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Microbiology In Nuclear waste Disposal

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DELIVERABLE 1.3

<Radiation and microbial degradation of bitumen>

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Publishable Summary

Work Package 1 (WP1) of the Microbiology in Nuclear waste Disposal (MIND) project addresses remaining key issues for the geological disposal of organic-containing intermediate level radioactive wastes. Specifically, its focus is on the long-term behaviour, fate and consequences of these organic wastes. This report (deliverable 1.3 of the MIND project) summarizes all results obtained by SCK•CEN regarding microbial degradation of salts and organic compounds leaching from bituminized waste in relevant conditions during geological disposal. In first instance, the effect of different organic compounds expected to leach from bituminized waste on the efficiency of nitrate reduction was investigated. Afterwards, the effect of pH on the nitrate reduction rates of two microbial communities was investigated.

In Belgium, an important fraction of the current stored volume of long-lived intermediate level radioactive waste is immobilised in a bituminous matrix as Eurobitum, which contains besides bitumen and radionuclides, large amounts of soluble salts with sodium nitrate as the most dominant. Geological disposal of this waste in a water-saturated sedimentary formation such as Boom Clay will induce water uptake by the hygroscopic salts present in the bituminized waste. The dissolution of these salts will result in a further osmosis-induced water uptake through the semi-permeable bitumen membrane and thus in swelling of the Eurobitum. Additionally, sodium nitrate will slowly leach from the waste. The nitrate plume in the clay water could cause a geochemical perturbation of the surrounding clay, possibly affecting the redox conditions, causing ionic strength effects and cation exchange processes, which might result in an increase in the mobility of the radionuclides through the host rock. However, it is known that nitrate can also be removed inside the disposal gallery or in the near-field by various processes. Abiotically, the reduction of nitrate can occur with H_2 (produced during radiolysis of bitumen or water and during corrosion of steel) and/or steel acting as electron donors and/or with the steel or even pyrite in the clay possibly serving as catalytic surface. These abiotic reactions would lead to the production of ammonium, which can sorb onto clay minerals and would therefore compete with some radionuclides for sorption. Biotically, nitrate can be consumed as electron acceptor by microorganisms, if proper growth conditions are provided. Respiratory microbial consumption of nitrate leads to the intermediate production of nitrite, and finally to nitrogenous gases. Most of the other leachates from bituminized waste (e.g. acetate, H_2) are biodegradable and can be used as electron donor, to fuel this microbial nitrate reduction. Depending on the electron donor used in this denitrification process, the final overall result could be a gas pressure decrease or increase. During disposal conditions, the microbial population will be exposed to hyperalkaline conditions originating from the pore water from the concrete lining of the waste monolith and the backfill material, which could affect the viability and activity and thus denitrification potential of the microbial population.

The efficiency of the Boom Clay borehole water microbial community to reduce nitrate leaching from regular and thermally aged non-radioactive Eurobitum in the presence or absence of known bitumen degradation products was investigated in different series of anoxic laboratory batch experiments. It was shown that acetate is the most preferred electron donor for microbial nitrate reduction and the highest nitrate reduction rates were observed in the presence of acetate. Formate seemed also to be an easy accessible electron donor, but related to the kinetics of the reaction, not as efficient as acetate to remove nitrate. On the other hand, oxalate was the least preferred electron donor for nitrate reduction and was only completely degraded in one out of three replicates. However, calcium oxalate crystals were formed, indicating that if oxalate is present, it will probably be less (bio)available compared to other organic compounds. Next to the added bitumen degradation products, the microbial community was able to use organics that leached from the Eurobitum blocks as electron donor to carry out nitrate reduction. Moreover, a clear biofilm formation on the solid

bitumen block was observed in all conditions, suggesting that microorganisms could enhance the degradation of Eurobitum.

Altogether, this study indicates that microbes can make biofilms on Eurobitum, and can reduce the nitrate leaching of the bitumen to nitrite and nitrous gasses. To carry out this nitrate reduction, the microbial community is able to use organics leaching from the bituminized waste. High alkaline pH (up to 12.5) alone is not sufficient to eliminate microbial presence in a geological repository, but it can induce a significant shift in the microbial community and inhibit microbial activity. Where a less alkaline pH will occur, e.g. at the boundary of the Boom Clay and the concrete liner of the disposal gallery, microbes can be present and are able to perform nitrate reduction.

Contents

1 Introduction

With its total volume of ~3000 m^3 and its high specific activities, radioactive waste immobilised in a bituminous matrix is a very important intermediate-level waste form in Belgium. There are three different types of bituminised waste in Belgium, depending on the bitumen used for immobilisation of the radioactive waste. One type is straight-run distillation (distilled, soft) bitumen, obtained as the bottom product after the distillation of asphaltic crude oils, in a first step at atmospheric pressure (340 °C), in a second step at underpressure (between 10 and 100 mm Hg; 400 °C). A second type is (semi- and fully) blown (oxidised, hard) bitumen, obtained by blowing air through the distilled bitumen, at temperatures between 200 and 260 °C. The third type is emulsified bitumen (liquefied bitumen), obtained by addition of anionic (alkaline soap), cationic (amine salt), or non-ionic aqueous solutions (emulsifiers) to the distilled bitumen. [1]. The waste immobilised in a blown bitumen matrix is called Eurobitum and is the most significant fraction of bituminised waste (± 80 %), hence primarily studied. Typically, Eurobitum contains 60 wt % bitumen Mexphalt R85/40 in which the waste (40 wt %) is homogenously dispersed [2, 3]. The waste contains soluble and insoluble salts such as 20 - 30 wt% $NaNO_3$, 4 - 6 wt% $CaSO_4$, and small amounts of CaF_2, $Ca_3(PO_4)_2$, $Ni_2[(Fe,Mn)(CN)_6]$, and (hydr)oxides of Fe, Zr, and Al. The residual amount of water present is between 0.5 - 1 wt%. The total radionuclides inventory (U and Pu isotopes, ^{241}Am, ^{244}Cm, ^{60}Co, ^{90}Sr, ^{137}Cs, ...) accounts for ~0.4 wt% for drums with an average activity but can be up to 1.15 wt % for drums with higher activities [4]. The radioactivity is only attribute to the inorganic fraction, and not the organic or C-compound fraction, of the bitumined waste.

The Belgian Agency for Radioactive Waste and Fissile Materials (Organisme National des Déchets Radioactifs et des Matières Fissiles / Nationale Instelling voor Radioactief Afval en Splijtstoffen or ONDRAF/NIRAS) proposes deep geological disposal for low- and intermediate-level long-lived radioactive waste (LILW-LL) (such as Eurobitum), vitrified high-level waste and spent nuclear fuel in a poorly indurated clay formation (Boom Clay or Ypresian clay) considering a depth of 200m, 400m or 600m. [5]. The disposal system for bituminized LILW-LL would consist of a combination of a natural and engineered barrier system. However, for long-term safety, the major contributor to the retardation and limitation of the release of radionuclides from the disposal system of Eurobitum will be the geological barrier, i.e. the host rock formation. Boom Clay could be suitable to serve as natural barrier for the disposal of radioactive waste because of its favourable properties to limit and delay the migration of the leached radionuclides to the biosphere over extended periods of time. Indeed, the Boom Clay is a homogenous clay layer that has a very low permeability and a low hydraulic conductivity and gradient. Consequently, transport is not influenced by the pore water movement but only by the concentration gradient of contaminants. In addition, Boom Clay has a strong retention capacity for radionuclides and chemical contaminants which delays migration throughout the clay layer. Another important feature is that is has a self-sealing capacity that enables it to respond to mechanical or other damages [6]. The current design of the geological disposal facility consists of horizontal galleries, lined with concrete wedge blocks, in which the waste monoliths are stored. A monolith is a prefabricated concrete container, which contains several primary waste drums (220 and 400 litre stainless steel drums). The voids between the drums will be filled with mortar and the monolith will be closed with a concrete lid. A concrete end plug is foreseen at the end of the concrete lined disposal galleries to avoid direct contact between the disposal waste package and the Boom Clay and remaining voids in the disposal galleries will be backfilled, probably with mortar. After the complete backfilling, the disposal galleries will be sealed [5, 6].

To ensure a safe geological disposal of the bituminized waste, a good understanding on the long-term behaviour of bituminised waste and its influence on the Boom Clay is of paramount importance. Under disposal conditions, two types of possible perturbations of the clay formation need to be considered [7]:

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- A geo-mechanical disturbance caused by an increase of the pressure in and around the waste due to the uptake of pore water by hygroscopic salts embedded in the waste followed by osmosis-induced water uptake and gas production by anaerobic corrosion of steel drums, radiolysis and possibly microbial activity.
- A geo-chemical disturbance caused by the leaching of large amounts of $NaNO_3$ and other soluble salts and by the leaching of water-soluble organic and potentially complexing agents from radiolytical-, chemical- and possible biodegradation of the bitumen.

In this report, an overview will be presented of the radiolytical and chemical degradation products from bitumen. In addition, the potential of microbes to reduce nitrate leaching from non-aged and thermally aged inactive bituminized waste in the presence or absence of relevant organic electron donors and the potential for microbial biodegradation of the bitumen was investigated more in detail.

2 Bitumen degradation

2.1 Ageing of bitumen

Bitumen is obtained by fractional distillation of crude petroleum and consists mainly of a complex mixture of high molecular weight hydrocarbons and a minor amount of structurally analogous heterocyclic species and functional groups containing nitrogen, sulphur and oxygen atoms. It also contains trace amounts of metals such as vanadium, nickel, iron magnesium and calcium present in the form of inorganic salts and oxides or in porphyrin structures [8]. The precise composition depends on the source of the crude oil from which the bitumen originates and on the modifications induced during the further production process and is therefore extremely complex. However, four broad chemical groups can be identified in bitumen, namely asphaltenes (5-25 wt %), saturates (5 - 20 wt %), resins (10 - 25 wt %) and aromatics (40 - 65 wt %). Typical chemical structures are presented in Figure 1.

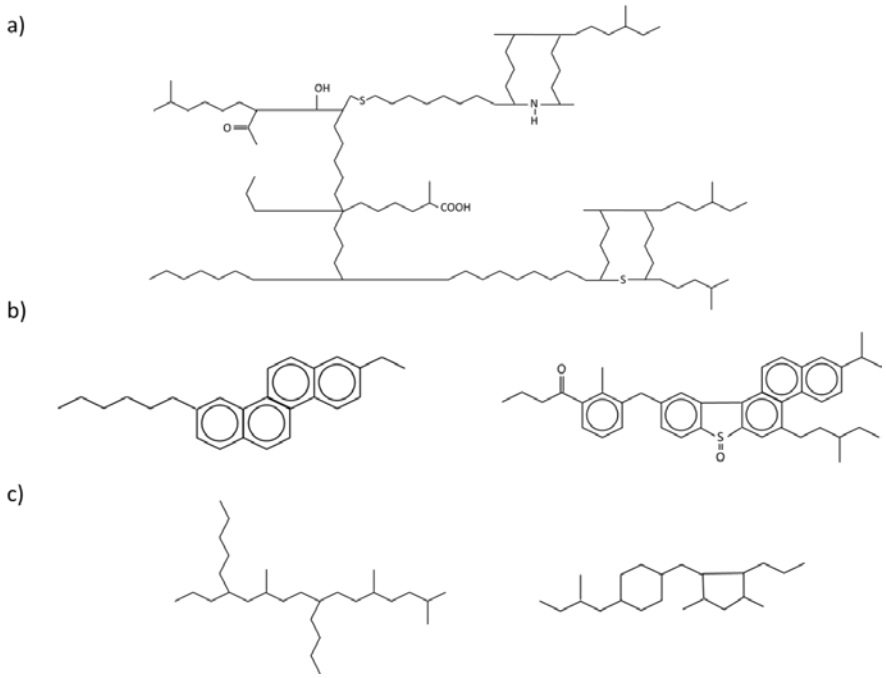

Figure 1: a) asphaltene structure, b) aromatic structures, c) saturate structures based on [8]

It is known that the rheological properties of bitumen change with time, a process called ageing. This results in harder bitumen, which tends to lose its binding capacity and becomes increasingly brittle. The most important chemical ageing process is oxidation, an irreversible process that results in an

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increase of the number of C=O and C=C groups together with an increase of the asphaltene content, a decrease of the saturates and aromatics content, while the amount of resins remains more or less the same. Furthermore, asphaltenes contain more hydrophilic groups, what might result – together with the expansion of (micro)fissures caused by ageing – in an increased infiltration of water into the aged bitumen matrix [8]. Oxidation is particularly important when bitumen is heated and/or irradiated in the presence of oxygen. Radio-oxidation was also observed in a 25 year old radioactive Eurobitum core, where the surface layer (upper 5 cm) was highly oxidized. When cutting the upper ~10 mm of the core with a firmly stretched steel wire, the loaded part of the sample disintegrated nearly completely into small crumbles. [7]. It is likely that with time this oxidation will extend deeper into the waste matrix, especially when oxygen can migrate to deeper layers. At this moment, the presence of oxygen deeper inside the waste is still under discussion. Most likely oxygen can infiltrate deeper in the waste via (micro)fissures formed in the bitumen caused by oxidation started from the surface. Microfissures are formed as a result of the internal stresses upon spatially cooling of the hot mixture after production and as a result of the increasing hardness due to oxidation. In addition, oxygen (or oxygen radicals) can be produced by the radiation of the $NaNO_3$ crystals. Although different studies have shown that there is an additional excitation (heating, dissolution in water) of the $NaNO_3$ crystals necessary to release the oxygen after γ irradiation [7, 9], a study of Hochanadel showed that α irradiation of $NaNO_3$ resulted in the production of oxygen levels that were 5 times higher compared to those after γ irradiation and still slightly higher compared to produced levels after γ irradiation at increased temperature [10]. However, it remains unclear whether this oxygen becomes available for reaction. Altogether, during geological disposal of bituminized waste, these changing properties of the bitumen matrix may affect the extent of the water uptake processes (causing swelling of the waste form or an increased pressure exerted on the gallery walls) and the leaching of $NaNO_3$ and radionuclides [2, 7, 11].

2.2 Radiolytical degradation of bitumen

The bitumen matrix will be constantly exposed to radiation during interim storage and deep geological disposal. The first 100 years, the main contributors to the total radioactivity will be β- and γ-emitters, while in the long term (up to 1 million years) α radiation becomes dominant. Between 100 000 and 1 million years, the nuclides belonging to the decay chain of ^{234}U and ^{206}Pb are predominantly present [4]. Based on data from Stankovskiy (2011) [4], the average dose rate for β/γ radiation will decrease from ~ 5 Gy/h after the first year to ~ 0.002 Gy/h after 100 000 years. For α radiation, the average dose rate will decrease from ~ 0.6 Gy/h after the first year ~ 0.05 Gy/h after 100 000 years (Table 1).

Table 1: Evolution of the average dose rates in the course of time in Eurobitum containers with average and maximum activity, calculated from data of Stankovskiy (2011) [4]

Time	**Containers with average activity**		**Containers with maximum activity**	
	alpha	beta/gamma	alpha	beta/gamma
1 y	0.6 Gy/h	5 Gy/h	1.6 Gy/h	20 Gy/h
100 y	0.8 Gy/h	1 Gy/h	2.2 Gy/h	5.5 Gy/h
100 000 y	0.05 Gy/h	0.002 Gy/h	0.1 Gy/h	0.008 Gy/h

Radiation of the organic compounds in the bituminised waste will lead to the formation of highly active free radicals such as $^{\bullet}COO^{-}$, R-H$^{\bullet}$, H$^{\bullet}$, $O^{\bullet -}$, $O^{\bullet 2-}$, OH$^{\bullet}$, $HO_2^{\bullet}$, $NO_3^{\bullet 2-}$, $NO_2^{\bullet}$, $N_2O_4^{\bullet}$. In the absence of oxygen (deeper inside the bituminised waste), the radicals are involved in cross-linking reactions in molecules and will lead to the production of gases, with H_2 making up approximately 90 to 95% of the total volume of gas produced. Other radiolytically produced gases are N_2, CH_4 and small hydrocarbons (1 - 4 C atoms) [9, 12]. As already stated, due to the fissures in the bitumen matrix that appear in time, oxygen can infiltrate deeper in the waste and consequently the type of radiolytically generated gases will change. The amount of small hydrocarbons will decrease, while next to H_2, N_2

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and CH_4, H_2O_2, CO and CO_2 are produced as well, but none of these carry any radioactivity [9, 12]. Besides gases, smaller and bigger water-soluble organic molecules can be formed from the reaction of free radicals.

During disposal conditions, chemical and radiolytical degradation processes in bitumen continue albeit in anoxic conditions. The bituminized waste gradually will be hydrated with cement-equilibrated Boom clay pore water resulting in the diffusion-controlled leaching of the produced water-soluble compounds, of which some putatively could complex radionuclides and enhance their mobility. In addition, radiolysis of water results in the formation of oxygen bearing radicals or O_2 which on their turn can promote oxidative ageing [13]. The presence of $NaNO_3$ saturated pore solution in the bitumen matrix however also results in the production of H_2, N_2O and H_2O_2 [14]. However, the extent of this process is not yet clear.

The type and amount of the organic molecules produced during chemical and radiolytical degradation products are mostly determined by leaching experiments, possibly following/while irradiation (e.g. bringing γ-irradiated inactive bitumen samples in contact with an aqueous solution). Experimental work has been performed with non-radioactive samples, to determine chemical degradation products and with radioactive Eurobitum or γ-irradiated inactive (Euro)bitumen [7, 15-18]. It was demonstrated that the leaching rate is influenced by temperature, the leachate composition, and the physical characteristics of the bitumen matrix [17]. Moreover, it has been shown that the amount of leaching products is dependent on several factors such as the presence or absence of oxygen [16], the quantity of the bitumen and the surface area/volume ratio [9]. In addition, the pH and ionic strength of the leachate are indicated as important factors [17]. The amount of leached dissolved organic carbon (DOC) increases strongly in function of the pH of the leachate. When a slightly alkaline solution of pH 8, reflecting the conditions of the pore water of the clay, is used as leachate during 8 weeks, the DOC is 5 times higher compared to ultra-pure water conditions. An increase in pH to 13.5, reflecting the near field conditions (i.e. young cementitious pore water) further increases the DOC 8 times [9]. When leaching experiments (70 hours) were performed with Eurobitum, the amount of DOC was also 2 times higher when cement-equilibrated clay water (pH 12.5) was used as leachate compared to synthetic Boom Clay water (pH 8.5) [19]. The observed differences can be explained by the higher solubility of certain types of organic molecules and on the chemical attack of the bitumen matrix at alkaline pH [9].

