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Nitrate reducing bacterial activity in concrete cells of nuclear waste disposal

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Abstract. Leaching experiments of solid matrices (bitumen and cement pastes) have been first implemented to define the physicochemical conditions that microorganisms are likely to meet at the bitumen-concrete interface (see the paper of Bertron et al.). Of course, as might be suspected, the cement matrix imposes highly alkaline pH conditions (10 <pH <11). The screening of a range of anaerobic denitrifying bacterial strains led us to select *Halomonas desiderata* as a model bacterium capable of catalyzing the reaction of nitrate reduction in these extreme conditions of pH. The denitrifying activity of *Halomonas desiderata* was quantified in batch bioreactor in the presence of solid matrices and / or leachate from bitumen and cement matrices. Denitrification was relatively fast in the presence of cement matrix (<100 hours) and 2 to 3 times slower in the presence of bituminous matrix. Overall, the presence of solid cement promoted the kinetics of denitrification. The observation of solid surfaces at the end of the experiment revealed the presence of a biofilm of *Halomonas desiderata* on the cement paste surface. These attached bacteria showed a denitrifying activity comparable to planktonic bacterial culture. On the other side, no colonization of bitumen could be highlighted as either by SEM or epifluorescence microscopy. Now, we are currently developing a continuous experimental bioreactor which should allow us a more rational understanding of the bitumen-cement-microbe interactions.

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1 Introduction

Before disposal, LILW-SL may be compacted to reduce its volume, or solidified by coating in a matrix of bitumen if it is a liquid [1,2]. Usually, it is placed in metal containers and then embedded in concrete. Thus, a package of LILW-SL is composed of 15 to 20% radioactive waste and 80 to 85% embedding matrix. During storage (Figure 1), driven by the combined effects of water resaturation and irradiation, bitumen and concrete are likely to release chemical species, especially soluble salts including, hydroxides, nitrates, organic matter (organic acids, phenols ...), gas and radionuclides [3-6].

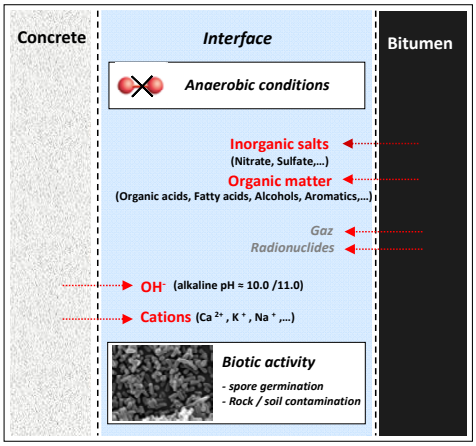


Figure 1: Schematic representation of the possible scenario at the concrete - bitumen interface after water resaturation in the disposal cells.

The presence of nitrates in the vicinity of waste packages may result in oxidising conditions favourable to the mobility of a series of radionuclides (Se, U, Tc, Pu, Np...)[7]. However, in the anoxic conditions prevailing in the cell, different phenomenon of redox reactions could lead to nitrates reduction and thus to reducing conditions favourable to the storage safety. Reduction of nitrate (NO_3^-) may occur from surface catalysis provided by the different type of steels present in the storage, and/or from biological catalysis through denitrifying bacterial activity, and may lead to the formation of nitrite (NO_2^-), gaseous nitrogen (N_2) and/or ammonium (NH_4^+), depending on the type of reaction [8-13]. The impact of this transition phase of oxidizing conditions depends mainly on the kinetics of the biotic reactions especially influenced by the extreme conditions imposed by the alkalinity of the concrete structure. Indeed, it is now perfectly supposed that microorganisms qualified as extremophiles due to their particular metabolisms are actually able to grow in the physico-chemical conditions specific to the storage of radioactive waste [14-15].

The objective of our work is **(i)** First to have a clear idea of the environmental conditions in terms of pH, (bio) availability of electron donors and acceptors, and salt concentrations found at the interface bitumen - concrete within the repository (reported in the paper of Bertron et al, NUCPERF 2012); **(ii)** By analysing the literature on extremophilic microbes, to identify a model bacterial strain that would have the properties required to grow under these conditions; **(iii)** To assess the behavior of the selected model strain in a simplified system, i.e. in a synthetic medium reproducing the conditions of pH, and concentrations of donors and acceptors of electrons, and soluble ions concentrations; **(iv)** And finally, to validate the hypothesis that this model bacterial strain can actually catalyse a denitrification reaction in conditions as close as possible the real conditions found at the bitumen-cement interface, i.e. in a solution containing solid matrices of bitumen and concrete. Bacterial growth in suspension as planktonic cells and also bacterial growth on surface ofsolid matrices as bacterial biofilms will be explored.

