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Survival of microorganisms in bentonite subjected to different levels of irradiation and pressure

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Publishable Summary

In the geological repository of radioactive waste, combined effects of high pressure, elevated temperature and a certain low-level radiation on the bacteria can be expected. Nowadays, we have reasonably good understanding of the effect of each particular variable separately, however their synergic effect and especially the effect of radiation and exceptionally long time frame remains rather unclear. Long-term studies on bentonite irradiation under realistic repository conditions are thus highly needed, although their conduction remains technologically very challenging. Our study aimed to improve the knowledge on the effect of Gama radiation and pressure on indigenous microbial community in bentonite inoculated by natural porewater. Therefore, we studied these effects using bentonite type BaM (Keramost, Czech Republic) enriched with granitic porewater VITA from Josef Undeground Research Center (Czech Republic), a natural source of anaerobic sulfate reducing bacteria.

The composition of microbial communities within the bentonite suspension samples was changing continuously during the irradiation experiment. At the beginning, indigenous microorganisms present in the underground VITA water and BaM bentonite went through bottleneck during adaptation to specific environment of bentonite suspension. Most of the bacterial genera occurring in the VITA water disappeared and both total biomass and species richness markedly decreased during this phase. Subsequently, gradual changes in microbial community composition were observed reflecting the prevailing conditions in the samples. In the samples influenced by the presence of oxygen, the community changes were driven by the available electron donors evolving from heterotrophs (chemorganotrophs) to autotrophs (chemolitotrophs) with the decrease of available organic material. In anaerobic control, limitation by both available electron acceptors and donors was identified. The communities evolved from heterotrophic facultative anaerobic nitrate reducers to autotrophic anaerobic iron or sulfur reducers and fermenting microorganisms. Community changes did not reflect species richness, which remained rather constant after the initial bottleneck. Microorganisms that adapted and survived under harsh conditions underwent further selection caused by the Gama radiation. Notably, application of 19,656 Gy absorbed dose at the constant dose rate 13 Gy/h did not completely eliminate bacteria in bentonite suspension. However, decline in total microbial biomass, but not species richness, was observed together with minor changes in the microbial community structure. Gram-negative non-spore-forming microorganisms dominated in the aerobic irradiated samples as well as aerobic controls although spore-formers are generally more radiation resistant. We ascribed this unexpected pattern to the presence of oxygen. In anaerobic control samples dominated Gram-positive spore-forming bacteria that are generally more resistant to radiation, thus we presume that even higher absorbed doses of ionizing radiation would be needed to eliminate microbial activity in anaerobic conditions. The results of irradiation experiment further showed a key role of iron reduction in the microbial processes occurring in the bentonite under anaerobic conditions. Ferric ions naturally occur in the bentonite molecular structure and their reduction might cause decrease in bentonite swelling capacity and thus negatively influence the safety of the repository.

Exposure to 2 and 5 MPa pressure did not markedly reduce the total microbial biomass nor did substantially change the composition of the microbial community present in the bentonite suspension.

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1 Introduction

The use of deep geological repositories has been internationally proposed as the safest option for the disposal of radioactive waste materials [1]. Long-lived high level radioactive waste will be buried several hundred meters below ground in stable geological formation in canisters made of corrosion resistant materials such as copper or stainless steel surrounded by a buffer of compacted bentonite or cement [2]. Bentonite is a soil material with a high content (up to 80 %) of a mineral montmorillonite, which in the contact with water swells. In a fixed total volume, the water uptake by bentonite results in the development of swelling pressure and reducing the volume of initially larger bentonite pores [3]. This osmosis driven swelling ability of montmorillonite is thus a crucial feature of bentonite in its use to construct engineered barriers in geological repositories for low-, intermediate- and high-level radioactive wastes [4]. Bacterial growth in swelling bentonite clay is generally expected to be limited due to the small pore size and to the scarcity of nutrients and electron acceptors necessary to maintain the bacterial population [5].

Although bentonite definitely represents an unusual and extreme environment, bentonite deposits are not sterile. Fukunaga et al. [6] detected microbial activity of mostly mesophilic bacteria at four sites in two different bentonite deposits in Japan and reported that the activity is lower than in typical soils and aquatic sediments. Similar results were reported by [7] in Belgian Boom clay. Fru and Athar [8] and Pedersen et al. [9] reported natural occurrence of the spore-forming Gram-positive bacteria in MX-80 bentonite. Svensson et al. [10] searched various natural bentonite samples for the presence of microorganisms with special attention to aerobic heterotrophs, anaerobic sulphate and iron reducers and autotrophic acetogens, because of their abilities to affect the storages for nuclear waste in different ways. Various mesophilic and thermophilic microorganisms belonging to these groups were detected in all eleven studied types of bentonite, although the differences in microbial abundances and compositions were generally large.

Bacterial spores can be found in environmental samples obtained from all parts of the Earth and as was already mentioned, spore-forming bacteria are indigenous also in various bentonites [8,9]. Bacterial spores exhibit incredible longevity and resistance. The recovery and revival of spores from environmental samples as old as 100.000 years were reported [11]. Spore-forming enables bacterial cells to temporally escape from nutritionally unfavorable local conditions via dormancy. The developmental pathway leading from a vegetative growing to a spore forming is initiated by depletion of a readily metabolized nutrients from the bacterium's local environment. Nevertheless, spores continuously monitor the nutritional status of their surroundings, although they are not metabolically active, and they rapidly resume vegetative growth, when the conditions change to more appropriate [12]. Setlow [13,14] or Nicholson [12] reviewed the factors important in spore resistance to various extreme conditions. Spores are significantly more resistant to ionizing radiation, various chemicals, desiccation and both wet and dry heat than growing cells [12,15]. The reasons for this elevated spore resistance are not completely understood, but the important factors may be the low core water content together with the presence of specific spore compounds such as acid-soluble spore proteins or dipicolinic acid and the ability to perform the DNA repairs [12,14]. Natural occurrence of spore-formers in bentonite thus greatly increases uncertainty of assumptions on microbial safety of deep geological repositories.

The repository conditions in the near-field following high radioactive waste (HLW) deposition generally evolves from initially warm and oxidizing to cool and anoxic in the long term and consists of four phases summarized by King et al. [16]: (i) immediate post-placement, when the environment will be aerobic and the Gama radiation dose rate will be at its highest level. At this stage, which will be short (up to several months), the conditions will be extreme. For the Canadian concept, it was

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calculated, that the temperature at the canister surface will reach up to 95°C, the radiation dose rate will be about 52 Gy/h and the pressure in the buffer and backfill materials can rise up to 12 MPa [17]. The second stage is (ii) dry-out, whose duration will depend on the initial compacted bentonite moisture content and can take from several years (in case of crystalline host rock) to several hundreds of years (in case of sedimentary host rock). The bentonite will shrink partially during this second phase. Third stage, (iii) container re-wetting and buffer saturation is characterized by transition from a dry to a saturated near-field environment, and consumption of residual oxygen. The last stage (iv) is long-term anoxic phase, which begins once the near-field reaches full saturation. It is the period of the continued cooling and anoxic conditions.

Because of the described extreme conditions, most early analyses considered repository to be either a completely sterile environment, or at least not seriously threatened by bacterial activity [18]. However, many microorganisms are able to tolerate really extreme environments [19,20] and citations therein) implying that the conditions in early stages post deposition does not need to be as devastating as expected for all microorganism. Stroes-Gascoyne and West [18] reviewed microbial effects on ILW repository and concluded that combination of radiation and high temperature effects in the close proximity of ILW container could result in the creation of a zone with severely reduced microbial activity, which should however be relatively narrow (25-50 cm). The potential diffusion of corrosive microbial metabolic products to the container surface and the possible bacterial repopulation after the amelioration of conditions within the repository in the long-term horizon thus must be considered. Further, we discuss some of the most important factors influencing microbial activity in compacted bentonites under repository conditions.

1.1 Effect of pressure

In most of the waste disposal concepts, the deposited HLW canisters will be surrounded by compacted bentonite as one of the multiple barriers [2]. Upon contact with groundwater, the bentonite will expand and develop a mechanical pressure when constrained by the surrounding rock walls leading to the sealing of the repository [21]. Depending on the mineralogy of the specific bentonite type and salinity of the groundwater, different swelling pressures are produced at the same wet density [3].

A high density is believed to have an inhibiting influence on bacterial activity of the natural bacterial populations in the bentonite clays. In the various waste disposal concepts, the desired swelling pressure is at least 5 MPa, which requires a clay dry density of >1,600 kg/m^3 [22]. Prokaryotic cells can compensate for the mechanical pressure (as in compacted bentonite) by turgor pressure. Published data on turgor pressure in prokaryotic cells mention pressures between 0.08 MPa and 2 MPa in most species, which means that cell integrity is generally possible at bentonite swelling pressures below 2 MPa [22,23]. However, there are many species of prokaryotes specialized to survive in high pressure environments, such as deep ocean. These organisms are known as piezophiles and were reviewed by [20,24]. Furthermore, endospores of any spore-forming bacteria can survive high pressure for reasonably long time. Neither the reason for spores' high resistance to high pressure nor the exact mechanism by which pressure kills spores is known, but efficient killing of spores by high pressure generally requires high temperatures, though these temperatures are significantly lower than needed to kill spores on its own [13]. In accordance with this, spore-forming bacteria were not completely eliminated after 15 months at the repository conditions, although their numbers were significantly reduced [25].

When Choi and Oscarson [26] compacted two types of bentonite to dry density 1,300 kg/m^3, Na-bentonite saturated with water developed pore sizes of around 0,01 µm with only very few larger pores present, which is about 100 times smaller than size of an average bacterium [18]. The tested Ca-bentonite on the other hand tended to have more large pores than Na-bentonite at the same density,

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which was ascribed to the generally larger quasicrystal size of the Ca-bentonite clay. This feature is further responsible for higher diffusion coefficients of Ca-bentonite compared to Na-bentonite [26]. Transport of bacterial nutrients and metabolic products in compacted bentonites is diffusion limited due to the low porosity of buffers and backfill. Bacterial activity will be consequently diffusion limited, because the rates of diffusion are probably very slow as was demonstrated for sulphide [4,22]. As a result bacterial growth will stop and their metabolic activity will cease. However, the bacterial viability may be (and often is) preserved [22].

Although cultivable bacteria were reported in compacted bentonite of different density both in case of indigenous bacteria [25,27,28] or cultivable bacteria introduced to bentonite prior compaction [25,29], there is strong experimental evidence, that bacterial activity is negatively affected by compactness of bentonite. Various kinds of non-spore-forming bacteria inoculated in compacted MX-80 bentonite (wet density 2,000 kg/m^3, swelling pressure about 5MPa) were significantly eliminated in 15 months under repository conditions [25]. Microbial sulphide-producing activity has been found to decrease with increasing density of MX-80 bentonite [9,30,31]. The sulphide-production results (by infested SRB) for the three types of bentonite (MX-80 and Asha belonging to Na bentonites) and Calcigel (Ca bentonite) indicated intervals between 1,740 and 1,880 kg/m^3 in wet densities within which sulphide-producing activity dropped from high to very low or below detection [29]. When studying indigenous bentonite microflora, sulphide-producing bacteria could be cultivated from following compacted clay samples: Wyoming Volclay MX-80 (USA), Asha (India), Calcigel (Germany), GMZ (Gaomiaozi, China, Rokle (Czech Republic) and FEBEX clay (Switzerland). Their numbers decreased over wet density (1,400 to 2,000 kg/m^3) for some but not all tested clays with the density threshold for metabolic activity (detected by the production of radioactively marked Cu_2S) varying between 1,690 kg/m^3 (Rokle) and over 2,000 kg/m^3 (Boom Clay). Moreover, acetate formation from natural organic matter was observed at all wet densities indicating presence of bacterial activity even at the higher bentonite densities, when sulphate production could not be detected [22]. Similarly, Bengtsson and Pedersen [32] reported no clear cut-off in Boom clay density with respect to presence and activity of cultivable SRB, sulphide production was increasingly possible at or below 1,800 kg/m^3.

These results indicate, that density alone does not control bacterial activity in clays, but the other variables such as clay type, temperature, water content, presence of organic matter and other energy sources, pore space or pore water composition must be also taken in consideration and need further attention [22,29].

1.2 Effect of temperature

The detrimental effect of temperature on microbial activity was demonstrated by several studies. The surface temperature of the waste canisters is expected to be about 90-95°C, in some concepts will even exceed 100°C, and high temperature is expected to be one of the important protections from microbial life [2]. In accordance with this, Pedersen et al. [25] reported, that at 80°C, the introduced spore-forming SRB were the only surviving bacteria in 28 weeks' time of the experiment in compacted MX-80 bentonite. Similarly, Lydmark and Pedersen [33] described that the higher temperatures (up to 85°C) led to decrease in the bacterial abundance and activity in repository experiment and Lucht and Stroes-Gascoyne [34] detected strong effect when heating bentonite samples at 90°C for 30 min, although heat resistant bacteria remained in the samples even after heating for 3 h.

Results of other experiments were however less encouraging. Heat treatment of the bentonite at 110°C for 170 h failed to eradicate SPB in the bentonite and intensive sulphide-producing activity and large numbers of cultivable SPB were observed in the heat-treated MX-80 bentonite samples [29]. Masurat et al. [30] in similar experiment detected loss of SRB viability after treatment of the bentonite at 120°C for 20 h, but Bengtsson and Pedersen [32] reported the presence of indigenous

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SRB, which survived heat treatment of 120°C for 48h. Unifying element of all of these studies is presence and survival of spore-forming microorganisms, which are capable of remarkable endurance. High resistance to wet heat seems to be a special feature of spores, some of which can survive temperatures higher that 100°C for extended periods of time [14]. In water, spores are resistant to 40°C higher temperatures than are growing cells of the same species, and to about 30°C higher temperatures in dry state. The mechanisms of spore killing by wet and dry heat are different, with damage to spore core proteins likely killing spores exposed to wet heat, while DNA damage is a major mechanism whereby spores are killed by dry heat [13].

Moreover, the elevated temperature can also have negative effect on bentonite sealing capability. Svensson et al. [10] detected a significant difference in swelling pressure between bentonite exposed to high temperatures and reference untreated material for two of the investigated bentonite materials, Asha 505 and Deponit CAN. The bentonite samples which were closer to the heater were affected more by the swelling pressure decrease. These results show the need for the further detailed studies concerning the effect of temperature on bacterial survival (with the special emphasis to spore-forming microorganisms) while taking into account the physical features of particular bentonite clays.

1.3 Effect of desiccation

In contrast to nutrient, energy, pressure, temperature and radioactivity constraints, few bacteria can tolerate removal of water from the cell [28]. The availability of H2O for microbial processes can be represented by the water activity (a_w). Most bacteria grow well at an a_w around 0.98 but relatively few species can grow at an a_w of 0.96 or lower [28]. The most tolerant bacteria can grow at a_w of 0.85 and halophilic archaea even at a_w of 0.75 [35].

In non-saline soils, water availability is determined by the thickness of the water film around soil particles. In the soils with low water content, substrate diffusion and cell motility reduces microbial activity and these conditions are more detrimental to microbes than osmotic stress in saline soils [36]. Reducing the water activity of buffer material either by the high compactness of the bentonite (>1600 kg/m^3) or/and by high porewater salinity (>100 g/L) thus may be a potential approach to limiting microbial activity in the vicinity of containers [37]. In accordance with this, Motamedi [28] evaluated the effect of water activity on survival of introduced SRB under repository conditions and found that water activity lower than 0.96 was lethal for the studied microbial species. Similarly, Aoki et al. [27] demonstrated, that the number of viable aerobic heterotrophs in compacted bentonite well correlated with the water content, which was affected by the temperature (presence of the heater).

However, many bacteria can withstand desiccation either by the biofilm formation at the repository interface between the bentonite and the surrounding environment [38] or by spore formation or even as vegetative cells in dry state in case of some desiccation-tolerant bacteria [12,39]. It was shown, that microbial activity was sustained at desiccated bentonite-air interfaces at 75 % relative humidity but was completely suppressed at 30 % relative humidity [40]. Surviving the desiccation by biofilm formation thus seems to have its limits. On the other hand, the maximum time of bacterial survival in dormant states is however unknown [39]. It is known, that the rates of microbial metabolism in the deep aquifers are at least 10^3-10^5 times slower than those in modern surface sedimentary environments [41]. As a result, many subsurface microorganisms have mean generation times of up to thousands of years [42] and even higher values can thus be expected for metabolically inactive spores.

Cell dehydration during desiccation process causes severe disruption of enzymes and electron transport chains, which results in the accumulation of free radicals causing DNA damage [39]. Similarly, free-radical-mediated DNA damage also occurs under ionizing radiation exposure [43]. Both desiccation and ionizing radiation thus cause a similar pattern of DNA damage and double-strand breaks. This together with the obvious lack of naturally highly radioactive environments on Earth led

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Mattimore and Battista [44] to hypothesize, that the resistance to desiccation can unintentionally increase the resistance to ionizing radiation, because both conditions cause similar cell damage and requires similar defensive and restoration mechanisms. In accordance with this, Musilova et al. [24] found a strong correlation between desiccation and radiation resistance across many bacterial species, indicating that an increase in the desiccation resistance of 5 days corresponds to an increase in the room-temperature irradiation survival of 1 kGy.

Montmorillonite with its high affinity for water could efficiently extract water from the bacterial cells, when surrounded by bentonite, leaving them in a desiccated (and possibly well preserved) state, because the bacterial cell membrane is water permeable [22]. Slow desiccation is a common process for bacterial preserving in microbiology [45] and spores easily survive multiple cycles of desiccation and rehydration, while growing bacteria are often killed by a single desiccation treatment [13]. Although Stroes-Gascoyne and West [18] concluded, that the presence of SRB might not pose a high risk to waste containers, if a_w of the saturated bentonite is kept low, the evaluation of the behavior of microorganism and especially the spore-forming bacteria under repository conditions seems to be crucial.

1.4 Effect of irradiation

Ionizing radiation causes various changes in cells. One of the most important is cytoplasmic water radiolysis which produces reactive oxygen species (ROS) - hydroxyl radical (·OH), ionized water (H_2O^+), hydrogen radical (H·) and hydrated electrons (e-) followed by secondary ROS products superoxide ($O_2\cdot^-$) and hydrogen peroxide (H_2O_2) formed within one picosecond (10^{-12} s) after irradiation. Subsequent chemical cascades generate additional cell-damaging molecules, which might degrade DNA by broad range of base damage and double strand breaks or other crucial cell biomolecules and cause cell death [43,46,47]. Although the amounts of DNA damage caused by given doses of ionizing radiation for different bacteria are very similar, most bacteria encode conventional enzymatic DNA repair mechanisms, which results in very variable and species specific dose necessary to kill the cell [48].

The radiosensitivity of microorganisms has been intensively studied since the World War II, because ionizing irradiation became a common sterilization method especially in food and pharmaceutical industry or human medicine [49,50]. The radiation resistance of a microorganism is commonly measured by the decimal reduction dose (D_{10}), which is defined as the radiation dose (kGy) required to reduce the number of that microorganism by one log (reducing 90 % of the total number). Although this value depends on several factors described below and is thus not the single variable of radiation dose, it is commonly used to compare the species radiation sensitivity. The average D_{10} dose for most vegetative microorganisms was calculated to be 0.420 kGy, although the variation between species and even strains is very high [15].

Microbial sensitivity to radiation depends on various intrinsic and extrinsic factors [15,51]: to intrinsic factors belong physiological and genetic features of particular microbial cell (e.g. cell water content, complexity of the cell, size and structure of DNA, etc.), bacterial ability to employ various defensive systems (changes in intracellular cation concentration, excellent DNA repair systems, and efficient enzymatic and non-enzymatic antioxidant systems) [52] or vegetative state of the microbial cells. The spores exhibit on average five times higher radiation tolerance, than vegetative cells [15]. For example radiation resistant spores of *Bacillus antracis* require doses 29.8 - 46.2 kGy for complete inactivation without subsequent recovery [53].

Environmental conditions represent the extrinsic groups of factors. Reduction of water content and subsequent desiccation cause very similar cellular damage as Gama irradiation [44], synergic effect of desiccation and radiation is however complicated. Microorganisms generally exhibit higher radiation tolerance in dry environments, because of their general adaptation to desiccation, which

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cause similar cell damage as radiation [44] (see below). However, the resistance of spores to radiation was shown to increase with increasing environmental moisture content. DNA damage (strand breaks) decreases with increasing water activity of the spores [54], inactivation of *Bacillus atropheus* spores dry powder required 25 kGy, while inactivation of *Bacillus atropheus* spores in liquid suspension required 35 kGy with dose rate 333 Gy/min [55]. Next important factor is the presence of oxygen. It was shown, that the radiosensitivity of microorganisms is highly pronounced by the presence of oxygen even at very low concentrations [56]. Under completely anaerobic conditions, the D_{10} value of some vegetative bacteria increases by a factor of 2.5–4.7 [51], in comparison with aerobic conditions. This might be significant factor in the anaerobic conditions of HLW repositories, where the lack of oxygen in the latter phases past disposal can result in increased tolerance of microorganisms to radiation compared to aerobic conditions. Further, irradiation at elevated temperatures synergistically enhances the lethal effect of radiation on microorganisms. Van Gerwen et al. [15] reported clear decrease of D_{10} values with increasing temperature across 360 vegetative bacterial species studied. Irradiation at very low temperature below zero on the other hand significantly increases the microbial radiation resistance [24]. Finally, environmental composition, species adaptation to it and post-irradiation conditions plays also very crucial role in microbial radiation sensitivity. It is well known, that the radiosensitivity depends on the media type [15] and higher radiation doses are necessary to kill the microorganisms in soils, than in pure cultures [57]. The unfavorable environmental conditions can increase both the radiation sensitivity but also tolerance depending on the past evolutional processes. Adu-Gyamfi et al. [58] detected decrease in D_{10} values in mixed cultures compared to single cultures under same experimental conditions, which might indicate reduced resistance to Gama radiation as a result of competition among the isolates. On the other hand, effective adaptation to extreme environmental conditions might accidentally increase the radiation tolerance [44,59,60]. The environmental conditions seem to be also crucial past irradiation, where bacterial recovery can be much enhanced in the optimal conditions [61].

