Halomonas desiderata as a bacterial model to predict the possible biological nitrate reduction in concrete cells of nuclear waste disposals

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A B S T R A C T

After closure of a waste disposal cell in a repository for radioactive waste, resaturation is likely to cause the release of soluble species contained in cement and bituminous matrices, such as ionic species (nitrates, sulfates, calcium and alkaline ions, etc.), organic matter (mainly organic acids), or gases (from steel containers and reinforced concrete structures as well as from radiolysis within the waste packages). However, in the presence of nitrates in the near-field of waste, the waste cell can initiate oxidative conditions leading to enhanced mobility of redox-sensitive radionuclides (RN). In biotic conditions and in the presence of organic matter and/or hydrogen as electron donors, nitrates may be microbiologically reduced, allowing a return to reducing conditions that promote the safety of storage. Our work aims to analyze the possible microbial reactivity of nitrates at the bitumen – concrete interface in conditions as close as possible to radioactive waste storage conditions in order (i) to evaluate the nitrate reaction kinetics; (ii) to identify the by-products (NO_2^-, $NH4^+$, N_2, N_2O, etc.); and (iii) to discriminate between the roles of planktonic bacteria and those adhering as a biofilm structure in the denitrifying activity.

Leaching experiments on solid matrices (bitumen and cement pastes) were first implemented to define the physicochemical conditions that microorganisms are likely to meet at the bitumen-concrete interface, e.g. highly alkaline pH conditions ($10 < pH < 11$) imposed by the cement matrix. 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 particular conditions of pH.

The denitrifying activity of *H. desiderata* was quantified in a 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 h) and 2–3 times slower in the presence of bituminous matrix (pH 9.7). The maximal rate of denitrification was approximately 0.063 mM h^{-1} and some traces of nitrite were detected for a few hours (<2%). Overall, the presence of solid cement promoted the kinetics of denitrification. The inspection of the solid surfaces at the end of the experiment revealed the presence of a biofilm of *H. desiderata* on the cement paste surface. These attached bacteria showed a comparable denitrifying activity to planktonic bacterial culture. However, no colonization of bitumen was observed either by SEM or by epifluorescence microscopy.

1. Introduction

The intermediate level, long-lived waste (MAVL) considered in this publication was a mixture of inorganic salts embedded in a bituminous matrix. The waste was the result of waste separation, for which the first step of the recycling procedure, known as PUREX (Plutonium–Uranium Extraction), is the dissolution of the fuel rods in a hot nitric acid medium followed by selective solvent extraction (Nikitenko et al., 2010). A variety of oxyanions are added during waste effluent treatment, in particular sulfate and nitrate in acid or salt form for the co-precipitation of radionuclides. To give some examples: addition of $Ti(SO_4)_2$ or $Ba(NO_3)_2$ triggers precipitation of

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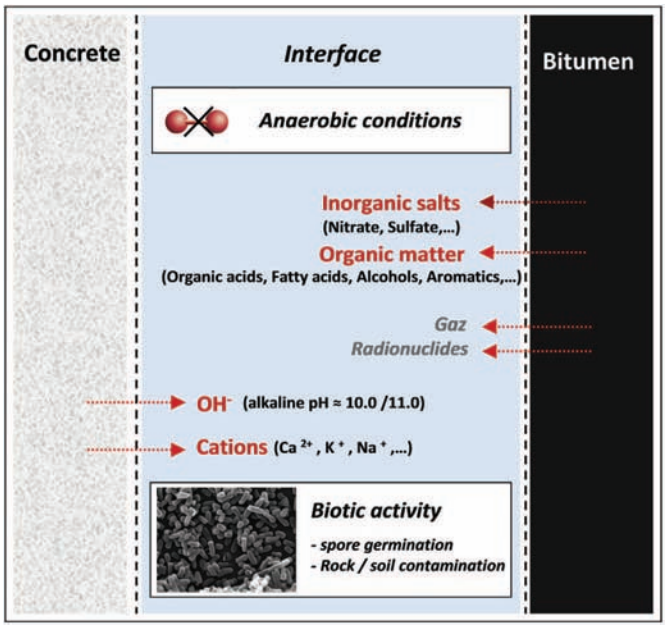

Fig. 1. Schematic representation of the physicochemical conditions at the concrete–bitumen interface in the disposal cells.

titanium ($Ti(OH)_4$) or barium ($BaSO_4$) respectively, the former along with Sb, the latter with Sr; addition of $CoSO_4$ induces precipitation of cobalt (CoS) together with Ru. Bituminization is used to stabilize the waste in metal packages which are grouped within steel-reinforced concrete overpacks. These are transferred to concrete-lined tunnels, called the waste cells, of a repository.