2 Materials and methods

2.1 Materials

2.1.1 Concrete

CEM V/A 42.5 (S-V) N CE PM-ES-CP1 cement pastes (Airvault Calcia factory) were made with a water/cement ratio of 0.40. They were cast in cylindrical plastic moulds 50 mm high and 27 mm in diameter without demoulding oil and were vibrated to evacuate air voids. The specimens were taken out of their moulds 24 hours after pouring and stored in water at 20°C for 28 days. They were then subjected to the leaching tests described below. In parallel, some control specimens were kept in water at 20°C.

2.1.2 Bitumen

Bitumen type Asalt 35-50 is packaged in a metal pot sealed and kept in the freezer. Fragments are shaped manually at room temperature. The average exchange surface of fragments is between 0.5 and 1.5 cm2.

2.2 Bacterial cultivation and bioreactors

2.2.1 Alkalophilic bacterial model

Halomonas desiderata DSM 9502 was obtained from the stains collection of DSMZ (Leibniz Institut DSMZ Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Germany)

2.2.2 Culture medium

The culture medium of *Halomonas desiderata*_DSM 9502 was prepared from solutions 1 and 2 as described in Table 1. These solutions should be sterilized separately by autoclaving and then mixed at room temperature (≈ 20-25 ° C). The final pH is adjusted to 9. to 10.0 at 30 ° C if necessary.

Table 1 : Composition of the culture medium for the growth of Halomonas desiderata DSM 9502

Solution 1		**Solution 2**	
Acetate	0,35 g	Na_2CO_3	5,40 g
$MgCl_2$, 6 H_2O	0,20 g	$NaHCO_3$	4,20 g
KH_2PO_4	1,00 g	water	100 mL
KNO_3	0.62 g		
water	900 mL		

2.2.3 Batch Bioreactors

Glass bioreactors containing 1L of culture medium, are kept hermetically sealed with plastic plugs and metal caps which ensure tightness. Inoculation of bioreactors was carried out with 1 mL of Halomonas desiderata preculture. An anaerobic atmosphere is created by degassing the culture medium with N_2 for 10 to 15 minutes. The bioreactors were then incubated at 30 ° C with shaking (150 rpm). Regular samples are performed using sterile needles and syringes for analytical monitoring: 1 mL was collected for immediate measurement of optical density at 600 nm and 2 mL

were collected, filtered to 0.2 µm in Eppendorf tubes, and then stored in a freezer at - 18 ° C for analyzes of ionic species.

2.3 Analytical methods

2.3.1 Ions analysis (Ca^{2+}, K^{+}, acetate, nitrate, nitrite)

Concentrations of anions (acetate, oxalate, nitrate and nitrite) and of cations (calcium and potassium) have been measured by High Performance Ion Chromatography coupled to conductimetric detector fitted with chemical suppressor (Dionex ICS-2000 and ICS-3000). Analytical conditions are summarized in Table 2.

Table 2. Conditions for HPIC analysis

	Eluant (1 mL/min)	**Precolumn**	**Chromatographic column**	**Suppressor**
Anions	KOH (1 10^{-3} mol/L) Elution gradient: 10% mobile phase to 60% mobile phase in 25 min.	NG1 (4 x 50 mm, Dionex) + IonPac AG11-HC (4 x 50 mm, Dionex)	IonPac AS11-HC (4 x 250 mm, Dionex)	ASRS 300 (4 mm, Dionex) + CRD 200 (4 mm, Dionex)
Cations	Methylsulfonic acid (30 10^{-3} mol/L) Isocratique (30 min)	NG1, (4 x 50 mm, Dionex) + IonPac CG16 (4 x 50 mm, Dionex)	IonPac CS16 (4 x 250 mm, Dionex)	Suppressor CSRS 300 (4 mm, Dionex)

Liquid samples were filtered at 0.2 µm (Minisart PES, Fisher Scientific) to remove suspended solid matter.

2.3.2 SEM analysis

Solid samples (i.e. cement and bitumen matrices) are post-treated before SEM observation following a specific method developped for the observation of biological samples. In a first step, the samples were fixed by immersion in a solution of 2% glutaraldehyde in 0.4 M phosphate buffer for 1 h. Then they were washed twice with the same buffer supplemented with 0.4 M sucrose. Finally, the samples were gradually dehydrated in acetone-water solutions before finishing with a solution of pure HMDS until total evaporation.
The SEM observation was performed on a “low vaccum” scanning electron microscope JEOL JSM 6380L (60 Pa, 15kV) equipped with an EDX detector (RONTEC Xflash 3001).

