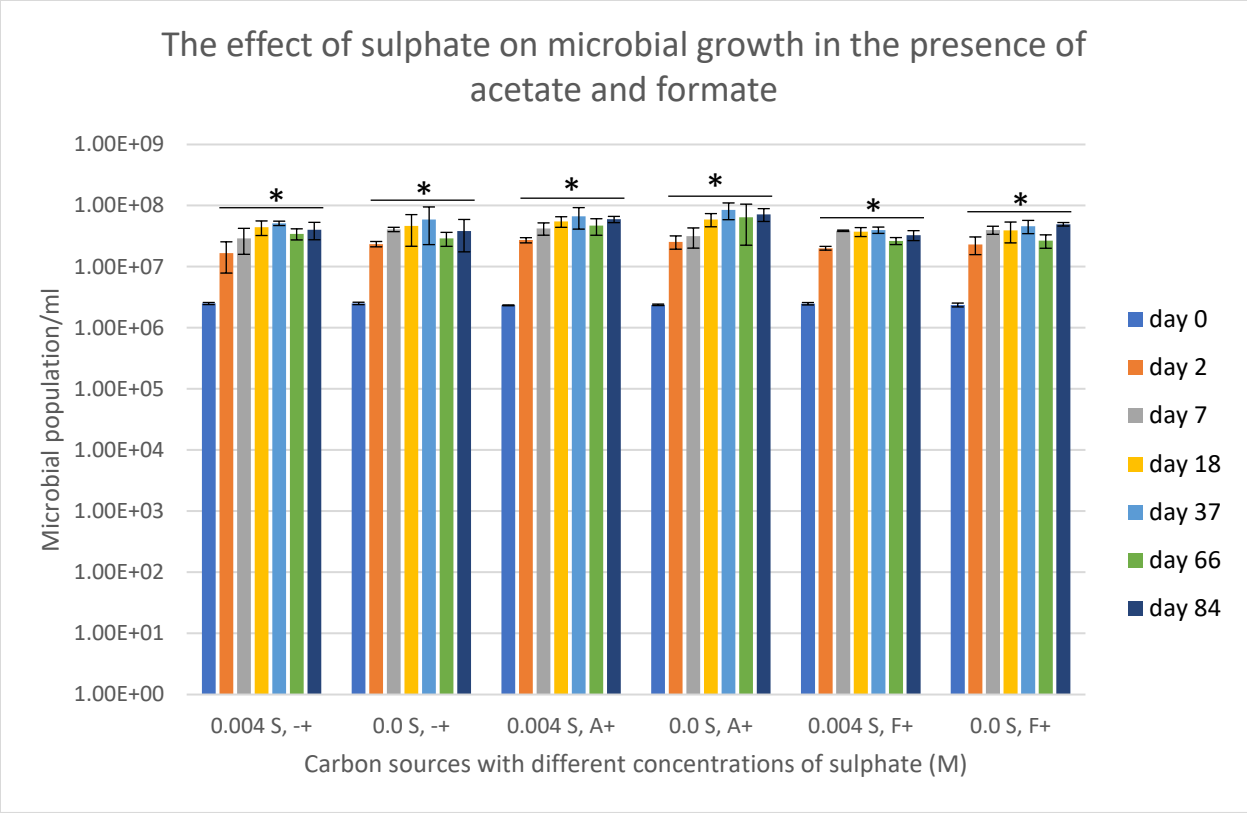

Figure 6: The microbial population of the cells analyzed by flow cytometer at different days. +: with MF23, -: without MF23, A: Acetate, F: formate, 0.004 S: 0.004M Na_2SO_4, 0.0 S: 0.0M Na_2SO_4. *: $p<0.05$. The data were analyzed with the student's T-test

3.2.2 Intracellular ATP-measurement

The intracellular ATP was analyzed at different time points to assess the active cells. The intracellular ATP of the microorganisms in the presence of formate significantly increased at day 7 in the 0.004M Na_2SO_4 sample (fig. 7). A significant increase was also observed for the day 2 sample without a carbon source and 0.004M Na_2SO_4 (fig. 7). No significant increase was observed in the equivalent active cells per ml in all the other samples at day 0 compared to the other days. The 0.004M Na_2SO_4 and 0.0M Na_2SO_4 without any addition of MF23 were used as controls in the samples and the active cells was below the detection limit of the kit and were assumed to be sterile.