We focused our research on the transition zone between the bitumen and the concrete over-pack (Fig. 1), initially ignoring the presence of the steel of the primary waste container. After waste-cell closure, resaturation leads to the release of chemical species from the waste, especially soluble salts, including hydroxides, nitrates, organic matter (organic acids, phenols, etc.), gas and radionuclides (Van Loon and Kopajtic, 1990; Van Loon and Hummel, 1995; Libert and Walczak, 2000; Walczak et al., 2001).

The presence of nitrates in the vicinity of waste packages may result in oxidizing conditions favorable to the mobility of a series of radionuclides such as Se, U, Tc, Pu, Np. (Albrecht et al., 2012). However, because of the presence of reducing substances in the cell (zero-valent Fe, H_2, organic matter), different redox reactions could lead to nitrate reduction and thus re-establish reducing conditions favourable to storage safety. Reduction of nitrate (NO_3^-) may occur from surface catalysis provided by the different types of steels present in the cell, 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 (Devlin et al., 2000; Truche and Berger, 2010; Libert et al., 2011; Alquier et al., 2012; Libert et al., 2012; Truche et al., 2013). The length and impact of this oxidizing transition phase depends mainly on the kinetics of the biotic reactions, which are especially influenced by the harsh conditions imposed by the alkalinity of the concrete environment. Microorganisms known as extremophiles are likely to be able to grow in physicochemical conditions comparable to those in the waste cell, because of their particular metabolisms (Sarethy et al., 2011; Sorokin et al., 2012).

The objective of our work was (i) 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 bitumen-concrete interface within the repository, (ii) by analyzing 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. a synthetic medium reproducing the conditions of pH, concentrations of donors and acceptors of electrons, ion concentrations …, and (iv) to validate the hypothesis that this model bacterial strain can actually catalyze denitrification under conditions as close as possible to those found at the bitumen-cement interface, i.e. in a solution containing solid matrices of bitumen and concrete. Bacterial growth, both in suspension as planktonic cells and directly on solid matrices as bacterial biofilms, was explored.

2. Materials and methods

2.1. Bacterial strain, culture medium, and solid matrices

2.1.1. Alkalophilic bacterial model

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

2.1.2. Culture medium

The culture medium of *H. desiderata* DSM 9502 was prepared from solutions 1 and 2 as described in Table 2. These solutions were sterilized separately by autoclaving (121 °C – 20 min) and then mixed at room temperature (≈20–25° C) under aseptic atmosphere. The pH was adjusted to 9.0–11.0 at 30° C.

2.1.3. Concrete matrix

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 h 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. The external exchange surface of a concrete sample had an area of approximately 38 cm^2.

2.1.4. Bitumen matrix

Asalt 35-50 bitumen was packaged in a metal pot, sealed and kept in the freezer. Fragments were shaped manually at room temperature. The average exchange surface area of fragments was between 0.5 and 1.5 cm^2.

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

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

Table 2
Conditions for chemical analysis by High Performance Ionic Chromatography (HPIC).

Solution 1		Solution 2	
Acetate	0.35 g	Na_2CO_3	5.40 g
$MgCl_2$, $6H_2O$	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. Leaching experiments

2.2.1. Reactor

The reactor, filled with 1 L demineralized water, notably comprised an outlet for solution sampling during leaching cycles, a gas inlet with check valve for N2 bubbling to impose anaerobic conditions inside the reactor (conditions that would prevail inside the cell), a gastight tap for a pH probe and a hermetically closed lid fitted with a gas vent. The pH probe was connected to a data acquisition system (Consort, D230 Data Acquisition System, v1.1.13). The solution in the reactor was continuously agitated using a magnetic barrel. The cement solid matrix was suspended in the solution by a PTFE thread. The solid/liquid volume ratio was about 3%. The experimental device was kept in an air-conditioned room during the whole experiment.

2.2.2. Method

Leaching solutions were renewed daily for 5 days. During the first day of exposure, 4 solution samples (20–25 mL each) were collected. The solid/liquid ratio was not modified much. On days 2–5, only one daily liquid sample was taken from the reactor, just before the solution was renewed. Concentrations of Ca^{2+}, K^+, acetate, nitrate, and nitrite were measured on each liquid sample.

2.3. Measurement of bacterial denitrifying activity

2.3.1. Batch bioreactors

Glass bioreactors containing 1 L of culture medium were kept hermetically sealed with plastic plugs and metal caps. The bioreactors were inoculated with 1 mL of *H. desiderata* preculture (active growing culture, optical density of 0.3 at 600 nm). An anaerobic atmosphere was created by degassing the culture medium with N_2 for 10–15 min. The bioreactors were then incubated at 30 °C with shaking (150 rpm). Samples were taken regularly using sterile needles and syringes for analytical monitoring: 1 mL was collected for immediate measurement of optical density at 600 nm and 2 mL was collected, filtered to 0.2 μm in Eppendorf tubes, and then stored in a freezer at −18 °C for analyses of ionic species.