As already mentioned, the radiation sensitivity among bacteria is a very variable feature. There are specific radiation highly resistant bacteria, most of which belong to Gram-positive microorganisms [15,62,63]. *Deinococcus radiodurans* is a non-spore forming bacteria that can grow under high chronic Gama radiation (50 Gy/h) or recover from acute doses greater than 10 kGy [64]. The average D_{10} dose for this species is 10,4 kGy [15]. As we already mentioned in the previous chapter, ionizing radiation resistance is probably a consequence of microbial adaptation to desiccation, as both hazards cause very similar cellular damage [44]. *Deinococcus* has the capacity to restore its broken chromosomes by homologous recombination in only hours [65]. Musilova et al. [24] corroborated this hypothesis by founding a strong correlation between desiccation and radiation resistance across many bacterial species. Similarly, Beblo et al. [59] detected surprisingly high tolerance against ionizing radiation in nine out of fourteen hyperthermophilic microorganisms belonging to Archaea and phylogenetically deep-branching thermophilic bacteria, two species were even able to survive 20 kGy resembling radioresistance comparable to *D. radiodurans*. The extraordinary UV resistance of one *Rhodobacter* sp. isolated from high altitude Altiplano was also ascribed to the cellular adaptation of this species to osmotic stress encountered in the hypersaline environment from which it was originally isolated [60]. Tolerance to radiation therefore might be a common phenomenon among thermophilic microorganisms or extremophiles in general, which must have evolved mechanisms to survive very extreme environmental conditions. This might be very important for the evaluation of HLWR concept safety.

On the other hand, dissimilatory metal-reducing bacterium *Shewanella oneidensis* was reported to be extremely sensitive to radiation although it encodes more complex DNA repair systems that one in *D. radiodurans* [64]. These results indicate direct DNA damage is not the major lethal effect

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of irradiation in this case, but there are other factors, such as oxidative stress, protein damage, and prophage-mediated cell lysis, that cause the lethal effect of irradiation [66]. Species very resistant to radiation often accumulate exceptionally high intracellular manganese and low iron levels. Manganese ions might serve as antioxidants that reinforce enzymatic systems which defend against oxidative stress during recovery. Fe-rich and Mn-poor cells of *Shewanella* might suffer from the release of Fe(II) from proteins during irradiation, leading to additional cellular damage by Fe(II)-dependent oxidative stress [64].

Microbial response to radioactivity is crucial question also for the HLW repository concepts and their long-term safety. Swedish KBS-3 HLW repository concept expects the maximum dose outside the canister to be less than 0,5 Gy/h [67] - the radiation field outside canister has been calculated about 100-150 mGy/h at deposition [68]. On the other hand, Canadian HLW concept predict the maximum dose rate 52 Gy/h at the surface of a Titan waste canister or dose rate 15Gy/h for the surface of the copper canister [69]. The gamma and neutron radiation are expected to be significant during approximately 1,000 years past deposition [67] and it will take 100,000 years for the radioactivity to decrease to similar levels to those of natural uranium [70]. Survival data from pure culture studies may not be fully applicable to relatively nutrient limited environment of proposed HLW, where different species of the community will be competing, and where radiation is highly probably not the only selective stress. There are three major ways, which could help us to understand the effect of irradiation on repository environment: a) irradiation studies conducted on environmental samples of soils, rocks or sediments, b) studies on environmental samples from radioactive contaminated sites and c) irradiation studies specifically targeted to bentonite environments.

1.4.1 Irradiation studies on environmental samples of soils, rock or sediments

These studies might help us to understand, how natural soil (sediment) microbial ecosystem reacts on the presence of radioactivity. McNamara et al. [71] in the literature review stated that Gama irradiation of the soil samples generally eliminates actinomycetes, fungi and invertebrates at the 10 kGy dose, the majority of soil bacteria are eliminated by 20 kGy, but a dose higher than 70 kGy may be required to kill certain radio-resistant bacteria. These approximated values well agree with the dose reaction of microorganisms described in further studies. Pitonzo et al. [72,73] exposed the microbes indigenous to the rock site of a proposed repository to low Gama radiation doses (1.63 Gy/min) and discovered, that the indigenous bacteria were capable to survive in a viable-but-nonculturable state even after applying maximum of 9.34 kGy total dose [72]. They further showed, that irradiated bacteria (up to 9.34 kGy total dose) might be resuscitated to the completely cultivable state in the course of time when environmental conditions become more favorable. Brown et al. [48] studied the impact of low-dose chronic gamma irradiation (8 weeks, 0.5 or 30 Gy/h) on a sediment microbial community during irradiation and throughout a subsequent recovery stage. All acetate and lactate amended sediment microcosms displayed nitrate and iron reduction, although the rate of Fe(III) reduction was decreased in 30 Gy/h treatments. These systems were dominated by fermentation processes. In systems containing no added electron donor nitrate, iron or sulphate reduction was not restricted by radiation, iron reduction was even stimulated at the 0.5 Gy/h radiation dose. The bacteria showed good ability to recover after receiving the total dose of 38.6 kGy, although the microbial composition of samples naturally changed. Contrary to these results, Bunzl and Schimmack [74] did not detect any significant microbial biomass recovery in 20 days past irradiation of several kinds of soils and clay with total absorbed doses of 40 and 80 kGy.

All these results indicate that biogeochemical processes does not have to be restricted by low dose rates in natural rock, soil or sediment environments, and some electron accepting processes may even be stimulated by radiation [48]. The native microbiota seems to be capable of surviving long-term

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low dose Gama irradiation and under proper conditions retains its metabolic activity [48,73]. On the other hand, the experimental evidence also suggests the existence of threshold in total absorbed radiation dose, which effectively eliminate the bacterial presence in various soil materials [71,74].

1.4.2 Studies on environmental samples from radioactive contaminated sites

It has been long-known that short-term exposure of microbes to physical or chemical deleterious agents can lead to the survival and selection of resistant mutants with an enhanced capacity to synthesize DNA-repair enzymes [75,76] and similar or even more pronounced effect can be expected under chronic low-dose irradiation, which will be more comparable to conditions expected in HLW repositories, because lower long-term dose rates may allow species to evolve and adapt over the geological timescales involved [48]. Studies on response of microbial ecosystems to long-term impact of radioactivity are thus very important to understand the possible microbial evolution under repository conditions.

Chernobyl in Ukraine, where the tragic accident in nuclear reactor happened in 1986, or various radioactive waste storage places represent very valuable testing sites to answer these questions. Chapon et al. [77] and Theodorakopoulos et al. [78] analyzed microbial composition in one of the trenches near Chernobyl power plant with relatively high levels of radioactivity (61-750 Bq/g ^{137}Cs, recalculated on ^{137}Cs: 0.36-4.45 Gy/y), where the radioactively contaminated wastes were buried past accident. Complex bacterial community structures were observed in both highly and weakly contaminated samples [77]. Subsequent analysis based on NGS revealed profound changes in community composition at the phylum and OTUs levels and higher diversity in the trench soils as compared to the outside [78]. These results demonstrate that the prokaryotic assemblages in soil are influenced by the total absorbed dose rate and also to a lesser extent by the high organic matter content of the trench [78]. Zavilgelsky [79] studied radiation sensitivity of bacteria from near the Chernobyl nuclear power station (radiation levels of 12-590 µGy/h = 0.1-5.2 Gy/y). They showed, that many isolates of the same species from near the power station and the radioresistant *E. coli* mutant SA236 were more resistant to X-rays, UVC and 4NQO compared with isolates from the natural radioactivity level control site and the *E. coli* parent strain.

Similarly to previous study, Bruhn et al. [62] tested radiation sensitivity of bacterial species from spent fuel storage pools (activity about 7 GBq/ml) exposed to three different total radiation doses. Many of the indigenous bacteria proved to be similarly resistant as radioresistant *Deinococcus radiodurans*. Upon exposing the microbes to the dose of $6x10^4$ Rad (600 Gy, 7.1 Gy/min) biphasic variation in sensitivity of bacteria to radiation was detected resulting in two distinct groups of bacteria – one radiation resistant and the other displaying radiation intolerance. At highest doses ($1.1x10^5$ Rad, 1100 Gy, 14.7 Gy/min), Gram-positive bacteria showed generally higher radiation resistance than Gram-negative bacteria, similarly to results of other studies in the radioactive environment [15,62,63]. Interestingly, two Gram-negative SRB isolates belonged to highly radiation tolerant microorganisms, which was ascribed to the lower effect of irradiation in the lack of oxygen in the anaerobic environment typical of SRB community [56].

Further, Fredrickson et al. [63] studied radiation sensitivity of bacteria indigenous to sediments from a high-level nuclear waste plume originated from a leak of a waste tank, characterized by high concentrations of radionuclide (up to 0.37 GBq/kg). The populations of cultivable aerobic heterotrophic bacteria from plume samples were generally low (from below detection to ~104 CFU/g), but viable microorganisms were recovered from 11 of 16 samples, including several of the most radioactive ones. Only Gram-positive and/or organisms high in G+C content were cultured from the most highly radioactive sediments. Isolated bacterial cultures from plume samples were subsequently exposed to 2.5, 5, or 20 kGy dose (radiation dose not stated). Two isolates closely related to

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Deinococcus radiodurans were able to survive acute doses approaching 20 kGy. Many of the Gram-positive isolates were resistant to lower doses of gamma radiation (2.5 or 5 kGy).

Results of mentioned studies demonstrate that bacteria, with clear domination of Gram-positive strains, indigenous to various long-term radioactivity contaminated sites show increased resistance to extreme physical and chemical stress associated with radioactive waste presence.

1.4.3 Irradiation studies specifically targeted on bentonite environments

Studies on radiation effect in bentonite are surprisingly rare considering the importance of bentonite as one of the key factors limiting metabolic activity of bacteria in future repositories. Long-term irradiation might have some direct effect on the bentonite properties, because the total absorbed doses over time will be high. Experimental studies were thus conducted to estimate the effect of repository conditions (pressure, temperature, and irradiation) on the bentonite quality and performance. MX-80 bentonite with density of 2050 kg/m^3, was heated to 90-130°C and irradiated for one year with a total radiation dose of 3.27x10^7 Gy, which is orders of magnitude more than what will be the case in the repository. Results showed no significant change in the montmorillonite quality and quantity in either of the samples, but there were changes in some of the accessory bentonite minerals caused by irradiation [80]. Similarly, Galamboš et al. [81] detected no changes in structure and quality of Slovak bentonites with absorbed doses of 10^4 and 10^5 Gy and only insignificant changes with absorbed doses of 1 MGy of Gama radiation. The direct effect of Gama radiation on bentonite property during the repository lifetime is thus possibly negligible. More detail information about the influence of radiation on clay mineral properties can be found in Allard and Calas [82].

Stroes-Gascoyne et al. [69] studied the radiation sensitivity of naturally occurring microorganisms in bentonite. The D_{10} dose (total radiation dose resulting in reduction of bacterial viability to 10 %) at dose rate 100 Gy/min was between 0.65 and 1.68 kGy for natural microbiota depending on bentonite type and the radiation survival responses decreased linearly with increasing total dose. Using these values and values of dose rates predicted at the canister surface post emplacement, authors estimated, that it would take 9-33 days (depending on the canister type) after emplacement to completely sterilize the outer container surface. Formation of biofilm and resulting MIC of canister surface will thus be rather improbable at the early repository phase. However, sterilization of 25cm thick buffer barrier by low radiation doses outside the canister is not likely and the repopulation of canister surface during the repository lifetime is thus real possibility.

In subsequent experiment Lucht and Stroes-Gascoyne [34] studied effect of Gama radiation at dose rates 90.7 Gy/min a 5.6 Gy/min (up to 10 kGy maximum dose) together with the effect of heat treatment (30°, 60° or 90°C) on natural biota in buffer system. Authors detected no general effect of dose rate on the radiation sensitivity, the total dose being the most important factor with the values similar to previous experiment. The combined effect of temperature and irradiation at dose rates 90,7 Gy/min produced biphasic dose responses in all bentonite buffers tested indicating presence of two naturally present differently heat and radiation resistant types of bacteria in bentonite, one type is temperature sensitive and shows the decrease of radiation resistance with increasing temperature. The other type showed increase in radiation resistance with increasing temperature possibly due to synergic desiccation effect of both factors. Similar biphasic reaction to radiation was also detected by [62] in microorganisms from stored nuclear fuel pools to radiation. Radiation resistant bacteria were isolated from bentonite and the isolate survived up to 5 kGy total dose proving the presence of naturally occurrence of radiation and heat resistant bacteria in natural bentonite samples [34].

Published evidence imply the possible natural presence of radiation resistant bacteria in bentonite buffer systems, however, their behavior and especially evolution under long-term low-level

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irradiation remains completely unknown. Long-term experiments under repository conditions including the effect of irradiation are thus highly needed.

1.5 Studies under repository conditions

Up to date, there is no study performed under all the extreme conditions present within a real HLW repository and at the reasonably long time scale, so the evaluation of evolution within repository conditions and possible microbial effect on it is still a challeng. In most cases, we have to rely on investigation performed under realistic repository conditions without radiation performed in underground labs [33,83,84] or on mathematical modeling approaches [85,86].

There are many studies evaluating the effect of different compaction densities on bacterial survival in the bentonite under repository conditions without radiation. Pedersen et al. [25] tested viability of various introduced aerobic and anaerobic bacteria in compacted bentonite under high-level radioactive waste repository conditions in the period of 15 months. Their results confirmed the detrimental effect of swelling pressure on bacterial survival in the compacted bentonite with the temperature being further limiting factor in bacterial growth and proliferation. The spore-forming microorganisms were the only one, which survived high pressure and high temperature conditions in repository for 15 months but in much lower numbers, than inoculated.

Stroes-Gascoyne et al. [87] studied the response of buffer to resaturation by groundwater over a 6.5-year period by placing 2.4 m^3 of clay-based buffer (18 % moisture content, density not stated, developed total pressure 1.2–2.1 MPa at the end of the test) in a borehole at the 240 m level of AECL's Underground Research Laboratory. Results suggested presence of the viable population of cells in the buffer. The constrictive and nutrient-poor conditions in buffer environment resulted in the loss of microbial activity and culturability but not viability. The interfaces between buffer and surrounding environment may be preferred sites for microbial activity and transport, the microbial populations at interfaces were an order of magnitude larger than inside the bulk buffer. Authors further detected the presence of culturable SO^4-reducing bacteria, although sulphate reduction was not a dominant process, and they detected slight reduction in oxygen level near the surface but not deeper inside the buffer. The overall results suggested that microbial processes were depressed in the buffer but may have been more active near the concrete/buffer interface. No significant evolution towards reducing conditions occurred during the duration of the experiment.

Aoki et al. [27] performed 440 day long experiment resembling repository conditions using bentonite, OT-9607, with mineral composition similar to Kunigel V1. The underground compacted bentonite block included internal heater. The bentonite was heated for 260 days (to 100°C) followed by the cooling phase of 180 days. The sampling of bentonite at various depths and distances from the heater was performed at the end of each phase and authors searched for the presence of microbes. Although, they were able to detect only the aerobic heterotrophs due to methodical obstacles in the work with bentonite samples, they found strong correlation between the presence of living heterotrophs and water content (influenced by heat and desiccation). No living heterotrophs were detected in places with the water content below 12 %. These results confirms the hypothesis that microbial activity might be severely limited near waste container in the vault for some time after disposal, due to desiccation as a result of the heat output of the waste container.

Lydmark and Pedersen [33] performed analysis of microbial diversity in compacted MX-80 bentonite (density over 2,000 kg/m^3) after five years under authentic KBS-3 storage conditions in the rock. The only source of microbial life in bentonite could be indigenous bentonite microorganisms or bacteria naturally present in groundwater. They detected presence of viable and active sulphate-reducing bacteria, acetogenic bacteria and heterotrophic aerobic bacteria in collected bentonite

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samples by means of cultivation experiments, measurements of sulphide and acetate production and ATP measurements. Detected numbers were lower than in underground water surrounding repository. Most of the bacteria were detected in the bentonite buffer close to the rock but in a few samples also in bentonite close to the copper canister. Bacteria from various distances from the rock and the inside of the bentonite were viable and active, but the higher temperatures in some parts of the repository (up to 85°C), obviously made it more difficult for the bacteria to survive.

In the subsequent experiment Arlinger et al. [83] reported low numbers of bacteria in buffer and on canister surfaces, but they detected presence of anaerobic thermophilic bacteria and other thermophilic aerobic bacteria with the potential to form spores in buffer areas with high water saturation and low density and traces of (probably unviable) SRB on canister surface. Authors hypothesized that these bacteria must have been dormant since the formation of the bentonite blocks, but viable when given appropriate growing conditions.

Finally, Stroes-Gascoyne et al. [37]´s short term study and eight years long study of Jalique et al. [84] with highly-compacted Wyoming MX-80 bentonite under repository conditions revealed that a dry density ≥ 1.6 kg/m^3 (resulting in a swelling pressure above 2,000 kPa, a_w < 0.96 and an average pore size <0.02 µm) suppressed microbial culturability (and presumably activity and growth). Viable cell counts were similar to those in the original non-compacted bentonite and most of the detected bacteria belonged among Gram-positive spore-formers.

1.6 Conclusion

In the repository, synergic effect of long time, high pressure, elevated temperature and low-level radiation can be expected. However, the particular magnitudes and thus effects of each of these factors on microbial composition in the repository will change during the evolution of repository conditions after waste disposal. Nowadays, we have reasonably good understanding about the effect of each particular variable separately, however their synergic effect and especially the effect of radiation and the long time frame remains rather unclear. The long-term studies under realistic repository conditions including radiation are thus highly needed, although their conduction remains technologically very challenging.

2 Materials and methods

2.1 Bentonite and VITA water characteristics

Bentonite BaM is a Ca-Mg bentonite exploited in the northern part of the Czech Republic and produced by the Keramost company, Obrnice plant as Bentonite-B. It consists of 78.2 % of montmorillonite and its natural water content is up to 8 % by weight. The elemental composition analysis showed, that BaM bentonite includes 7.59 % of Fe, 2.48 % of K, 2.46 % of Mg and 1.22 % of Ca by weight [88].

Natural groundwater (called VITA) was collected at the Josef Underground Research Centre, Czech Republic a day before performing of each experiment (Figure 2-1, Figure 2-2). VITA water is anaerobic water with high concentration of sulphate. Such natural conditions promotes the growth of sulphate reducing bacteria (SRB) and fermenting bacteria, which form important part of the indigenous microbial community of this water (see below). The anaerobically collected VITA water was poured into sterile bottles and the contact time with the air was minimized during the handling. The VITA water was transported to the laboratory and kept under anaerobic condition inside the glove box until the experimental beginning.

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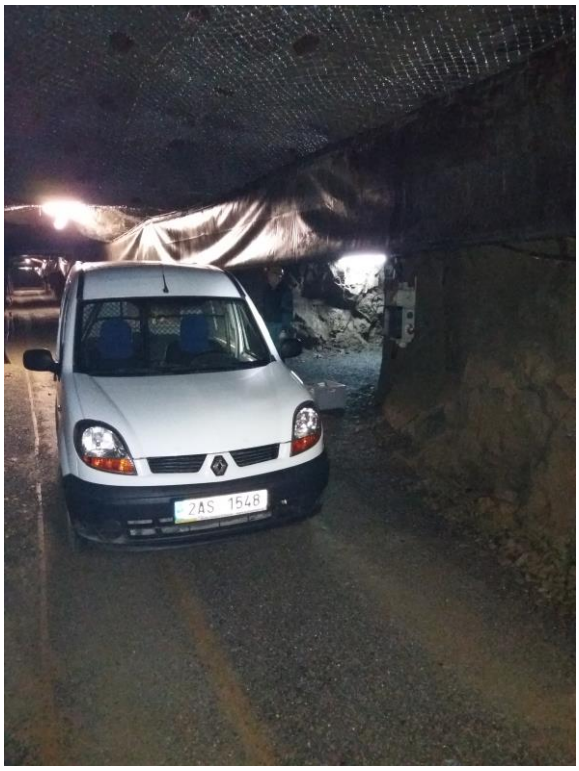

Figure 2-1: Transport of VITA water in the Josef URC tunnel

Figure 2-2: Main tap of VITA water source under 2,5 bar

2.2 Irradiation experiments

2.2.1 Sample preparation and experimental design

All the samples were prepared under anaerobic conditions in a glove box (argon; conc. O_2 < 1ppm) at room temperature (Figure 2-3). BaM powder was kept in the glow box for a week prior the beginning of the experiment to remove all oxygen. VITA water was always collected fresh one day before the

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beginning of the experiment as described above and was kept under anaerobic conditions in the glove box overnight to deoxygenize. Because of destructive power of ionizing radiation to most organic materials or rubber, we used all-glass reagent bottles tightly sealed with several layers of parafilm membrane for samples in the irradiation chamber. Control samples were kept in common glass bottles with a plastic cap.

Constant dose rate of 13 Gy/h was used in all our experiments, which enabled us to obtain biologically relevant results comparable to the condition expected within repository in reasonably long experimental times.

Figure 2-3: Preparation of samples for irradiation experiments in anaerobic box.

Preliminary experiments

Several short preliminary irradiation experiments were conducted prior to the main irradiation experiment to test the experimental conditions, the following two will be described in more detail.

In the first experiment, 500 ml of the mixture of VITA water and sterilized tap water (1:1) was irradiated for 30 days by the dose rate 13 Gy/h to describe the reaction of natural VITA water microflora. The total absorbed dose for this experiment reached 9360 Gy. Samples were taken after 14 and 30 days for qPCR and cultivation analysis as described below.

In the second experiment, we irradiated 150 g of BaM bentonite in VITA water suspension (500 ml) for 13 days reaching the total absorbed dose of 4056 Gy. The experiment was sampled at day 1, 2, 3, 9 and 13 for qPCR.

Because the sequencing data from long-term irradiation experiment revealed problems with maintaining anaerobic conditions in irradiated samples (see below), we subsequently also sequenced samples from preliminary experiments to search for the signal of presence of oxygen and to analyze the microbial community changes due to irradiation.