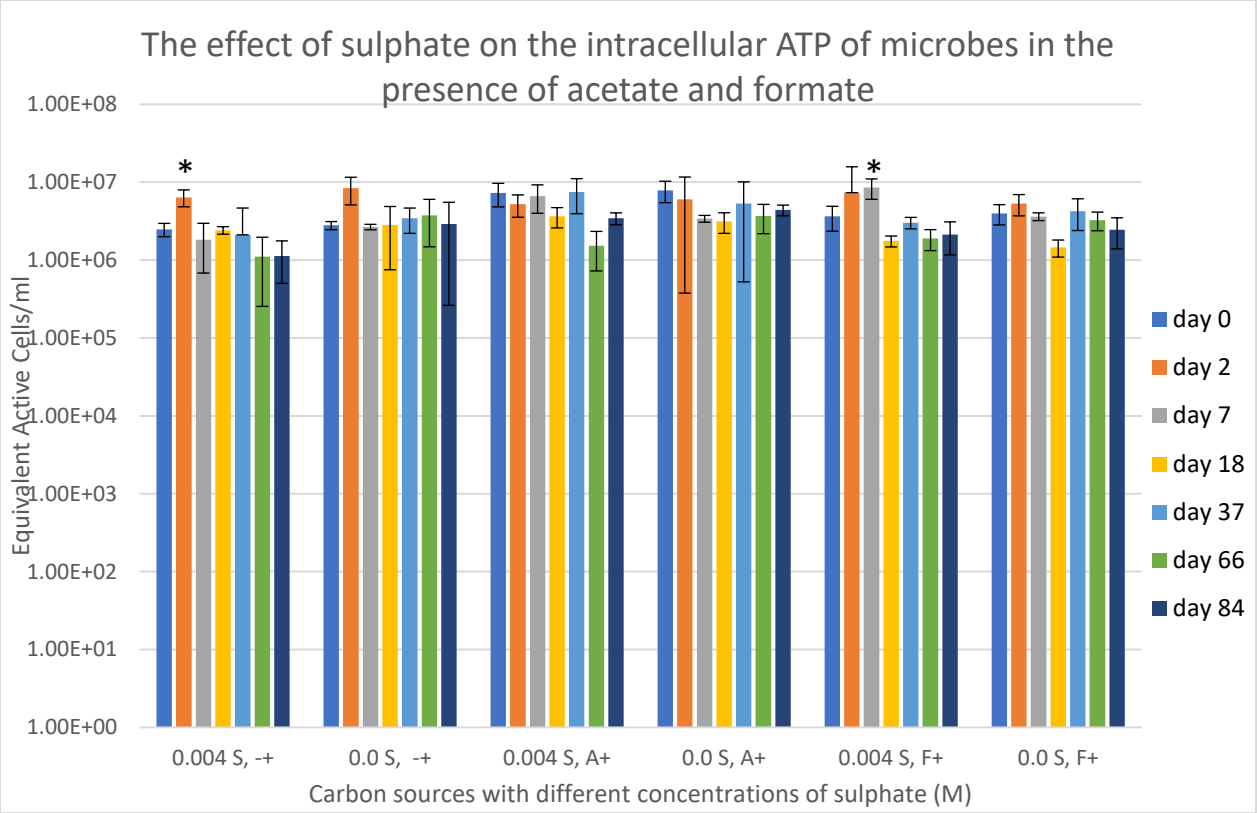

Figure 7: Intracellular ATP analysis of the microbial active cells in the samples with days. +: with MF23, -: without MF23, A: Acetate, F: formate, 0.004 S: 0.004M Na_2SO_4, 0.0 S: 0.0M Na_2SO_4, *: $p<0.05$. The data were analyzed with the student's T-test.

3.3 Spectrophotometric analysis of the carbon sources

3.3.1 Formic acid analysis

Formic acid was measured for samples containing sodium formate at different time points (day 0, 2, 7, 18, 37, 66 and 84). All these samples initially (from day 0) contained 15mM sodium formate. A significant decrease in the concentration of formic acid was observed from day 2 (fig. 8) in samples containing 0.004M Na_2SO_4 and MF23 (0.004S, F+). The concentration of formic acid also significantly decreased from day 37 in samples containing 0.0M Na_2SO_4 and MF23 (0.0SO_4 F+) (fig. 8). The concentration of formic acid in samples without MF23 did not significantly decrease with days. In addition to the measurement of formic acid in the samples that initially contained sodium formate, the concentration of formic acid was also asses in all the non-formate containing sample (i.e. samples containing acetate and samples without a carbon source) as a control. And it was confirmed from the formic acid measurement that the non-formate containing samples had 0.0M formic acid.