Due to the heterogeneity in experimental setups and the consequent variable results, it is difficult to predict the real concentrations of soluble compounds specifically relevant for Eurobitum. Nevertheless, next to the high amount of salts (mainly $NaNO_3$) the following groups of molecules have been identified after leaching experiments of bitumen and bituminised waste in alkaline conditions [9, 16]:

- Carboxylic acids, essentially lower linear acids such as formic acid and acetic acid but also longer mono- and polycarboxylic acids such as propanoic acid, butanoic acid, pentanoic acid, hexanoic acid, heptanoic acid and octanoic acid.
- Glycol, essentially ethylene glycol
- Aromatic compounds such as naphthalene, alkyl substituted naphthalene and polysubstituted phenol
- Oxidised compounds such as
    - Linear alcohols containing between 8 and 19 carbon atoms
    - Linear carbonyls containing between 7 and 13 carbon atoms
    - Aromatics
- Sulphur compounds
- Nitrogen compounds

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Combined chemical degradation and radiolytic degradation experiments were performed as well. Here, it is again difficult to predict the real concentrations of the soluble compounds. Moreover, most experiments study non-radioactive bitumen and irradiate it with an external gamma source, while in the real scenario the dose absorbed by alpha radiation will be more significant. In addition, the dose rates used (kGy/h) are in most cases much higher than the real dose rates (Gy/h) to obtain realistic total absorbed doses within a reasonable time frame, and the amount of degradation compounds depends on the type of set-up (with or without oxygen, whether water is present already during the irradiation or not). Nevertheless, from the performed experiments it seems that the defined soluble compounds are in general very similar in nature to the ones found in case of purely chemical degradation. Additionally identified compounds are ketones and phenols [9, 19] and other types of carboxylic acids [19]. Although only found in some experiments, another bitumen degradation product worth mentioning is oxalate as it is a known complexing agent for radionuclides (discussed in MIND deliverable 1.1 [20]). Oxalate is often found when carboxylic acids are irradiated as a consequence of the reaction of two $^{\cdot}COO^{-}$ radicals. It is expected that oxalate will only be produced in the presence of oxygen [19, 21].

2.3 Biodegradation of bitumen

The possible degradation of bitumen by micro-organisms is already known and studied since at least 1935 [22]. Biodegradation rates depend on the chemical composition of the bitumen substrate, the metabolic character of the microorganisms involved and various biological and physico-chemical parameters [23]. In aerobic conditions, biodegradation rates are much higher compared to anaerobic conditions [24, 25]. The biochemical mechanisms involved in anaerobic degradation of hydrocarbons are completely different and more diverse compared to those involved in aerobic degradation. In addition, known organisms capable of anaerobic hydrocarbon degradation belong to different genera than the aerobic degraders [26, 27]. The most frequently isolated organisms capable of aerobic degradation of hydrocarbons belong to the genera *Pseudomonas, Acinetobacter*, *Flavobacterium*, *Corynebacterium*, *Alcanivorax* and *Arthrobacter*, all common soil or freshwater bacteria, but a wide range of other genera has been identified [27]. The anaerobic hydrocarbon-degrading bacteria comprise among many others the genera *Thauera*, *Azoarcus*, *Aromatoleum, Georgfuchsia*, *Geobacter, Desulfobacula* and *Desulfococcus* [26]. Therefore, the low degradation rates observed in anaerobic conditions in some of these experiments can putatively be explained by the inadequate cultures used in the degradation experiments. Nevertheless, both in aerobic and anaerobic conditions a distinct production of N_2 and CO_2 was observed and all short strain carboxylic acids (C_5-C_{10}) leached from the bitumen were degraded by microorganisms, showing a clear activity of microorganisms [25]. In addition, in aerobic as well as anaerobic growth conditions biofilm formation on the bitumen was often observed [23, 24, 28]. It was shown that blown 'hard' bitumen is more sensitive to biodegradation compared to a distilled 'soft' bitumen, comprising a lower amount of saturates [23]. This is in agreement with the fact that the resins and asphaltenes are much more resistant to biological degradation compared to the saturates, although organisms able to use asphaltenes as carbon source are reported several times [29-31].
In conclusion, the biodegradation of the bitumen itself can occur, although the biodegradation rates will probably be low. However, as already mentioned above, the chemically and radiolytically produced degradation products can be easily used as carbon source by microorganisms.

2.4 Consequences of leaching of bitumen degradation products and $NaNO_3$ during deep geological disposal

Some of the chemically and radiolytically produced water-soluble organic degradation products may form complexes with radionuclides, thereby increasing their solubility and decreasing their sorption [17, 19, 32]. Furthermore, the nitrate plume originating from the high amounts of $NaNO_3$ released from bituminised waste, might affect certain properties of the Boom Clay: hydraulic conductivity, anion exclusion phenomena, sorption potential, microstructure, but also the redox conditions

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present in the Boom Clay. The latter might cause a decrease of its reducing capacity and putatively an increase of the mobility of redox-sensitive radionuclides (Se, Tc, U, Np, Pu) [33]. Scoping conservative calculations indicate that, close to the waste drums the $NaNO_3$ concentration can reach up to a few molar and that during the first ~300 to ~1000 years the $NaNO_3$ concentration within the monolith will remain above 1 M and 0.4 M, respectively [34]. Maximum NO_3^- concentrations reach between 0.5 and 1 M at the gallery interface, and decrease also fast within the host formation (e.g. 0.1 M at a distance of 5 m in the Boom Clay) [34]. A purely chemical reaction between nitrate and redox-active components of the Boom Clay (dissolved organic matter, pyrite, kerogen) is not expected to occur or characterized by very slow kinetics [35]. In contrast, the oxidation of pyrite and dissolved organic matter by nitrite (resulting from the radiolysis of $NaNO_3$), was observed [36, 37] However, it is assumed that the impact will be limited due to the slow reaction kinetics and the expected low concentrations of nitrite leaching from the bituminized waste [36, 37]. However, hydrogen that will be produced in the disposal facility and/or steel can act as electron donor for abiotic nitrate reduction, with the steel or even pyrite serving as catalytic surface [38-41]. These reactions lead to the production of ammonium which can sorb onto clay minerals and therefore compete with some radionuclides for sorption (e.g. Ni, Pd, Cs), but could also affect the solubility of certain radionuclides (e.g. Pd, Ni) [32, 42-44].

3 Microbial nitrate reduction processes

Microorganisms will be present in the repository either as autochtonous species that are already present in the host formation (although no consensus has been reached yet) or as species brought into the repository during its construction or putatively via the waste monolith [45]. In anoxic conditions, nitrate is the most energetically favourable electron acceptor. Consequently, after oxygen is consumed and when nitrate becomes available, the microbial population near the disposal facility of bituminised waste will likely evolve to nitrate and/or nitrite reducing prokaryotes that could augment nitrate reduction in the repository. To fuel the nitrate reduction, water-soluble organic bitumen degradation products can be used as electron donor, thereby reducing the amount of possible complexing agents for radionuclides. The first step in dissimilative nitrate reduction (DNRN) is the production of nitrite. This can be further reduced to to gaseous nitrogen compounds (NO, N_2O, N_2) by denitrification or to ammonium by DNRA (dissimilative nitrate reduction to ammonium). On its turn, nitrogen fixating species can reduce the N_2 to ammonia and then assimilate this into organic forms, such as amino acids and nucleotides and a few bacterial genera are able to oxidize ammonium to nitrogen gas, a process called anammox (anoxic amonium oxidation). On the other hand, in certain conditions, nitrite will not be further reduced, resulting in an accumulation of nitrite [46]. (Figure 2).

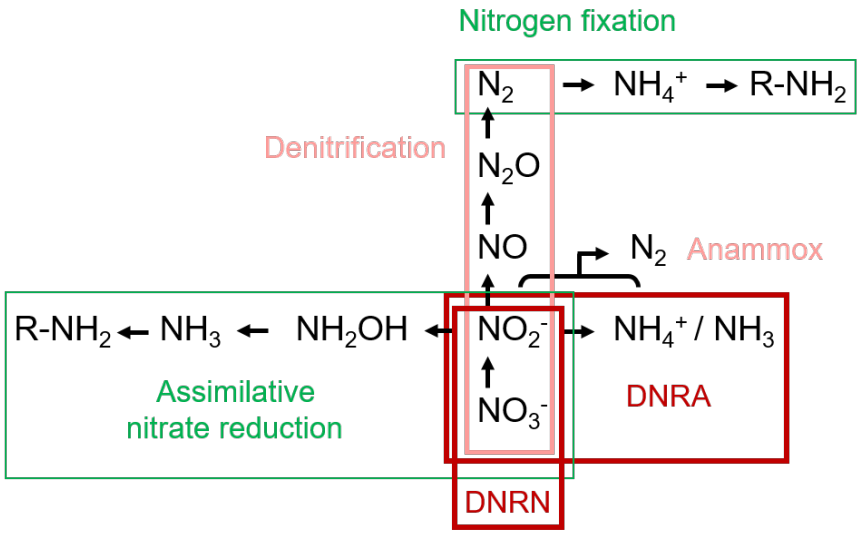

Figure 2: Biological pathways involved in the anoxic reduction of nitrate [36].

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Denitrification and DNRA are two processes that compete for the same electron acceptor. It has been shown that in soils, the redox status and the C/N ratio (*i.e.* the ratio of available carbon used as electron donor over nitrate-nitrogen) are key parameters in regulation of the processes. The general hypothesis is that DNRA may outcompete denitrification in more reducing conditons and at low or limiting nitrate conditions and an excess of available carbon, thus a high C/N ratio [47]. However, due to low biodegradability of DOM in the Boom Clay and the expected slow release of water-soluble bitumen degradation products from the bituminised waste [2, 19, 48], it is expected that nitrate reduction to nitrite and denitrification will be the preferred processes in the repository. As already mentioned above, nitrite accumulation can lead to the oxidation of redox-active Boom Clay components, which could (locally) decrease the reducing capacity of the clay formation [36, 37]. Thus incomplete denitrification and netto nitrite production can affect the host rock safety functions. Full denitrification could lead to a net gas pressure increase or decrease, depending on the electron donor used in the denitrification process. If dissolved organic matter present in the clay formation or organic compounds leaching from the bituminized waste are used as electron donor, a net gas production is observed [49]. If the concentration of the produced nitrogen gases would exceed the solubility limit of the gases, a separate gas phase could be formed, putatively causing fissuring of the host rock, hence resulting in the formation of preferential pathways for radionuclide migration [50]. On the other hand, the use of hydrogen as electron donor results in a net gas consumption, preventing a gas pressure build-up in the disposal facility and the associated gas-related perturbation of the clay, but it can result in the production of ammonium [49], which can sorb onto clay minerals and therefore compete with some radionuclides for sorption (e.g. Ni, Pd, Cs), but could also affect the solubility of certain radionuclides (e.g. Pd, Ni) [32, 42-44].

In this report, the effect of different organic additives on the nitrate reduction rate leaching from regular and thermally aged Eurobitum was investigated more in detail. In addition, the effect of pH on the nitrate reduction was studied and biofilm formation on the Eurobitumen itself in the different conditions was analysed.

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4 Materials & Methods

4.1 Characteristics of the Boom Clay Borehole water

The HADES (High-Activity Disposal Experimental Site) underground research facility is located at a depth of 225 meters in the core of the Boom Clay (Figure 3). Boom Clay is a marine sediment from the Rupelian period (approximately 35 million years ago), dominated by illite and smectite clay minerals. It is a silty clay or argillaceous silt and presents a layered structure with (limited) variations in grain size (alternating clayey silt and silty clay layers), organic matter content and carbonate content. These variations are the consequence of depositional processes (sedimentary, from marine origin) and subsequent limited burial history. The band thickness ranges between 10 centimetres and 2 meters. A detailed overview of the geology is given in [51].

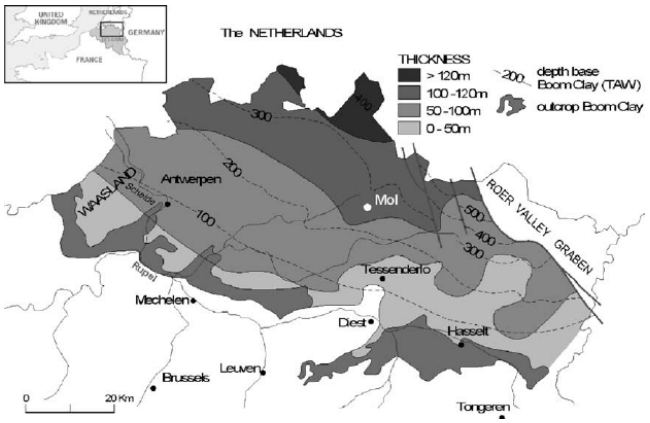

Figure 3: Occurrence and thickness of the Boom Clay formation in Belgium. The HADES underground research facility is indicated in white [52]

The bulk mineralogy of the Boom Clay is shown in Table 2. Qualitatively, the mineralogy is the same over the entire Boom Formation but the quantities of the minerals present at each clay layer vary according to the precise sedimentological build-up of the deposit. Organic matter from marine origin is present is small quantities (ca. 0.5 %) while organic particles from terrestrial origin can reach a few percentage (3-5 %) in some horizons [53]. The clay contains about 20 wt % of water that mainly consists of about 15 mM $NaHCO_3$ and 150 mg/l dissolved organic carbon. At a temperature of 16 °C, it has a slightly alkaline pH around 8.5 and a pCO_2 of $10^{-2.62}$ bar (Table 3)[33].

Table 2: Mineralogical composition of the Boom Clay [53].

Mineral	Amount
Quartz	20-60 %
Clay minerals	22-56 %
Illite	5-18 % of bulk
Smectite	7-24 % of bulk
Mixed ilite-smectite	7-23 % of bulk
Plagioclase	10-15 %
Potassium-feldspar	10-15 %
Kaolinite	10-15 %
Chlorite	1-4 %
Pyrite	0-3 %
Carbonates	
Siderite	0-1 %
Dolomite	0-1.5 %
Calcite	0-4 %
Anatase	> 1 %

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Table 3: Average Boom Clay pore water composition at a temperature of 16°C [33]. Concentrations are presented in mg/l

Component	Concentration	Component	Concentration
HCO_3^-	880	SO_4^{2-}	2.2
Na^+	359	Ca^{2+}	2
Cl^-	26	Mg^{2+}	1.6
K^+	7.2	Br^-	0.6
Si	3.4	Fe	0.2
F^-	3	Al	0.0006

The water used to carry out the experiments was borehole water from the piezometer called TD-116E (previously reported as SPRING). The piezometer is placed horizontally in the Boom Clay, and is located in the Test Drift part of the HADES URF, at ring 116, pointing towards the east. It is entirely made of stainless steel and contains in total four large surface filter screens made from high porosity seamless filter tube made by "Krebsöge", quality: SIKA R5, material: 1.4404 (AISI 316 L/B), pore size distribution: 7 to 16 µm. This piezometer was designed in 1999 to provide sufficient quantities of representative Boom Clay pore water as feed and reference material for laboratory experiments [33]. The chemical composition of the pore water used in this study is presented in Table 4.

Table 4: Composition of Boom Clay pore water from piezometer TD-116E used as background water for batch experiments 1 and 3. Concentration are in mg/l unless indicated otherwise.

Component	Concentration	Component	Concentration
pH	8,56	Alkalinity (meq/l)	15,5 meq/l
TIC	169	TOC	72
Na^+	357 ± 36	SO_4^{2-}	2.78 ± 0.32
Cl^-	21.5 ± 1.4	Ca^{2+}	3.65 ± 0.38
K^+	11.4 ± 1.5	Mg^{2+}	3.07 ± 0.31
Si	4.4 ± 0.5	Br^-	0.94 ± 0.23
F^-	2.86 ± 0.29	Fe	0.282 ± 0.035

The inoculum for the experiments was collected from another piezometer. In a recent study, the microbial population present in borehole water collected via a vertical piezometer TD-11D (previously reported as MORPHEUS) was characterised in detail [54]. The piezometer, installed in 2001, was designed to study the variability of the Boom Clay pore water composition underneath the HADES research facility. It allows pore water sampling at 12 different stratigraphic levels of the Boom Clay (clayey/silty, organic rich/poor, carbonate rich/poor). All the porous filter screens of this piezometer are made out of "Schumatherm" filters, used for its chemically inert characteristics [33]. The chemical composition of the pore water used during this study is presented in Table 5. The microbial population present in borehole water from filter 23 was determined as the most representative for the microbial community present in that piezometer [54], hence it was selected as inoculum for the experiments carried out in this study (Figure 4).

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Table 5: Composition of Boom Clay pore water from filter TD-11D-23 used as in inoculum for all batch experiments performed in this study. Concentration are in mg/l unless indicated otherwise.

Component	Concentration	Component	Concentration
pH	7,88	Alkalinity (meq/l)	15,0 meq/l
TIC	166	TOC	142
Na^+	389 ± 39	SO_4^{2-}	80.9 ± 3.6
Cl^-	28.2 ± 1.8	Ca^{2+}	2.86 ± 0.30
K^+	8.6 ± 1.3	Mg^{2+}	2.54 ± 0.25
Si	5.8 ± 0.6	Br^-	1.11 ± 0.23
F^-	2.98 ± 0.30	Fe	0.53 ± 0.06

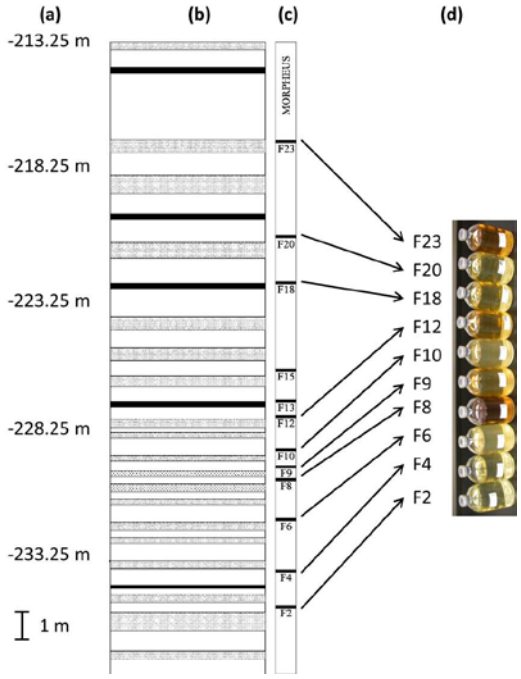

Figure 4: Schematic view of the vertical piezometer TD-11D, used for sampling Boom Clay borehole water, indicating depht below sea level (a), Boom Clay stratigraphic layers (b) (white = clayey, grey = silty, black = including septaria, very silty), piezometer filters, as indicated by Fxx (c) and corresponding samples in septum bottles (d), as indicated by arrows.