Long-term experiment

Two sets of samples (A and B) were prepared, each one consisting of 30 samples (60 samples in total). Each sample in Set A contained 100 ml of VITA water, 100 ml of filter-sterilized tap water and 60 g of BaM bentonite powder. Samples in set B contained 100 ml of VITA water, 100 ml of filter-sterilized tap water, 60 g of BaM powder and 10 mM acetate, 2.46 mM sulphate and 0.29 mM nitrate to test the reaction of bacteria to radiation in the environment with higher nutrient availability.

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The concentration of acetate was approximated from [48], the concentrations of sulphate and nitrate was calculated to five times exceed the measured natural concentration of these nutrients in BaM and VITA suspension (5 mg/l nitrate and 70 mg/l sulphate). VITA water had to be diluted using sterile filtered and degassed tap water due to limited amount of VITA water in the Josef URL source collector and a high number of samples.

We put 16 samples of Set A and 16 samples of set B as controls (named cA and cB) into anaerobic box. The remaining 28 samples from set A and B (named A and B) were placed in the irradiation chamber and were irradiated up to 9 weeks by the dose rates of 13 Gy/h resulting in the maximum total absorbed dose of 19,656 Gy (Figure 2-4). The experiment was sampled each week based on the schedule in Table 2-1.

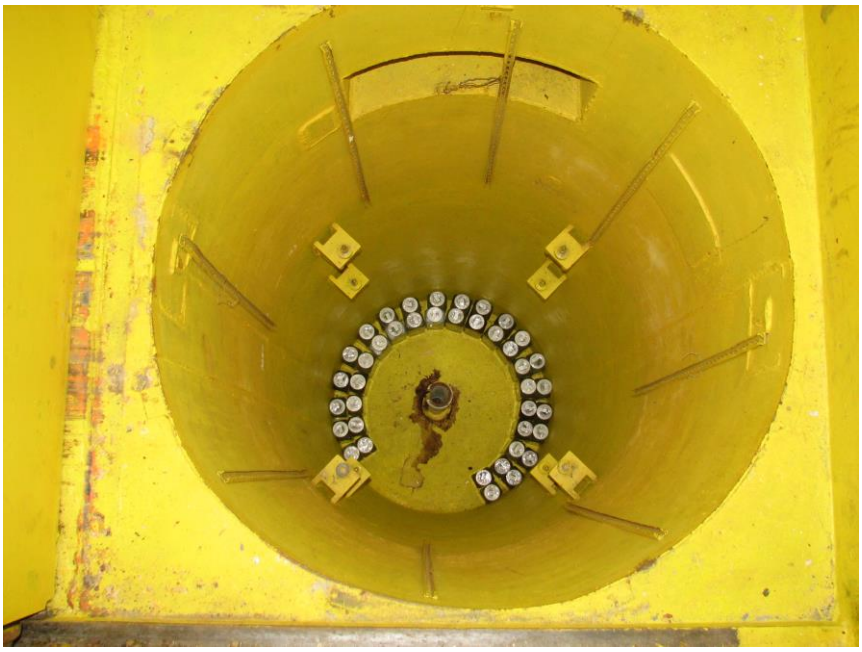

Figure 2-4 Samples inside the irradiation chamber, diameter ca 100 cm

Table 2-1: Sampling schedule of irradiated samples and controls and cumulative doses of Gama radiation

		Irradiated samples		**Controls**	
	Cumulative dose (Gy)	**A**	**B**	**cA**	**cB**
09.10.2018	0	0	0	2	2
16.10.2018	2,184	2	2	2	2
23.10.2018	4,368	2	2	2	2
30.10.2018	6,552	2	2	2	2
06.11.2018	8,736	2	2	2	2
13.11.2018	10,920	2	2	2	2
27.11.2018	15,288	2	2	2	2
11.12.2018	19,656	2	2	2	2
Number of samples		**14**	**14**	**16**	**16**

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Unexpectedly, the microbial community composition within irradiated samples indicated the presence of oxygen, which means that we failed to keep anaerobic condition during irradiation. The subsequent sequencing of the samples from the two preliminary experiments revealed similar failure (they were originally analyzed only by means of qPCR). Because the non-irradiated controls were kept under anaerobic conditions in the glove box and were thus not comparable with the irradiated samples and repeating of the irradiation was not possible due to time and economic reasons, we decided to prepare a new set of 16 non-irradiated control samples without nutrients similar to controls cA, but aerobic (named new cA). These additional control samples were prepared similarly to original irradiated samples in glass reagent bottles sealed with parafilm membrane in glove box, but further were kept in refrigerator at 12°C and in the presence of air, which best simulated the conditions in irradiation chamber (except for radiation). The microbial community composition of VITA water and BAM is generally uniform, which enabled comparison of such ad hoc control with the original irradiated samples. The sampling followed similar time schedule as in the original control samples (see Table 2-2). Due to the time reasons, only four sampling points were analyzed for the purposes of this report, but the experiment continues and will be completed in February.

Table 2-2: Schedule of additional control (new cA) sampling. DL - Last sampling for the purposes of this report.

	Control to cumulative dose (Gy)	New cA controls
14.12.2018	0	2
20.12.2018	2,184	2
28.12.2018	4,368	2
5.1.2019	**6,552**	**2 (DL)**
11.1.2019	8,736	2
18.1.2019	10,920	2
1.2.2019	15,288	2
15.2.2019	19,656	2
Number of samples		**16**

2.2.2 Microbial analysis

Sample processing

Defined amounts (500-1000 ml) of VITA water samples were filtered through sterile 0.22 µm GV Durapore® filter membrane and the filters were stored in a deep freezer and subsequently used for DNA extraction as described below.

Bentonite suspension samples were homogenized properly and 1 ml of the sample was used to determine number of cultivable bacteria. Subsequently, 50 ml of each sample was centrifuged at 11500 × g for 15 minutes. The supernatant (40 ml) was used for chemical analyses (described below), the remaining pellet was weighed and used for DNA extraction (described below). The supernatant and pellet were stored in deep freezer (-80°C) until further processing.

Cultivable bacteria

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One ml of bentonite suspension was diluted in 9 ml of physiological saline (dilution 1×10^{-1}). The procedure was repeated five more times (up to dilution 1×10^{-6}). We performed both aerobic and anaerobic cultivations on agar plates using PCA medium. We prepared samples in dilutions from 1×10^{0} to 1×10^{-6} for aerobic cultivation (seven plates per sample) and from 1×10^{0} to 1×10^{-4} for anaerobic cultivation (five plates per sample). Samples for the anaerobic cultivations were sealed in the anaerobic jars (Figure 2-5) with oxygen binding medium and oxygen indicator inside. We cultivated the plates under aerobic conditions for 4 days and plates in anaerobic conditions for 6 days. We expressed the resulting number of cultivable bacteria as colony forming units (CFU) per 1 ml of bentonite suspension.

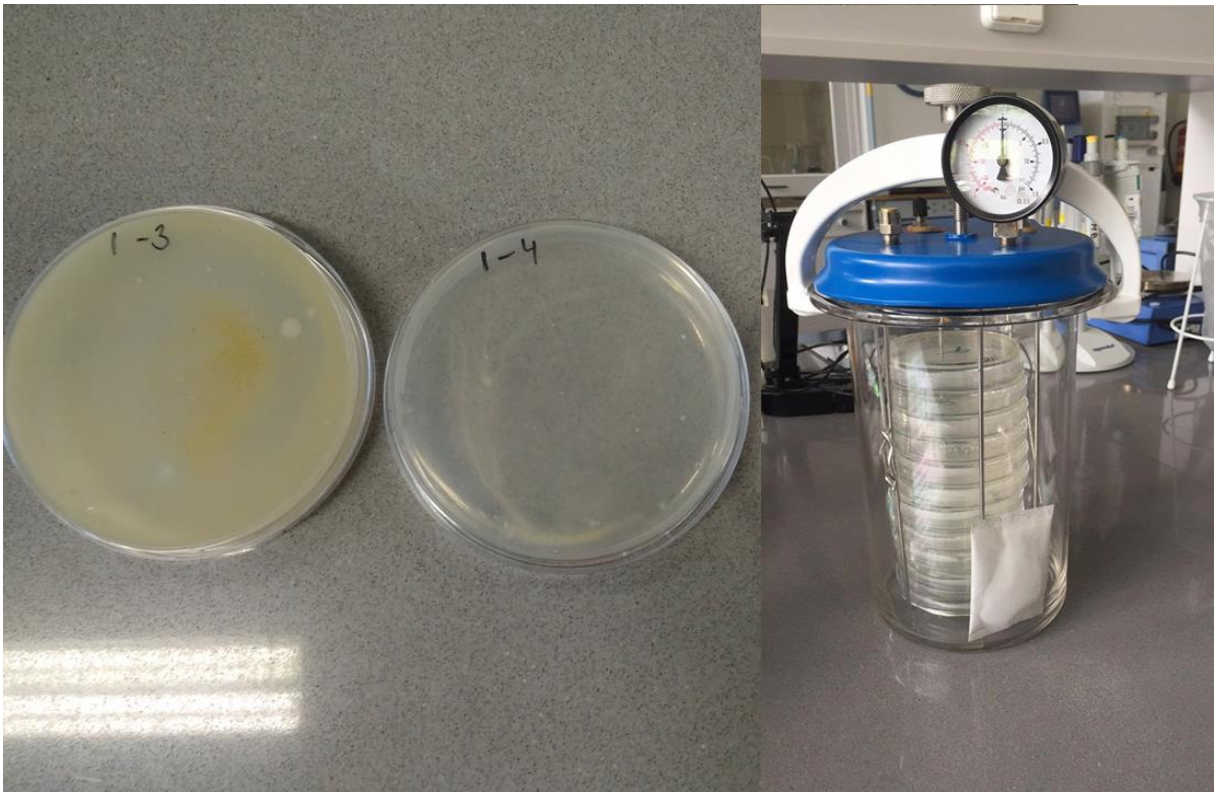

Figure 2-5: Examples of CFU on agar plates cultivated aerobically (1×10^{-3} and 1×10^{-4} dilution) on the left, and the agar plates in the anaerobic jar with oxygen indicator on the right.

DNA Extraction

The DNA from VITA water biomass concentrated at 0.22 µm GV Durapore® filter membranes (see above) was extracted by means of PowerWater® DNA Isolation Kit from Mo Bio (QIAGEN) according to manufacturer's protocol.

We used DNeasy® PowerMax® Soil Kit from QIAGEN according to manufacturer's protocol for extraction of DNA from bentonite pellets. Concentration of isolated DNA was subsequently measured by Qubit® 2.0 Fluorometer (Invitrogen, Life Technologies, USA) according to the manufacturer's protocol.

Quantitative PCR (qPCR)

The method of qPCR was used to describe relative changes in bacterial abundance in the bentonite samples. Because obtaining standards fully reliable to our environmental samples is very difficult and absolute quantification without standardized calibration curve in not possible, we used relative quantification (RQ) ΔΔCq calculation method. It estimates the magnitude of difference in Cq values between the sample zero state at the beginning of experiment and the sample after treatment (or in

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time in no treatment control (NTC) samples) using the formula RQ=effectivity $^{(-\Delta Cq)}$. PCR effectivity for each marker was estimated beforehand by measuring the slope of curves constructed from a serial dilution of template DNA from five internal environmental standards. The measured Cq values were normalized by the sample mass used for DNA extraction prior calculations.

We used markers specific for various bacterial groups (such as total Eubacteria, sulphate-reducing bacteria, nitrate-reducing bacteria or Geobacteraceae). These markers were amplified using primers described in Table 2-3 on a LightCycler ® 480 Instrument (Roche Biochemicals, USA). Reaction mixtures were prepared in 10 µl of reaction volume. The mixture contained 2 µl of DNA template, 5 µl KAPA SYBER FASTqPCR kit (Kapa Biosystems. Inc., MA, USA), 0.4 µl of µM forward and reverse primer mixtures (Generi Biotech, Czech Republic, IDT, US) and 2.6 µl ultra-pure water (Bioline, UK). For each DNA sample, qPCR reaction was performed in duplicate along with negative control where DNA template was replaced by nuclease free water. Reaction conditions consisted of an initial 5 min incubation at 95°C, followed by 45 cycles of denaturation at 95°C for 10 s, annealing at 60°C for all primer except for Geobacteraceae which was 55°C for 15 s and extension 72 °C for 20 s with final extension at 72°C for 3 min. Finally, a melting curve was set for 5 s at 95°C, 1 min at 65°C and final ranging from 60 to 98°C, with a temperature gradient of 40°C per 10 s. Purity of the amplified fragment was determined through observation of a single melting peak. Crossing point values were obtained using the 'second derivative maximum' method included in the LightCycler® 480 Software.

Table 2-3: qPCR primers. SRB – sulphur reducing bacteria, NRB – nitrate reducing bacteria, IRB – iron reducing bacteria.

Primer	Sequence 5´- 3´	Specifity	Description	Reference	Annealing temp.
16SqPCR-F	TCCTACGGGAGGCAGCAGT	All bacteria	Gen for 16S rRNA	[89]	60°C
16SqPCR-R	GGACTACCAGGGTATCTAATCCTGTT				
RH1-dsr-F	GCCGTTACTGTGACCAGCC	SRB	Functional bio-marker gene dsrA	[90]	60°C
RH3-dsr-R	gGTGGAGCCGTGCATGTT				
RH1-aps-F	CGCGAAGACCTKATCTTCGAC	SRB	Functional bio-marker gene apsA	[90]	60°C
RH2-aps-R	ATCATGATCTGCCAgCGgCCGGA				
nirK 1F	GGMATGGTKCCSTGGCA	NRB	NirK gene for Nitrite reductase	[91]	60°C
nirK 5R	GCCTCGATCAGRTTRTGGTT				
nirS cd3AF	GTSAACGTSAAGGARACSGG	NRB	NirS gene for Nitrite reductase	[91]	60°C
nirS R3cd	GASTTCGGRTGSGTCTTGA				
nosZ-F	CGYTGTTCMTCGACAGCCAG	NRB	NosZ gene for N_2O reductase	[91]	60°C
nosZ 1622R	CGSACCTTSTTGCCSTYGCG				
Geo494F	AGGAAGCACCGGCTAACTCC	Geobacteraceae (IRB)	Amplifying specific region of 16S rRNA	[92]	55°C
Geo825R	TACCCGCRACACCTAGT				

Library preparation and next generation sequencing

The next-generation sequencing (NGS) was used to study microbial diversity in bentonite samples. Primers 530F and 802R (Table 2-4) were used for amplification of variable V4 region of 16S rDNA gene

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for sequencing of amplicons. The size of the amplicon was kept below 400 bp to cover as much microbial diversity as possible by performing In silico analysis of primers [93].

Two consecutive PCR reactions per sample were performed during library preparation. We used primers 530F and 802R in the first PCR reaction and the PCR conditions were as follows: 95°C for 3 min; 15 cycles at 98°C for 20 s, 50°C for 15 s and 72°C for 45 s; and a final extension at 72°C for 1 min. Subsequently we preformed second PCR reaction with tagged barcode fusion primers. We used 21 differently tagged bar code fusion primers in one library preparation, which enabled us to sequence up to 20 samples (plus mock up) in one run. The second PCR was performed as follows: 95°C for 3 min; 35 cycles at 98°C for 20 s, 50 °C for 15 s and 72 °C for 45 s; with a final extension at 72°C for 1 min. The quality of the library product was checked by gel-electrophoresis technology. The PCR products were purified using the Agencourt Ampure XP system (Beckman Coulter, Brea, USA), and the concentration of the purified PCR products was measured with a Qubit 2.0 fluorometer (Life Technologies, USA). Following this step, the barcode-tagged amplicons from different samples were mixed in equimolar concentrations.

Sequencing of the amplicons was performed on an Ion Torrent PGM (Thermo Fisher Scientific, USA) using the Ion PGM Hi-Q Sequencing Kit with the Ion 314 Chip following the manufacturer's instructions (Thermo Fisher Scientific).

Table 2-4: Primers for amplicon sequencing of 16S rRNA gene.

Primer	Sequence 5´- 3´	Coverage			Reference
		Archaea	Bacteria	Eukaryotes	
530F	GTGCCAGCMGCNGCGG	54,9	96,9	94,0	[94]
802R	TACNVGGGTATCTAATCC	91,8	92,5	0,9	[95]

NGS data processing

Raw reads were split to particular samples by Mothur software [96]. The split samples were subsequently processed by the DADA2 software package [97]. Low quality and short reads were removed as well as chimeric sequences. Taxonomy classification by the DADA2 package used SILVA database (version 13, www.arb-silva.de). The accuracy of classification was verified and evaluated against a predefined artificial MOCK community sample with 4 different taxa: *Enterococcus*, *Bacillus*, *Klebsiella* and *Staphylococcus*. Dada2 output was transformed to a Phyloseq object in R and subsequent bioinformatics analyses were performed in the R software using the Phyloseq library [98]. Rarefaction curves were created using the Phyloseq and vegan packages in the R software. The relative frequency of OTUs was visualized by a heat-map showing only OTUs with the mean of relative frequency higher than 0.03 %. Further, MDS/PCoA cluster analysis was performed using the Phyloseq library using Bray-Curtis dissimilarly.

In subsequent metabolic profile analysis, we selected 50 most abundant OTUs for each sample and from these most frequent OTUs only OTUs successfully determined to particular genera were used for metabolic characterization the microbial community within the samples. On average 84 % of OTUs were used in final metabolic analysis. For each OTU successfully determined to particular known genus, we searched through the literature and listed the information about Gram stain, spore-forming, trophy, used electron acceptors, donors and oxygen requirement in this genus and we used these categories to characterize the bacterial community and compare it with prevailing physico-chemical condition within the samples.

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Chemical analyses

Supernatant of each sample was analyzed by ion spectroscopy to detect the concentrations of nitrate, sulphate and acetate in the samples. The anion concentration (sulphate, nitrate, acetate) was determined using a Dionex ICS 90 chromatograph (ThermoFisher Scientific, USA) with 8 mM K_2CO_3 a 1 mM $KHCO_3$ as the mobile phase in a Dionex IonPac AS14A column. Flow rate of mobile phase was 1ml/min and 10 µl of the sample was always injected.

2.3 Pressure experiments

2.3.1 Sample preparation and experimental design

These experiments were conducted to evaluate the effect of increased pressure on survival of bacteria in system containing VITA water and BaM bentonite simulating effect of swelling pressure in compacted bentonite.

Pressure experiments were conducted by means of two steel high pressure laboratory autoclaves AMAR EQUIPMENTS PVT. LTD. (India) with the inner volume of 750 ml up to 100 bars of pressure resistance (Figure 2-6 Stainless steel high pressure laboratory autoclaves (750 ml) for Figure 2-6). These autoclaves were pressurized by argon and enabled testing of two different pressure values simultaneously during each pressure experiment. No pressure control (NC) was carried out outside. We performed four different pressure experiments (Table 2-5). At the beginning, three-week experiments under laboratory temperature were carried out to test the functionality and behavior of the complete system. Later on, we continued by final 30-day experiment performed under decreased temperature (12°C) optimal for VITA water microorganisms and also better simulating the deep underground repository conditions. The particular values of the pressure for all these experiments were approximated from [32].

For each of these experiments, suspension of 150 g of BaM bentonite and 500 ml of VITA water was prepared anaerobically in the glove box similarly as described in 2.2.1 and sample of mixed suspension as well as the original VITA water and BaM were taken as the zero state samples. Prepared suspension samples were poured inside the autoclaves or kept in glass reagent bottles as negative controls Figure 2-6 Stainless steel high pressure laboratory autoclaves (750 ml) for as indicated in Table 2-5. Autoclaves were assembled and dismantled in the anaerobic glove box, pressurizing and the whole experiment was due to technical reasons performed outside the box. Negative controls for each experiment (NC) were kept without pressure under anaerobic conditions in glove box (Experiment 1-3) or in anaerobically prepared and tightly sealed bottles in refrigerator under 12°C (Experiment 4). The oxygen content in the inert atmosphere (Ar) was below 1 ppm. Autoclaves were kept under required pressure continuously during the whole experiment. Experiment 4 was carried out in the fridge at 12°C. Samples were processed immediately after finishing the experiments as described in 2.2.2 and stored in deep freezer (-80°C) until analysis.

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Figure 2-6 Stainless steel high pressure laboratory autoclaves (750 ml) for pressure experiments

Table 2-5: Overview of pressure experiments

Experiment	Pressure	Temperature	Duration	NC
1	1 and 2 MPa	Tlab	7 days	yes
2	2 and 5 MPa	Tlab	7 days	yes
3	3 and 5 MPa	Tlab	7 days	yes
4	2 and 5 MPa	12°C	30 days	yes

2.3.2 Sample analyses

The one-week preliminary experiments (Experiment 1-3) were evaluated only by means of qPCR to easily check the changes in bacterial abundance under various pressures. The samples from Experiment 4 were evaluated by means of molecular methods (qPCR and NGS) and cultivations. All these methods were described in 2.2.2.

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3 Results and discussion

3.1 Effect of Irradiation

3.1.1 Preliminary short experiments

Effect on microbial community in VITA water

The VITA water irradiated for 30 days up to 9360 Gy total absorbed dose (Figure 3-1) showed clear decrease in total microbial biomass (16S rRNA) during the first 14 days, it started to recover and slightly increased in the second half of the experiment, but it did not reach the initial abundance. Similar trend was visible in all other monitored markers except for Geobacteraceae, which was more or less similar to initial values. SRB-dsrA marker was not amplified in any of the tested samples.

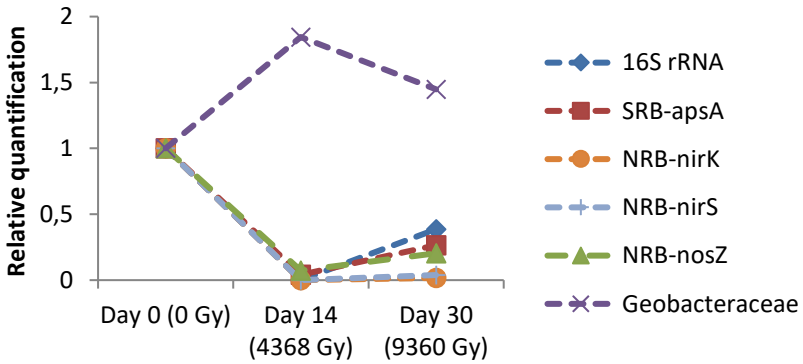

Figure 3-1: Relative quantification of 1:1 VITA: tap water irradiated by 9360 Gy total absorbed dose in 30 days

The analysis of sequencing data showed that only non-sporulating Gram-negative microorganisms were present in all studied samples of VITA water. The VITA water was dominated by heterotrophic facultative anaerobic genera *Polaromonas* and *Pseudomonas* at the beginning of experiment, but the community composition markedly changed during the course of experiment. The sample irradiated by 4368 Gy absorbed dose was dominated by auto/heterotrophic genus *Acidovorax* utilizing organic compounds as well as molecular hydrogen as electron donor, while the final sample irradiated by 9360 Gy was dominated by genus *Paracoccus* (Figure 3-2), which can use a wide scale of electron donors such as ammonia or reduced sulphur compounds.