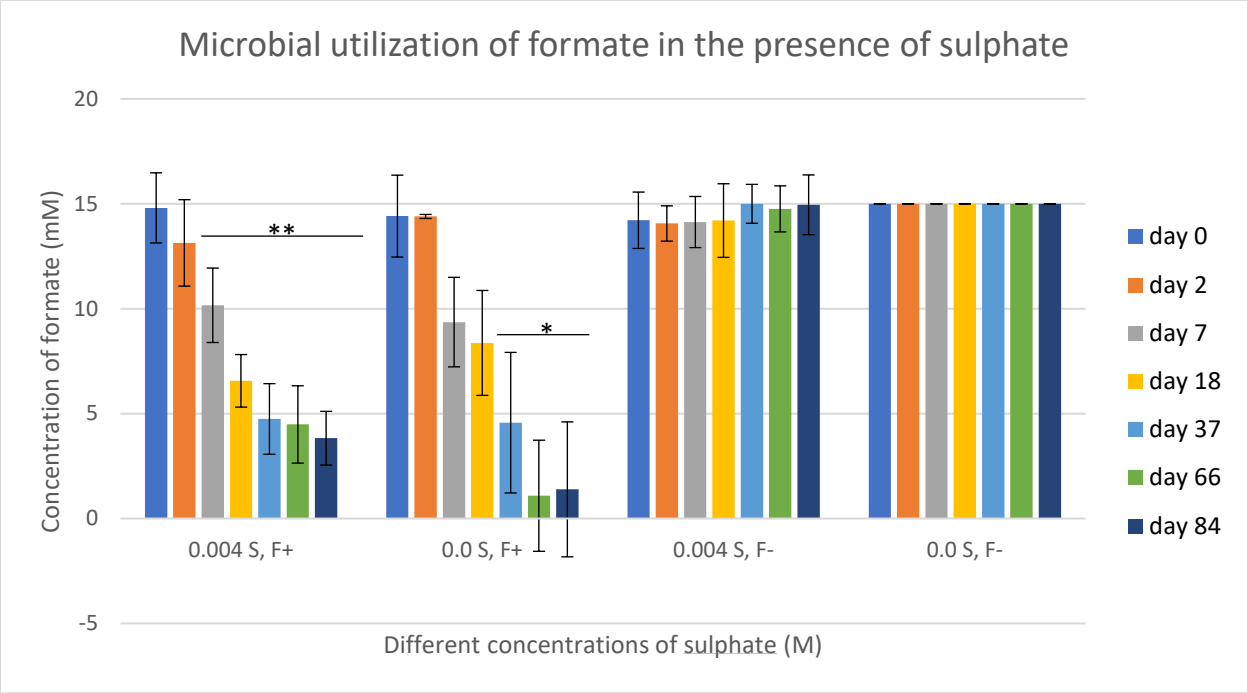

Figure 8: The concentrations of formic acid at different time points in samples containing; MF23, sodium formate, Na_2SO_4 and water environment. +: with MF23, -: without MF23, F: formate, 0.004 S: 0.004M Na_2SO_4, 0.0 S: 0.0M Na_2SO_4, *: $p<0.05$, **: $p<0.001$. The data were analyzed with the student's T-test.

3.3.2 Acetic acid analysis

Acetic acid was measured for samples containing sodium acetate at different time points (day 0, 2, 7, 18, 37, 66 and 84). All these samples from day 0 contained 15mM sodium acetate. None of the samples had a significant decrease in the concentration of acetic acid (fig. 9). In addition to the measurement of acetic acid in the samples that initially contained sodium acetate, the concentration of acetic acid was also asses in all the non-acetate containing samples (i.e. sample containing formate and samples without a carbon source) as a control. And it was confirmed from the acetic acid measurement that the non-acetate containing samples had 0.0M acetic acid.

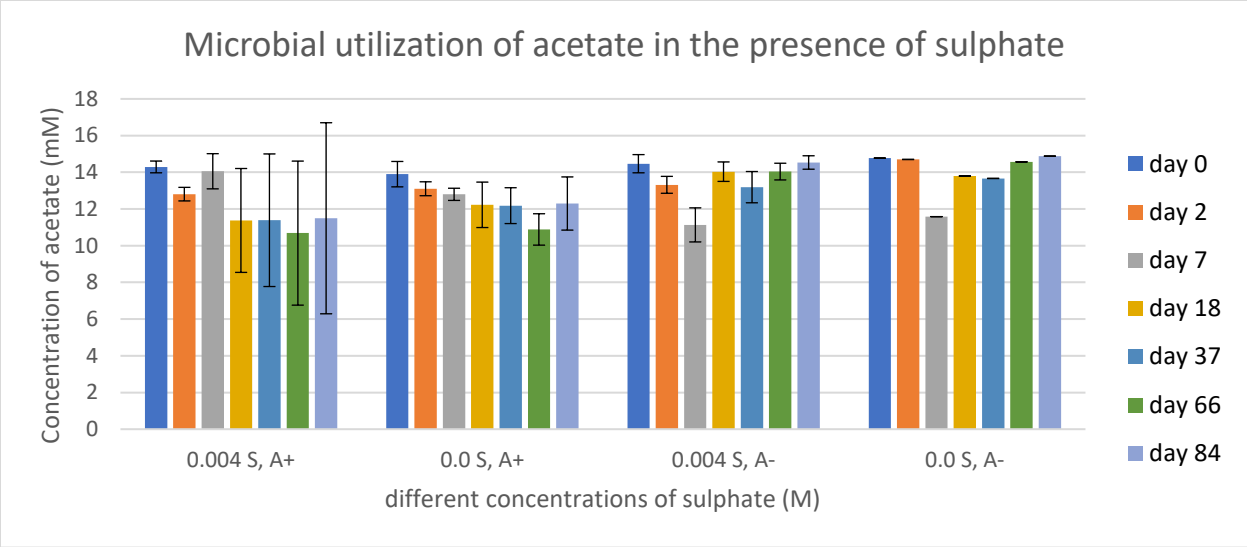

Figure 9: The concentrations of acetic acid at different time points in substances containing; MF23, sodium acetate, Na_2SO_4 and water environment. +: with MF23, -: without MF23, A: Acetate, 0.004SO4: 0.004M Na_2SO_4, 0.0SO4: 0.0M Na_2SO_4, *: $p<0.05$, **: $p<0.001$. The data were analyzed with the student's T-test.

3.4 Polymerase chain reaction of microorganisms used for DGGE analysis

3.4.1 Polymerase chain reaction for bacteria presence used for DGGE analysis

PCR was performed to examine for the presence of bacteria in samples containing formate (fig. 10a, b), acetate (fig. 10c, d) and samples without a carbon source (fig. 10e, f). The PCR analysis was successful for the samples that contained 0.004M Na_2SO_4 (fig. 10a, c, e) and 0.0M Na_2SO_4 (fig. 10b, d, f) for the samples of day 0 (1, 2, 3), day 18 (4, 5, 6) and day 84 (7, 8, 9) as indicated by the bands on the gel. Positive control bacteria (*Cupriavidus metallidurans* CH34) was loaded in band C, while water was used as negative control (blank) in band B and the ladder in band L (fig. 10). Samples were analyzed according to their sizes compared to the positive control bacteria and the ladder. It was observed that all the samples were in the same alignment with the positive control bacteria sample (i.e. at 500bp). It was observed that all the samples that initially had MF23 responded positively in the presence of bacteria as analyzed by PCR which will be used for DGGE (fig. 10).