3 Results and discussion

Considering physico-chemical conditions determined in the bitumen-cement interface: pH≈10.6 / 0.50 mM acetic acid / 0.33mM nitrates (described in the paper of Bertron et al, NUCPERF 2012), we focused our attention to then identify a model microorganism capable of (i) growing in (hyper-) alkaliphilic conditions, (ii) using nitrate as electron acceptor and catalyzing its reduction to the step of nitrogen gas, and finally (iii) oxidizing simple organic substrates (electron donors) such as carboxylic acids with short aliphatic chains (acetate, butyrate, oxalate ...). The majority of identified denitrifying strains operates in a pH range between pH 6 and 10. Only 2 are known to reduce nitrate at more alkaline pH (pH 11-12): *Halomonas campisalis* DSM 15413 [16, 17] and *Halomonas desiderata* DSM 9502 [18]. The first one, *Halomonas campisalis*, is not only alkalophilic, it also has the distinction of being halophilic, i.e. it develops especially in salty environments (NaCl), which is outside our field of study. Our choice of model strain is then related to *Halomonas desiderata* DSM 9502.

3.1 Bacterial growth and denitrification kinetic in alkaline model media (pH 9.7)

As previously described by Berendes et al. [18], the optimum pH for the growth of *Halomonas desiderata (Hd)* DSM 9502 is 9.7. A first experiment was therefore to cultivate Hd at pH 9.7 in a mineral synthetic medium containing acetic acid and nitrates used respectively as sole sources of electron donor and acceptor. Acetic acid was provided at a concentration of 350 mg/L (5.8 mM) and nitrate was added in excess (620 mg/L or 10 mM).

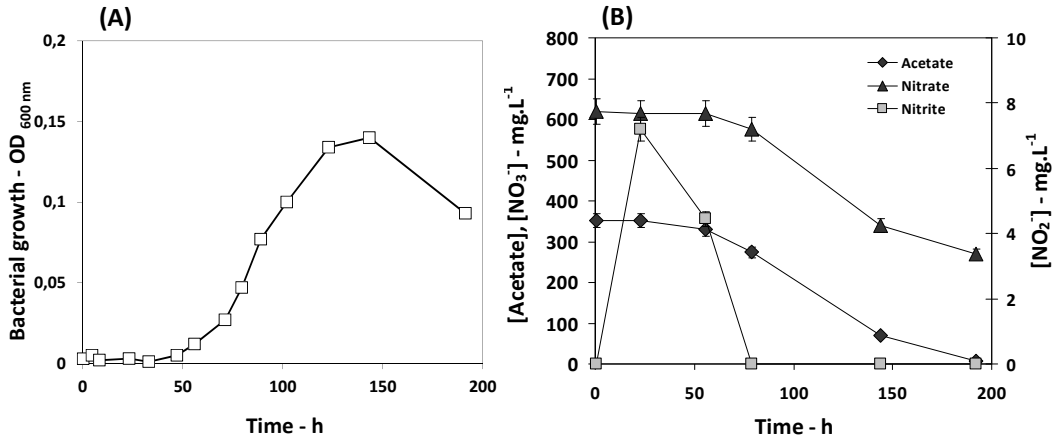


Figure 2: Bacterial growth of *Halomonas desiderata* (A) and analytical monitoring of species in solution during the bacterial denitrification reaction (B) in the presence of acetate (350 mg/L) and an excess of nitrate (620 mg / L) at pH 9.7. Note, an OD at 600 nm of 0.15 corresponds to an average of 5.5 10^7 cells/mL and a corresponding biomass of 230 mg of bacteria per liter (expressed in dry mass).

In figure 2.A, the bacterial growth in the presence of acetate and nitrate started after a lag phase of about 48 hours. This lag phase is the time required for the bacterial cells they adapt to new environmental constraints, particularly here by switching their metabolisms from oxygen respiration (aerobic pathway) to nitrate respiration (anaerobic pathway). During the first 48 hours, the concentrations of acetate and nitrate did not (apparently) evolve; only traces of nitrite were detected (Figure 2.B). This low production of nitrite was temporary; it irepresented up to 1.3% of initial nitrate (8 mg/L or 0.13 mM). It wais probably due to the enzymatic conversion of nitrate into nitrite via the nitrate reductase (nar) synthesized constitutively in *Hd* or induced by the presence of nitrates [19]. Nitrites disappeared (re-used) when bacterial growth was actually initiated (time> 50h).