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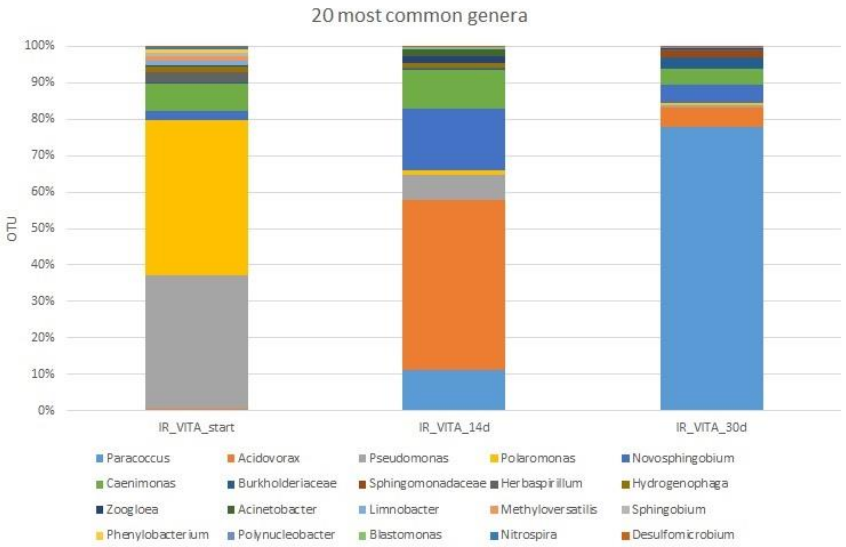

Figure 3-2: Changes in genera composition within samples of VITA water irradiated for 14 and 30 days compared to the original state. 20 most common genera including 5 most frequent in each sample are presented.

Analysis of the metabolic profiles helped us to better understand the causes of detected changes in the microbial composition and abundance and to infer the prevailing conditions inside the sample ecosystem. The oxygen requirement of the detected microorganisms and their preferred electron acceptors revealed, that the samples were probably influenced by oxygen as the ecosystem was dominated by facultative anaerobes respiring oxygen or nitrate in its absence and only very few obligate anaerobes were detected (Figure 3-3).

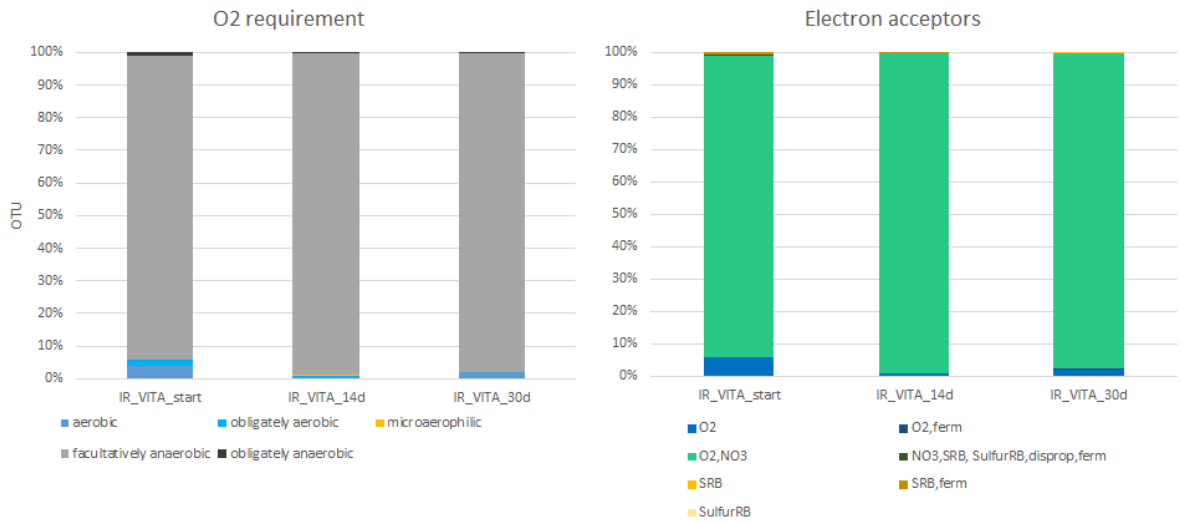

Figure 3-3: Composition of microbial ecosystems in irradiated samples of VITA water categorized by O_2 requirement or preferred electron acceptors of detected microorganisms.

Further, the VITA water was dominated by the microorganisms utilizing organic material as electron donors at the beginning of the experiment, but we detected a shift towards using molecular hydrogen and even reduced sulfur compounds as preferred electron donors during the course of experiment (Figure 3-4). This result indicates the deficiency of available organic material (and later of molecular hydrogen as well) in the system leading to the proliferation of microorganisms oxidizing

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sulfur compounds. VITA water is very poor in organic material, but rich in sulphate so the resulting proliferation of SRB could cause an increase in the availability of reduced sulfur compounds for other microorganisms. In accordance with this finding, we detected a gradual shift in the community structure from the dominance of heterotrophs at the beginning of experiment towards their decrease and a replacement by autotrophic genera at the end (Figure 3-4).

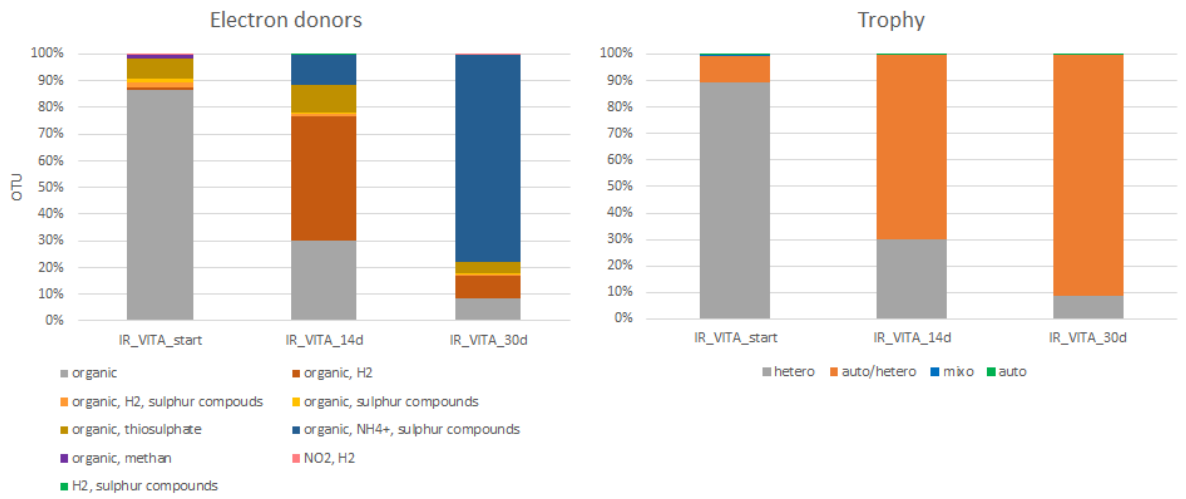

Figure 3-4: Composition of microbial ecosystems in irradiated samples of VITA water categorized by preferred electron donors and trophism of detected microorganisms.

All the detected changes in the microbial community composition and resulting shifts in preferred electron acceptors and donors as well as the qPCR results indicate that the applied total absorbed dose of 9360 Gy did not eradicate natural microflora of the VITA water, although such dose is referred to be lethal for most microorganisms except very radiation-tolerant ones such as *Deinococcus radiodurans* [15]. The natural VITA water microflora thus seems to be capable of adaptation to relatively low levels or ionizing radiation (applied irradiation dose 13 Gy/h), although there were no Gram-positive or sporulating microorganisms, which are generally more radiation resistant compared to Gram-negative and non-sporulating microorganisms (Bruhn et al., 1999; Fredrickson et al., 2004; van GERWEN et al., 1999). The overall decline in the microbial abundance after 14 days detected by qPCR was most probably caused by the synergic effect of both Gama radiation and the reduction of availability of the organic material, which is a preferential electron donor for most microorganisms. However, non-irradiated aerobic control sample that would enable us to distinguish between effects of both factors was not included in this preliminary experiment. The subsequent increase in the microbial abundance might be caused by the distinct microbial groups that were able to survive the radiation and metabolize other available nutrients than organic material.

Effect of irradiation on microbial community in bentonite - VITA water suspension

Total absorbed dose of up to 4056 Gy slowed the growth of bacterial community compared to non-irradiated control samples, but did not lead to decrease of total bacterial biomass. The detected quantity of the total bacterial biomass (16S rRNA gene) in irradiated samples was more than four times lower compared to the untreated control samples (Figure 3-5). We also detected an increase in quantity of all studied metabolic microbial groups in both irradiated samples and controls where the increase was generally higher or at least faster in controls (Figure 3-5). DsrA gene failed to be amplified in most of the samples (data not shown).

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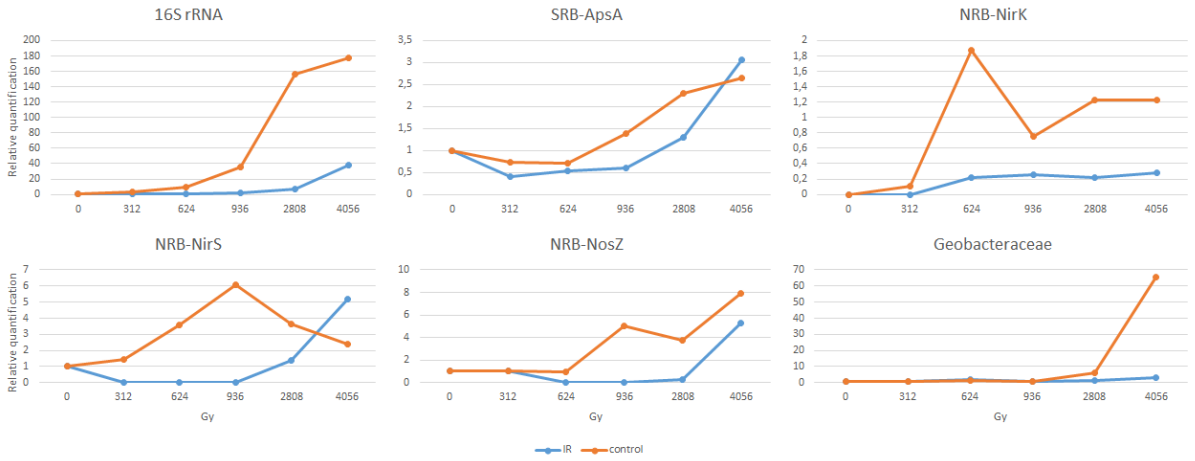

Figure 3-5: Relative quantification results from irradiation experiment reaching 4096 Gy cumulative absorbed dose.

First, we visualized NGS data using rarefaction analysis to see the overall diversity in the samples. This analysis showed that the species richness was dramatically reduced in the bentonite suspensions during the experiment compared to the original state and this reduction was similar for both irradiated samples as well as for control samples. It implies ongoing harsh selection (caused by the effect of irradiation) of microorganisms, which were present in the bentonite environment at the beginning of experiment.

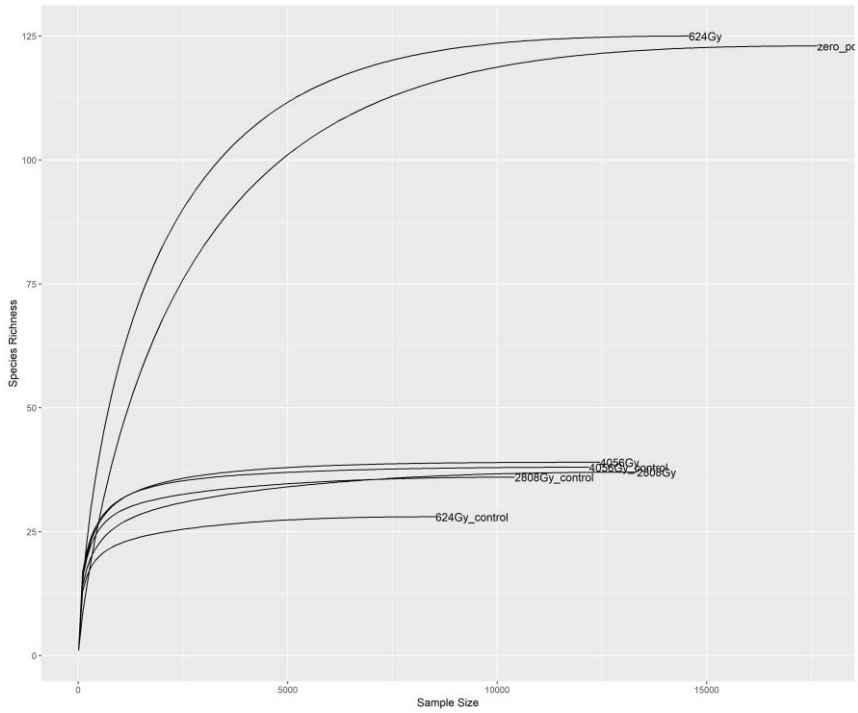

Figure 3-6: Rarefaction analysis of the preliminary irradiation experiment.

The analysis of sequencing data revealed vast changes in microbial community composition both within the irradiated samples with increasing absorbed dose and also between irradiated and control samples (Figure 3-7).

The zero point sample was dominated by genus *Aquabacterium* similarly to the 624 Gy irradiated sample. The aerobic irradiated samples evolved towards the dominance of facultative

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anaerobic heterotrophs *Pseudomonas* and *Paenibacillus*, while the anaerobic control samples evolved towards dominance of microaerophilic genus *Azoarcus,* facultatively anaerobic *Pseudomonas* and obligately anaerobic iron reducing *Geobacter*.

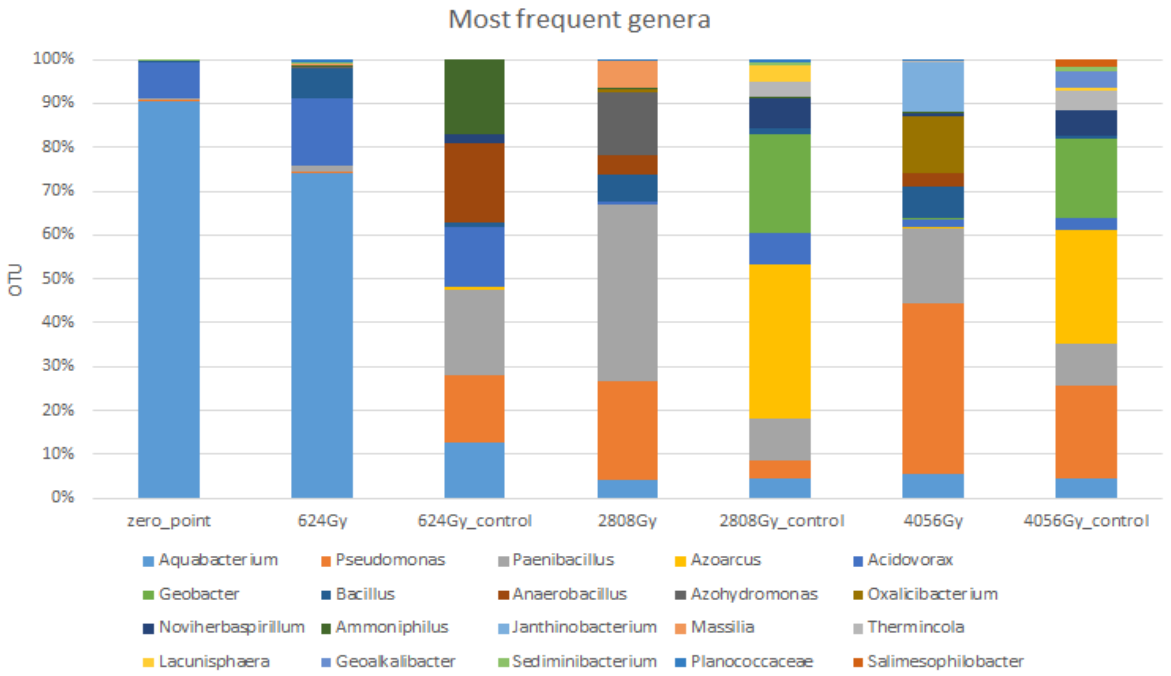

Figure 3-7: Composition of most frequent genera in preliminary irradiation experiment samples.

The zero point bentonite suspension was dominated by Gram-negative non-sporulating microorganisms, but the proportion of Gram-positive or Gram-variable sporulating microorganisms rapidly increased up to more than 50 % in the 624 Gy control or 2808 Gy irradiated sample. The proportion of Gram-positive or Gram-variable sporulating microorganisms than decreased to about 15-25 % at the end of experiment. The sequencing data suggest that the detected peak of Gram-positive sporulating microorganisms occurred earlier in control samples (624 Gy) than in irradiated samples (2808 Gy).

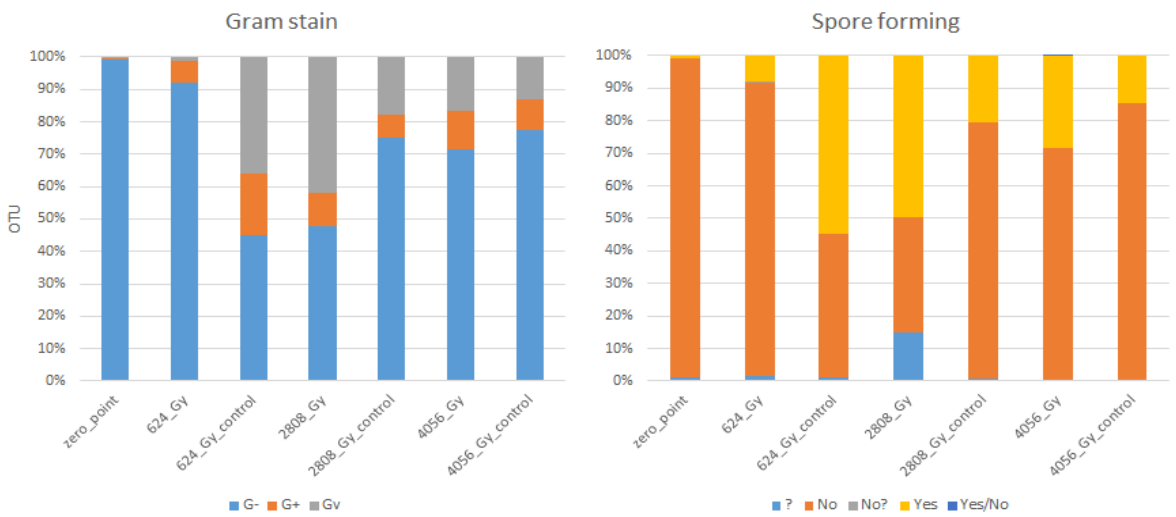

Figure 3-8: Composition of the microbial ecosystems in irradiated samples and controls of bentonite-VITA suspension categorized by Gram staining and spore forming ability of detected microorganisms.

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The analysis of the oxygen requirement and preferred electron acceptors of detected microorganisms within the samples suggested the presence of oxygen within the irradiated samples similarly to the VITA water irradiation experiment above and long-term experiment described below. We assume that the presence of oxygen in irradiated samples and its absence in strictly anaerobic controls probably caused the main differences between the control and irradiated samples. The irradiated samples were dominated by facultative anaerobes (or microaerophilic/facultatively anaerobic microorganisms at the beginning), which respire nitrate or ferment in the absence of oxygen (Figure 3-9). Although facultative anaerobes were also dominant in the control samples, there was also increasing proportion of obligatory anaerobic microorganisms that respire ferric iron (iron reducing bacteria - IRB) or ferment (Figure 3-9). The IRB started to proliferate in about the middle of the experiment (2808 Gy control), which might indicate that the available oxygen and nitrate was respired and ferric ion started to be reduced as alternative electron acceptor leading to increase in Geobacteraceae in control samples Figure 3-5). Ferric ions are common in BaM bentonite [88] similarly to other bentonites [10]. Their good availability was most probably the reason, why we detected only very little proportion of SRB in the sequencing data of control samples, although SRB are very common in VITA water and the qPCR suggested their slight increase. We expected that SRB would start to proliferate if the concentration of ferric ion will be significantly reduced either by direct metabolic reduction by bacteria [99] or by indirect reduction through microbially produced sulfide [4]. This presumption was further addressed in long-term irradiation experiment.

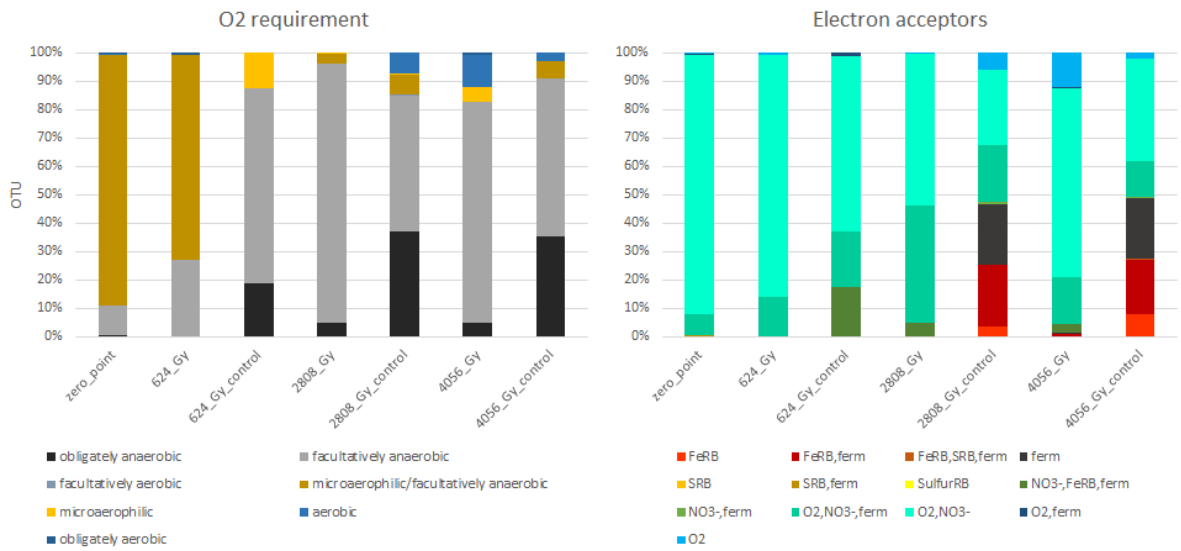

Figure 3-9: Composition of microbial ecosystems in irradiated samples and controls of bentonite-VITA suspension categorized by oxygen requirement and preferred electron acceptors of detected microorganisms.