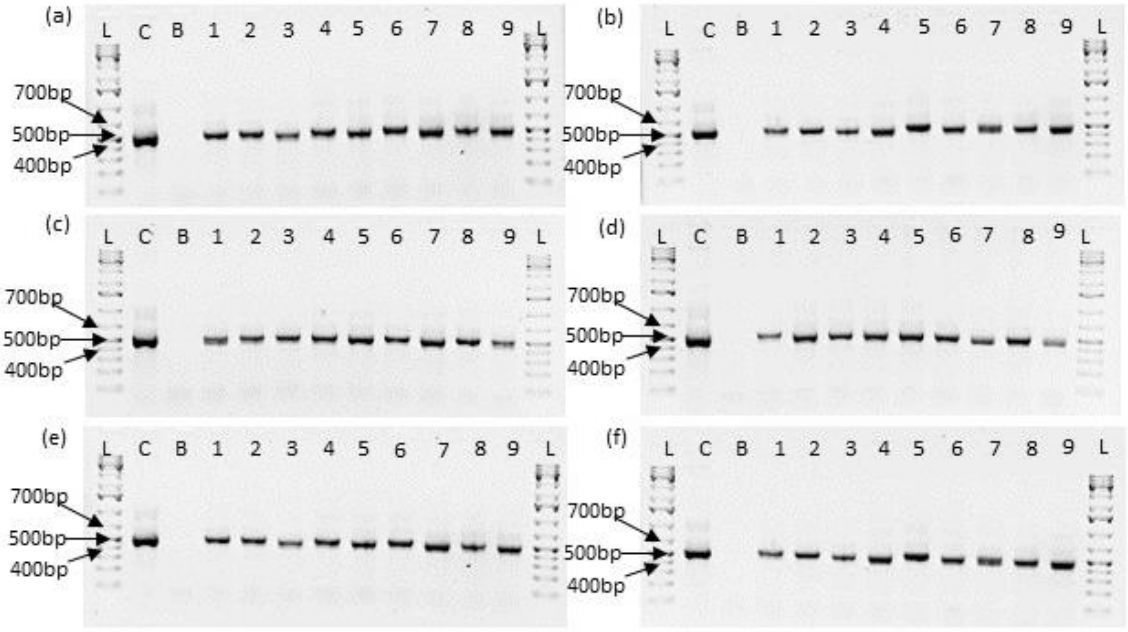

Figure 10: PCR showing the presence of bacteria in the samples; PCR was performed for samples containing formate (a, b), acetate (c, d) and samples without a carbon source (e, f) for day 0 (1, 2, 3), day 18 (4, 5, 6) and day 84 (7, 8, 9). Positive control bacteria (C) with 500 base pairs and negative control bacteria (B) as compared with the observed band position of the ladder (L). Bands: L, C, B, 1, 2, 3, 4, 5, 6, 7, 8, 9.

3.4.2 Polymerase chain reaction for archaea presence used for DGGE analysis

Archaea were also detected in some of the samples upon PCR analysis (fig. 11). The PCR was performed for samples containing formate (fig. 11a, b), acetate (fig. 11c, d) and samples without a carbon source (fig. 11e, f). Samples with 0.004M Na_2SO_4 (fig. 11a, c, e) and 0.0M Na_2SO_4 (fig. 11b, d, f) were analyzed as indicated by the bands for samples collected at day 0 (1, 2, 3), day 18 (4, 5, 6) and day 84 (7, 8, 9). Positive control archaea (*Haloferax volcanii* DSM 3757) are indicated by band C, negative control bacteria (blanc, water) by band B and the ladder by band L (fig. 11). Samples were analyzed according to the expected band length of approximately 650bp. It was observed that archaea were present in the samples with formate containing 0.004M Na_2SO_4 (fig. 11a) for samples collected at day 0 (band 1, 2, 3) and day 84 (band 7, 8, 9) and those of 0.0M Na_2SO_4 (fig. 11b) for samples collected at day 0 (band 1, 2, 3), day 18 (band 5) and day 84 (band 8, 9). It was also observed that archaea were present in the samples with acetate containing 0.004M Na_2SO_4 (fig. 11c) for day 0 (band 1, 2, 3), day 18 (band 4, 5) and day 84 (band 7, 8) samples, and those containing 0.0M Na_2SO_4 (fig. 11d) for day 0 (band 1, 2, 3), day 18 (band 5) and day 84 (band 7, 8, 9) samples. Archaea was present in samples without a carbon source

containing 0.004M Na_2SO_4 (fig. 11e) collected at day 18 (band 4, 5) and day 84 (band 7, 8, 9) and those containing 0.0M Na_2SO_4 (fig. 11f) collected at day 84 (band 8, 9).