4.2 Characteristics of the bitumen samples

Non-radioactive reference Eurobitum used in this study originates from a drum (identification code 0-2 CR15/16) that was produced during the inactive start-up phase of the Belgian reprocessing facility Eurochemic/Belgoproces. The production method and composition of this non-radioactive reference Eurobitum are similar to that of the radioactive Eurobitum, except for the presence of the radionuclides. This reference Eurobitum is characterized by a density of 1.31 g/ml and it is composed of 38.9 % salts of which 28.5 % is $NaNO_3$ and 0.39 % water. Cylindrical specimens were sampled using a sampling tube designed at SCK•CEN with inner diameter of 52 mm, which were further sliced in cylindrical samples with a height of 10 mm [7]. These cylindrical specimens were further cut in blocks with dimensions of ca. 7 mm x 7 mm x 10 mm. Thermally aged Eurobitum was prepared by heating crushed non-radioactive reference Eurobitum 0-2 CR15/16 at 180°C and manually mixing them for 8 h to maximise the exposure of the bulk of the material to air. Afterwards, the aged Eurobitum was cooled down slowly to ambient temperature. In the initial stage of the cooling process, the sample was occasionally stirred to reduce air bubbles formed at the bottom of the sample [7]. From the pieces of this thermally aged Eurobitum, cylindrical samples of 10 mm thickness were sliced as for the non-aged Eurobitum, which were further cut in blocks with dimensions of 7 mm x 7 mm x 10 mm. To work as sterile as possible, all cutting materials were as good as possible disinfected with 70 vol % ethanol.

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4.3 Culture conditions

All batch experiments were performed in anoxic conditions. To this end, all preparations were performed in an anaerobic glove box with a manually controlled atmosphere of 99% argon and 1% hydrogen. Acetate, formate and oxalate were selected as representative degradation products from bitumen and added as electron donor and/or C source.

In **the first series of batch experiments**, 250 ml glass bottles were filled with 90 ml 0.22 µm filter sterilized, autoclaved and deoxygenated **natural Boom Clay pore water** collected from piezometer TD-116E supplemented with 15 mM sodium acetate, 15 mM sodium formate or no organic additives. Important to note is that in this way, part of the dissolved organic matter present in the Boom Clay pore water could be oxidized. Three blocks of regular or thermally aged non-radioactive bituminised waste were added, with a total weight varying from 2.51 g to 2.83 g bitumen. Control samples in which no Eurobitum was added were also included. Samples were inoculated with 10 ml Boom Clay borehole water from piezometer TD-11D-23 providing the microbial community or with 10 ml sterile Boom Clay borehole water from piezometer TD-116E. All conditions were performed in triplicate. The glass bottles were hermetically closed with a butyl stopper to maintain anoxic conditions and incubated at **30°C** without shaking.

In **the second series of batch experiments,** 250 ml glass bottles were filled with 90 ml 0.22 µm filter sterilized, autoclaved and deoxygenated **Synthetic Boom Clay water (SCW)** supplemented with 15 mM sodium acetate, 15 mM sodium formate, 15 mM sodium oxalate, a mixture of 5 mM sodium acetate, 5 mM sodium formate and 5 mM sodium oxalate or no organic additives. SCW is a mixture of 15 mM $NaHCO_3$ and 1.5 mM Na_2CO_3 and represents the main components of Boom Clay porewater except for the presence of DOM. Three blocks of regular or thermally aged non-radioactive bituminised waste were added, with a total weight varying from 2.41 g to 2.65 g bitumen. Control samples in which no bituminised waste was added were also included. Samples were inoculated with 10 ml Boom Clay borehole water from piezometer TD-11D-23 providing the microbial community or with 10 ml SCW. All conditions were performed in triplicate. The glass bottles were closed and incubated at **room temperature** without shaking in the anaerobic glove box (99% Ar/1% H_2) to maintain anoxic conditions throughout the experiment.

The consumption of nitrate, nitrite, acetate, formate and oxalate, and the microbial cell count was monitored by taking subsamples regularly. At each sampling time, subsamples of 2 ml were retrieved using a sterile syringe and needle pushed through the septum of each bottle.

4.4 Chemical analysis

Nitrate, nitrite, acetate, formate and oxalate were measured with a Dionex ion chromatography system equipped with a Dionex IonPac AS11-HC anion exchange column, a Dionex IonPac AS11-GC guard column and conductivity detector. Before measuring, samples were diluted 40 times and filtered using 0.45 µm Acrodisc® PSF syringe filters. The eluent gradient program was 1 mM sodium hydroxide for 8 min, increasing to 15 mM sodium hydroxide during the following 10 min, increasing to 30 mM sodium hydroxide in the following 10 min, in the next 10 min increasing to 60 mM sodium hydroxide and finally hold for 2 min at 60 mM sodium hydroxide. The chromatograms were collected and processed with the Chromeleon version 6.5 software.

TOC/TIC analysis was performed with a Shimadzu TOC-L CPH analyzer. Total carbon (TC) analysis is measured after placing the sample in a combustion tube, which was filled with an oxidation catalyst and heated to 680 C. The sample was burned, by which all carbon components were converted to carbon dioxide. Carrier gas (oxygen) carried the combustion products to a non-dispersive infrared (NDIR) gas analyzer, where the carbon dioxide was detected. Total inorganic carbon (TIC) analysis was performed by introducing each sample into an IC-reactor filled with a dilute phosphoric acid solution (pH less than 3). In the IC-reactor, carbonates and hydrogen carbonates of the sample were converted to carbon dioxide. Carrier gas (oxygen) purged out the carbon dioxide from the IC-reactor

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and lead it to a non-dispersive infrared (NDIR) gas analyzer, where the carbon dioxide was detected. The total organic carbon (TOC) was calculated as the difference between the TC and TIC analysis values.

4.5 Microbiological analysis

Flow cytometry was used to count the total amount of microbial cells present in the borehole water. Samples were diluted in 0.22 µm filter sterilized Evian potable water. Double stranded DNA was stained with SYBR® Green I (10 000 x concentrate in 0.22 µm filtered dimethyl sulfoxide) (ThermoFisher Scientific, Belgium) (final concentration of 1x concentrate) and incubated in the dark for 20 min at 37°C. Flow cytometry was performed using a C6 Accuri™ flow cytometer (BD Biosciences, Belgium), which was equipped with four fluorescence detectors (530/30 nm, 585/40 nm, >670 nm and 675/25 nm), two scatter detectors and a 20-mW 488-nm laser. The flow cytometer was operated with Milli-Q filtered water (Merck Millipore, Belgium) as sheath fluid. Samples were analysed in a fixed volume mode of 50 µl and the threshold was fixed on the green fluorescence (FL1-H at 1000). Dilutions which had between 200 and 2000 events per µl were considered representative and used for the calculation of cell counts. After flow cytometry measurement, data were extracted under the Flow Cytometry Standard format, imported in R (v.3.4.4) and analysed with the Phenoflow package developed by Props *et al.* (2016) [55].

DNA was extracted from the samples using the QIAamp DNA mini kit (Qiagen, Belgium). Concentration of dsDNA was determined using the Quant-iT™ PicoGreen® dsDNA Assay Kit according to the manufacturer's protocol (Promega, the Netherlands) and measured with the Clariostar monochromater microplate reader (Isogen Life Sciences, the Netherlands). Fluorescence was measured using 483-14 nm for excitation and 530-30 nm for emission and an automated gain and focus adjustment was performed. PCR analysis of the V3-V4 region of the bacterial 16S rDNA (341F and 785R primer) and subsequent sequencing using illumina MiSeq V3 technology was performed by LGC genomics (Germany). The obtained raw DNA sequences were treated with an in-house developed bioinformatics pipeline, optimised to pre-process the output of different high throughput sequencing methods. This pre-processing includes trimming, denoising [NoDe [56] or IPED [57]] and chimera removal [CATCh [58]]. Using this software, the obtained DNA sequences were clustered into operational taxonomic units or OTU [59]. Each OTU contains a number of reads indicating the relative abundance of the OTU in the analysed sample. The 50 most abundant OTUs were further characterised. Each of the consensus DNA sequences of these OTU was used to find the closest matching bacterial species by BLAST against the GreenGenes database with a minimum of 90% sequence similarity [60].

4.6 Scanning electron microscopy (SEM)

One of the Eurobitum blocks present in the samples was used to perform SEM analysis. The Eurobitum block was washed with 10 mM $MgSO_4$ for 10 min to remove residual microbial cells that are not attached to the Eurobitum. Samples were fixated overnight with solution containing 0.3 M gluteraldehyde and 0.132 M sodium cacodylate. Afterwards, excess gluteraldehyde was removed by washing the filter with sodium cacodylate solution (0.150 M) twice. Subsequently, cells were dehydrated using an ascending graded series of ethanol solutions (30%, 50%, 70%, 90%, 95% v/v), followed by a final solution of 100% ethanol which was replaced two times (minimum 10 min between each solution). Drying of the samples was performed twice with hexamethyldisilazane for 2 min, followed by air drying for at least one hour. Finally, a small piece of Eurobitum was sliced with a sterile razor blade and mounted on a copper stub using carbon conducting tape and nail polish. Samples were sputter-coated with gold (20 nm) in one cycli of 200 s (4 mbar Argon, 50 mA, 1 kV) (Scancoat Six, BOC Edwards B.V., Dongen, The Netherlands). SEM analysis was performed on a Phenom ProX (Phenom-World, The Netherlands), equipped with a backscatter electron detector at a working distance of 20 mm and a 10 or 15 kV acceleration.

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5 Results

5.1 Results of the first series of batch experiments

5.1.1 $NaNO_3$ leaching from regular and thermally aged bitumen under free swelling conditions

Figure 5 represents the time evolution of the $NaNO_3$ concentration leached from regular and thermally aged non-radioactive bituminized waste under free swelling conditions in abiotic conditions. Initially, $NaNO_3$ is leaching fast from the bitumen, from pores close to the surface, but afterwards the leaching process proceeds much slower. $NaNO_3$ leaching from deeper layers within the bituminised waste is a diffusion controlled process – where less interconnecting pores or cracks are present – is limited by the diffusion rate through the leached bitumen layer at the surface of the sample [61]. At day 3, the concentration of $NaNO_3$ leached from thermally aged Eurobitum was significantly (shown with Mann–Whitney U test) higher compared to that of regular Eurobitum, demonstrating that ageing of bitumen can enhance $NaNO_3$ leaching. This leaching behaviour and the total amount of $NaNO_3$ leached from the samples is in agreement with previously published results from a water uptake and leaching experiment with Eurobitum at free swelling conditions (though with different geometry and normalised for the initial hydrated surface) [61]. Note also that ± 40 % of the total amount of $NaNO_3$ had leached within these 50 days.

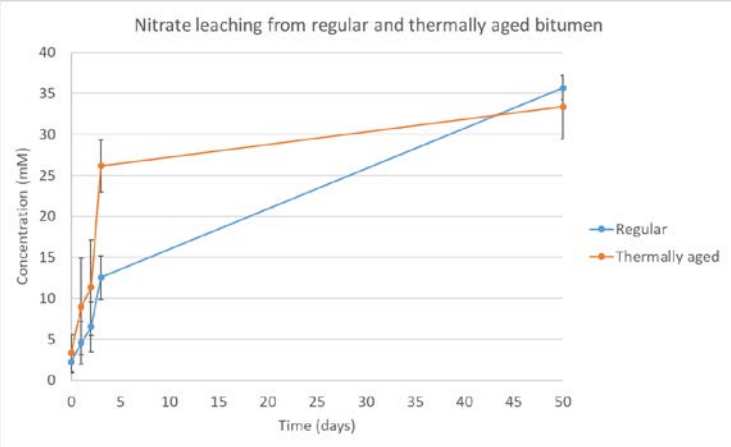

Figure 5: Time evolution of $NaNO_3$ concentrations leached from regular (blue line) and thermally aged non-radioactive bituminized waste (orange line) under free swelling conditions. Results represent the averages and standard deviation from all conditions where no microbial inoculum was added.

5.1.2 Impact of the electron donor on microbial nitrate reduction

To calculate nitrate reduction rates, nitrate concentrations in the biotic samples were compared with the abiotic conditions. When no additional electron donors were added, the nitrate concentration did not decrease during 50 days of incubation (Figure 6) and no production of nitrite was observed. This is in agreement with previous studies demonstrating that the natural dissolved organic material present in Boom Clay borehole water is not an easy accessible electron donor [35, 48]. Additionally, although not measured via TIC/TOC analysis in this experiment, this suggests that no significant amount of organics leached from the bitumen or they were not used as electron donor to stimulate nitrate reduction. When 15 mM HCOONa was added as additional electron donor, ca 15 % of nitrate that leached from the regular bitumen was reduced after 50 days, while 6 % of nitrate that leached from the thermally aged bitumen was reduced after 50 days. This corresponds to respectively 0.10 mM NO_3^-/day and 0.04 mM NO_3^-/day. In that time frame, respectively 5.7 and 6.6 mM formate was used as electron donor or C-source and 2.8 and 3.3 mM NO_2^- was produced. On the other hand, when CH_3COONa was added as additional electron donor, about 42 % of the nitrate that leached from the regular bitumen was reduced after 50 days and 41 % of the nitrate that leached from the

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thermally aged bitumen was reduced after 50 days. This correspond to 0.3 mM NO_3^-/day for both types of bitumen. The 15 mM CH_3COO^- was completely oxidised and 10 and 16 mM NO_2^- was produced for respectively regular and thermally aged bitumen. The maximum removal rate was observed after 3 days for both types of bitumen and was ~ 3.2 and ~ 3.8 mM NO_3^-/day for respectively regular and thermally aged bitumen. Overall, there seems no difference between regular and thermally aged bitumen.

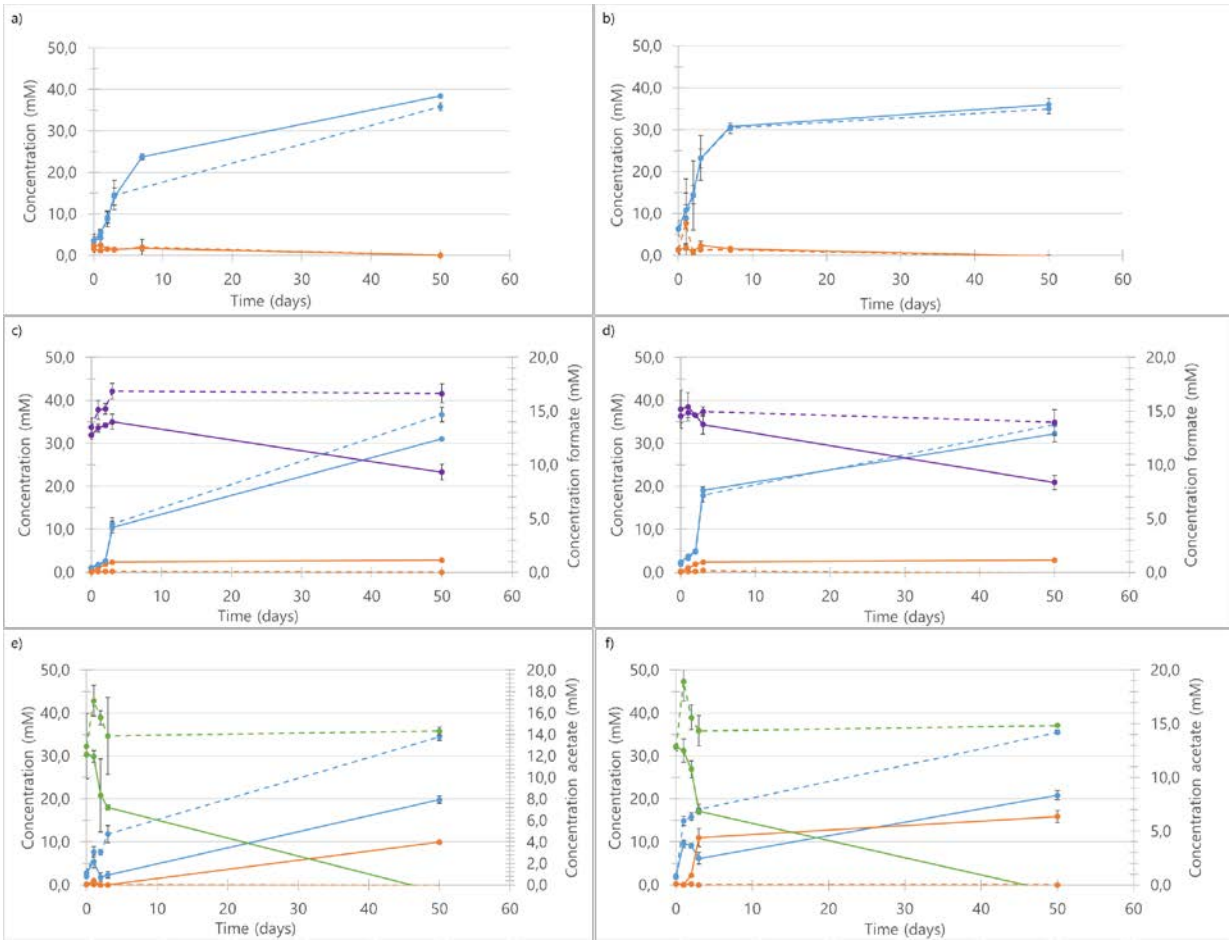

Figure 6: Evolution of nitrate (bleu), nitrite (orange), formate (purple) and acetate (green) concentrations during 50 days of incubation for regular (left) and thermally aged (right) non-radioactive bituminized waste with a), b) no additional electron donors added; c), d) 15 mM HCOONa added as additional electron donor and e), f) 15 mM CH_3COONa added as additional electron donor. Values represent the average and standard deviation of three replicates of samples with Boom Clay borehole water with inoculum (full line) or without microorganisms (dashed line).