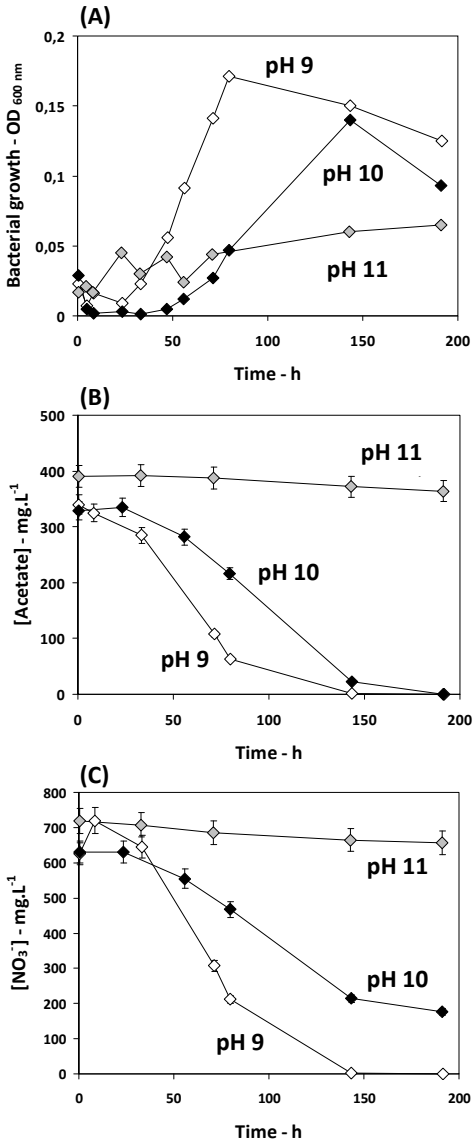
During the exponential growth phase, observed from 70 to 120 hours, the generation time or "doubling time" (g), i.e. the average time required for the cells of the bacterial culture, to double, was 0.36 hours. Meanwhile, the consumption profile of the acetate and nitrate was quite similar. Reaction rates were respectively 3.13 and 3.65 mg/L/h for acetate and nitrate, corresponding to a ratio acetate / nitrate of 0.85. Taking into account the biomass production, complete denitrification reaction forming nitrogen gas is as follows [20]:

$$0{,}819\ CH_3COOH + NO_3 \rightarrow 0{,}068\ C_5H_7O_2N + HCO_3 + 0{,}466\ N_2 + 0{,}301\ CO_2 + 0{,}902\ H_2O$$

The ratio acetate / nitrate determined experimentally (0.85) was quite close to the theoretical ratio (0.82) and confirmed the idea that almost all the nitrate were converted into nitrogen gas during the growth of *Hd*. Also, control experiments performed in the absence of nitrate in the medium under anaerobic conditions did not lead to any bacterial growth. The ammonium concentration measured with a colorimetric kit was negligible throughout the reaction. Therefore, denitrification by microbial respiration of nitrogen oxides (catalysed by the *Hd* strain) was the only possible and feasible way of nitrate reduction under the conditions of this study.

3.2 Effect of the pH

The pH at the interface between the solid matrices of bitumen and concrete was evaluated as being close to pH 10.6 - previously determined in experiments of leaching / release of solid matrices of CEM V cement paste (see Bertron et al., this conference).



To be as close as possible to the real conditions of the disposal, the bacterial growth of *Hd*, and consequently its denitrifying activity, were studied for 3 different pH around pH 10.6: pH 9, pH 10 and pH 11.

The growth of the *Hd* strain started after a lag phase that depends on the pH (Figure 3.A). More alkaline was the pH, the higher the latency period was long (24 hours at pH 9 to 72 hours at pH 11). Similarly, the kinetics of the microbial growth was strongly influenced by the pH. More the pH was alkaline more the growth kinetic of *Hd* was slow. There was almost a factor 2 between the growth kinetics of *Hd* at pH 9 and at pH 10 (the difference is 44% exactly). Although it was strongly inhibited at pH 11, the growth of *Hd* seemed possible in extreme conditions of such as low pH because a small increase in OD at 600 nm was observed on the 200h of the study.

Also, low consumption of substrates evaluated respectively at 0.25 mg / L / h and 0.36 mg / L / h for acetate and nitrate (or 4.1 10^{-3} mM / L / h and 5.8 10^{-3} mM / L / h), could be observed at pH 11(Figure 3. B and C).