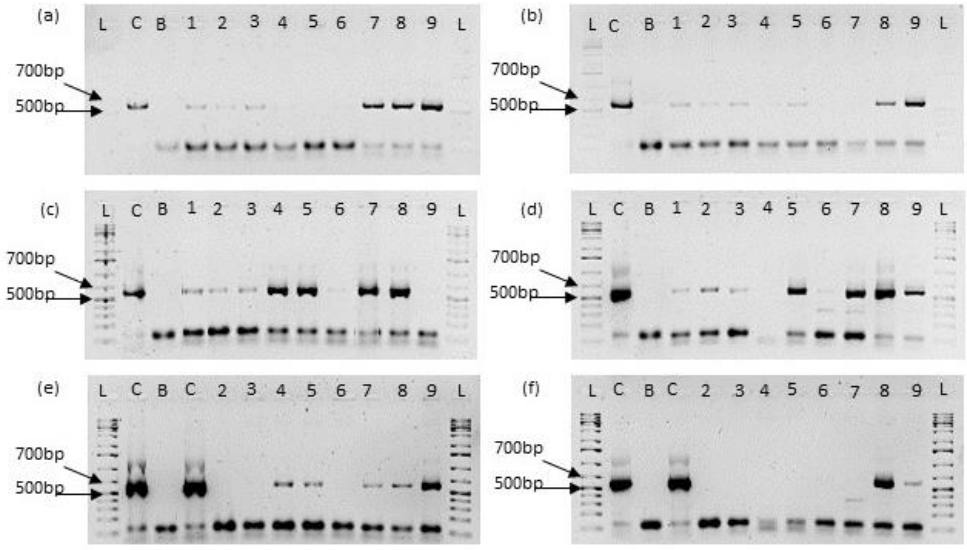

Figure 11: PCR showing the presence of archaea in the samples; PCR was performed for samples containing formate (a, b), acetate (c, d) and samples without a carbon source (e, f) for day 0 (1, 2, 3), day 18 (4, 5, 6) and day 84 (7, 8, 9). Positive control archaea (C) between 500-700 base pairs and negative control archaea (B) as compared with the observed band position of the ladder (L). Bands: L, C, B, 1, 2, 3, 4, 5, 6, 7, 8, 9.

3.5 DGGE analysis for changes in the bacteria population

DGGE was performed for some selected samples to analyzed the changes in the bacteria population with time. The selected samples were those of the day 0 (lane 1, 6, 11) and day 84 (lane 2, 3, 5, 7, 8, 9, 10, 12, 13) with bands which represent different bacteria species (example b, c, d) (fig. 12). Samples containing acetate and 0.004M Na_2SO_4 (lane 2, 3); samples with acetate and 0.0M Na_2SO_4 (lane 4, 5); samples with formate and 0.004M Na_2SO_4 (lane 7, 8); samples with formate and 0.0M Na_2SO_4 (lane 9, 10); samples with no carbon source and 0.004M Na_2SO_4 (lane 12); samples with no carbon source and 0.0M Na_2SO_4 (lane 13) (fig. 12). The day 0 samples (lane 1, 6, 11) containing acetate, formate and no carbon source respectively had similar bacteria species (a). While in the day 84 samples the were observed changes in the microbial population, example the bacteria specie (b) is present in the samples with acetate and Na_2SO_4 (lane 2) and not present in the samples with acetate and 0.0M Na_2SO_4 (lane 4, 5) nor in the day 0 sample (lane 1). Also, there is a bacteria specie (c) is present in the day 84 samples with formate and Na_2SO_4 (lane 7, 8) and not present in the samples with formate and 0.0M Na_2SO_4 (lane 9, 10) nor in the day 0 sample (lane 6). In addition, sample of day 84 (lane 13) with no carbon source and 0.0M Na2SO4 had a bacteria specie (d) which is not present in the sample (lane 12) with no

carbon source and Na_2SO_4. Summarily, there is a change in the bacteria population in the samples with days, as well as a change among the replicate samples.

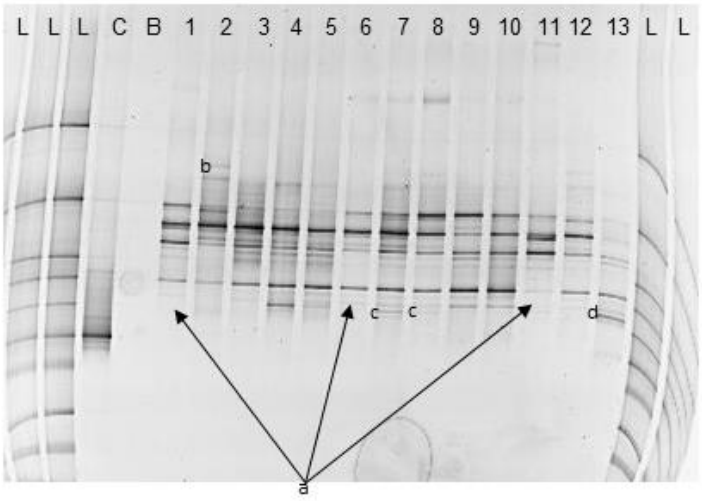

Figure 12: DGGE analysis for the bacteria population present in the samples. The bands represent different bacteria species (example, b, c, d). Day 0 (1, 6, 11 with similar bacteria species (a)) samples containing acetate, formate and no carbon source respectively. The day 84 samples are as follows; samples with acetate and 0.004M Na_2SO_4 (2, 3); samples with acetate and 0.0M Na_2SO_4 (4, 5); samples with formate and 0.004M Na_2SO_4 (7, 8); samples with formate and 0.0M Na_2SO_4 (9, 10); samples with no carbon source and 0.004M Na_2SO_4 (12); samples without a carbon source and 0.0M Na_2SO_4 (13). C: Positive control archaea; B: Negative control (blanc); L: Ladder.