In general, the microbial cell count increased slightly during the first 3 days of incubation. Afterwards, it stayed constant over the course of the experiment. The number of microbial cells in the samples without the addition of microbial inoculum, increased fast in case of thermally aged bitumen, while in the other two conditions, it remained low until 50 days of incubation (Figure 7).

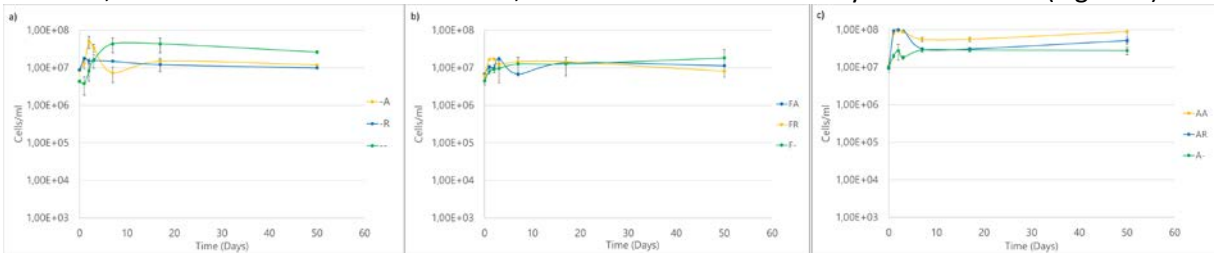

Figure 7: Evolution of microbial cell count for a) conditions were no electron donor was added b) formate as electron donor and c) acetate as electron donor. Values represent the average of three replicates and standard deviation. Results for regular bitumen are shown in blue, thermally aged bitumen are shown in red and samples without bitumen are shown in green.

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5.1.3 Microbial community

A metagenomics study based on the 16S rDNA gene sequences of the microbial community present in the Boom Clay borehole water filter TD-11D in the presence of non-radioactive regular and bituminised waste and in the presence of different electron donors was performed. Community profiling was performed at different time points during the batch experiments to investigate possible shifts in the community.

To investigate whether sufficient sample was collected to perform 16S rDNA gene amplicon sequencing, rarefaction curves for each sample, showing the number of OTUs in function of the number of reads were made (Figure 8). Curve shape indicates community diversity while the maximum values indicate observed community richness. If the curve flattens towards the right, sufficient individual samples have been analysed and it is unlikely to find many new additional species by increasing the number of samples [33], which was the case for all samples (Figure 8).

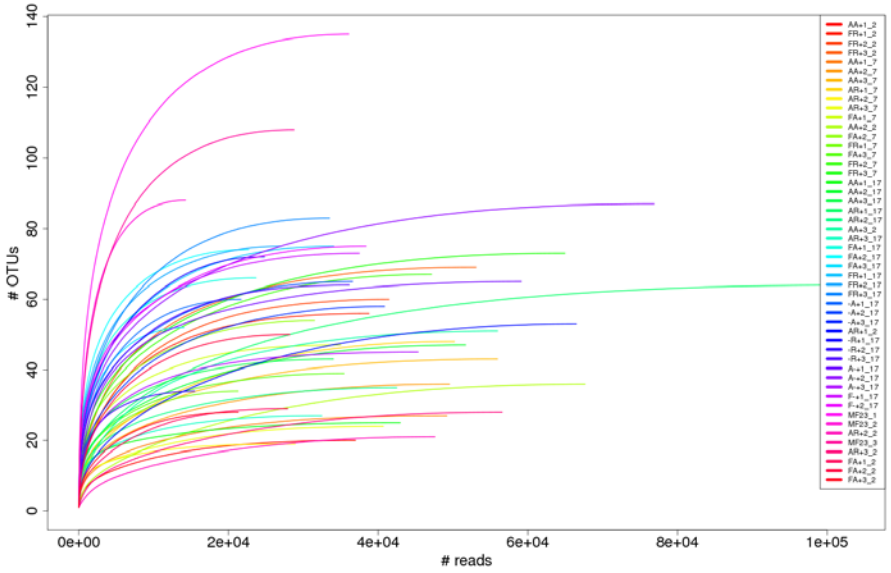

Figure 8: Rarefaction curves for the Illumina® 16S rDNA gene amplicon sequencing data showing for each sample the number of OTUs in function of the number of reads.

Taxonomical characterization of the OTUs from the Boom clay borehole water filter TD-11D used as inoculum for the batch tests, indicated the presence of 5 abundantly represented (sub)phyla (OTU contains at least 1% of total sequences in all 3 replicates), namely the Proteobacteria, Chloroflexi, Firmicutes, Actinobacteria and Bacteriodetes (Figure 9). Proteobacteria represent 82% of the microbial community with similar abundance of β- and γ-proteobacteria (38%), while α-proteobacteria where slightly less abundant, representing 24% of the proteobacteria. Within each class, there was one highly prominent genus present, namely *Sphingomonas, Achromobacter* and *Pseudomonas* representing respectively 96% of the α-proteobacteria, 71% of the β-proteobacteria and 100% of the γ-proteobacteria.

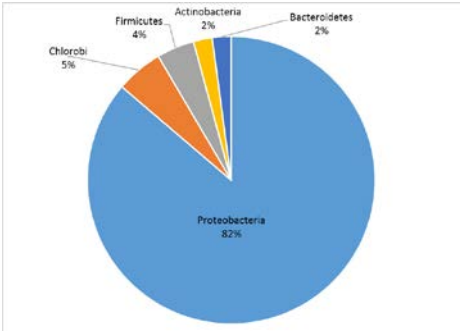

Figure 9: Pie chart showing the relative abundance of bacterial phyla present in the Boom Clay boreholewater filter TD-11D based on the classification of representative sequences of OTUs.

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A more detailed overview of the distribution of the different OTUs is given in Figure 10. Overall, the distribution of OTUs in all 3 replicates is highly similar. Eleven OTUs contain at least 1% of the total sequences in all three replicates (Table 6). The most abundant OTUs represent the following genera: *Pseudomonas* (31%), *Achromobacter* (22%), *Sphingomonas* (19%), *Acidovorax* (8%), *Ignavibacterium* (5%) and *Acetobacterium* (3%).

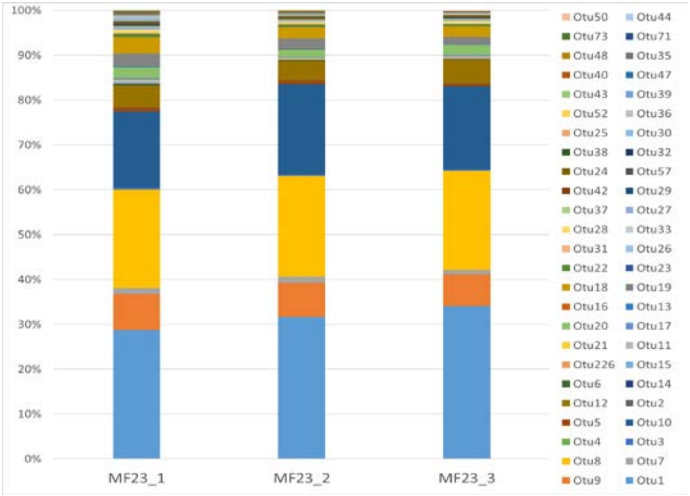

Figure 10: Distribution of OTUs present in three replicates of Boom Clay borehole water filter TD-11D (MF23).

Table 6: Taxonomic characterization of the OTUs that contained at least 1 % of the sequences present, as function of abundancy, in 3 replicates of Boom Clay borehole water filter TD-11D (MF23)

OTU	Phyla	Class	Family	Genus	Abundance
OTU1	Proteobacteria	Gammaproteobacteria	*Pseudomonadaceae*	*Pseudomonas*	31%
OTU8	Proteobacteria	Betaproteobacteria	*Alcaligenaceae*	*Achromobacter*	22%
OTU10	Proteobacteria	Alphaproteobacteria	*Sphingomonadaceae*	*Sphingomonas*	19%
OTU9	Proteobacteria	Betaproteobacteria	*Comamonadaceae*	*Acidovorax*	8%
OTU12	Chloroflexi	Ignavibacteria	*Ignavibacteriaceae*	*Ignavibacterium*	5%
OTU18	Firmicutes	Clostridia	*Eubacteriaceae*	*Acetobacterium*	3%
OTU19	Actinobacteria	Actinobacteria	*Coriobacteriaceae*	*unclassified*	2%
OTU20	Bacteroidetes	unclassified	*unclassified*	*unclassified*	2%
OTU7	Proteobacteria	Betaproteobacteria	*Comamonadaceae*	*Acidovorax*	1%
OTU5	Proteobacteria	Alphaproteobacteria	*Rhizobiaceae*	*Rhizobium*	1%
OTU28	Firmicutes	Clostridia	*Clostridiaceae*	*Proteiniclasticum*	1%

When Boom Clay borehole water is incubated 17 days in the presence of bitumen without the addition of an electron donor, there is a shift in the microbial population towards Proteobacteria (Figure 11). No OTUs that contain more than 1 % of the reads representing other (sub)phyla are present. *Pseudomonas*, *Acidovorax*, *Achromobacter*, *Mesorhizobium* and *Sphingomonas* are the most abundant genera (Table 7). Between regular and thermally aged bituminized waste, only very subtle differences can be observed. Similar OTUs are present, they only differ slightly in their abundance (

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Figure *11*

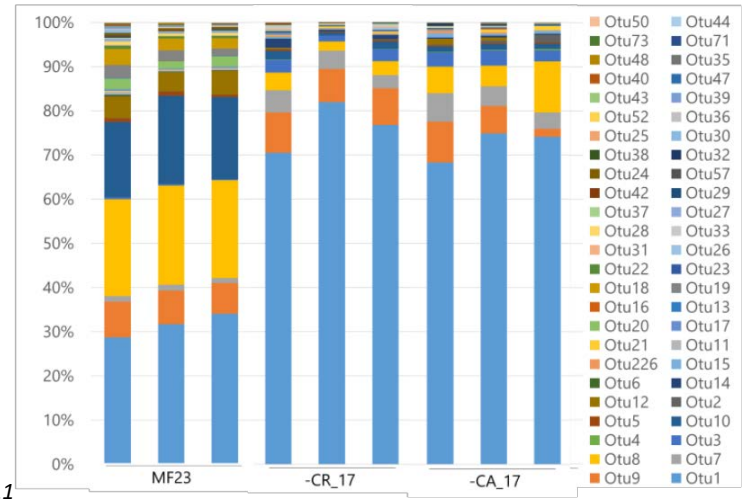

Figure 11).

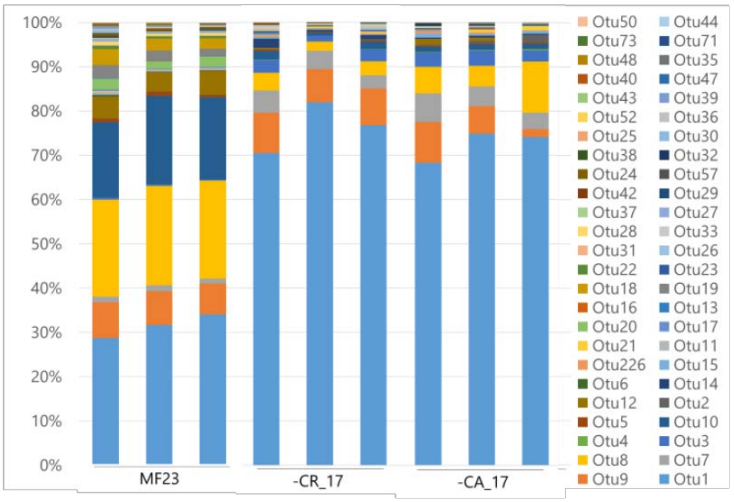

Figure 11: Community structure dynamics in Boom Clay borehole water filter TD-11D (MF23) and after 17 days incubation in the presence of regular and thermally aged bituminized waste without the addition of an electron donor (respectively -CR+ and -CA+). Each coloured bar represents the relative abundance of a single species (OTU). Three biological replicates are analysed for each condition and are underlined.

After 2 days of incubation of inactive Eurobitum with 15 mM sodium formate added as electron donor, OTUs representing only Proteobacteria and Bacteriodetes were present. *Pseudomonas* and *Acidovorax* were highly prominent genera. After 7 days of incubation, the microbial community remained quite constant. Overall, *Pseudomonas* became slightly less dominant, while the genus *Mesorizhobium* became more abundant and the genus *Ignavibacterium*, belonging to the phylum of the Chloroflexi was found as well. This trend was more pronounced after 17 days of incubation, where *Acidovorax*, *Mesorizhobium* and *Pseudomonas* were almost equally dominant. Also here, no clear differences were observed between regular and thermally aged non-radioactive bituminized waste. The microbial community present in Boom Clay borehole water with sodium formate as electron donor but without addition of bitumen, was highly similar to that of day 17 with Eurobitum and formate. Only 1 unclassified genus belonging to the Bacteriodetes was more abundant and within one of the replicates even more than *Pseudomonas* and *Acidovorax* (Figure 12, Table 7).

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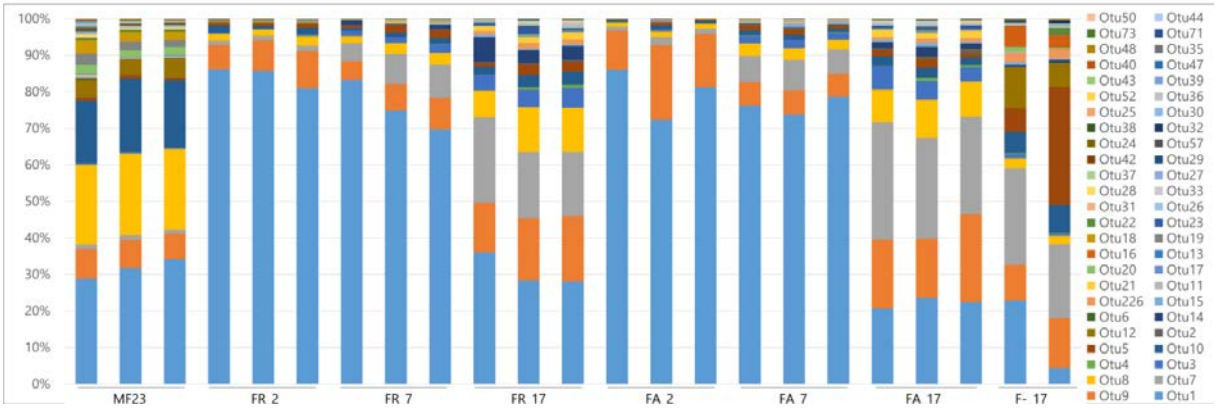

Figure 12: Community structure dynamics in Boom Clay borehole water filter TD-11D (MF23) and after 2, 7 and 17 days incubation in the presence of regular and thermally aged bituminized waste with 15 mM sodium formate added as electron donor, indicated with FR and FA, respectively and after 17 days incubation with 15 mM sodium formate added as electron donor but without the addition of bitumen (F-)). Each coloured bar represents the relative abundance of a single species (OTU). Different biological replicates were analysed and are underlined.

With sodium acetate as electron donor, the microbial community shifted towards *Pseudomonas* after 2 days. Similar as with sodium formate as electron donor, the dominant presence of *Pseudomonas* dissapeared with time. After 7 days, one OTU representing the genus *Rhizobium* became more prominent. After 17 days, a difference could be observed between the community in the presence of regular and thermally aged bitumen. In case of the regular bitumen, *Pseudomonas*, an unclassified member of the family of *Erysipelotrichaceae*, Mesorhizobium and *Acholeoplasma* were the most dominant genera, while in the presence of thermally aged bitumen, *Pseudomonas*, *Rhizobium* and *Mesorhizobium* were the most abundant genera. Without the addition of bitumen, the microbial community shifted towards *Pseudomonas*, *Alkaliphilus* and another unclassified genus of the family of *Clostridiaceae* in 2 of the 3 replicates, while in the other replicate 94% of the reads matched with *Pseudomonas* (Figure 13,Table 7).

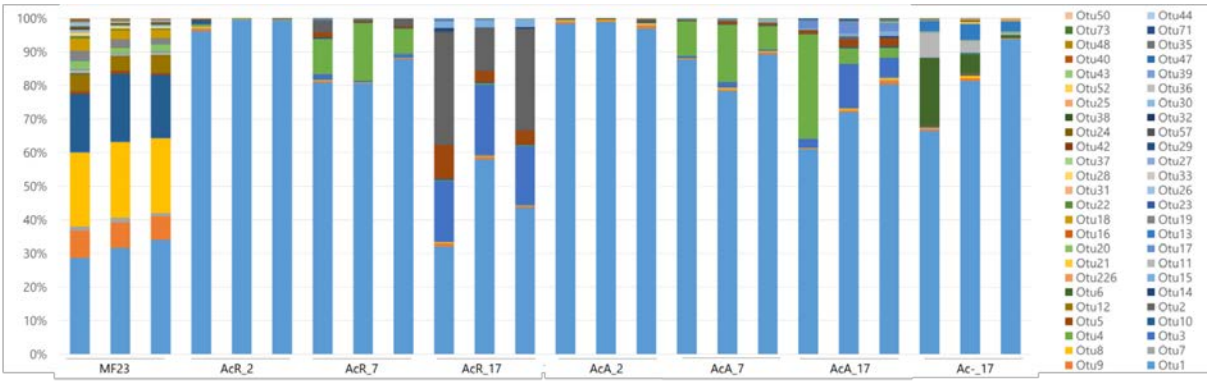

Figure 13: Community structure dynamics in Boom Clay borehole water filter TD-11D (MF23) and after 2, 7 and 17 days incubation in the presence of regular and thermally aged bituminized waste with 15 mM sodium acetate added as electron donor, indicated with AcR and AcA respectively and after 17 days incubation with 15 mM sodium acetate added as electron donor but without the addition of bitumen (Ac-). Each coloured bar represents the relative abundance of a single species (OTU). Three biological replicates are analysed and are underlined.