Both irradiated and control samples were dominated by heterotrophs metabolizing organic substrates and their proportion was rather similar in all the irradiated samples. However, we observed an increase in the proportion of the genera that contain both auto- and heterotrophs or that are mixotrophs in the control samples in the second half of experiment (2808 and 4056 Gy control) (Figure 3-10). In these samples the molecular hydrogen of thiosulphate became preferred electron donor. This result indicates the limitation in availability of organic substrates that can be used as electron donors in the control ecosystem in the later phases of experiment similarly to the situation observed in irradiated VITA water. The lack of this effect in irradiated samples can be explained by the lower microbial population density (Figure 3-5) that has probably not managed to oxidize all the available organic electron donors.

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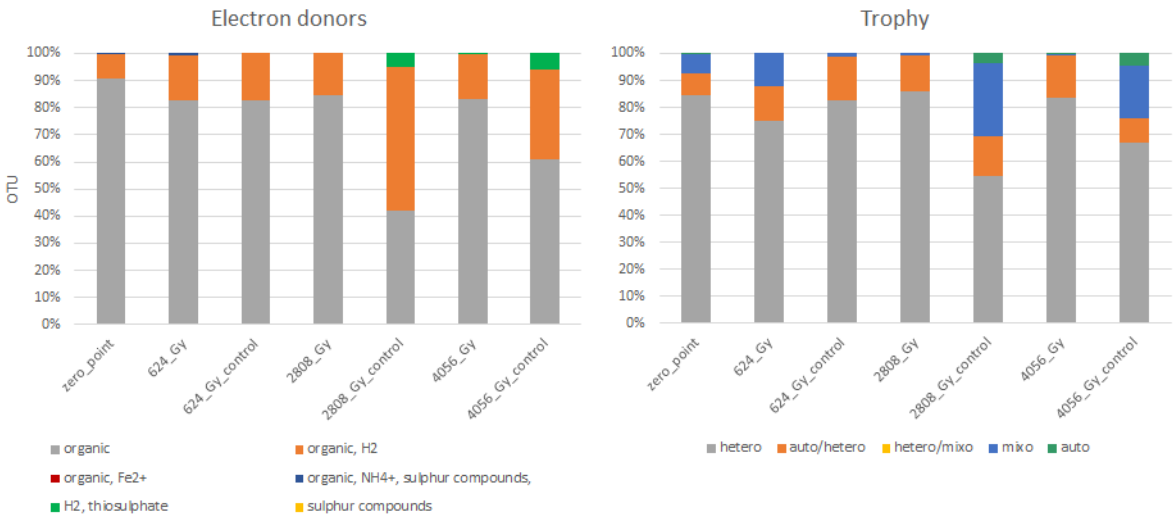

Figure 3-10: Composition of microbial ecosystems in irradiated samples and controls of bentonite-VITA suspension categorized by preferred electron donors and trophy of detected microorganisms.

We can conclude that exposure to ionizing radiation with the total absorbed dose 4056 Gy was not sufficient to eradicate present microorganisms, although decrease in growth was detected. Availability of accessible electron acceptors and donors together with exposure to radiation determined the final microbial community composition.

3.1.2 Long-term experiment

All the performed analyses revealed treatment-dependent pattern, similarly to the results in preliminary irradiation experiments. As described in methods, contrary to expectations, we failed to maintain fully anaerobic condition within irradiated samples. As a result, the irradiated samples A and B were probably influenced by the presence of oxygen, while the non-irradiated controls cA and cB kept in the glove box stayed anaerobic. For this reason we prepared an additional control named “new cA”, which was purposely aerobic and thus better comparable with irradiated samples.

Effect on specific microbial markers (qPCR)

In the anaerobic control set cA (without added nutrients) we detected a rapid decline in the microbial abundance during the first week followed by a small increase in the second week, and from this point the bacterial abundance was gradually decreasing for the rest of the experiment. The metabolic group specific markers further revealed, that the abundance of nitrate reducers (NRB) was highly variable during the experiment with the highest peak around fifth week. The iron reducers (Geobacteraceae) proliferated gradually since the beginning and had highest abundance in fourth week. Subsequently, we detected the increase in SRB in the second half of the experiment and their slight reduction at the last sampling point.

In the anaerobic control set cB (with nutrients) we detected the highest total microbial biomass across all the treatments and the abundance was gradually increasing during the whole experiment. We have not detected any decline in total biomass towards the end as we did in controls cA. The specific markers showed proliferation (also with very variable values) of NRB in the middle of the experiment with highest peak in the third and fourth week. Iron reducers started to proliferate in the second week and their highest density was detected in the seventh week. We detected a slight decrease in IRB at the last sampling point. On the other hand, we detected an almost gradual increase

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in SRB since the beginning of the experiment with the highest values reached at the end of the experiment.

The total biomass in the aerobic irradiated samples (both A and B) was clearly lower than in both controls cA and cB during the whole experiment. The microbial community in irradiated samples was dramatically reduced within the first week in both irradiated samples A and B, but then we detected a slight increase in the abundance in both sets up to the similar values as in the initial state. The microbial growth reached the peak after applying 8736 Gy cumulative dose (fourth week) followed by gradual decrease towards the end. The total biomass in samples A was slightly higher than in B. Similarly to control samples cA and cB, we detected increase in the abundance of NRB in the middle of the experiment with the highest peaks at second and fourth week (4368-8736 Gy cumulative dose), but their abundance was then dramatically reduced. Contrary to control samples, we did not detect an increase in IRB and we recorded only a very slight increase in SRB at the end in both irradiated samples A and B. These results are not very surprising, as the IRB and SRB are obligatory anaerobes or at least microaerophiles [100]. The absence of these microbial metabolic groups in the irradiated samples can be thus ascribed to the likely prevailing aerobic condition in the samples rather that to the effect of irradiation. Even though analyzed data only from first four sampling points, the results of qPCR in aerobic new cA controls, which are generally very similar to the results of irradiated samples (Figure 3-11), confirm this hypothesis.

Although the quantity of the total biomass in irradiated samples at the end of the experiment (19,656 Gy cumulative absorbed dose) was clearly reduced compared to the state at the beginning of the experiment, the applied dose was obviously not sufficient to completely eradicate all bacteria in the bentonite-VITA suspension. Although the values of RQ decreased to zero (especially in A) (Figure 3-11), the Cq values of samples at the last sampling point remained high (about 25 cycles) implying that remaining proportion of bacteria was in fact high and the detected decline was only relative compared to the original state. Moreover, we will be able to better distinguish between the effect of time itself (the total population decline was detected also in non-irradiated control cA during the course of experiment) and irradiation after we analyze new cA control samples after nine weeks.

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Figure 3-11: Relative quantification of changes in microbial abundance in irradiated and control samples. A – irradiated samples, no nutrients added, B – irradiated samples with nutrients, cA – non-irradiated control to A, cB – non irradiated control to B, new cA – non-irradiated aerobic control to A. w – week.

Species richness

We visualized the NGS data by means of heat-map and rarefaction analysis to see the overall diversity and OUT frequency in the samples. In the rarefaction analysis we can see clear difference between the original samples of VITA water and BaM bentonite, where the diversity is much higher than in the samples of bentonite suspension (Figure 3-12), although the total number of detected OTUs in the samples is rather low (Figure 3-13). Furthermore, the species richness was unexpectedly low in the zero-point suspensions cA0 and cB0 compared to new cA control with a very similar composition of source VITA and BaM and it also clearly differed from the samples of other zero suspensions used in different experiments (Figure 3-6, Figure 3-27). Zero point sample cB0 with the smallest species richness was for this reason completely omitted from following metabolic analysis and we used only

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cA0 as zero point for the irradiation experiment (zero_point_susp). We also included the NGS data of VITA and BaM used for zero suspension preparation in subsequent analysis of microbial metabolic profiles. The low species richness in these samples could be caused either by problems during the isolation of DNA, which is improbable as DNA yields per gram of sample were similar to the other samples of bentonite suspension including new cA zero suspension, or rather during library preparation and NGS. Therefore, these samples will be sequenced again.

Detected species richness was much lower in the bentonite suspension samples since the first sampling point compared to VITA, BAM and zero point sample new cA0. Such dramatic decline in species richness implies ongoing rapid microbial selection on prevailing conditions in the samples during the first week(s) and we can expect severe community bottlenecks at this phase. Dramatic decline in overall microbial densities in most of the samples during the first week was also detected by qPCR (Figure 3-11).

Interestingly, species richness was rather similar past the bottleneck in most of the samples of bentonite suspensions, although the number of sequenced OTUs differed between samples (Figure 3-13). We also did not see any difference neither between the irradiated and control samples nor between the samples belonging to one treatment in time. This shows that radiation probably has not significantly reduced the total number of present genera during the course of experiment.

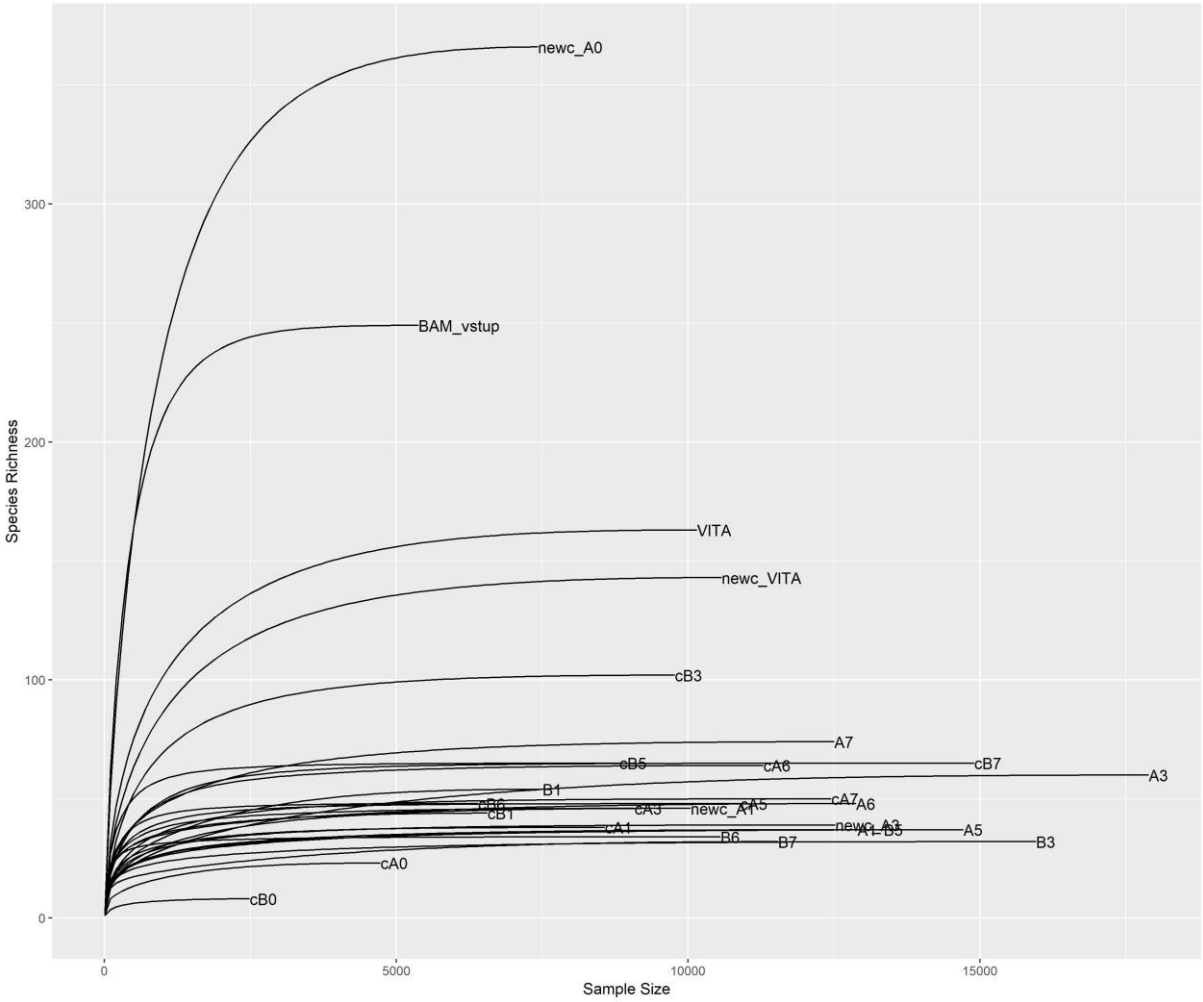

Figure 3-12: Rarefaction curves estimating species richness in the samples during long irradiation experiment.

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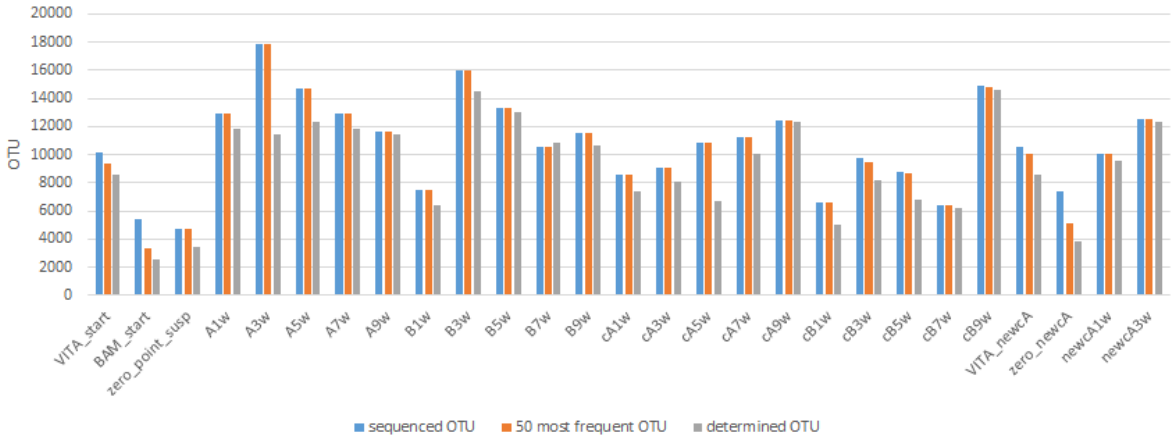

Figure 3-13: Number of sequenced OTUs, number of OTUs belonging to 50most frequent OTUs and OTUs successfully determined to particular genera. A, B – irradiated samples without and with nutrients, cA, cB – non-irradiated anaerobic control samples to A and B, newcA – non-irradiated aerobic control, w – week.

Microbial metabolic profiles

The metabolic profile analysis of genera detected by NGS agreed well with the conclusion we have driven from the qPCR data analysis. We detected large difference between the control samples and irradiated samples that we ascribe to the presence of aerobic conditions in irradiated samples but anaerobic in control samples.

Figure 3-14 shows that both samples of VITA water (VITA_start and VITA_newcA) as well as zero point suspensions (zero_point_susp and zero_newcA) were clearly dominated by Gram-negative non-sporulating microorganisms at the beginning of the experiment contrary to the microbial community composition of the dry BaM powder, where Gram-positive sporulating microorganisms formed nearly half of the detected OTUs. This composition is similar to the preliminary experiments.

While the proportion of Gram-negative non-sporulating microorganisms in most aerobic irradiated samples (both A and B) remained high, the anaerobic control samples cA and cB were rather dominated by Gram-positive spore-formers. The aerobic controls new cA were similarly to irradiated samples clearly dominated by non-sporulating Gram-negative bacteria. These results imply that it is the oxygen presence and not the presence of Gama radiation that selects for the Gram-negative non-spore-formers in irradiated bentonite suspensions. This result is very surprising, because spores generally exhibit five times higher radiation tolerance than vegetative cells (van GERWEN et al., 1999) and we expected to see the selection for spore-forming microorganisms in irradiated samples. New irradiation experiment at strictly anaerobic conditions is thus needed to study the effect of irradiation on spore-forming microorganisms.

We have not observed almost any effect of nutrition addition on the composition of Gram-stain or spore-forming microorganisms in the studied samples. The only difference was higher proportion of Gram-positive spore-forming bacteria in the irradiated sample B9w compared to similarly irradiated sample A9w. However, further data are necessary to determine, if this difference is any sign of possible future community change.

The bacterial composition in the samples from the first (up to three) weeks usually differed from the state at the beginning and in the subsequent part of experiment. Similar pattern is visible across various analyses, see below. We assume that rapid microbial selection on prevailing conditions occurs in the samples during the first week(s) as was also suggested by dramatic decrease in species richness by the rarefaction curves (Figure 3-12).

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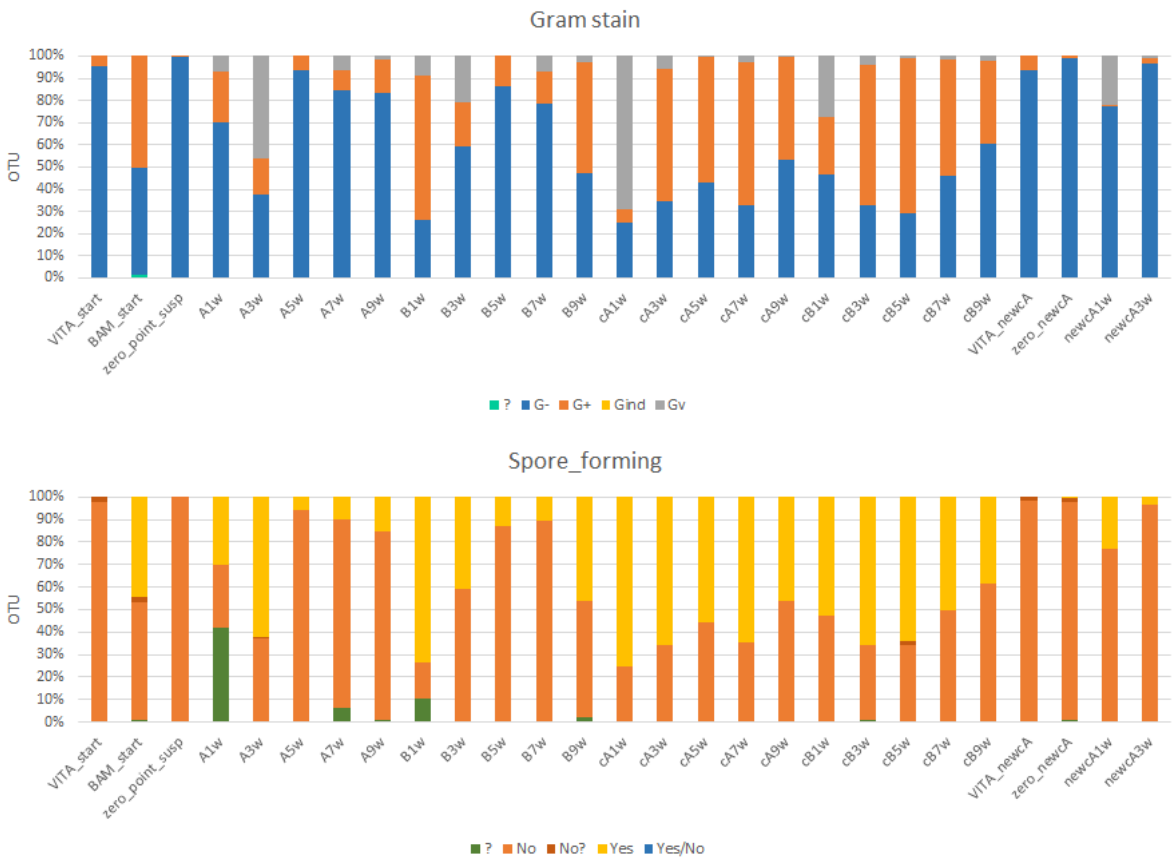

Figure 3-14: Composition of microbial ecosystems in irradiated and control samples of bentonite suspension categorized by Gram staining and spore forming ability of detected microorganisms. A – irradiated samples, no nutrients added, B – irradiated samples with nutrients, cA – non-irradiated control to A, cB – non irradiated control to B, new cA – non-irradiated aerobic control to A. w – week.

The prevailing aerobic or anaerobic conditions within irradiated and control samples are clearly visible when we analyzed the oxygen requirement and preferred electron acceptors of detected microorganisms within the samples (Figure 3-15). Obligatory anaerobic microorganisms that belong either to iron reducers or fermenting microorganisms dominated in the anaerobic control samples (cA and cB). On the other hand, aerobic irradiated samples (A and B) were massively dominated by facultative anaerobes that preferentially respire oxygen, but can switch to nitrate reduction or fermentation in the absence of oxygen. Similar pattern was visible in aerobic controls new cA. In the constant presence of oxygen we cannot expect any changes in the preferred electron acceptors. In accordance, we observed no difference among aerobic samples and thus also no effect of irradiation on oxygen requirement and the preferred electron acceptors of present microorganisms.