3.6 DGGE analysis for changes in the archaea population

The changes in the archaea population for some selected samples with time, was analyzed by DGGE. The selected samples were those of day 0 (lane 1, 6, 11) and day 84 (lane 2, 3, 5, 7, 8, 9, 10, 12, 13) (fig. 13). The day 84 sample are the samples containing acetate and 0.004M Na_2SO_4 (lane 2, 3); samples with acetate and 0.0M Na_2SO_4 (lane 4, 5); samples with formate and 0.004M Na_2SO_4 (lane 7, 8); samples with formate and 0.0M Na_2SO_4 (lane 9, 10); samples with no carbon source and 0.004M Na_2SO_4 (lane 12); samples with no carbon source and 0.0M Na_2SO_4 (lane 13) (fig. 13). The day 0 samples (lane 1, 6, 11) containing acetate, formate and no carbon source respectively had archaea present (a), while in the day 84 samples the archaea are absent (fig.13).

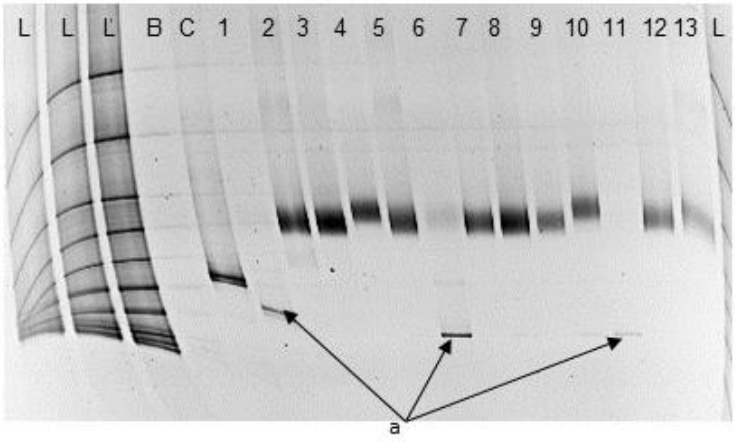

Figure 13: DGGE analysis for the archaea population present in the samples. Day 0 (1, 6, 11) samples containing acetate, formate and no carbon source respectively. The day 84 samples are as follows; samples with acetate and 0.004M Na_2SO_4 (2, 3); samples with acetate and 0.0M Na_2SO_4 (4, 5); samples with formate and 0.004M Na_2SO_4 (7, 8); samples with formate and 0.0M Na_2SO_4 (9, 10); samples with no carbon source and 0.004M Na_2SO_4 (12, 13); samples with no carbon source and 0.0M Na_2SO_4 (4, 5). C: Positive control archaea; B: Negative control (blanc); L: Ladder; a: Archaea present in samples (1, 6, 11).

4 Discussion

In many countries, deep geological disposal of radioactive waste has been considered as a safe and sustainable solution. In Belgium, Boom Clay has suitable physico-chemical characteristics to serve as a natural barrier for the disposal of LILW-LL. It has been reported that Boom Clay borehole water contained microorganisms (15, 16) and its infiltration into the waste drum can reduce sulphate (which leaches from the bituminised waste) in the presence of relevant electron donors. However, the extent at which sulphate-reducing microorganisms in the Boom Clay borehole water degrades the bituminised waste (formate and acetate) is unknown. Here, an approach was used that mimicked a similar condition in the repository in years to come. Samples were prepared in anoxic septum bottles, with Boom Clay borehole water as water environment and inoculated with MF23 (Boom Clay borehole water) together with sulphate as the electron acceptor, and formate or acetate as the electron donors.

4.1 Optimization experiments

Before the start of the sulphate batch experiment, different methods of DNA extraction were compared and the optimization for the measurement of the concentration of oxalic acid was attempted. This was done because the method currently used in our group is cumbersome and with the number of samples to be analyzed, a faster and less cumbersome method was necessary.

4.1.1 DNA extraction

The nature of Boom Clay borehole water makes it difficult to extract DNA. This is probably because Boom Clay borehole water still contains particles from the Boom Clay, these particles contain RNA and DNA. In addition, Boom Clay borehole water contains different types of chemical substances which could have an impact as well (table. 1) (6). Thus, a customized method was implemented at SCK.CEN to extract DNA from the solid Clay. For example, in a study on deep subsurface clay borehole water, this customized protocol was used to extract the DNA from the microorganisms (15). As earlier mentioned, this customized method is cumbersome, and complex matrices are used to extract the DNA from the microorganisms. However, in this study, before the sulphate batch experiment, a different customized method was additionally compared with a more standard method (the Qiagen method). Among the three different DNA extraction methods that were compared, the most reliable method was further use in the experiment. The concentration of the extracted DNA by the different methods had a lot of variations when repeatedly measured on a Nanodrop. Due to that, we improvised a selection criteria for the preferred method in the

batch experiment. The selection criteria were validated based on the results obtained from the analyses of PCR and DGGE. The Qiagen DNA extraction method was considered the best. This is because the MF23 samples that was tested contained bacteria and archaea with the expected band length of 500bp and approximately 650bp, respectively. Although the customized PEX extraction method was also successful, Qiagen method of DNA extraction was preferred over it because Qiagen protocol is less time consuming (1 day) while PEX extraction is time consuming (2 days). In addition, it is the most standard protocol to work with (a kit), instead of protocols which are designed and optimize DNA extraction from complex matrices that can influence the yield of DNA.