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Table 7: Taxonomic characterization of the 50 most abundant OTUs, ordered as function of abundancy present in the batch experiments

OTU	Phyla	Class	Family	Genus
OTU1	Proteobacteria	Gammaproteobacteria	*Pseudomonadaceae*	*Pseudomonas*
OTU9	Proteobacteria	Betaproteobacteria	*Comamonadaceae*	*Acidovorax*
OTU7	Proteobacteria	Betaproteobacteria	*Comamonadaceae*	*Acidovorax*
OTU8	Proteobacteria	Betaproteobacteria	*Alcaligenaceae*	*Achromobacter*
OTU3	Proteobacteria	Alphaproteobacteria	*Phyllobacteriaceae*	*Mesorhizobium*
OTU4	Tenericutes	Mollicutes	*Acholeplasmataceae*	*Acholeplasma*
OTU10	Proteobacteria	Alphaproteobacteria	*Sphingomonadaceae*	*Sphingomonas*
OTU5	Proteobacteria	Alphaproteobacteria	*Rhizobiaceae*	*Rhizobium*
OTU2	Firmicutes	Erysipelotrichia	*Erysipelotrichaceae*	*unclassified*
OTU12	Chloroflexi	Ignavibacteria	*Ignavibacteriaceae*	*Ignavibacterium*
OTU14	Bacteroidetes	unclassified	*unclassified*	*unclassified*
OTU6	Firmicutes	Clostridia	*Clostridiaceae_2*	*unclassified*
OTU15	unclassified	unclassified	*unclassified*	*unclassified*
OTU226	Proteobacteria	Betaproteobacteria	*Comamonadaceae*	*unclassified*
OTU11	Bacteroidetes	Bacteroidia	*Porphyromonadaceae*	*unclassified*
OTU21	Proteobacteria	Gammaproteobacteria	*unclassified*	*unclassified*
OTU17	Bacteroidetes	Bacteroidia	*Porphyromonadaceae*	*unclassified*
OTU20	Bacteroidetes	unclassified	*unclassified*	*unclassified*
OTU13	Firmicutes	Clostridia	*Clostridiaceae_2*	*Alkaliphilus*
OTU16	Proteobacteria	Alphaproteobacteria	*Caulobacteraceae*	*Phenylobacterium*
OTU19	Actinobacteria	Actinobacteria	*Coriobacteriaceae*	*unclassified*
OTU18	Firmicutes	Clostridia	*Eubacteriaceae*	*Acetobacterium*
OTU23	Actinobacteria	Actinobacteria	*Propionibacteriaceae*	*Propionibacterium*
OTU22	Firmicutes	Clostridia	*unclassified*	*unclassified*
OTU26	Bacteroidetes	unclassified	*unclassified*	*unclassified*
OTU31	Bacteroidetes	unclassified	*unclassified*	*unclassified*
OTU33	Proteobacteria	Betaproteobacteria	*unclassified*	*unclassified*
OTU28	Firmicutes	Clostridia	*Clostridiaceae_1*	*Proteiniclasticum*
OTU27	unclassified	unclassified	*unclassified*	*unclassified*
OTU37	Chloroflexi	Anaerolineae	*Anaerolineaceae*	*unclassified*
OTU29	Bacteroidetes	Bacteroidia	*Porphyromonadaceae*	*Petrimonas*
OTU42	Actinobacteria	Actinobacteria	*Intrasporangiaceae*	*Tetrasphaera*
OTU57	Bacteroidetes	Bacteroidia	*Porphyromonadaceae*	*unclassified*
OTU24	Firmicutes	Clostridia	*Incertae_Sedis_XI*	*Dethiosulfatibacter*
OTU32	Proteobacteria	Betaproteobacteria	*unclassified*	*unclassified*
OTU38	Proteobacteria	Alphaproteobacteria	*unclassified*	*unclassified*
OTU30	Proteobacteria	Gammaproteobacteria	*Enterobacteriaceae*	*Escherichia_Shigella*
OTU25	unclassified	unclassified	*unclassified*	*unclassified*
OTU36	Proteobacteria	Deltaproteobacteria	*Syntrophaceae*	*Smithella*
OTU52	unclassified	unclassified	*unclassified*	*unclassified*
OTU39	Actinobacteria	Actinobacteria	*Coriobacteriaceae*	*unclassified*
OTU43	Firmicutes	Clostridia	*unclassified*	*unclassified*
OTU47	Firmicutes	Negativicutes	*Veillonellaceae*	*unclassified*
OTU40	unclassified	unclassified	*unclassified*	*unclassified*
OTU35	Verrucomicrobia	Opitutae	*Opitutaceae*	*Opitutus*
OTU48	Actinobacteria	Actinobacteria	*Microbacteriaceae*	*Microbacterium*
OTU71	Actinobacteria	Actinobacteria	*Nocardioidaceae*	*Nocardioides*
OTU73	unclassified	unclassified	*unclassified*	*unclassified*
OTU44	unclassified	unclassified	*unclassified*	*unclassified*
OTU50	Chloroflexi	Ignavibacteria	*Ignavibacteriaceae*	*Ignavibacterium*

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Clustering analysis based on the abundance of OTUs in each condition indicates that the samples can be divided into two groups based on the electron donor that is added. The conditions in which acetate was used as electron donor cluster together and the conditions without electron donor group together with formate as electron donor, except after 2 days of incubation which cluster together with acetate (Figure 14).

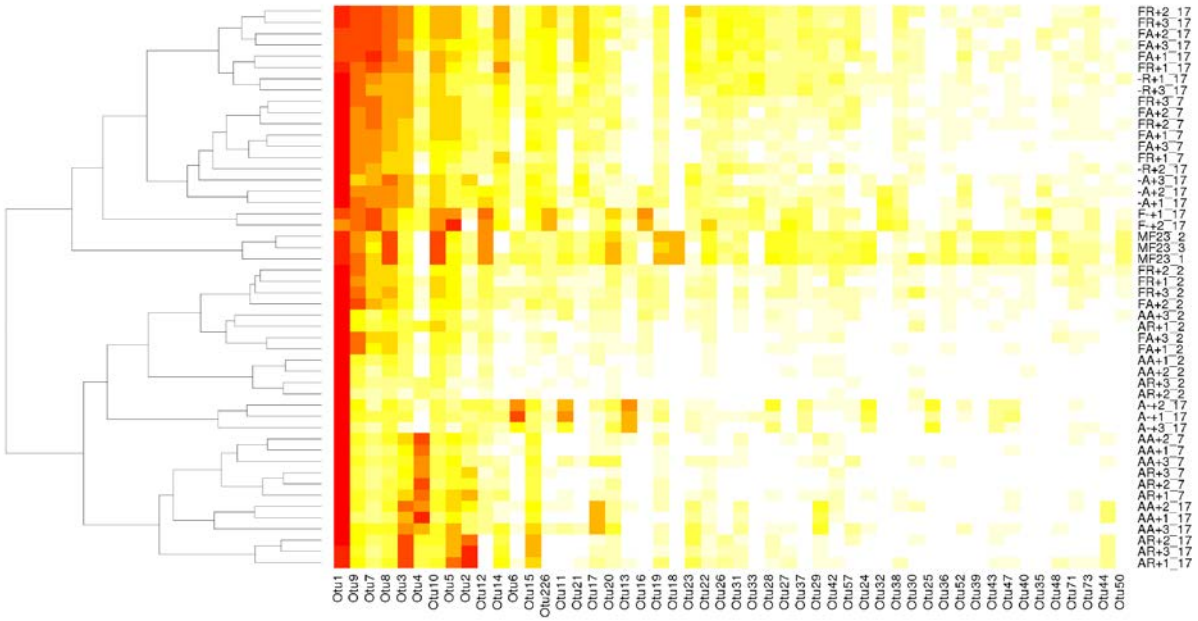

Figure 14: Graphical representation and clustering analysis of different OTUs present in Boom Clay borehole water filter TD-11D exposed to different conditions. The abundance of each OTU in each condition is reflected by a colour: the darker, the more abundant.

5.2 Results of the second series of batch experiments

This experiment differs from the first, more in particular: synthetic boom clay water (thus lacking natural DOM) was used as background medium, flasks were incubated at room temperature (instead of 30°C), and besides formate and acetate, also oxalate, and a mixture of all 3, was tested.

5.2.1 $NaNO_3$ leaching from regular and thermally aged bitumen under free swelling conditions

Initially, $NaNO_3$ leaching from the Eurobitum is fast, from pores close to the surface, but afterwards the leaching process proceeds much slower. After one month, there is again a re-increase in the leaching rate which can be attributable to the formation of interconnecting pores and (micro)cracks in the bitumen matrix in response to its increasing deformation [61] (Figure 15**Error! Reference source not found.**). Notably, it seems that nitrate leaching was higher in conditions where oxalate was added as organic additive compared to the other conditions. The trend was visible up to 74 days compared to the samples without organic additive and up to 31 days in the samples supplemented with formate and acetate. However, the adjusted P-values are not significant hence more replicates should be tested to confirm this.

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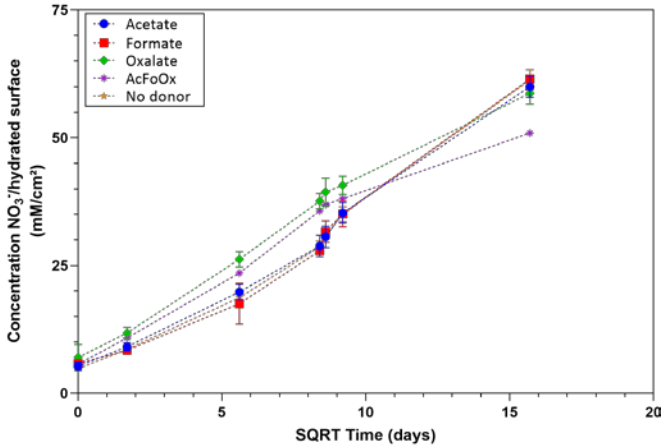

Figure 15: Time evolution of $NaNO_3$ concentrations in abiotic samples leached from thermally aged non-radioactive bituminized waste under free swelling conditions. Blue circles represent conditions where 15 mM sodium acetate is added as organic additive, 15 mM sodium formate is represented by red squares, 15 mM sodium oxalate by green diamonds, the combination of 5 mM the three organic additives (AcFoOx) is presented by purple asterisks and the conditions without additional organic additives is shown as orange stars. Values represent the average and standard deviation of three replicates.

Table 8: P-values and adjusted P-values obtained with Kruskal-Wallis test followed by a Dunn test. P-values were adjusted with the Benjamini Hochberg method [62]. Significant P-values are marked in bold.

		No organic additive	Formate	Oxalate versus Acetate	Mixture
Day 1	P-value	0,083	**0,003**	**0,014**	0,273
	adjusted P-value	*0,207*	*0,035*	*0,069*	*0,390*
Day 2	P-value	**0,036**	**0,008**	**0,018**	0,523
	adjusted P-value	*0,119*	*0,081*	*0,088*	*0,747*
Day 31	P-value	**0,011**	**0,008**	**0,028**	0,411
	adjusted P-value	*0,053*	*0,081*	*0,095*	*0,588*
Day 71	P-value	**0,003**	0,465	0,083	0,315
	adjusted P-value	*0,035*	*0,517*	*0,207*	*0,450*
Day 74	P-value	**0,028**	0,411	0,235	0,411
	adjusted P-value	*0,285*	*0,588*	*0,588*	*0,686*

5.2.2 Impact of the electron donor on nitrate reduction

Contrary to the first batch experiment, nitrate reduction seems to be limited during the first 71 days of the experiment (Figure 16). Only in the presence of formate, there is a decrease in nitrate concentration observed in the presence of the Boom Clay borehole water community. Nevertheless, after 71 days, nitrite production is observed in all conditions except in the presence of oxalate, indicating that nitrate reduction did occur. The highest nitrite concentrations are observed in all conditions with acetate added as organic additive and concentrations are ~ 2 times higher than conditions with formate or without organic additive. Based on the decrease in acetate and formate and the presence of nitrogen species (Figure 18), the microbial community seemed to use these organic additives as electron donor to carry out nitrate reduction according to the following reactions:

$CH_3COO^- + 4\ NO_3^- \rightarrow 4\ NO_2^- + 2\ HCO_3^- + H^+$ ($\Delta G^{0'}$ (pH 7) = -69 kJ/mol) (1) [46]

$HCOO^- + NO_3^- \rightarrow NO_2^- + HCO_3^-$ ($\Delta G^{0'}$ (pH 7) = -161.9 kJ/mol) (2) [63].

When the medium is supplemented with all three additives, acetate is used first, followed by formate (Figure 16), indicating that the Boom Clay borehole water community prefers acetate as electron donor to reduce nitrate.

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Similarly, microbial activity seems limited during the first 71 days in conditions without Eurobitum and thus without nitrate. On the other hand, the present microbial community does not seem to use acetate as electron donor in those conditions. Instead, formate seems to be the preferred electron donor as only formate is used when all three organic additives are present (Figure 16). In addition, in samples supplemented with only formate, there seems to be no rate limiting factor as 60 % formate is used after 71 days. Interestingly, the decrease in formate is associated with an increase in acetate concentrations (Figure 16). In addition, acetate concentrations also increase in conditions without organic additive and in conditions supplemented with acetate (Figure 16). This will be discussed further in the report.

The total cell number did not increase during the first 71 days, putatively indicating that there was a rate limiting factor present during that period (Figure 17). Therefore, 0.2 mM NaH_2PO_4 was added to all conditions. This concentration was similar as the amount supplemented in a mineral salts medium frequently used in our lab [64]. The total cell number immediately increased 1- 1.5 log in conditions with thermally aged Eurobitum but remained quite constant in conditions without bitumen (Figure 17). This demonstrates that phosphate can be a limiting factor in such batch tests with artificial pore water to allow growth of the microbial community when other factors such as electron donor and electron acceptor are present in sufficient amounts. Without Eurobitum, there is no nitrate and bicarbonate is the sole electron acceptor, hence this could be an additional limiting factor to allow microbial growth.

Adition of phosphate stimulated acetate dependent nitrate reduction in SBCW tremendously: all 15 mM acetate was completely oxidized after 3 days concomitantly with an additional reduction of ± 50 % nitrate and NO_2^- concentrations doubled in all three replicates (Figure 16). At that moment, ~ 85 % of the nitrate was reduced to nitrite, indicating that acetate dependent nitrate reduction (1) to nitrite was still the most dominant reaction (Figure 18). At the end of the experiment, after 248 days, the microbial community had reduced ~ 90 % of the nitrate, equivalent to an additional reduction of ~ 0.27 mM NO_3^-/day after oxidizing all acetate. The total nitrogen species from NO_3^- and NO_2^- are lower in the presence of the Boom Clay borehole water microbial community (Figure 18), suggesting that in addition to nitrate reduction to nitrite, denitrification to nitrogen (or DNRA) occurred or was inoculated as biomass.

No other conditions showed a similar response to the addition of phosphate as in the presence of acetate. In the samples without organic additive, it seemed that adding phosphate did not stimulate the microbial community as no additional nitrate reduction was observed during the first 14 days. After 248 days, 50 % of the nitrate was reduced, corresponding to ~ 0.23 mM NO_3^-/day after the addition of phosphate. The mass balance of the nitrogen species remained similar with or without microbial community, demonstrating that the production of nitrogen gases or ammonium are negligible. Addition of phosphate seems to inhibit formate consumption, as concentrations did not decrease for 14 days. Possibly, this inhibition is an indirect effect caused by the rapid-growing species which activity is boosted by the addition of phosphate, thereby suppressing the slow-growing formate consuming species. Formate was completely consumed at the end of the experiment. The nitrate reduction rate remained quite stable throughout the entire period. The mass balance of nitrogen species and the decrease in formate suggest that in the presence of formate, nitrate reduction to nitrite (2) remains the most dominant reaction. After 248 days, ~ 60 % of the nitrate was reduced corresponding to ~ 0.19 mM NO_3^-/day. The total nitrogen species from NO_3^- and NO_2^- appeared lower in the presence of the Boom Clay borehole water microbial community (Figure 18), suggesting that in addition to nitrate reduction to nitrite, denitrification to nitrogen (or DNRA) occurred or it was used as biomass. In the presence of oxalate, only ~ 6% nitrate is removed up to 14 days after the addition of phosphate (~ 0.18 mM NO_3^-/day in those 14 days). After 248 days, ~ 66% of the nitrate was reduced, hence comparable with the samples supplemented with formate but without the production of nitrogen gas or ammonium (Figure 18 c). Contrary to other organic additives, there is more variation in oxalate concentration and it is only completely removed in one

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of the replicates, while in the other replicates only ~ 3 mM was removed compared with the sterile samples, indicating that oxalate is not preferred as electron donor. Notably, nitrate reduction seems not associated with the consumption of oxalate as the sample where all oxalate was removed, had the highest nitrate and lowest nitrite concentration of the three replicates. The oxalate concentration also decreases with ~ 33% in the negative controls so, presumably also a chemical reaction occurred. Putatively oxalate coprecipitates with Ca^{2+} leaching from the Eurobitum as $CaSO_4$, resulting in the formation of CaC_2O_4 precipitates. Therefore, although more difficult to metabolize, oxalate will be less bioavailable.

Addition of phosphate to the samples supplemented with a mixture of organic additives, did not stimulate nitrate reduction after 3 days. After 14 days, there was still no difference in nitrate concentration compared to the negative controls, nevertheless nitrite production raised up to ~ 0.8 mM NO_2^-/day implying nitrate reduction. After 248 days, 58 % of the nitrate was reduced to nitrite (Figure 18 e), corresponding to ~ 0.30 mM NO_3^- per day.

In the absence of Eurobitum, phosphate addition does seem to affect the microbial community. Acetate production continues at a rate of ~0.05 mM/day in the absence of organic additives and ~ 0.10 mM/day in the presence of formate or acetate (Figure 16).