When we focus on the development of microbial community composition in anaerobic control, we can see that after less than three weeks obligatory anaerobic iron reducers started to dominate (Figure 3-15), similarly to the results obtained in preliminary short experiment described above. Bentonite is rich in ferric ion and IRB can obviously thrive on this substrate for several weeks. There was slight decrease in the proportion of IRB at the last sampling points in cA and cB, and similar decline of Geobacteraceae in the second half of experiment was detected by qPCR (Figure 3-11). At the same time, we detected faster growth of the SRB since the fifth week (Figure 3-11). These results might indicate the decrease in available Fe^{3+} in the suspension causing the gradual shift in microbial composition from IRB to SRB. Bentonite suspension is rich in sulphate, which would become preferred

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electron acceptor, when the ferric ions are unavailable. Unfortunately, our laboratory is not equipped with the devices necessary for the detection of iron reduction by Ferrozine-method optimized by [101], so we could not measure its changes in time. However, the similar microcosm experiment with bentonite B36 showed massive ferric ion reduction in the samples during 14 weeks with the highest peak in sixth week [102] and we can expect similar processes in BaM bentonite. At least, we monitored the concentration of available sulphate, which is described below, and we will continue analyzing the control samples further in time to see whether the microbial composition will change toward SRB as expected and as it was experimentally proved by [103] in one-year microcosm experiment.

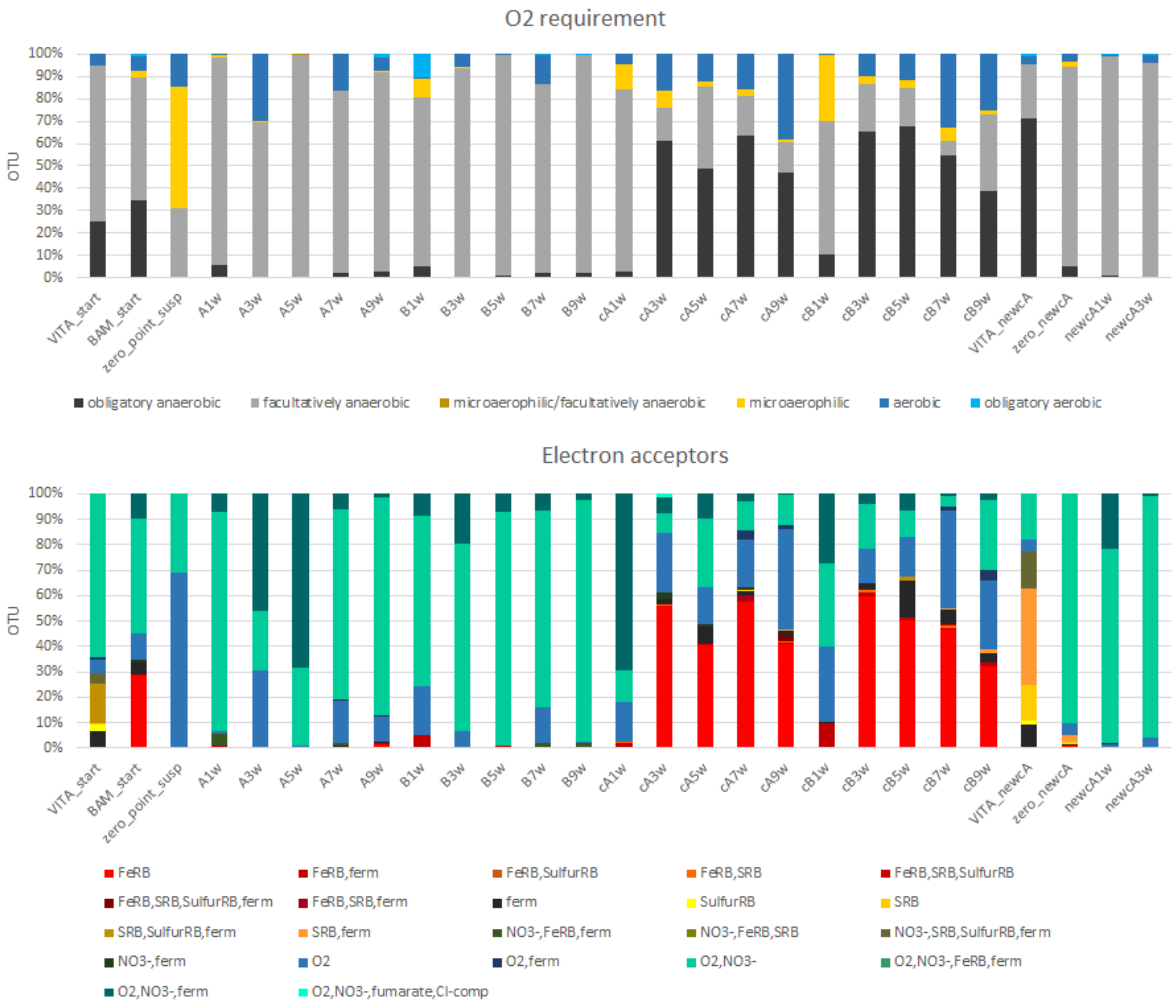

Figure 3-15: Composition of microbial ecosystems in irradiated and control samples of bentonite suspension categorized by oxygen requirement and preferred electron acceptors of detected microorganisms. A – irradiated samples, no nutrients added, B – irradiated samples with nutrients, cA – non-irradiated control to A, cB – non irradiated control to B, new cA – non-irradiated aerobic control to A. w – week. FeRB – iron reducing bacteria, SRB – sulphate reducing bacteria, SulfurRB – oxidized sulfur compound reducing bacteria, ferm – fermenting bacteria.

Analysis of preferred electron donors and trophism of microorganisms detected in studied samples revealed big difference between irradiated and control samples similarly to previous analyses (Figure 3-16).

About a half of the proportion of detected genera in VITA water contained both autotrophic, heterotrophic or mixotrophic genera that use organic compounds, molecular hydrogen

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or reduced sulfur compounds as electron donors. Except for dominating heterotrophs using organic compounds, there was a big proportion of autotrophic genera utilizing molecular hydrogen and thiosulphate as electron donors in BaM powder.

The irradiated samples A and B were clearly dominated by heterotrophs metabolizing various organic materials and we detected an increase in auto/heterotrophs that are able to use also molecular hydrogen, ammonia or sulphur compounds as electron donors. This shift indicates that the availability of organic material in the samples was limited. Interestingly, the shift towards auto/heterotrophs was much more prominent in aerobic new cA samples, where these genera dominated since the first week.

Contrary to the aerobic samples, the anaerobic control samples cA and cB were dominated by autotrophs using molecular hydrogen or thiosulphate as electron donors since the third week (6,552 Gy cumulative dose). As organic material should be energetically preferred electron donor, our results imply its fast reduction in the control samples. Interestingly, proportion of these autotrophs in anaerobic control samples was gradually decreasing during the second half of experiment at the expense of heterotrophs, especially in samples B (Figure 3-16). Such shift might indicate an increase in the availability of organic material for example from dead cells.

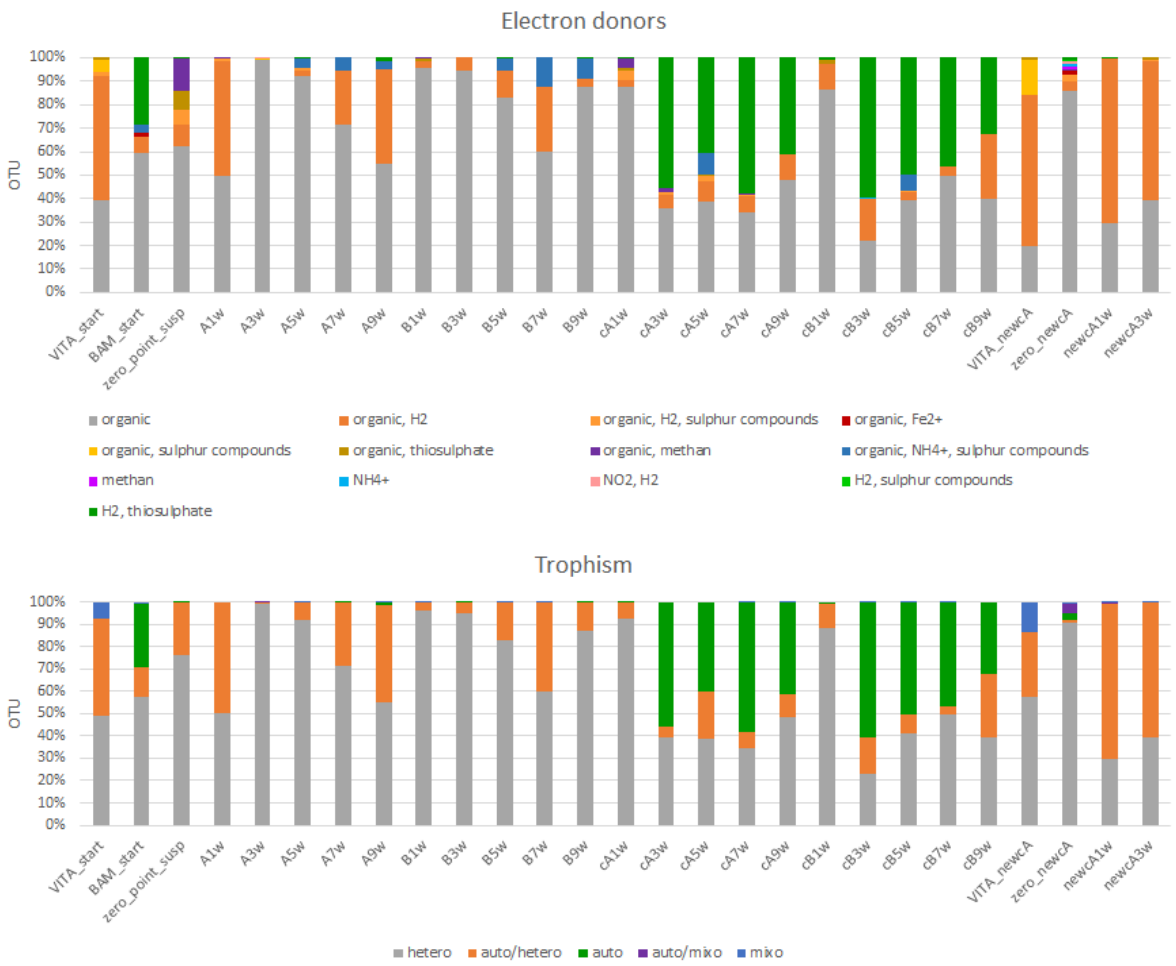

Figure 3-16: Composition of microbial ecosystems in irradiated and control samples of bentonite suspension categorized by preferred electron donors and trophism of detected microorganisms. A – irradiated samples, no nutrients added, B – irradiated samples with nutrients, cA – non-irradiated control to A, cB – non irradiated control to B, new cA – non-irradiated aerobic control to A. w – week.

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Lastly, we evaluated the microbial community composition within the studied samples. The most frequent genera in VITA water were generally rather constant (Table 3-1), although their relative abundance is variable (Figure 3-17). In this study, we present only a single sequencing result for BaM bentonite powder, because previous results revealed very stable microbial community composition.

Table 3-1: Ten most frequent genera in initial samples of VITA water (VITA start, VITA new cA), BaM bentonite and BaM-VITA suspension (zero point susp, zero new cA).

	VITA_start	VITA_new cA	BAM_start	zero_point_susp	zero_new cA
1	*Acidovorax*	*Desulfovibrio*	*Noviherbaspirillum*	*Azospira*	*Delftia*
2	*Polaromonas*	*Desulfobulbaceae*	*Thermincola*	*Methyloversatilis*	*Ralstonia*
3	*Desulfomicrobium*	*Desulfomicrobium*	*Pseudomonas*	*Noviherbaspirillum*	*Acinetobacter*
4	*Desulfovibrio*	*Acidovorax*	*Delftia*	*Caenimonas*	*Desulfovibrio*
5	*Acidovorax*	*Herbinix*	*Paenibacillus*	*Hydrogenophaga*	*Hyphomicrobium*
6	*Desulfobulbaceae*	*Acidovorax*	*Planococcaceae*	*Sphingobium*	*Blastocatellaceae*
7	*Herbinix*	*Lacunisphaera*	*Rhodocyclaceae*	*Polaromonas*	*Hyphomonadaceae*
8	*Caulobacter*	*Sphingobium*	*Massilia*	*Caulobacter*	*Pseudomonas*
9	*Desulfurivibrio*	*Desulfurivibrio*	*Sphingomonas*	*Novosphingobium*	*Gallionella*
10	*Hydrogenophaga*	*Christensenellaceae*	*Paracoccus*	*Lysinibacillus*	*Aquabacterium*

The composition of VITA water is generally very different from the composition of BaM bentonite with only a few genera being present in both environments. Interestingly, the composition of genera in resulting initial suspensions was highly variable (Table 3-1, Figure 3-17), which was probably caused by the lower DNA quality or sequencing problems of zero samples showed by rarefaction analysis (Figure 3-12). It points out to the necessity to always calibrate the data by presenting the sequencing data from VITA and BaM together with the zero point suspension. High variability in microbial composition was also found in the first sampling points (1-3) of irradiated samples and controls. We ascribe this stochastic pattern to the rapid changes during this transition phase.

The irradiated samples A and B started to evolve similarly in both sets with a few genera clearly dominating the samples such as *Massilia*, *Noviherbaspirillum*, *Pseudomonas* or *Bacillus*. All these genera belong to facultative anaerobes that use organic material (or H_2 in case of *Noviherbaspirillum*) as electron donors. Except for *Massilia* detected both in VITA and BaM, all remaining dominating genera originated from BAM. However, the final samples of set A and B (after applying 19,656 Gy cumulative dose) differs. In A9w the composition remains rather similar as previously described with *Massilia* and *Bacillus* being the dominant genera, but *Massilia* was generally less pronounced in set B and completely disappeared in the final sample B9w, where microaerophilic heterotrophic *Azospira* together with facultatively anaerobic heterotrophic *Bacillus* became a dominant genus. All the genera dominating in the irradiated samples can be considered as highly radiation tolerant with the ability to survive at least 20 kGy total absorbed dose of Gama radiation.

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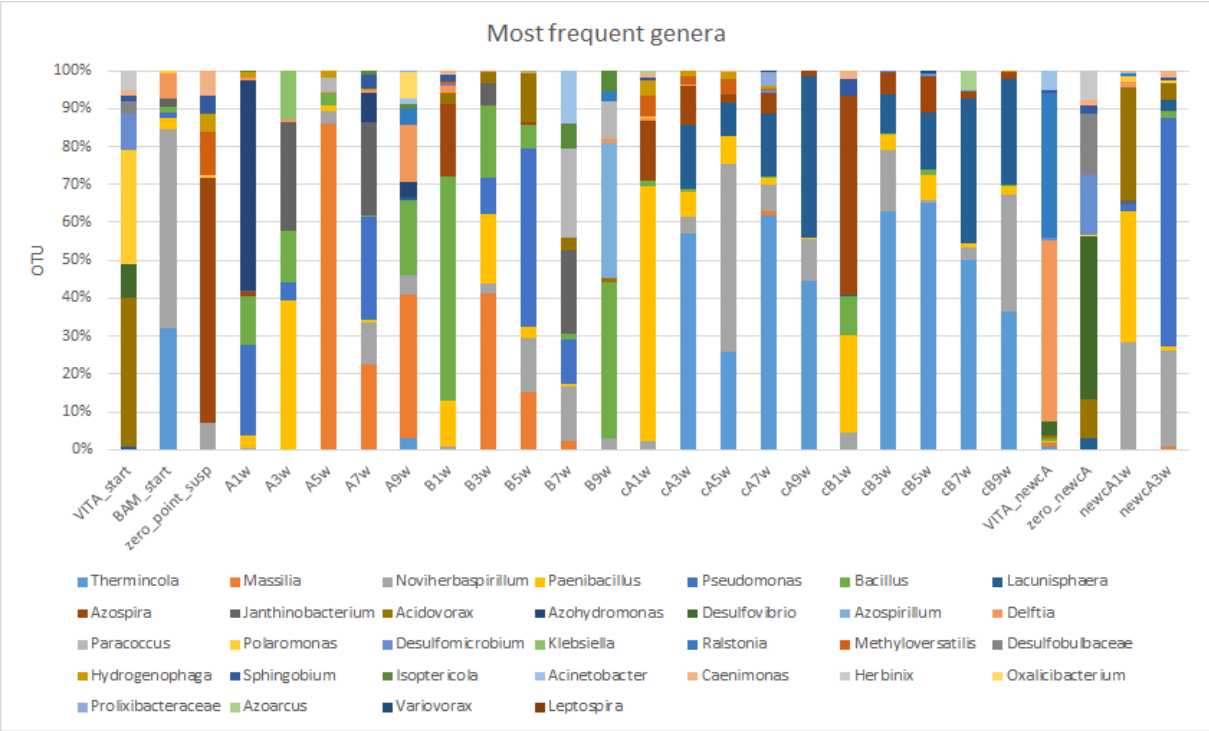

Figure 3-17: Composition of microbial ecosystems in irradiated and control samples of bentonite suspension – 5 most common genera in each sample are included. A – irradiated samples, no nutrients added, B – irradiated samples with nutrients, cA – non-irradiated control to A, cB – non irradiated control to B, new cA – non-irradiated aerobic control to A. w – week.

In both anaerobic control samples cA and cB, we can clearly see the dominance of the anaerobic IRB genus *Thermincola* beginning since the third week and its presence well agrees with the qPCR detected peak of Geobacteraceae (Figure 3-11). Other frequent genera are auto/heterotrophic facultatively anaerobic *Noviherbaspirillum* and aerobic heterotrophic *Lacunisphaera*. Because we are sure, that the control samples were strictly anaerobic, we assume, that present representative of the genus *Lacunisphaera* species must be capable of anaerobic respiration or fermentation, which is unknown feature of this new genus (see Rast et al., 2017). There was no clear signal in new cA controls, which had very variable compositions similarly to the first week's samples of sets A, B, cA and cB. Facultatively anaerobic genera *Pseudomonas* and *Noviherbaspirillum* dominated the sample after three weeks, but we assume that analysis of samples in following weeks will better reveal pattern general for aerobic non-irradiated controls.

Statistical analyses

The differences in microbial ecosystems detected by qPCR and metabolic profile analysis were confirmed also by statistical methods. It independently shows, that the irradiation of samples with relatively high absorbed doses of ionizing radiation influenced the composition of microbial ecosystems less, that the unintentional difference in oxygen presence within the samples.

NMDS analysis clustered almost all the aerobic (irradiated and new control) samples into one cluster while the anaerobic control samples formed second cluster. The samples of BaM, VITA and zero points for new control new cA cluster with aerobic samples, but some of the samples from the first sampling point and original zero points cA0 and cB0 are not assigned to neither of the detected groups, which again agrees with the expected pattern. The result of MDS/PCoA shows, that the

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irradiation with relatively high total absorbed dose has lower effect on microbial composition in the samples than the presence oxygen in the samples.

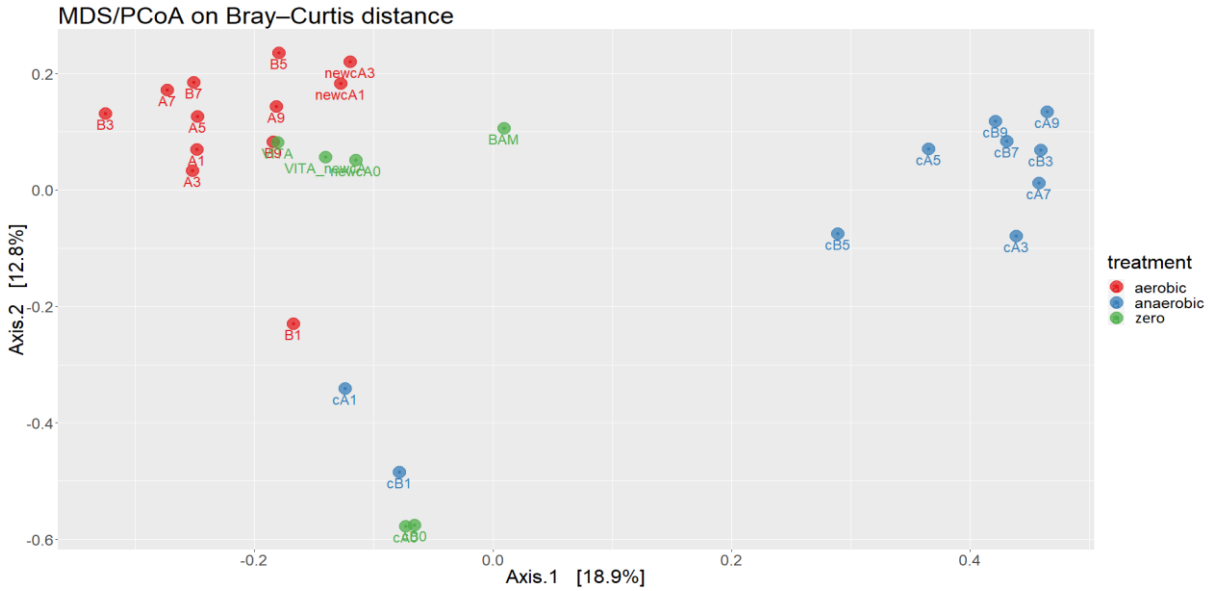

Figure 3-18: MDS/PCoA analysis visualizing shared variability in the NGS data in the samples. The first two axes describing highest proportion of variability in the data are shown. A – irradiated samples, no nutrients added, B – irradiated samples with nutrients, cA – non-irradiated control to A, cB – non irradiated control to B, new cA – non-irradiated aerobic control to A. Number 1-9 – weeks of sampling.

Heat-map computed from the NGS data similarly to MDS/PCoA tend to group samples from anaerobic controls into one cluster, while most of the samples from samples that were probably influenced by oxygen and some of the zero points were grouped to the second cluster (Figure 3-19). Zero point samples cA0 and cB0 together with first sampling points cA1 and cB1 similarly as in MDS/PCoA stayed outside these two major groups.