Local bacterial development of *Hd* in areas where the pH is even more alkaline (higher pH vs. 10.6), i.e. very close to the vicinity of the cement matrix even in direct contact with it, is a serious track to investigate during longer campaigns of study (weeks to months).

Figure 3: Influence of the pH on the bacterial growth of *Halomonas desiderata* (A) and on the acetate (B) and nitrates (C) concentrations during the bacterial denitrification reaction.

3.3 Denitrifying activity in presence of solid matrices of bitumen and concrete

The growth of the alkalophilic denitrifying strain (*Hd*) and its kinetic of denitrification were investigated in the presence of both solid matrices of cement and bitumen, usually present in the storage device. The influence of additions of solid matrices into the reaction medium was tested individually (cement or bitumen added alone) or simultaneously (combination of two types of solid matrices). Specifically, experiments were carried out in a reaction medium consisting of distilled water, 25% mineral culture medium for denitrifying bacteria in the presence of acetate (300 mg / L or 5 mM) and nitrate (500 mg / L or 8 mM). The cement matrix was introduced into the reactor in the form of fragments of CEM V pastes coarsely crushed (15 g of solid per 100 ml of liquid medium). The bitumen was introduced in the form of solid granules (about 5 g/100 ml of the liquid medium). The initial pH at the start of the experiments, were 9.6 in the presence of bitumen alone, 10.5 in the presence of cement paste and 9.6 in the presence of cement + bitumen.

It was therefore impossible to actually follow the growth of bacteria at 600 nm, because a strong reaction disorder appears rapidly explained by af massive release of solid matrices. But we also found that this strong disorder reaction has no impact on the kinetics of bacterial denitrification. Indeed, the kinetic is the same in the presence of cementitious matrix alone and in the presence of both bituminous and cement matrices. By cons, microbial growth and therefore the denitrification kinetics are significantly slowed or inhibited in the presence of solid bitumen matrix alone. In the absence of cement matrix to maintain the pH of the reaction medium at an alkaline pH, the pH of the medium in the presence of bituminous matrix tends to become less alkaline (final pH close to 8.5). This would explain the acidification growth inhibition of *Hd*.

Kinetics of acetate consumption correlated well with the kinetics of nitrate reduction. Indeed, the oxidation of the acetate and the reduction of the nitrate took place simultaneously and the whole of these two substrates was consumed in less than 100 hours (except in the presence of bitumen alone). Small amounts of acetate and nitrate appeared at the end of experiment, after perhaps desorption of species associated with solid matrices at the start of the reaction. The pH changed during the reaction, it increased from 10.5 to 12.3 at the end of the experiment in the medium containing only cement matrix, from 9.6 to 12.5 for the solution containing the two solids, because of the release of hydroxide by the cementitious matrix over time.

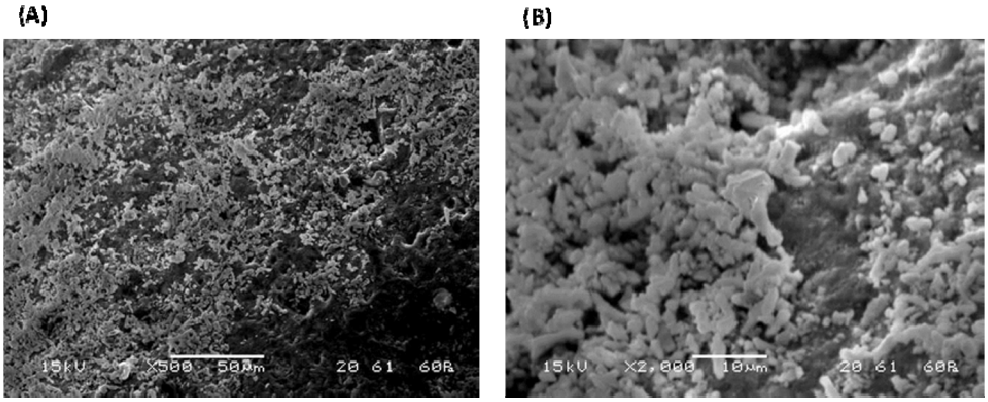


Figure 4: SEM observation of cement pastes exposed for 400 hours in a culture of a model alkalophilic and denitrifying bacteria: *Halomonas desiderata*.

Bituminous and cementitious matrices exposed to bacterial cultures of *Hd* for 400 hours were observed by SEM in order to search for possible bacterial deposits (or real biofilms). The observation of the surface of the cement paste revealed the presence of bacterial deposits in significant amounts (Figure 4). Conversely, matrices of bitumen were completely free of bacteria.

Acknowledgments

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