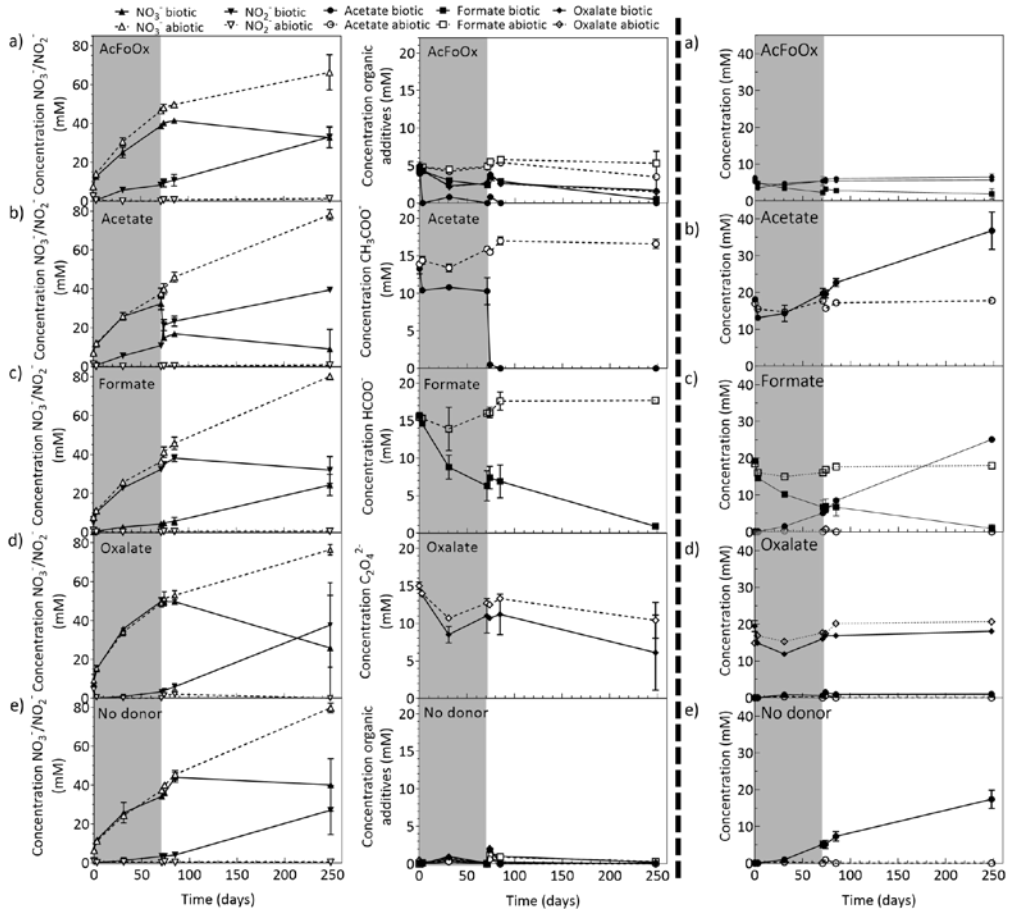

Figure 16: Evolution of nitrate (upward triangle), nitrite (downward triangle), acetate (circle), formate (square) and oxalate (diamonds) concentrations during 248 days of incubation in synthetic Boom Clay borehole water in the presence of thermally aged Eurobitum (left of the dashed line) or in the absence of thermally aged Eurobitum (right of the dashed line). Different organic additives were added: a) a mixture of 5 mM HCOONa, 5 mM $Na_2C_2O_4$ and 5 mM CH_3COONa; b) 15 mM CH_3COONa; c) 15 mM HCOONa; d) 15 mM $Na_2C_2O_4$; and e) no additional organic additives added. Values represent the average and standard deviation of three replicates of samples with Boom Clay borehole water added as inoculum (full line) or abiotic controls (dashed line). The first 71 days, no additional phosphate was added to the cultures (grey part of the curve). At day 71, 0.2 mM Na_2HPO_4 was added to all conditions (white part of the curve).

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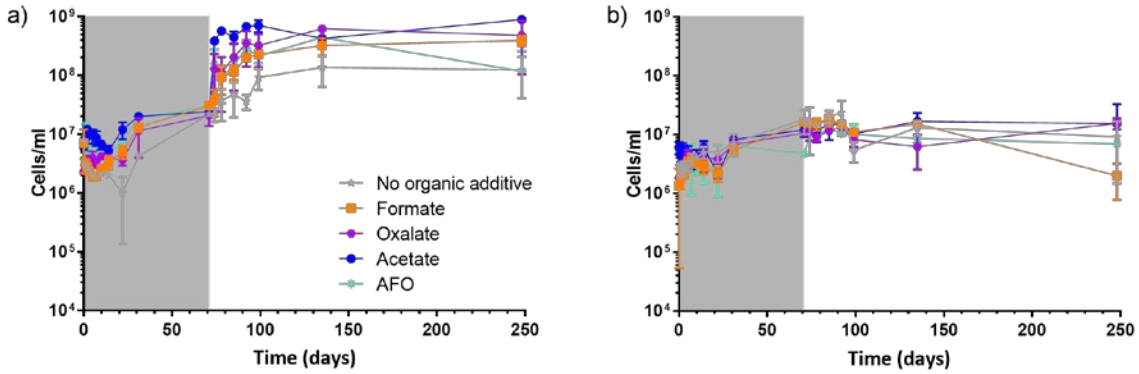

Figure 17: Total cell numbers measured with flow cytometry of Boom Clay borehole water a) In the presence of thermally aged Eurobitum and b) without thermally aged Eurobitum. Values represent the mean and standard deviation of three replicates. Samples where no organic additive was added are shown as grey stars, samples with formate are presented as orange squares, samples with oxalate are shown as purple hexagons, blue circles represent samples with acetate and samples with a combination of the three additives are shown as light blue asterisks. The first 71 days, no additional phosphate was added to the cultures (grey part of the curve). At day 71, 0.2 mM Na_2HPO_4 was added to all conditions (white part of the curve).

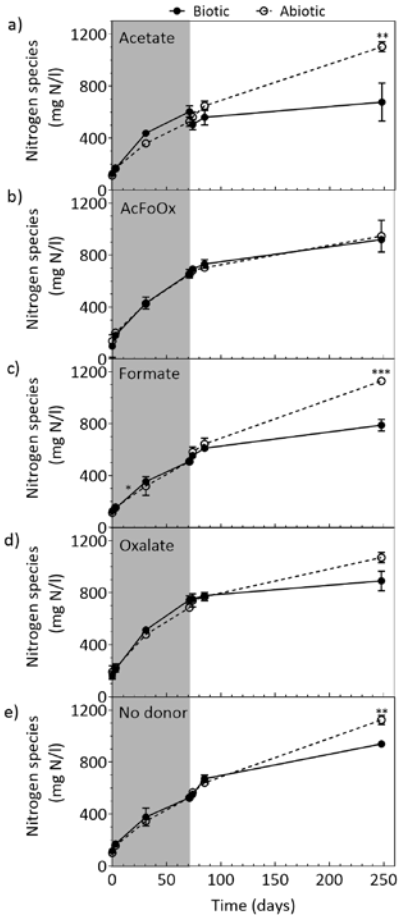

*Figure 18: Evolution of the mass balance of N species (NO_3^- and NO_2^-) during the experiment with a) 15 mM sodium acetate; b) a mixture of 5 mM sodium acetate, 5 mM sodium formate and 5 mM sodium oxalate; c) 15 mM sodium formate; d) 15 mM sodium oxalate and e) without organic additives added. N species in the presence of the Boom Clay microbial community are shown as full circles connected with a full line, while N species in the abiotic samples are presented as open circles connected by a dashed line. Results are shown as mean and standard deviation of three replicates. Significant differences between biotic and abiotic samples are indicated with * for P < 0.05, ** for P < 0.01 and *** for P < 0.001. The first 71 days, no additional phosphate was added to the cultures (grey part of the curve). At day 71, 0.2 mM Na_2HPO_4 was added to all conditions (white part of the curve)*

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In all conditions, the present microbial community is able to fulfil nitrate reduction in the absence of organic additives. This indicates that there is another source of electron donor to perform nitrate reduction. Some energy could originate from the organic material present in the fraction of Boom Clay borehole water that was added as inoculum, from the organics leaching from Eurobitum or from the 1 % H_2 present in the anaerobic glove box that putatively leaks in the glass bottles. Total organic and inorganic measurements of day 248 of one replicate were performed to enable to elucidate this hypothesis more in detail. Results show that without microorganisms, TIC concentrations had halved or decreased even more (Figure 19). This could be attributed to the formation of $CaCO_3$ precipitates resulting from the leaching of $CaSO_4$ from the Eurobitum. In the samples supplemented with oxalate, this decrease was not that prominent, presumably to the formation of CaC_2O_4 precipitates instead of $CaCO_3$. SEM pictures of thermally aged Eurobitum in the presence of the different organic additives also demonstrate this difference (Figure 20).

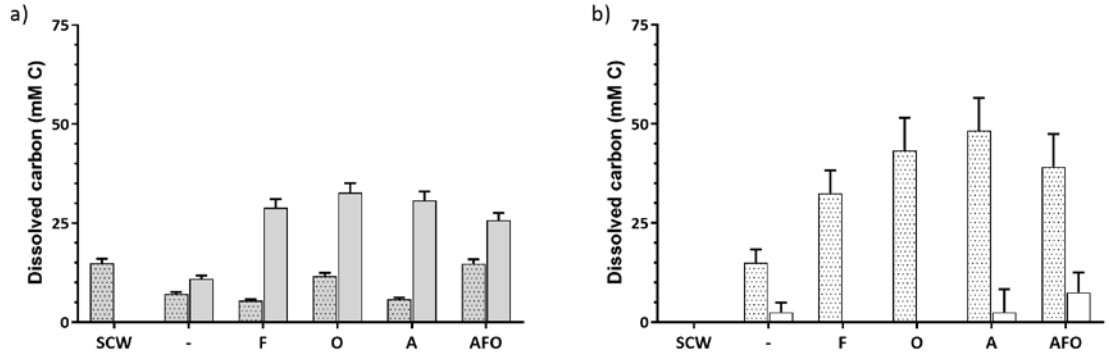

Figure 19: a) Total dissolved inorganic carbon (grey bars) and b) total dissolved organic carbon (white bars) measured after 248 days in one of the replicates in samples in the presence of thermally aged Eurobitum without organic additive (-), supplemented with formate (F), oxalate (O), acetate (A) or a mixture of the three organics (AFO). The composition of Synthetic Clay water used as background medium is indicated by SCW. Samples inoculated with the Boom Clay borehole water microbial community are shown as full bars, while negative control samples are presented as dotted bars. Data represent the result of the measurement and the measurement uncertainty.

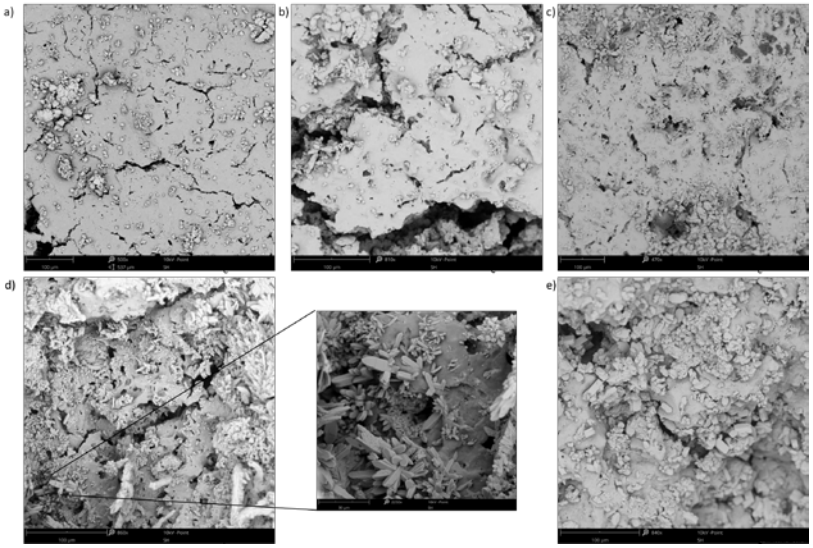

Figure 20: SEM pictures of thermally aged Eurobitum without microbial community a) in the absence of organic additives; in the presence of b) formate; c) acetate; d) oxalate and e) a mixture of formate, acetate and oxalate.

In the samples without microorganisms nor organic additives, the TOC concentration increased up to 15 ± 3.3 mM compared to that of SCW used as inoculum. No Boom Clay water was added to this sample thus suggesting the leaching of organic material from the thermally aged Eurobitum. TOC concentrations in the sterile samples supplemented with organic additives were also higher than expected from the added concentration of organic additives. The difference between the expected

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and measured TOC was similar to the concentration measured in the samples without organic additive (Figure 19). In the presence of the Boom Clay borehole water microbial community, the TOC concentration decreases to values close to or under the detection limit, demonstrating the use of the added organics and additionally, the use of organics leached from the thermally aged Eurobitum as electron donor for nitrate reduction. Indeed, the chromatogram of the sterile samples without organic additive show some peaks, however the samples were highly diluted to measure nitrate concentrations as accurate as possible, hence concentrations of organics were below the detection limit and it remains unclear to which organic compound the peaks correspond to. Concomitantly with the decrease in TOC, TIC concentrations increase, indicating oxidation of organic material to CO_2 (HCO_3^-). The remainder in difference between TIC and TOC could have been released as CO_2 gas, or incorporated into biomass (Figure 19). Note that for oxalate, the replicate was measured were all oxalate was removed, which is confirmed with the TIC/TOC analyses. One remark could be that in the samples inoculated with Boom Clay borehole water, a fraction of organic material present in Boom Clay borehole water was added. The water used as inoculum contained 11,8 mM dissolved organic carbon. In these experiments, this was diluted 10 times and therefore, ~ 1 mM organic carbon originating from Boom clay borehole water was added. This concentration falls under the detection limit of the measurement method and therefore it is not possible to draw conclusions regarding the ability to use the organic matter present in Boom Clay as electron donor. Nevertheless, it has been shown before that this is highly recalcitrant for microbial degradation under nitrate reducing conditions[48].

In contrast, when Boom Clay pore water is cultured in the absence of thermally aged Eurobitum, TIC concentrations decreased in conditions without organic additive, in the presence of formate and with acetate. This indicates that the microbial community uses CO_2 or HCO_3^- as electron acceptor. Additionally, TOC concentrations in those samples are higher compared to the negative controls. The observed increase is similar to the increase in acetate as shown in Figure 16 (left). A possible reaction that resulted in the formation of acetate could be:

$2\ HCO_3^- + 4\ H_2 + H^+ \rightarrow CH_3COO^- + 4\ H_2O$ ($\Delta G^{0'}$ (pH 7) = -105 kJ/mol e^-) (3) [46]

However, based on this equation and the amount of available bicarbonate in the sample (~ 15 mM), only ~ 8 mM acetate is expected to be formed, while ~ 12 mM and ~ 26 mM acetate is observed without additional organic additive and in the presence of acetate, respectively. In the presence of formate, acetate concentrations increased with decreasing formate concentrations and ~ 19 mM acetate was produced (Figure 16 b, left) although TIC concentrations decreased only by ca. 7.5 mM (Figure 21). Putatively, reactions (4) or (5) occurred followed by reaction (3). Further analysis of the microbial community could differentiate which reactions did occur. Although from an energetic point of view, reaction (5) is the least favourable, this reaction could probably have occurred if methanogens were inhibited.

$4\ HCOO^- + H^+ \rightarrow CH_4 + 3\ HCO_3^-$ ($\Delta G^{0'}$ (pH 7) = -130 kJ/mol) [65] (4)

$HCOO^- + H_2O \rightarrow HCO_3^- + H_2$ ($\Delta G^{0'}$ (pH 7) = + 1.3 kJ/mol) [66] (5)

Consequently, there should be an alternative route resulting in the production of acetate. One possibility is through fermentation of organic material present in the samples. However, the source of this organic material is not yet clear. Indeed, in the negative controls, only SCW containing no organic material was added, but the TOC concentrations are substantially high (Figure 21). On the other hand, the control sample taken from the original SCW water used to prepare the experiments, showed no organic carbon. For practical reasons, the medium and all experiments with Eurobitum were performed in Schott glass bottles where no unusual concentrations of TOC were measured, while the samples without Eurobitum were prepared in septum bottles. The TOC contamination seems similar for all conditions as in the presence of organic additives, the amount of TOC diminished with the amount in the negative control corresponds to the concentration of organic additive added to the samples. Therefore, it is possible that there was some organic material present in the septum bottles at the start of the experiment, which was fermented later on.

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With oxalate as organic additive, there is no difference compared to the negative control (Figure 21) and the concentration of oxalate does not decrease in time (Figure 16), indicating that it is not used as electron donor or carbon source by the Boom Clay borehole water microbial community. Also in conditions with a combination of organic additives, TIC concentrations are similar to SCW water and TOC does not suggest acetogenesis or acidogenic fermentation (Figure 19).

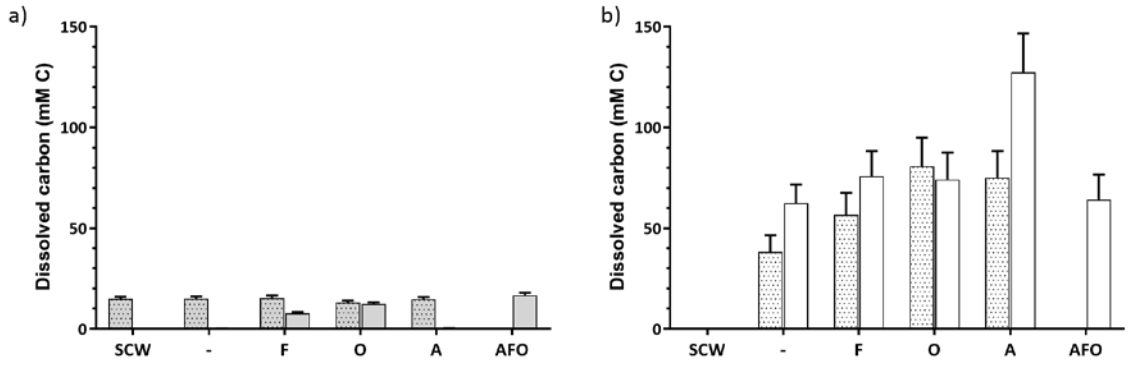

Figure 21: Total dissolved inorganic carbon (grey bars) and total dissolved organic carbon (white bars) measured after 248 days in one of the replicates in samples in the absence of thermally aged Eurobitum without organic additive (-), suppelemented with formate (F), oxalate (O), acetate (A) or a mixture of the three organics (AFO). The composition of Synthetic Clay water used as background medium is indicated by SCW. Samples inoculated with the Boom Clay borehole water microbial community are shown as full bars, while negative control samples are presented as dotted bars. Data represent the result of the measurement and the measurement uncertainty.

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5.2.3 Biofilm formation on thermally aged Eurobitum in the presence of different organics

SEM analysis of thermally aged Eurobitum show that after 248 days without shaking, a clear biofilm is formed on the Eurobitum in all conditions (Figure 22).