4.1.2 Oxalic acid measurement:

The optimization experiment for measuring the concentration of oxalic acid was not successful. This was because of the challenges to obtain a good standard curve from where the concentration of oxalic acid was to be calculated. A kinetic spectrophotometric method was also used to measure the concentration of oxalic acid as described by Ensafi. A *et al*., 2001 (40) , although the reproduction of this method was not successful in this case. The Kinetic spectrophotometric method is based on the measurement of the absorbance of safranin which is inversely proportional to the concentration of oxalic acid. Based on the principle of the Kinetic spectrophotometric method, the protocol was further modified by varying the concentration of safranin in the reagent preparation, although we could still not obtain a good standard curve. We therefore focused only on the measurement of the concentration of acetic acid and formic acid as the carbon electron donors in the experiment.

4.2 Sulphate batch experiment

The ability of microbes to reduce sulphate was investigated in the presence of formate and also in the presence of acetate as electron donors. Negative control samples were also analyzed, whereby some samples were prepared without any addition of a carbon source and to some samples without an inoculum.

4.2.1 Sulphate reducing microbes in the presence of formate as the electron donor

The microbial population from the flow cytometric analyses in the samples containing formate significantly increased from day 2, but remained constant with time in the samples with or without Na_2SO_4. This suggests that the increase in the microbial population from day 2 is probably not due to the presence of Na_2SO_4 (electron acceptor) as it was also the same case observed for the samples without a carbon source. From the metabolic activity based on the intracellular ATP

analyzed, it was equally observed that the metabolic activities of the microorganisms in the samples with or without Na_2SO_4 were within the same equivalent active cells per ml (logarithmic range within 10^6). Hoehler *et al*., 2013 (44) showed that when considering basal power requirements for microbial active cells (e.g. 10^6 EAC per ml), the magnitude could be many order higher. It is confirmed that microbial cells in extreme conditions are able to survive in low but stable metabolic rate, with low production of ATP over time (45). This could be one of the reasons why there was no difference in the magnitude (log of EAC per ml) of the active cells in all the samples with and without Na_2SO_4. However, there was a significant increase in the intracellular ATP in the sample with Na_2SO_4 at day 7 and not in the other days. Hence, it cannot be concluded that the metabolic activity of the cells is the same in the samples with and without Na_2SO_4.

During microbial metabolism, microorganisms is expected to reduce sulphate (electron acceptors) by consuming the carbon sources (formate). Thus, a decrease in the concentration of the carbon source is expected with time as the microbial population increases. In this study, a significant decrease in the concentration of formic acid in the samples with Na_2SO_4 was observed from day 2 onward, while those without Na_2SO_4 significantly decreased from day 37 onward. This implies that the sulphate-reducing microorganisms were metabolically active since they consumed formic acid earlier compared to those without Na_2SO_4. The reduction in the concentration of formate is in conformity with a study that was performed to analyze the function of formate dehydrogenase in *Desulfovibrio vulgaris* a sulphate reducing bacteria (46). In that study, it was also reported that in the absence of sulphate as electron acceptor, the *Desulfovibrio vulgaris* could not degrade formate which is contrary to our findings. It was unexpected, but Interesting that the microorganisms could degrade formate in the absence of Na_2SO_4. Boom Clay borehole water contains sulphate (table 1), although the concentration is low, which could have an influence on the result. This could probably account for the decrease in the concentration of formate in the samples without sulphate. The unexpected increase in the microbial population in the control samples without a carbon source, may also be as a result of some relevant electron donors present in the Boom Clay borehole water. Since $NaNO_3$ is also present in the bituminized waste, and from the electrochemical series, it is known that nitrate is higher than sulphate, meaning that nitrate will displace sulphate, rendering sulphate less active as an electron acceptor in the repository (47). Therefore, the rate at which formate will be degraded by sulphate-reducing microorganisms may probably be lower in the actual condition.

4.2.2 Sulphate-reducing microbes in the presence of acetate as the electron donor

The microbial cells from the flow cytometric analyses in the samples containing acetate also significantly increased from day 2, but remained constant with time in the samples with or without Na_2SO_4. From this observation, it could be assumed that the microbial population increase from day 2 is not due to the presence of Na_2SO_4 (electron acceptor) as it was also the similar observation for the samples without a carbon source. In addition, from the intracellular ATP analyses, it was also equally observed that the metabolic activity of the microorganisms in the samples with or without Na_2SO_4 were within the same equivalent active cells per ml (logarithmic range within 10^6). This probably implies that the metabolic activity of the cells is similar in the samples with and without Na_2SO_4. It could be true because there was no significant decrease in the concentration of acetic acid in all the samples with or without Na_2SO_4. During the experiment, it was however noticed that in one of the triplicate samples containing Na_2SO_4, there was a drastic reduction in the concentration of acetic acid with days. That is why an increase in the standard error of mean (SEM) was observed (fig. 6). Other studies have shown that sulphate-reducing microorganisms are able to degrade acetate (48, 49) in the presence of sulphate. Since the was no observed significant increase in the microbial population nor an observed significant increase in the metabolic activities (from intracellular ATP measurements) and also, no significant decrease in the concentration of acetic acid. This implies that acetate could still be a potential electron donor and likely, more time is required for sulphate reducing microorganisms to degrade acetate.