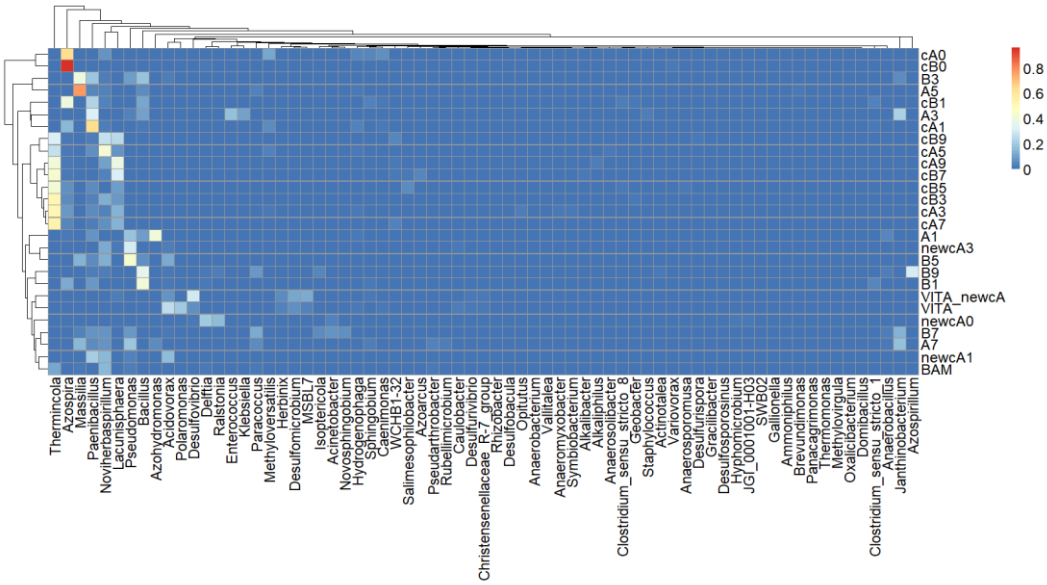

Figure 3-19: Heatmap of NGS data from irradiation experiment showing the relative frequency of most frequent OUTs in the samples. A – irradiated samples, no nutrients added, B – irradiated samples with nutrients, cA – non-irradiated control to A, cB – non irradiated control to B, new cA – non-irradiated aerobic control to A. Number 1-9 – weeks of sampling.

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Chemical analysis

One of the aims of our study was to evaluate, whether better nutrient availability significantly influences the microbial composition of irradiated samples. By addition of nitrate we intended to promote the total biomass as most of the microorganisms are able to respire it and with addition of sulphate and acetate we wanted to promote the proliferation of SRB, which are frequent in the source VITA water. However, the added concentration of nitrate was too low and was consumed within first week of experiment in all samples with no significant effect (Figure 3-20). On the other hand, both natural and added concentrations of sulphate were probably sufficient to promote bacterial growth, but this energetically less favorable electron acceptor was not exploited in the presence of oxygen in aerobic irradiated samples and in presence of ferric ion in anaerobic controls as discussed above. We detected a slight decrease of available sulphate at the end of experiment in aerobic irradiated samples B and in anaerobic controls cB with high sulphate concentration (Figure 3-20) suggesting that probably a longer time frame is required to observe a higher microbial sulphate consumption.

In irradiated samples B slight increase in SRB abundance was detected by qPCR at the end of experiment (Figure 3-11), which might indicate the existence of sulphate reduction probably in microclimatically suitable conditions in irradiated samples. The noticeable increase in the quantity of SRB detected by qPCR in anaerobic controls cB together with the detected decrease of available sulphate indicate the possible beginning of transition from IRB community to SRB community. Further data are needed to confirm this hypothesis.

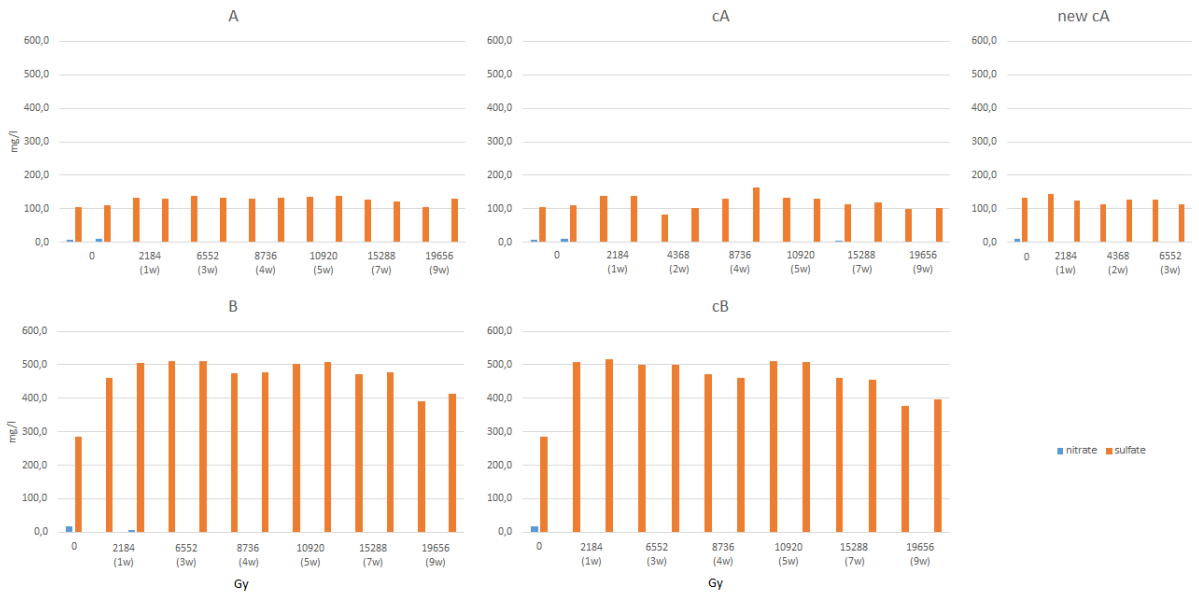

Figure 3-20: Concentrations of sulphate and nitrate during the irradiation experiment. For each sampling point duplicates were analyzed. A – irradiated samples, no nutrients added, B – irradiated samples with nutrients, cA – non-irradiated control to A, cB – non irradiated control to B, new cA – non-irradiated aerobic control to A. w – week.

Acetate is a major product of fermentation processes and it is an important substrate for sulphate reducing bacteria [105]. The natural concentration of acetate in samples A, cA and new cA was about an order of magnitude lower than in acetate enriched samples in B and cB and its level remained low during the whole course of experiment (Figure 3-21).

The acetate concentration in aerobic irradiated samples B was relatively stable for the first three weeks and later on it started to decline implying that acetate was used as electron donor by some microorganisms. On the other hand, the acetate concentration was much less stable in anaerobic

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controls cB and visibly increased in two peaks – one smaller in the third week and second larger in the seventh week of experiment (Figure 3-21). Also the pH values at times corresponding to these peaks visibly declined (Figure 3-22). This might indicate ongoing acetogenesis either by formation of acetate by the reduction of CO_2 or rather the formation of acetate from oxidation of organic acids produced by fermenting microorganisms [106]. We detected presence of fermenting microorganisms in the anaerobic samples (Figure 3-15).

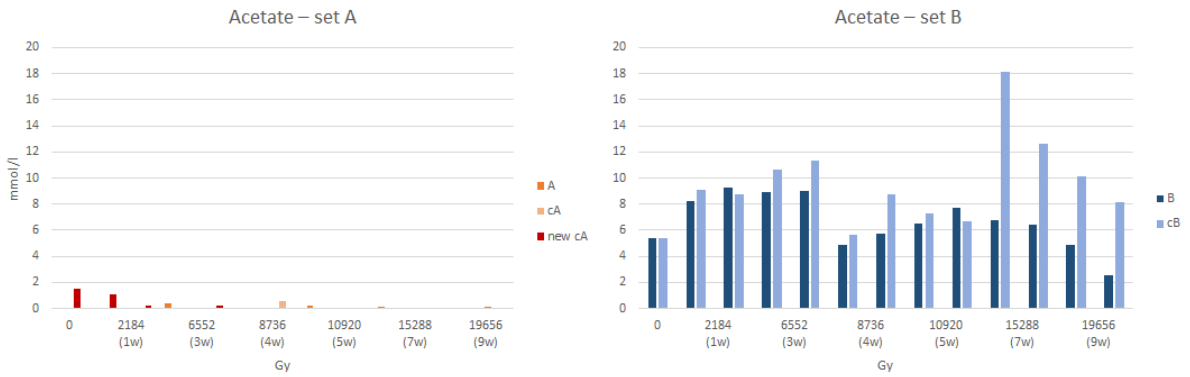

Figure 3-21: Acetate concentration during the experiment. A – irradiated samples, no nutrients added, B – irradiated samples with nutrients, cA – non-irradiated control to A, cB – non irradiated control to B, new cA – non-irradiated aerobic control to A. w – week.

The pH values were generally higher in irradiated samples A than B, and in controls cA than cB, which can be explained by the presence of acetate in B and cB. However, values of pH were also higher in aerobic irradiated samples A and B and new cA control than in anaerobic control samples cA and cB (Figure 3-22). The latter can be explained either by the detected higher population density in control samples (Figure 3-11) together with different microbial composition in both samples (Figure 3-15) or by different chemical processes ongoing under aerobic and anaerobic conditions.

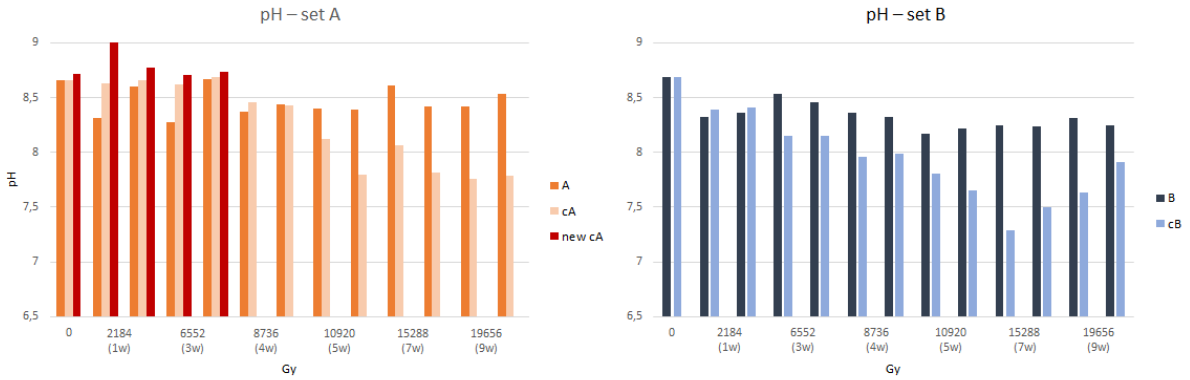

Figure 3-22: Changes in pH during the experiment. A – irradiated samples, no nutrients added, B – irradiated samples with nutrients, cA – non-irradiated control to A, cB – non irradiated control to B, new cA – non-irradiated aerobic control to A. w – week.

Cultivations

Cultivations (both aerobic and anaerobic) of irradiated samples and controls gave very unambiguous results during first five weeks of experiment that do not correspond well with the results of other

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analyses. We thus decided not to perform further cultivations as they are very time consuming and obviously inaccurate compared to other methods used.

Table 3-2: Cultivation results of irradiation experiment.

Sample	Aerobic cultivations (CFU)	Anaerobic cultivations (CFU)
zero susp.	$1.97x10^6$	$2.1x10^2$
A5	$1.67x10^5$	$3.2x10^2$
cA5	$2.68x10^5$	$3.2x10^2$
B5	$8.2x10^5$	$6.8x10^2$
cB5	$1.79x10^5$	27

3.2 Conclusions - irradiation experiment

The composition of microbial communities within the studied samples was changing continuously in all studied samples. Microorganisms indigenous to VITA and BAM mixed in bentonite suspension obviously undergo a dramatic reduction in terms of both the absolute biomass as well as the species richness at the beginning of experiment. This bottleneck event occurred similarly in irradiated (aerobic) and non-irradiated (aerobic and anaerobic) samples at the beginning of experiment and it is probably caused by the inevitable adaptation to the specific environment of the bentonite suspension. In accordance with this, we detected, that most of the bacterial genera unique to VITA water (not present in BAM) were not able to adapt to this new environment and they were not detected in any of the suspension samples after first week. The indigenous bacteria from BaM thus formed a crucial part of the microbial community in bentonite suspensions.

After this initial bottleneck event, gradual changes in microbial community composition were observed. As we worked with the closed systems with limited energy sources, bacterial communities well mirrored the prevailing conditions in the sample. In the samples influenced by the presence of oxygen, in which microorganisms were not limited by the available electron acceptors, we observed the community changes caused mainly by the available electron donors. The aerobic communities generally tend to evolve from heterotrophs metabolizing organic substrates towards autotrophs (chemilitotrophs) capable of using molecular hydrogen, ammonia or various sulphur compounds as electron donors in the lack of organic material. On the other hand, in anaerobic conditions we observed a clear limitation by both available electron acceptors and donors. The communities evolved from heterotrophic facultative anaerobic nitrate reducers to autotrophic anaerobic iron reducers or fermenting microorganisms. The iron reduction is thus probably very important process in the bentonite under anaerobic conditions. At the end of the experiment we have detected the slight decline in iron reducing bacteria and increase in sulfur reducing bacteria, although the experiment was not long enough to capture this community shift in its full extent. Similar shifts in microbial community composition in closed microcosms under anaerobic conditions were described also by Brown et al. [48] or Matschiavelli et al. [102,103].

Interestingly, although we detected changes in the overall microbial abundances and in the community composition during the course of experiment, the species richness in the samples remained rather constant in time after initial bottleneck and was also similar in both aerobic (irradiated+control) and anaerobic (control) samples.

Our analysis showed no effect of added nutrients on microbial composition within studied samples. Concentration of nitrate was too low to cause any effect. Sulphate as energetically less favorable electron acceptor was not exploited either in the presence of oxygen in aerobic samples

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or in the presence of ferric ion in anaerobic controls. However, the beginning of community shift from IRB to SRB was detected at the end of experiment. Similar pattern was observed in the acetate consumption. The concentration of acetate (which is a preferential electron donor for many SRB) markedly increased in the anaerobic controls cB in the seventh weeks suggesting possible ongoing acetogenesis, but higher acetate concentration rapidly decreased in time which agrees with the start of increase in SRB population.

The effect of Gama radiation turned out to be far less important factor determining the microbial community composition compared to the effect of the bentonite environment itself or the availability of energy sources that select for the community composition primarily. Stroes-Gascoyne et al. [69] studied the radiation sensitivity of naturally occurring microorganisms in bentonite and estimated that 90 % of microorganisms will be eliminated by dose between 0.65 and 1.68 kGy depending on the bentonite type. On the other hand, McNamara et al. [71] showed, that majority of soil bacteria needs about 20 kGy dose of ionizing radiation to be eliminated, but a dose higher than 70 kGy may be required to kill certain radio-resistant bacteria. Although we can make conclusion only about the effect of irradiation in aerobic conditions, application of 19,656 Gy cumulative absorbed dose of Gama radiation was not sufficient to eradicate bacteria naturally present in the bentonite. Significantly higher doses are expected to be able to sterilize the bentonite suspension completely. The applied dose of ionizing radiation however caused apparent decline in total bacterial biomass in irradiated samples during the course of experiment.

Interestingly, the species richness did not change with the increasing absorbed dose. However, we detected some genera (namely *Massilia*, *Bacillus* or *Azospirillum*) which dominated the microbial community in the irradiated samples. This implies that although the whole microbial community was highly radiation resistant, some of the genera are probably more resistant than the others and outcompete them.

Microbial spores generally exhibit much higher radiation tolerance, than vegetative cells [15] and Gram-positive and/or microorganisms high in G+C content are reported to be more radiation resistant than Gram-negative bacteria [63]. Because Gram-positive sporulating microorganisms are dominant in BAM bentonite, we expected these bacteria to dominate in the irradiated samples. However, these samples were clearly dominated by Gram-negative non-spore forming bacteria and we ascribe this finding to the intrusion of oxygen in the samples (the aerobic non-irradiated controls have similar composition). On the other hand, sporulating Gram-positive bacteria formed about a half of the community in the anaerobic non-irradiated controls. Irradiation experiment under strictly anaerobic conditions will be necessary to estimate the effect of irradiation on the microbial community that evolves in the bentonite suspension under anaerobic conditions and that better corresponds to the repository conditions. Brown et al. [48], who studied the impact of Gama radiation on sediment microbial processes in the anaerobic closed microcosm, found that iron or sulfate reduction was not restricted by radiation even after receiving the total dose of 38.6 kGy and some electron accepting processes were even stimulated by low dose-rate radiation. We can expect that similar or even higher absorbed doses will be needed to eliminate the microbial activity in bentonite under anaerobic conditions.

Further irradiation experiments performed in strictly anaerobic conditions and exposed to higher total absorbed doses are necessary for better estimation of the irradiation effect on microbial community in bentonite.

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3.3 Pressure experiment

Pressure experiments aimed to evaluate the effect of increased pressure on survival of bacteria in system containing VITA water and BaM bentonite simulating effect of swelling pressure in compacted bentonite.

3.3.1 Results of preliminary experiments

The qPCR data from all preliminary week-long experiments were rather consistent among different experiments and showed that none of the targeted microbial groups nor the total bacterial biomass was significantly influenced by the effect of increased pressure for a week period (Figure 3-23 -Figure 3-25). There was clear increase in total biomass (measured by 16S rRNA) and in abundance of all studied metabolic groups of bacteria under all the tested pressure similarly to control samples. Marker NirS for NRB failed to amplify in all zero state samples of each preliminary experiment, which disabled us to calculate the relative quantification values, but its Cq values decreased in all treatment and control samples across all the experiments similarly to NirK and NosZ. Similarly, NirK and NosZ markers for NRB failed to amplify at zero sample in experiment 2, but their quantities were also increasing during the experiment in both treatment and control samples. In the cases of detected biomass decrease, similar trends were not confirmed by the results of other experiments (e.g. decreasing value of SRB quantity detected by ApsA gene marker in control sample of experiment 1, but not in experiment 2 or 3) and these results thus must be treated with caution.

As the week-long pressure experiments showed no significant effect on bacterial survival, we decided to perform a month-long experiment similarly designed to test the long-term exposal to elevated pressure.

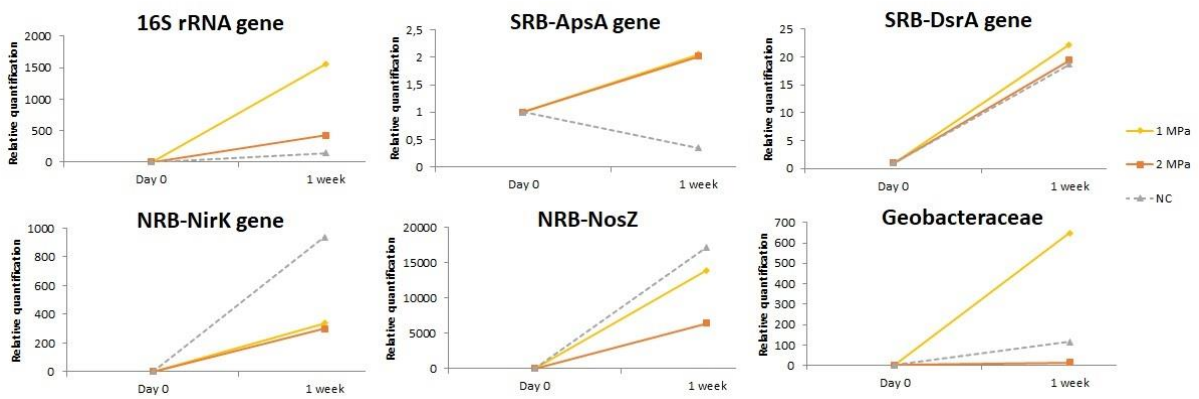

Figure 3-23: Relative quantification results from pressure experiment 1 (1 and 2 MPa).

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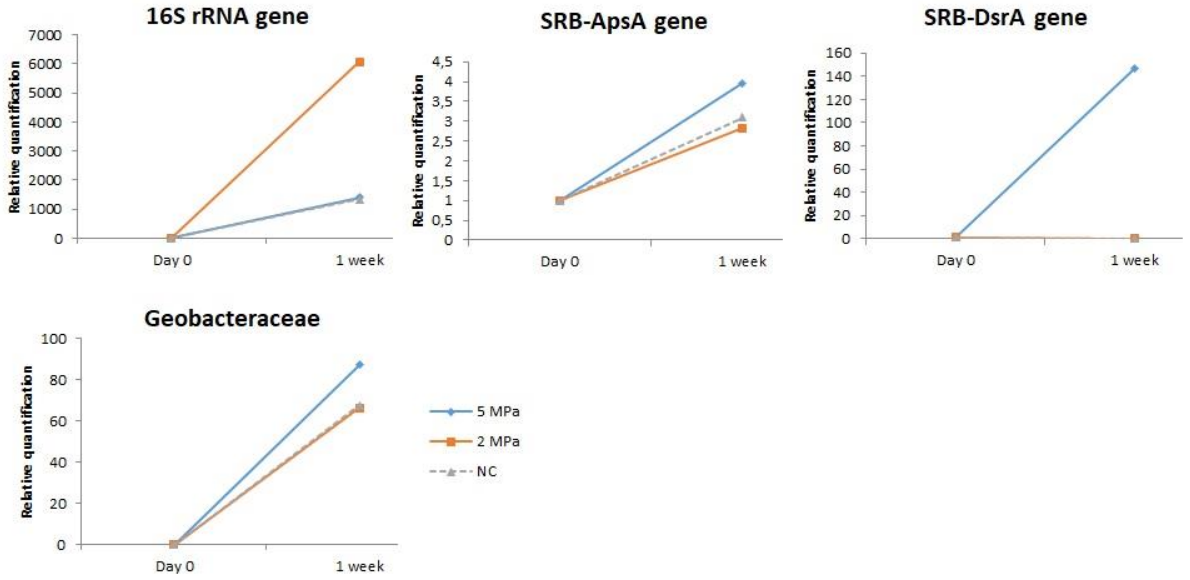

Figure 3-24: Relative quantification results from pressure experiment 2 (2 and 5 MPa).