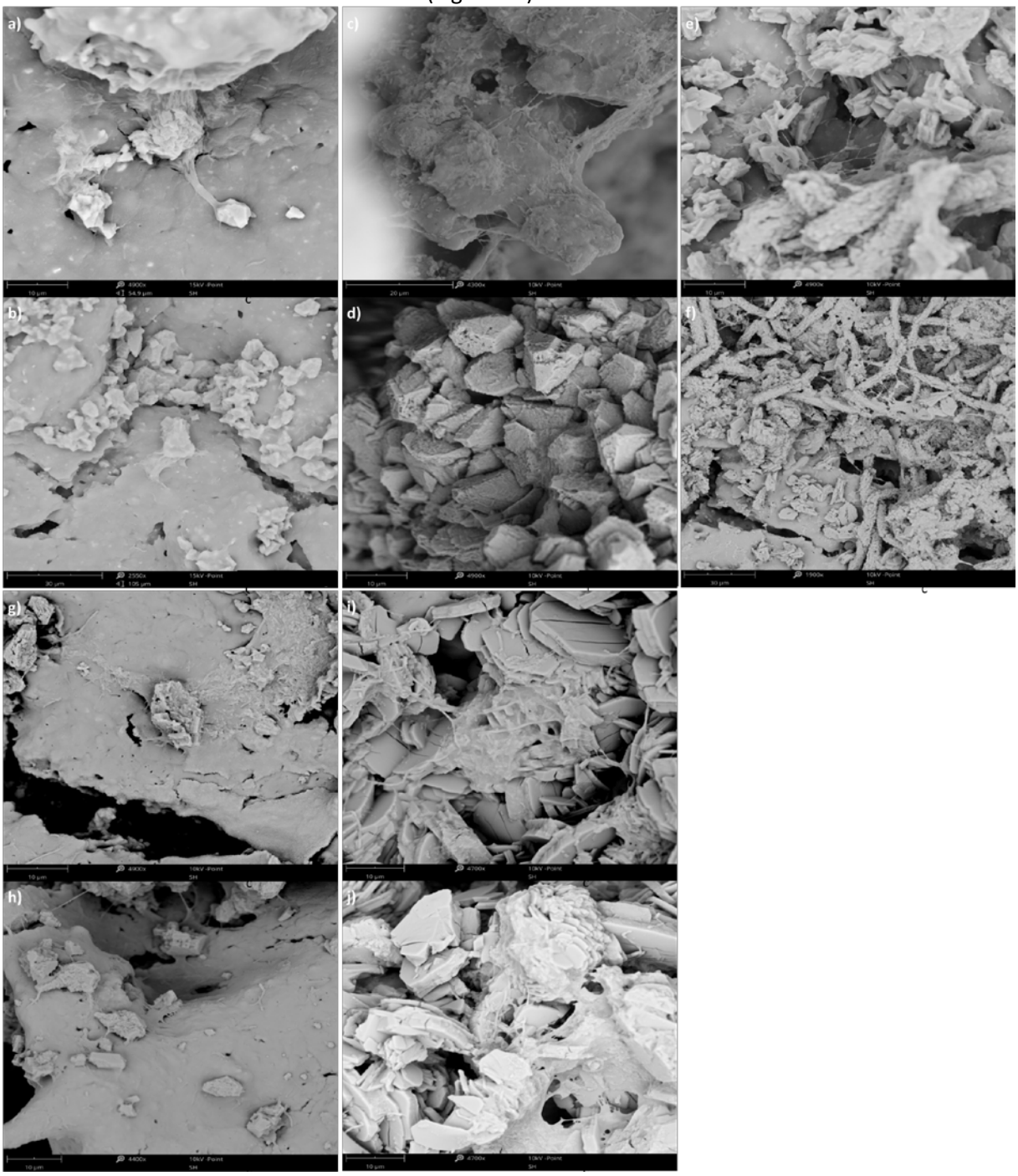

Figure 22: SEM revealing biofilm formation of the Boom Clay borehole microbial community on thermally aged Eurobitum a,b) in the absence of organic additives; in the presence of c,d) formate; e,f) oxalate; g,h) acetate and i,j) a mixture of organic additives.

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5.2.4 Impact of the electron donor on the microbial community present in Boom Clay borehole water

Recently, it was shown that diversity analysis based on flow cytometry fingerprints was highly suitable to determine interesting time points for 16S rRNA amplicon sequencing [55]. In general, a decrease in phenotypic diversity could indicate an enrichment for few species, while an increase could indicate more general conditions, favourable for more species. Our results show that overall, the phenotypic diversity of the microbial community seems comparable in all conditions and tend to evolve similarly in time (Figure 23). Only the dataset of formate was normally distributed, hence one way ANOVA analysis could be performed showing significant differences from day 74 up to day 99. In all other conditions, data were not normally distributed, hence non-parametric Kruskall-Wallis test was applied to investigate a significant effect of time. Although a significant effect of time was found in all conditions, post hoc test indicated no significant differences between successive time points. However, in samples without organic additives and in the presence of oxalate, a similar trend was observed, hence, studying more replicates is necessary to confirm this observation. Only in the presence of acetate, phosphate addition seems to induce a different pattern, in agreement with the changes in metabolic rate which was mainly observed for tests with acetate.

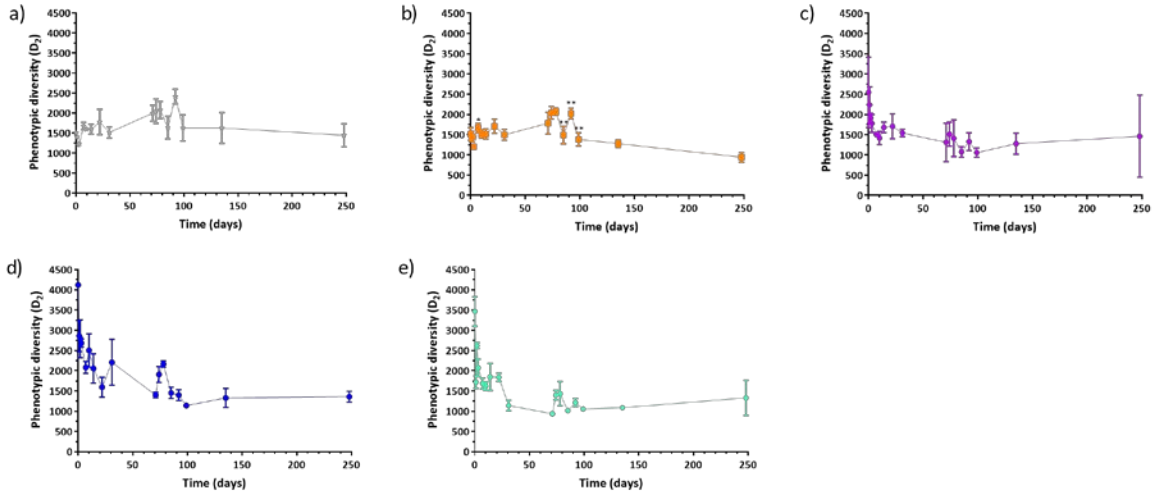

*Figure 23: Phenotypic alpha diversity metrics measured by flow cytometry for Boom Clay Borehole water in the presence of thermally aged Eurobitum a) without organic additive; in the presence of b) 15 mM $HCOO^-$; c) 15 mM $C_2O_4^{2-}$; d) 15 mM CH_3COO^- or a e) mixture of 5 mM of the three. Values correspond to the mean and standard deviation of alpha diversity (q=2) for 3 replicates. Significant changes compared to the previous sampling point are presented with an * if p< 0.05 and with ** if p<0.01. Samples with formate were analysed with one way ANOVA, while the other conditions were analysed with a Kruskall-Wallis multiple rank test followed by a DunnTest. Adjusted P-values were calculated with the Benjamini-Hochberg method [62].*

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In the absence of thermally aged Eurobitum, a similar profile is observed (Figure 24). Here it seems that the addition of phosphate results in a temporarily increase in phenotypic diversity, however, no significant differences were observed, again putatively due to too few biological replicates.

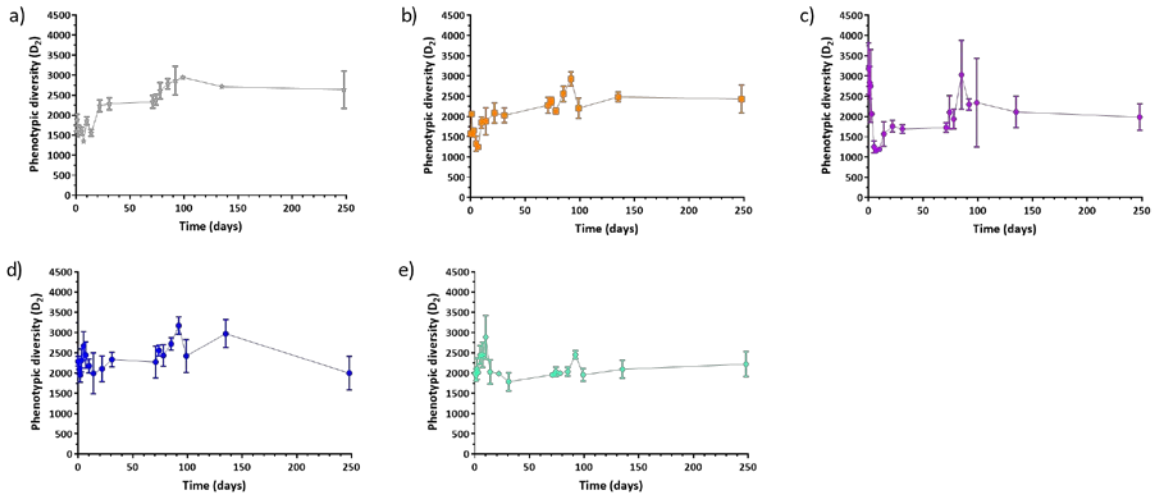

Figure 24: Phenotypic alpha diversity metrics measured by flow cytometry for Boom Clay Borehole water in the absence of thermally aged Eurobitum a) without organic additive; in the presence of b) 15 mM $HCOO^-$; c) 15 mM $C_2O_4^{2-}$; d) 15 mM CH_3COO^- or a e) mixture of 5 mM of the three. Values correspond to the mean and standard deviation of alpha diversity (q=2) for 3 replicates. Samples with acetate were analysed with one way ANOVA, while the other conditions were analysed with a Kruskall-Wallis multiple rank test followed by a DunnTest. Adjusted P-values were calculated with the Benjamini-Hochberg method [62].

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Beta diversity between all samples was calculated with principal component analysis (PcoA), a method to explore and to visualize similarities or dissimilarities of data. There seems no differentiation in the microbial community depending on the added organic additive. If an effect could be determined, it would be an effect of time as all samples are grouped together for each time point (Figure 25). However, more detailed analysis on the microbial community is in progress to elucidate which organisms are specifically enriched in the presence of a specific organic additive.

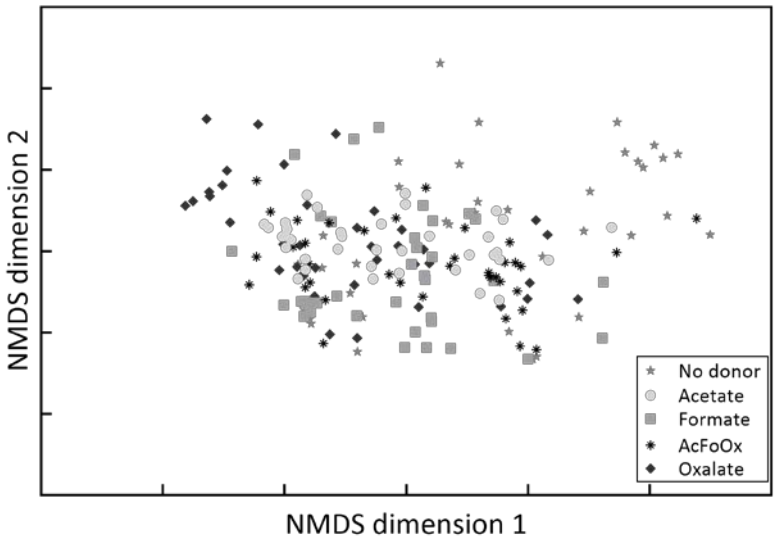

Figure 25: Community beta diversity assessment of the flow cytometric fingerprint data. Ordination occurred by non-metric multidimensional scaling (NMDS) based on the Bray–Curtis distance metric. Values represent each replicate at the different time points (day 3, 7, 10, 14, 22, 31, 71, 74, 85, 99, 135 and 248). Circles represent SBCW supplemented with thermally inactive Eurobitum where 15 mM sodium acetate is added as organic additive, 15 mM sodium formate is represented by squares, 15 mM sodium oxalate by diamonds, the combination of 5 mM of the three organic additives (AcFoOx) is presented by asterisks and the conditions without additional organic additives is shown as stars.

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5.3 Results of the third series of batch experiments: effect of pH

To investigate the effect of pH on microbial nitrate reduction, two microbial communities originating from different locations were compared. The first community is the microbial community of Boom Clay borehole water from filter TD-11D-23 and is identical as the one studied in the aforementioned batch experiments. The other microbial community originates from a sediment of the Harpur Hill site near Buxton (UK). Features and processes occurring at the Harpur Hill site (UK) are seen as analogous to some conditions present in a cementitious geological disposal facility. The site had been contaminated for decades by high pH legacy lime works. The leaching of residual lime (CaO) within the lime waste deposited at the site, by percolating rainwater and shallow groundwater, has led to the development of alkaline, calcium hydroxide dominated leachate with a pH of about 12.5 and the formation of an alkaline 'lagoon' of calcium carbonate tufa precipitate. However, the pH of the fluids at the site varies drastically, and is influenced by factors such as rainfall [67]. Consequently, the microbial community present in the Harpur Hill sediment is adapted to thrive in high pH conditions.

Batch experiments were performed in anoxic Boom Clay pore water to investigate the potential microbial reduction of nitrate leaching from thermally aged inactive bituminized waste at different pH (9 – 10.5 – 12.5). Boom Clay borehole water or the Harpur Hill sediment (natural analogue to a cementitious geological disposal facility) were used as inoculum and sodium acetate was added as electron donor and C source. At pH 9, the microbial community of the Boom Clay borehole water reduced ~3.8 mM nitrate per day during the first 5 days, which is twice as much as the microbial community from the Harpur Hill sediment. Nevertheless, after 110 days, a similar amount of nitrate was reduced by both communities. On the other hand, when the communities were exposed to pH 10.5, the nitrate reduction rates were almost 30 times lower during the first 5 days for the Boom Clay borehole water microbial community, while those of the Harpur Hill sediments were comparable with reduction rates observed at pH 9. In addition, at pH 10.5, the Harpur Hill community reduced almost 2.5 times more nitrate after 110 days compared to the Boom Clay borehole water community and oxidized all 15 mM acetate, while acetate concentrations only dropped by 7 mM when Boom Clay borehole water was used as inoculum. It seems that the Harpur Hill microbial community reduced nitrate to nitrogen gas (or ammonium), while the microbial community of the Boom Clay borehole water produced more nitrite. At pH 12.5, no nitrate or acetate was removed by neither communities. However, in the samples at pH 12.5 the total microbial cell counts of the Harpur Hill community increased in time, while that of the Boom Clay borehole water decreased. Based on flow cytometry community profiles, principal component analysis shows that flow cytometry profiles at pH 12.5 differ from those at pH 9 and pH 10.5, which are quite similar. In addition, at pH 12.5 flow cytometry profiles for the Harpur Hill community clearly differ from that of the Boom Clay borehole water community. Both communities were able to form a biofilm after 110 days on the inactive bituminized waste product at pH 9 and pH 10.5, but at pH 12.5 no biofilm was observed. Altogether, our data indicate that, although flow cytometry profiles are similar at pH 9 and 10.5, nitrate reduction rates differ between both microbial communities. Although exposure to pH 12.5 inhibits nitrate reduction, the Harpur Hill community was still viable at this high pH as total cell counts increased in time. Hence, it seems that high pH alone as stress factor will not eliminate all microbial presence in a geological repository, though it seems to provide enough stress to cause a significant shift in the microbial population and reduce its nitrate reducing activity.

6 Discussion

6.1 General comments on experimental procedures

Its good binding capacity, low solubility in and low permeability to water, and its good chemical and biological inertia resulted in the use of bitumen as immobilization matrix for long-lived low- and intermediate level radioactive waste [7]. Not only in Belgium, but also in other countries, bituminized waste is an important fraction of the national radioactive waste inventory [20]. With the advent of

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deep geological disposal as possible long-term solution for the treatment of radioactive waste, research into the compatibility of bituminized waste with those conditions increased tremendously. An important trait of Eurobitum – and other types of bituminized waste – is that it contains significant amounts of sodium nitrate. Leaching of sodium nitrate can result in geochemical perturbations of clay, which is seen as candidate host rock for the geological disposal of radioactive waste in several countries [7, 45]. Consequently, various studies regarding chemical and radiolytical degradation of (Euro)bitumen and the possible mechanical and chemical disturbance have been/are being carried out gathering substantial knowledge on this matter, which was recently reviewed in [2, 36, 68]. It was decided not to repeat such studies but rather use the obtained knowledge to select known bitumen degradation products to investigate within this project. Leaching of nitrate can enhance microbial activity as it is a well-known energetically favourable electron acceptor in anoxic conditions [46]. Various studies (e.g. the *in situ* Bitumen-Nitrate experiment at the Mont-Terri Rock laboratory in Switzerland, batch experiments with Boom Clay pore water) have been carried out to study nitrate reduction in the presence of known bitumen degradation products [49]. However, in the present study, other degradation products were investigated together with previously studied ones and the most significant difference with all other studies is that a solid fraction of inactive Eurobitum was added to investigate nitrate reduction rates leaching from inactive Eurobitum. Additionally, our experimental setup enabled the investigation of biofilm formation on the bitumen itself, possibly enhancing Eurobitum degradation. Additionally, the impact of exposure to high pH was investigated, a condition that will be more relevant to conditions present during geological disposal. Note that the concentration of organic additives that were added was probably not representative for geological disposal, but was selected to stimulated microbial activity.