4.3 PCR and DGGE

PCR and DGGE were performed to analyze the microbial changes in the samples with days. Some days (0, 18 and 84) of sample collection were selected to investigate these microbial changes. The DNA of the microorganisms (archaea and bacteria) was extracted for the samples with and without Na_2SO_4 for samples containing the carbon sources. PCR was performed for the presence of archaea and bacteria. From the PCR analysis, all the inoculated samples were confirmed by the presence of bacteria. Conversely, not all the samples that were inoculated showed the presence of archaea. The PCR was performed prior to the DGGE as PCR positive samples were used to perform the DGGE.

The DGGE was performed for all the selected samples, but the DGGE was not successful because of an obstruction noticed in the flow of the current during the electrophoretic step. During the optimization process the DGGE was successful and this problem of current obstruction flow was not encountered, also the same procedure of DGGE analyzes was performed in the batch

experiment. Since the optimization for DNA extraction was confirmed by the result obtained from the PCR and DGGE, we proceeded with a second DGGE analysis for some selected samples, which was finally successful. Although, only the day 0 (initial day), and the day 84 (final day) samples were chosen to analyze the changes in the microbial population from the initial to the final day of sample collection. From the DGGE analysis for archaea presence, all day 0 samples had archaea, while no archaea were present for most of the day 84 samples. Probably, archaea could not survive in the medium for a longer time. The DGGE analysis for bacteria presence showed that all the samples had a variation in the bacteria species present with days. Also, there was variation in the bacteria species among replicate samples. Changes in the microbial population was observed both for the samples with and without sodium sulphate for all the samples with acetate and formate. Even though not all the samples were used to perform the DGGE, we can assume that all the bacteria population changes with time.

This study is part of the MIND (Microbiology In Nuclear waste Disposal) project, a first of its kind European project to analyze microbial processes in nuclear waste disposal facilities. This experiment was designed initially to achieve three main objectives, but due to the bulk of work regarding the extensive nature of the project, it was further split into sub-objectives. Hence, only a portion of the project was analyzed. We could not also perform some of the experiments during the allocated time because of an unexpected shortage of Boom Clay borehole water. Therefore, the other aspects of the project need to be performed in the future. This includes; to investigate the effect of microbial processes on bitumen derivatives in the presence of relevant selection of electron acceptors (i.e. different concentrations of nitrate and nitrite) since nitrate also leaches from the bituminized waste. To investigate the effects of microbial processes on bitumen derivatives at different pH (8.5, 10.5) levels, because the disposal system is constructed with cementitious materials making it alkalinic. To investigate the effects of microbial process on bitumen derivatives in the presence of different types of solid phase bitumen, that is for the bitumen that have been prone to degradation (aged bitumen by heating) and the regular bitumen (not heated). After performing these objectives, experiments can then proceed to the actual disposal facility.

5 Conclusion

The study was performed to investigate the extent to which microorganisms interact with bituminous materials (acetate and formate) used in radioactive waste disposal. The focus was on sulphate as the electron donor and acetate or formate as the electron acceptors to the microorganisms. Oxalic acid (one of the relevant selected electron donors) was not included in the experiment because the optimization for its measurement was not successful. The Qiagen method for DNA extraction was preferred over the other customized method, because it is the most standard and requires lesser time.

The sulphate batch experiment was performed for a period of 84 days. It was observed that, the microorganisms present in Boom Clay borehole water can probably reduce sulphate. Formate was consumed by the microorganisms with a significant decrease observed from day 2 onwards. While in the control samples without sulphate, an unexpected significant decrease in the concentration of formate was observed from day 37 onwards. The concentration of acetate did not decrease significantly. The microbial population count nor the intracellular ATP did not significantly increase. However, there was an observed change in the microbial population from day 0 compared to the final day (day 84) of sample collection. From the DGGE results, all the samples showed the presence of archaea at day 0, but was not the case at day 84. Implying that the archaea probably died with time. Whereas, the bacterial population had an observed change for all the conditions, with variation in the species present. From this study, it implies that formate is a more favorable electron donor compared to acetate in these conditions.

This experiment was performed such that only the concentration of the carbon electron donors was analyzed. It will be important in the future to perform this experiment by measuring the concentration of sulphate as well. Although the concentration of acetate did not significantly decrease, it is also necessary to extend the time of measurement, probably with time they will be completely degraded.

As earlier mentioned, LILW-LL is immobilized in a bituminous matrix which and typically contains also a large amount of soluble salts with sodium sulphate as one of the important ones. In years to come, Boom Clay borehole water will infiltrate the waste drum, and this will cause the leaching and solubilisation of these salts (Na_2SO_4) and organic compounds (formate and acetate) from the waste. As a result of this, the redox conditions of Boom Clay could be affected and its reducing capacity may consequently decrease. This will in turn increase the rate at which radionuclides migrate through the host rock. Therefore, it is important to investigate this study in actual conditions.

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