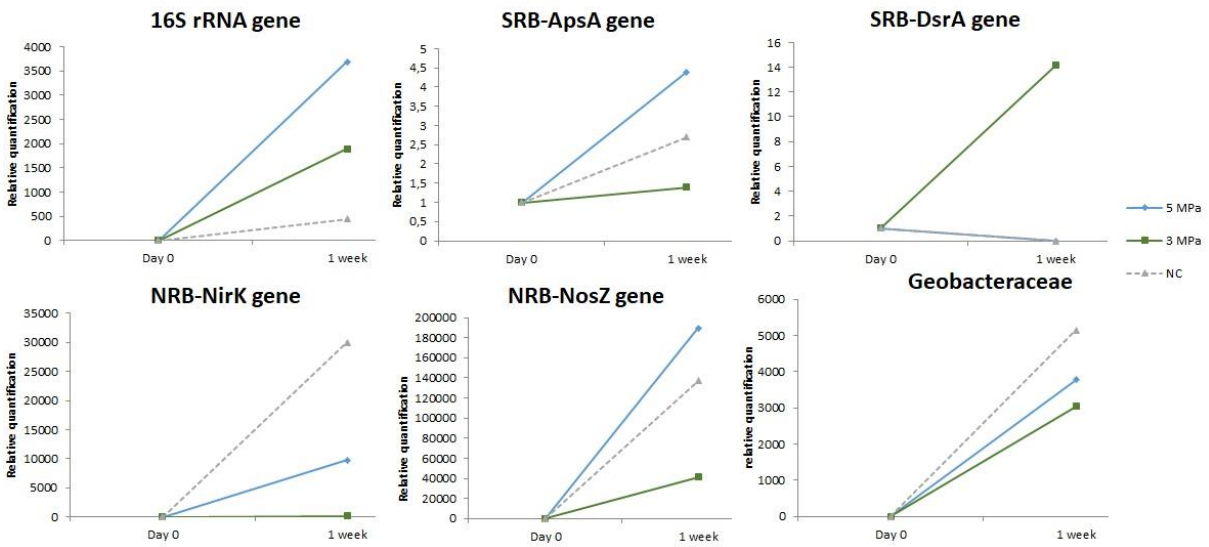

Figure 3-25: Relative quantification results from pressure experiment 3 (3 and 5 MPa).

3.3.2 Long term experiment

The results of month-long experiment under 2 and 5 MPa pressure (experiment 4) showed a pressure resistance of the total bacterial mass as well as of all the studied metabolic groups of bacteria (Figure 3-26), similarly to preliminary short experiments. However, increase in the relative quantity of total bacteria in 5 MPa treated sample was markedly lower compared to 2 MPa sample and was even lower than relative microbial quantity detected in control sample, which indicates negative influence of high pressure on bacterial proliferation.

Based on the data on turgor pressure, microbial cell integrity is generally possible at environmental pressures below 2 MPa in most microorganisms [22,23]. In accordance with this, the survival and proliferation of natural microflora from bentonite and deep underground water was not limited by the application of 2Mpa pressure, but the growth was slightly reduced by the 5 MPa treatment compared to no pressure control. Application of much higher pressures are probably needed to significantly limit bacterial growth and proliferation.

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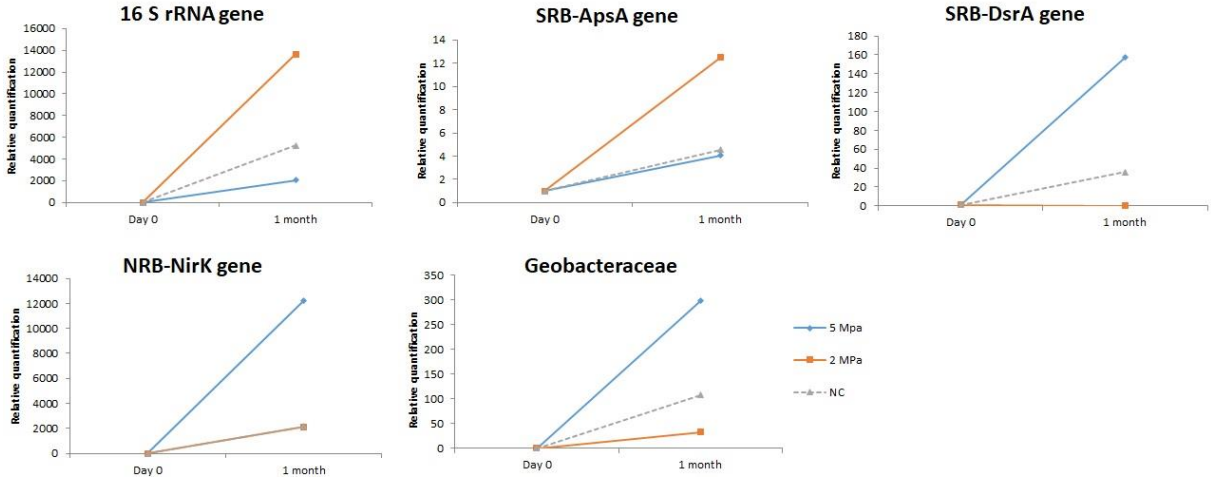

Figure 3-26: Relative quantification results from pressure experiment 4 (2 and 5 Mpa, one month).

Rarefaction curves showed us the overall species diversity in the samples. Similarly to IR experiments, we see highest species richness in zero point suspension followed by a dramatic reduction of species richness in suspension samples during the experiment. Species richness was slightly higher in the control sample than in both pressurized samples (Figure 3-27).

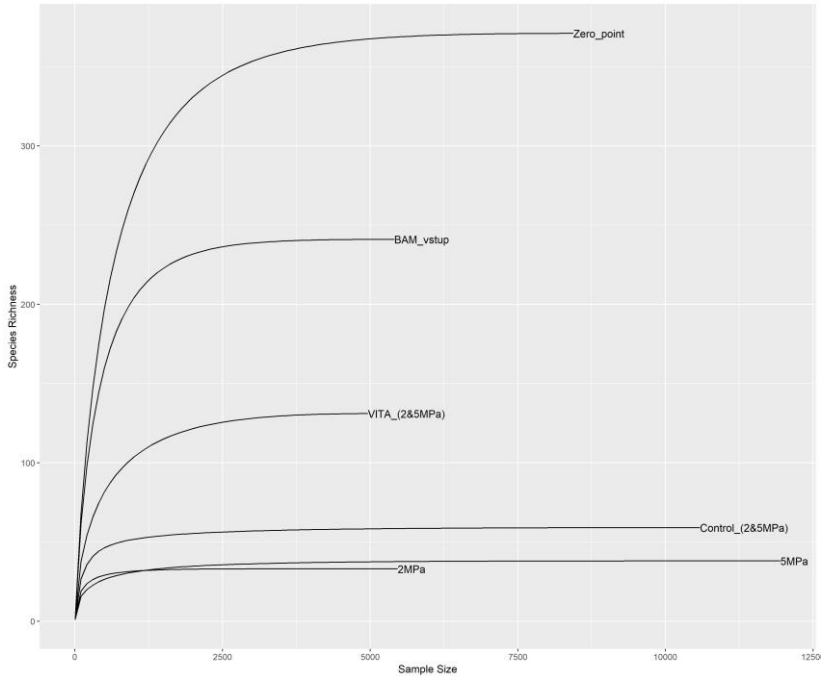

Figure 3-27: Rarefaction curves showing the species richness in samples from pressure experiment 4.

The sequencing data (Figure 3-28 - Figure 3-31) helped us to better understand the changes in microbial community during the course of pressure experiment. Both control and pressurized samples were clearly dominated by Gram-negative non-sporulating microorganisms (Figure 3-28). The proportion of Gram-positive microorganisms was quite similar in both treatment samples and did not vary from the zero state. However, the proportion of Gram-positive microorganisms slightly declined in the control sample, where on the other hand, Gram-variable microorganisms (mostly genus *Paenibacillus*) emerged. We detected a slight increase in proportion of spore-forming microorganisms in both treatment and control samples compared to zero state. Spore-

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forming microorganisms are typical of bentonite (Figure 3-28) and the harsh conditions in the bentonite environment thus select for them in time.

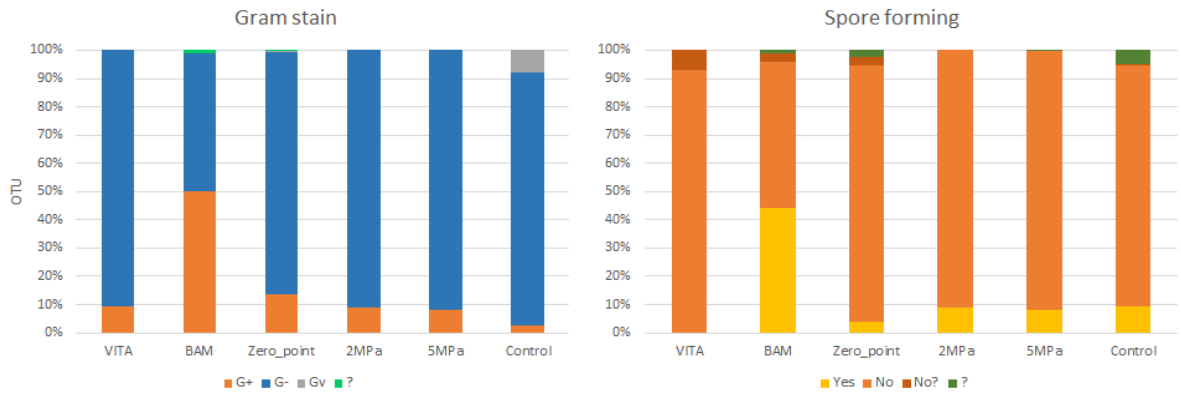

Figure 3-28: Composition of microbial ecosystems in controls and samples pressurized to 2 and 5 MPa categorized by Gram staining and spore forming ability of detected microorganisms.

Except for VITA water dominated by obligate anaerobes, all the treatment and control samples were clearly dominated by heterotrophic facultative anaerobes (respiring nitrates or fermenting in the absence of oxygen) (Figure 3-29). The results further confirmed the presence of all studied metabolic groups (SRB, NRB and Geobacteraceae) both in treatment samples as well as in controls and showed, that the environment of both treatment samples and controls was clearly dominated by NRB after a month period with the dominating NRB genera *Massilia* and *Pseudomonas* (Figure 3-29, Figure 3-31). On the other hand, although qPCR data indicated increase in SRB in both treatment and control samples in experiment 4 (Figure 3-26), these bacteria were relatively more abundant in the zero sample at the beginning of the experiment than in the treatment or control samples dominated by NRB (Figure 3-30). However, this discrepancy can be caused by the relative nature of ΔΔCq quantification method.

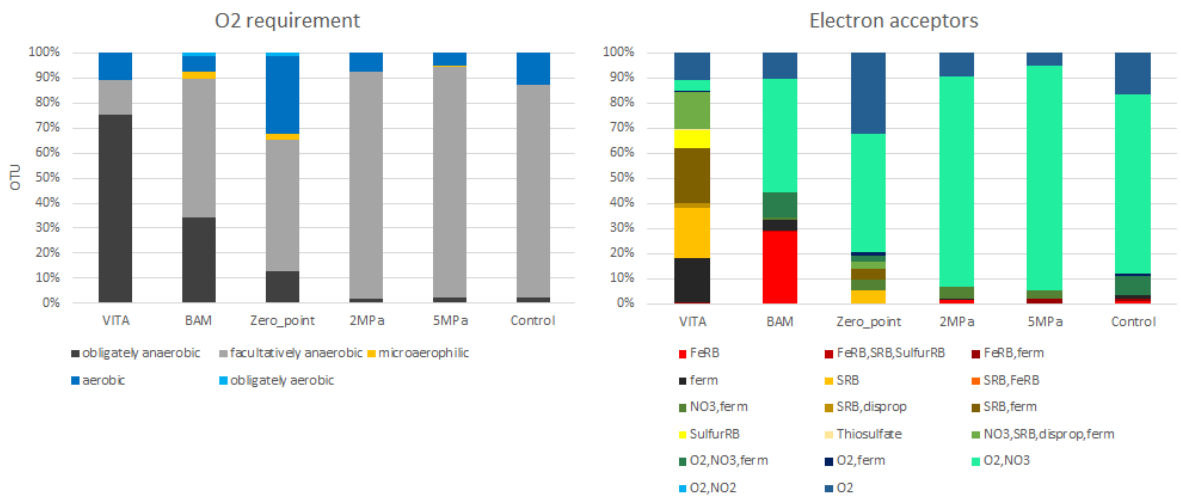

Figure 3-29: Composition of microbial ecosystems in controls and samples pressurized to 2 and 5 MPa categorized by oxygen requirement and preferred electron acceptors of detected microorganisms. FeRB – iron reducing bacteria, SRB – sulphate reducing bacteria, SulfurRB – oxidized sulfur compound reducing bacteria, ferm – fermenting bacteria.

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In control sample, we detected an increase of auto/heterotrophic or mixotrophic genera with the ability to use molecular hydrogen (Figure 3-30). This shift towards autotrophy indicates that the available organic substrates in the system were probably limited similarly to the results of IR experiments.

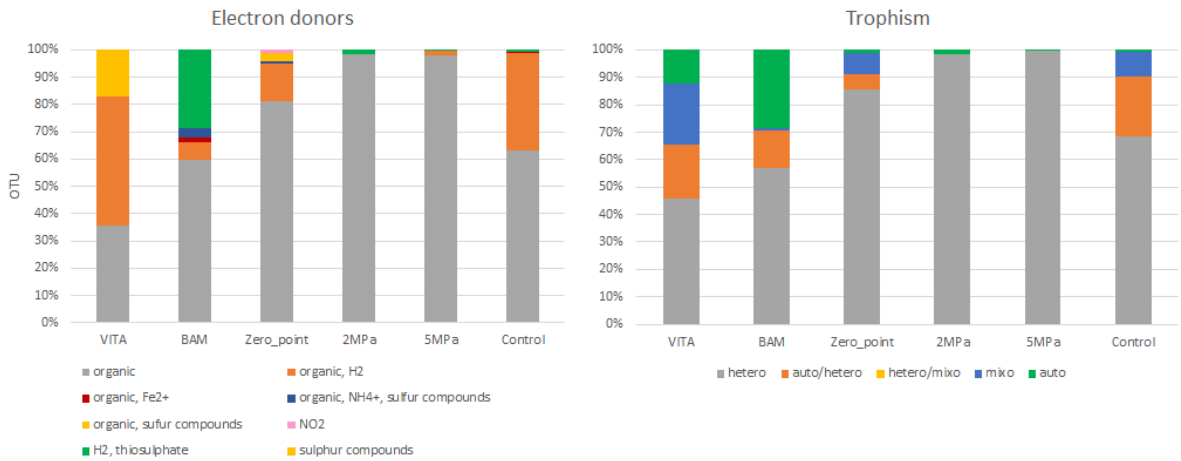

Figure 3-30: Composition of microbial ecosystems in controls and samples pressurized to 2 and 5 MPa categorized by preferred electron donors and trophy of detected microorganisms.

The composition of most frequent genera in zero_point suspension was similarly to the results of IR experiments above rather different from both source VITA water and BaM bentonite. The sample was dominated by genera *Pseudomonas*, *Massilia and Sphingomonas* (Figure 3-31). On the other hand, the genera composition of both pressurized samples showed higher dominance of *Massilia* and *Pseudomonas* than control, indicating, that these genera might be slightly more pressure resistant, than the other genera detected in bentonite suspension.

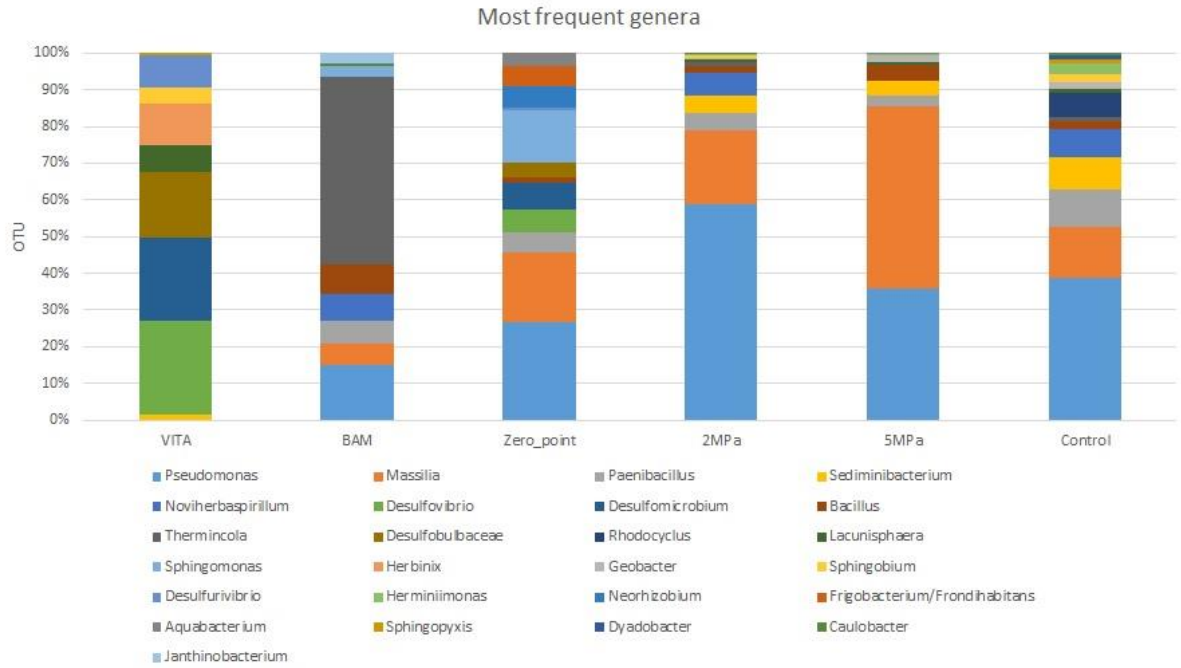

Figure 3-31: Composition of microbial ecosystems in controls and samples pressurized to 2 and 5 MPa. Ten most common genera in each sample are included.

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The cultivation results of experiment 4, summarized in Table 3-3, showed remarkable increase of cultivable bacteria in both treatment samples and control compared to zero state, which well agrees with the qPCR results. Further, the numbers of cultivable microorganisms were slightly lower for 5 MPa than for 2 MPa for both aerobic and anaerobic cultivations and cultivations from all pressurized samples resulted in lower numbers in cultivable bacteria (aerobic and anaerobic) than in control samples. Although the cultivation results are probably not fully reliable, they indicate week negative effect of pressure on the number of cultivable bacteria.

Table 3-3: Cultivation results

	CFU/ml	
Sample	**Aerobic**	**Anaerobic**
Zero point	9.5×10^2	1.6×10^3
2 MPa	3.3×10^6	1.25×10^3
5 MPa	1.2×10^6	1.11×10^3
Control 2+5 MPa	8.2×10^6	2.69×10^3

3.4 Conclusions - pressure experiment

Pressure experiments were performed to evaluate the effect of increased pressure on survival of bacteria in the environment of the bentonite suspension. In compacted bentonite, microorganisms have to deal not only with highly elevated pressure, but also with the related reduction of pore sizes (and thus very limited life space) and changes in bentonite physico-chemistry caused by high pressure [5]. Our experiment aimed to separate these effects and evaluate single effect of increased pressure on bacterial survival.

In the various waste disposal concepts, the desired swelling pressure of compacted bentonite is at least 5 MPa [22]. We have tested the effect of 2 and 5 MPa pressure on the microorganisms present in the bentonite suspension and we demonstrated that even 5 MPa pressure was not sufficient to markedly reduce the total microbial biomass nor to visibly influence the microbial composition. However, the total microbial biomass was slightly lower in 5 MPa sample compared to the control sample. Similarly to irradiation experiments, dramatic reduction of the species richness in the bentonite suspensions was observed compared to the samples of zero state suspension, BaM bentonite powder and VITA. This implies a strong bottleneck event caused by harsh conditions in the bentonite suspension that are obviously not optimal for most bacterial species detected in the beginning of the experiment. Although spores are much more pressure-resistant than vegetative cells [13], we have not detected any increase in the proportion of Gram-positive spore-forming bacteria in any of the pressurized samples.

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4 Conclusion and future prospects

The composition of microbial communities within the bentonite suspension samples was dramatically affected by the bottleneck during community adaptation to specific environment of bentonite suspension. Most of the bacterial genera unique to VITA water disappeared at this phase. Following this, gradual changes in microbial community composition were observed mirroring the prevailing conditions in the sample. In the samples influenced by the presence of oxygen the community changes were caused mainly by the available electron donors evolving from heterotrophs to autotrophs with the decrease of available organic material. In anaerobic conditions a clear limitation by both available electron acceptors and donors was observed. The communities evolved from heterotrophic facultative anaerobic nitrate reducers to autotrophic anaerobic iron reducers and fermenting microorganisms or sulfur reducing bacteria at the very end. Community changes were not reflected in detected species richness, which was relatively stable throughout the experiment. Presence of Gama radiation was less important factor determining the microbial community composition than bentonite environment or the availability of energy sources. Application of 19,656 Gy cumulative absorbed dose of Gama radiation did not eradicate naturally occurring bacteria in the bentonite suspension under conditions influenced by the presence of oxygen. It caused the decline in total bacterial biomass in irradiated samples but not in species richness. Gram-negative non spore-forming microorganisms dominated the irradiated samples, which we ascribe to the intrusion of oxygen into the samples. As anaerobic samples were dominated by spore-forming bacteria, known to be generally more resistant to radiation, even higher absorbed dose might be necessary to eliminate microbial activity in bentonite under anaerobic conditions.

The results of irradiation experiment confirmed potential key role of the iron reduction in the microbial processes occurring in the bentonite under anaerobic conditions. Ferric ions naturally occur in the bentonite and their reduction might cause decrease in bentonite swelling ability and thus negatively influence the performance and safety of nuclear waste repository.

To better estimate the effect of irradiation on microbial community changes in bentonite under repository conditions, subsequent irradiation experiments performed under strictly anaerobic condition and exposed to higher total absorbed doses are needed. For a more accurate quantification of changes in microbial abundance, absolute quantification (e.g. by digital droplet PCR) will be necessary in the future experiments.

The results of pressure experiments showed, that cultivation under 2 and 5 MPa pressure did not markedly reduce the total microbial biomass nor did substantially change the microbial composition. Although spores are more pressure resistant compared to the vegetative cells, the samples were dominated by non-sporeforming microorganisms.

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