In all independent experiments (except when oxalate was present), similar nitrate leaching rates were obtained during the first 70 days of the experiments, demonstrating the reproducibility of such experiments. In the second batch test, phosphate was added to the samples with SCW to stimulate microbial activity. However, polyphosphoric acid (PPA) (bruto formala $H_{2+n}P_nO_{3+n}$ with n>1) is a known chemical bitumen modifier and is often used as catalyst during the air-blown oxidation process [69, 70]. Addition of PPA to bitumen results in an increase in the asphaltene content and a decrease in saturates and resins [70]. The asphaltene fraction enhances PPA dissociation into PPA^- and H^+, disrupting the hydrogen bond network formed within the agglomerates of asphaltene micelles. This lowers the molecular weight of the asphaltene fraction and improves the distribution of asphaltenes in the remaining fractions (i.e., saturates, aromatics, and resins), shifting the bitumen towards a more elastic gel-type structure [70]. Consequently, addition of Na_2HPO_4 possibly enhanced degradation of Eurobitum, resulting in the production of hydrogen, an increased leaching of nitrate and organic compounds. However, the amount of Na_2HPO_4 added in our experiments was much lower compared to the 0.2 % - 1.2 % that are generally added to the bitumen [70]. In addition, leaching profiles were similar as previously demonstrated for Eurobitum in free swelling conditions in the experiments with Boom Clay pore water and also during the period without phosphate [61]. The faster re-increase in $NaNO_3$ leaching observed in this study (also observed in the experiments without additional phosphate) could be attributed to the use of much smaller bitumen blocks compared to the previous study, where this was only observed after 1-2 years of hydration due to the smaller contact surface [61]. Nevertheless, more samples after phosphate addition will be analysed and compared with the experiments without additional phosphate, to investigate the effect of phosphate in our experiments. Furthermore, in our experiments, leaching of $NaNO_3$ was independent of the leaching solution and of pH, confirming previous observations [71]. Leaching of Ca^{2+} and SO_4^{-2} was not followed up in detail in our experiments but it is expected that leaching is diffusion-controlled at slightly alkaline pH but suppressed in a cementitious environment by the low solubility of Ca^{2+} and SO_4^{2-} [71]. This could explain the stable pH of 12.5 when Eurobitum was exposed to Boom Clay pore water equilibrated with OPC CEM I, while in the other conditions Ca^{2+} leaching from the bitumen led to the formation of $CaCO_3$ precipitates resulting in a pH decrease. It seems that oxalate interacts with Ca^{2+} leaching from the bitumen to form insoluble CaC_2O_4 crystals.

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Consequently, oxalate will not be as bioavailable compared to other organic additives. Previous research also observed precipitation of calcium oxalate crystals when studying the interaction of oxalate and cementitious materials [40].

To calculate the nitrate reduction rates, the differences between the amounts of nitrate that leached from the bitumen in sterile conditions were compared to the non-sterile conditions. Not that not all Eurobitum pieces contained exactly the same amount of NO_3^-, hence the concentration of leached nitrate can vary between different replicates. Moreover, the Eurobitum pieces could not be sterilized as autoclaving induces thermally ageing and a chemical sterilization putatively affects the behaviour as well. Nevertheless, the blocks were prepared as sterile as possible, but in some cases contamination (especially during the first series of batch experiments) could not be prevented. Presumably contaminated samples were not considered in the calculations, but consequently less replicates were available, probably resulting in more variation. Therefore, the rates presented here should be seen as estimated rates and not absolute values. On the other hand, this demonstrates how easily contamination can occur and microorganisms are able to thrive in this environment, even though no consumption of the added organic additives (or nitrate) was always observed in these contaminated samples. Nevertheless, nitrate reduction rates among the different conditions can be compared and thus the most optimal and least optimal conditions could be defined.

6.2 Impact of electron donor on microbial nitrate reduction

It seems that in the presence of nitrate, acetate is the most favourable electron donor/C-source for the microbial population present in Boom Clay borehole water of filter TD-11D-23. When a combination of three organic additives (acetate, formate and oxalate) is supplemented to the medium, acetate is consumed first, followed by formate. Additionally, in the first series of experiments, acetate was consumed more rapidly compared to formate what resulted in higher amounts of nitrate reduction. Furthermore, although it seemed that no nitrate is reduced after 71 days, there was a clear nitrite production suggesting that nitrate reduction did occur. Therefore, microbial activity putatively evokes a local concentration gradient and as nitrate leaching is a diffusion controlled process, results in a faster leaching from the Eurobitum. To confirm this hypothesis, the total amount of nitrate that is still present in each Eurobitum piece should be measured and compared with the initial amount, hence this could not be investigated in our experimental setup. In both batch experiments carried out with 100 % natural Boom Clay pore water instead of SCW, the maximum NO_3^- reduction rate was similar and observed during the first week of the experiment. However, as stated before, this was not observed in the experiments with SCW, indicating that microbial activity was limited in those conditions. A major nutrient limiting microbial activity in those conditions was phosphate as addition of 0.2 mM Na_2HPO_4 immediately boosted NO_3^- reduction and all acetate was consumed after 3 days. Similarly, an instant increase in total cell counts was observed, suggesting an increase in biomass. In conditions with 100 % Boom Clay pore water, a sufficient amount of phosphate is available to carry out nitrate reduction and build additional biomass. Phosphate in Boom Clay is bioavailable in the mineral fraction such as apatite [33], hence it seems no limiting factor for microbial nitrate reduction in a radioactive waste repository.

Another difference in the results of the first and second batch experiments seems the biochemical reaction carried out by the microbial community. In the presence of Boom Clay pore water the microbial community is able to carry out a complete denitrification with acetate as electron donor, while in the presence of SCW acetate consumption was associated with dissimilatory nitrate reduction to nitrite (DNRN). Putatively, this difference can be attributed to the higher C/N ratios during acetate consumption in conditions with Boom Clay water. In the beginning of the experiment with natural Boom Clay water, the amount of nitrate leaching from the Eurobitum is still limited compared to the excess of organic additive that is added. On the other hand, in the experiment with SCW nitrate concentrations accumulated during the first 71 days of the experiments as significant acetate consumption only started afterwards when phosphate was added to the samples. This

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resulted in a decreased C/N ratio (~ 0.3) at the start of the nitrate reduction. Indeed, decreasing the C/N ratio below a certain threshold (depending on the microbial population and the type of organic compound) results in an accumulation of nitrite [48, 72, 73]. After 248 days, ca. 54 % of the nitrate was reduced to nitrite, while the remaining nitrate possibly was completely denitrified to nitrogen gas. This could putatively be explained by the lower availability of reducible nitrate concentrations, hence further reduction of nitrite to nitrogen gas is favoured. As all added acetate was already consumed, other electron donors – probably organics leaching from the Eurobitum as discussed earlier in this report – were used for this complete denitrification. In addition, nitrite can be reduced abiotically to nitrous gases, however the rate is expected to be low [36]. Futhermore, the production of ammonia was not monitored in these experiment and therefore DNRA cannot be ruled out either.

Next to acetate, formate seems an easily accessible electron donor for nitrate reduction, although not as efficient compared to acetate as at the end of the experiment, less nitrate is reduced. In contrast to what was observed in the presence of acetate, addition of phosphate did not seem to enhance nitrate reduction as the nitrate reduction rate was not increased during the first 14 days after phosphate addition. Detailed analysis of the microbial community carried out in the first batch experiments, indicates that in the presence of formate, *Pseudomonas* is the dominating genus during the first week, but afterwards, two species from the genus *Acidovorax* become the most dominant. In other experiments, it was shown that one particular species of *Acidovorax* had a longer lag phase and slower growth rate compared to one species of *Pseudomonas*. Therefore, when nitrate becomes available as electron acceptor, the fast growing species could evoke a relative suppression of the slower growing species. Putatively, addition of phosphate had a similar suppressive effect, *i.e.* promoting growth of fast-growing species over slow-growing. This can be confirmed with detailed analysis of the microbial community present during the course of the second batch experiment.

Oxalate is widely spread in nature as it is a metabolic end product of several plants, algae, fungi and bacteria [74]. Oxalate is known to chelate radionuclides which is explained in detail in deliverable 1.1 of the MIND project [20]. Mostly aerobic oxalothrophic bacteria have been identified and isolated, however, oxalate degradation in anoxic conditions is also described [74, #1918]}. The microbial community present in filter TD-11D-23 of the HADES URL seems to be able to use oxalate as electron donor, although results were not as reproducible as observed for acetate and formate. In addition, more nitrate was reduced in the samples that did not consume all oxalate compared to the sample where all oxalate was removed. Anoxic oxalate degradation without nitrate reduction was also observed in forest soils, but no microbial identification was carried out [75]. It would be interesting to elucidate the differences in the microbial community in the conditions with oxalate more in detail. In that way, the oxalate degrading community might be identified. Although the non-reproducible results make it difficult to draw proper conclusions towards what is expected in a geological radioactive waste repository, it seems that oxalate will be less bioavailable due to its precipitation as insoluble calcium oxalate crystals when calcium leaches from bituminized waste.

Importantly, in all conditions nitrate was mainly reduced to nitrite. It has been observed that nitrite can chemically oxidise pyrite and possibly dissolved organic matter from the Boom Clay host rock (Bleyen *et al.* 2017). Oxidation of these Boom Clay components, makes that its reducing capacity will be decreased and mobility of certain radionuclides can be increased. However, it is assumed that the impact will be limited due to the slow reaction kinetics and the expected low concentrations of nitrite leaching from the bituminized waste (Bleyen *et al.* 2017). But, if microbial activity is possible in repository conditions, they could significantly increase the nitrite concentrations, hence the oxidation effect of nitrite could be more pronounced.

6.3 Impact of pH on microbial nitrate reduction

In situ Boom clay water has a slightly alkaline pH of ~ 8.4 [33]. It is expected that the cementitious materials used during geological disposal of radioactive waste will induce an alkaline plume resulting in a pH > 12.5 for thousands of years. Afterwards the pH will gradually drop to pH 10. At the interface

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of Boom Clay with the disposal gallery, the pH will be not higher than pH 10.5. This alkaline plume reaches the first 1-3 meters from the concrete – Boom Clay interface, while further down in the Boom Clay, the pH will not change and will remain ~ 8.4 [76]. Therefore, different media were defined in order to investigate the effect of different pH on the efficiency of nitrate reduction performed by the microbial community in Boom Clay borehole water. In first instance, the most optimal conditions were provided, *i.e.* the most favourable electron donor was added to the samples. In addition, nitrate reduction at different pH by the microbial community present in the Harpur Hill sediment – shown to be metabolically active at high pH (see e.g. deliverable 2.1) – was also investigated. At pH 12.5 no signs of microbial activity were observed. However, the high pH did not completely eliminated the microbial community as intact cells were visible after resuscitation. Consequently, it seems that high pH alone as stress factor will not eliminate all microbial presence in a geological repository, though it seems to provide enough stress to cause a significant shift in the microbial population and reduce its nitrate reducing activity. However, several pH lowering processes can be present in a repository, resulting in small niches where microbial activity can be possible. In addition, it is clear that at the interface between the disposal gallery and the Boom Clay, the pH will not be sufficiently high to inhibit microbial nitrate reduction and organic compounds leaching from bituminized waste can be used as electron donor to fuel nitrate reduction. Besides pH, the prevailing high salinity conditions closer to the bituminized waste monolith (up to few molars), will provide an additional stress to the microbial community. Maximum NO_3^- concentrations are expected to reach between 0.5 M and 1 M at the gallery interface, and decrease also fast within the host formation (e.g. 0.1 M at a distance of 5 m in the Boom Clay). These values are quite conservative due to the assumption in the used model [34].

In all experimental conditions tested in this study a clear biofilm formation was observed on the Eurobitum samples. This could have enhanced the degradation of Eurobitum, resulting in an increased leaching of salts and organic compounds and to an increase in CO_2 production when these organic compounds are degraded. However, it is not yet clear to what extent this degradation could occur in disposal conditions due to the stress originating from the high salinity. Therefore, to assess possible microbial presence and activity in the disposal in gallery, it would be interesting to study the effect of the high salinity on the microbial community and its potential to form biofilm on the Eurobitum more in detail and the combination of both stress factors.

6.4 Impact of the experimental conditions on the microbial community

A previous study already showed that a diverse bacterial community is present and metabolically active *in situ* in the Boom Clay piezometer TD-11D [54]. However, it is not completely clear how this community is able to thrive in those conditions as in general anoxic conditions are maintained and Boom Clay pore water comprises 15 mM bicarbonate and ca. 12.5 mM dissolved organic carbon as (almost sole) redox-active species [33]. This DOM has been demonstrated to be highly recalcitrant for microbial degradation [48]. Bicarbonate as electron acceptor can be used by methanogic archaea or by acetogens, respectively reducing it to methane or acetate. 16S rRNA amplicon sequencing showed the presence of the genus *Acetobacterium*, a known acetogen [77], in the filter TD-11D-23 used in this study. Moreover, a preliminary study revealed the presence of the *mcrA* gene in several filters present in the HADES underground facility, including filter TD-11D-23, indicating the presence methanogenic archaea. Moreover, based on the *mcrA* sequence, species belonging to at least 3 classes of methanogens, including hydrogenothrophic and acetoclastic methanogens have been identified up to now in the borehole water of the HADES underground facility. In general methanogens are dependent of a fermenting bacterial community to provide a suitable donor [26, 78]. A fermenting microbial community commonly exists of different groups of cooperating bacteria: the first group degrades complex organic matter by extracellular hydrolysis in monomers and oligomers (simple sugars, amino acids or long fatty acid chains). Hydrolysis is followed by acidogenesis, where these monomers are fermented to volatile fatty acids, alcohols, H_2 and CO_2. Afterwards, volatile fatty acids are further fermented to acetate, H_2 and CO_2 [77, 78]. Acetate

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production has indeed already been found in piezometer water the HADES URL [48]. As a result of this fermentation process, all necessary compounds for methanogenesis are produced. 16S rRNA amplicon sequencing of the bacterial community present in filter TD-11D-23 indicated the presence of all the different groups of bacteria, suggesting that this community is able to digest complex organic material. Moreover, oxidation of dissolved organic matter which is unavoidable during the excavation of a borehole, can change the dissolved organic matter in lower molecular weight constituents [79, 80], thereby increasing the rate of fermentation. In summary, based on sequencing data, the *in situ* microbial population of Boom Clay borehole water in filter TD-11D-23 is expected to be a fermenting bacterial community, fuelling methanogenic archaea.

In the batch experiments without thermally aged Eurobitum, a clear acetate production was observed in conditions without organic additive and in conditions supplemented with formate or acetate as electron donor. This acetate production could originate from acidogenic fermentation of organic material that was present in the inoculum or produced through acetogenesis. The decrease in TIC implies the use of bicarbonate as electron acceptor, therefore either methanogenesis or acetogenesis occurred. Hydrogenothrophic methanogenic archaea and acetogens compete for a similar substrate, namely H_2 and as the formation of methane is thermodynamically more favourable, it is expected that acetogens are outcompeted [78]. Hydrogen is formed during fermentation reactions, but an important note is that the atmosphere of the anaerobic glove box contained 1% H_2. The bottles were not hermetically closed, hence this H_2 could be bioavailable. On the other hand, parallel to this decrease in TIC, an increase in TOC was observed, suggesting the reduction of CO_2 to acetate. No following decrease in acetate is observed, hence probably no active acetoclastic methanogens were present. However, without detailed 16S rRNA amplicon sequencing or amplification of the *mcrA* gene, it is difficult to conclude exactly which process occurred. In first instance, flow cytometry was used to monitor the microbial population as it was shown that phenotypic diversity analysis based on flow cytometry fingerprints was a suitable tool to determine which samples would be interesting for detailed analysis [55]. Flow cytometry can be used as a rapid, high-throughput technique to immediately observe a possible shift in the microbial population. After the data analysis, samples for further analysis could be selected more carefully, rather than analysing all samples. From the moment when acetate production becomes visible in the samples, beta diversity analysis based on the flow cytometry fingerprints indeed putatively suggests a grouping of those samples, while it is less clear when no acetate production is observed. This could indicate that indeed a similar population is enriched in those conditions. Nevertheless, additional identification of the microbial community is necessary and is still ongoing in this study.

In the conditions with thermally aged Eurobitum, nitrate is present as additional electron acceptor, hence methanogens and acetogens are expected to be outcompeted by nitrate reducing bacteria. In addition, nitrate can be coupled to the degradation of organic material. This was indeed confirmed in the first batch experiments, where 16S rRNA amplicon sequencing was performed at different time points during the experiment. As stated above, initially, fast growing genera such as *Pseudomonas* were observed, while afterwards, genera capable of using the added organic additive became more dominant. In case of acetate, species belonging to the genus *Pseudomonas* remained dominant, while in the presence of formate, *Acidovorax* became the dominant genus. Beta diversity assessment of flow cytometry fingerprints obtained in the experiments with SCW indicate no clear difference in the microbial community related to the organic additives, but rather suggests an effect of time and other chemical species (e.g. phosphate). Further analysis to elucidate which genera are enriched in different conditions is in processes.

7 Conclusions

Eurobitum bituminised radioactive waste is subject to chemical and radiolytical degradation which results in important ageing of the bitumen waste matrix and in the generation of radiolysis gases. Moreover, a nitrate plume in the clay water arises that could cause a geochemical perturbation of

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the surrounding clay, possibly affecting the redox conditions, causing ionic strength effects and cation exchange processes, which might result in an increase in the mobility of the radionuclides through the host rock. However, next to some abiotic reactions, nitrate reduction is a well-known microbial process. This study demonstrated the metabolic diversity of the Boom Clay microbial community present in filter TD-11D-23. Although no nitrate is present in current *in situ* conditions in the HADES underground laboratory, the nitrate leaching from Eurobitum rapidly stimulated microbial nitrate reduction. Different rates were observed depending on the organic compounds that were used as electron donor to fuel the nitrate reduction process. Microbial nitrate reduction resulted in a significant production of nitrite, shown to be able to chemically oxidise pyrite and possibly dissolved organic matter from the Boom Clay host rock. Oxidation of these Boom Clay components, makes that its reducing capacity will be decreased and mobility of certain radionuclides can be increased. Moreover, it was shown that microbial nitrate reduction was possible up to pH 10.5, reflecting conditions at the interface of the Boom Clay and the concrete liner of the disposal gallery. On the other hand, pH conditions that are expected in the disposal gallery seem to inhibit microbial nitrate reduction. Nevertheless, microbial presence can be expected under these conditions, hence if niches arise with local pH decrease, microbial nitrate reduction can be induced.

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