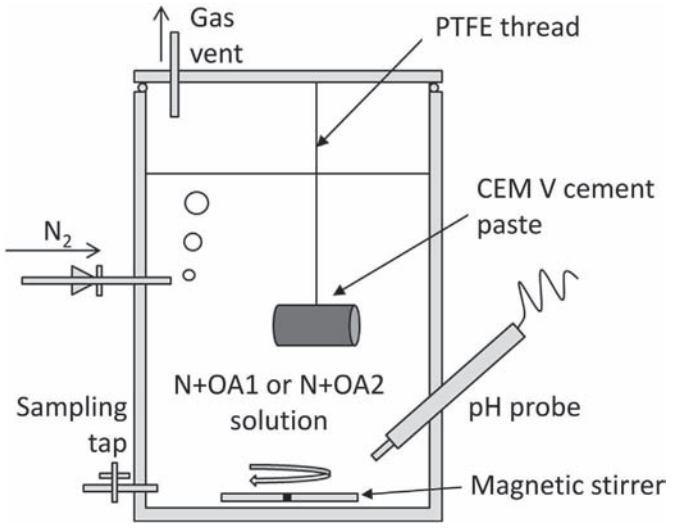

Fig. 2. Experimental set-up of the leaching experiment described in (Bertron et al., 2013) for determining evolution of ions concentrations and pH into leachates solutions.

2.3.2. Experiments in bacterial growth medium

As previously described by Berendes et al. (1996), the optimum pH for the growth of *Hd* is 9.7. A first experiment therefore cultivated *Hd* at pH 9.7 in a minimal synthetic medium (Table 2) containing acetic acid and nitrates used as sole sources of electron donors and acceptors respectively. Acetate was provided at a concentration of 350 mg/L (6 mM) and nitrates were added in excess compared to acetate (based on the stoichiometric Equation (1) of the denitrification reaction) (620 mg/L, 10 mM). *Hd* growth was measured using optical density at 600 nm and the concentrations of acetate, nitrate and nitrite were monitored regularly over time.

2.3.3. Experiments in presence of solid matrices

Experiments were carried out in a liquid medium containing 75% distilled water and 25% mineral culture medium for denitrifying bacteria in the presence of acetate (300 mg/L or 5.5 mM) and nitrate (500 mg/L or 8.6 mM). The cement matrix was introduced into the reactor in the form of coarsely crushed fragments of CEM V pastes (15 g of solid per 100 mL of liquid medium). The bitumen was introduced in the form of solid granules (about 5 g of solid per 100 mL of the liquid medium). The pH at the start of the experiments was 9.6 in the presence of bitumen alone, 10.5 in the presence of cement paste and 9.6 in the presence of both cement and bitumen. The pH was not adjusted or buffered. Only release phenomena or reactions could lead to a pH change.

2.4. Chemical analysis (Ca^{2+}, K^+, acetate, oxalate nitrate, nitrite)

Concentrations of anions (acetate, oxalate, nitrate and nitrite) and of cations (calcium and potassium) were measured by High Performance Ion Chromatography coupled to a conductimetric detector fitted with a chemical suppressor (Dionex ICS-2000 and ICS-3000). The analytical conditions are summarized in Table 1.

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

2.5. SEM analysis

Solid samples (i.e. cement and bitumen matrices) were post-treated before SEM observation following a specific method developed 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 vacuum” scanning electron microscope JEOL JSM 6380L (60 Pa, 15 kV) equipped with an EDX detector (RONTEC Xflash 3001).

3. Specification of physicochemical conditions at the bitumen-cement interface

Uncertainties remain regarding the release of organic and inorganic matter by bitumen–salt mixtures. Experimental studies have shown that the degradation of bitumen causes the release of

Table 3
Excerpt of the inventory of the literature on alkaliphilic heterotrophic bacteria capable of reducing nitrate.

Strains	References	Isolated from	Nitrate reduction	pH and temperature	Aérobic/anaérobic	Other information
Virgibacillus (Bacillus) halodenitrificans DSM 10037	Denariaz et al., 1989	Marine solar saltern	Incomplete: production of gas (N2O)	5.8 < pH < 9.6 pH_{opt} = 7.4	Facultatively anaerobic	no inhibition at high nitrate concentration (65 g/l)
Pseudomonas stutzeri DSM 5190	Van Niel et al., 1952	Soil, mud, standing water	Complete (N_2)	pH_{opt}~7 pH_{max}. 9 T_{opt} ~ 35 °C	Facultatively anaerobic	
Halomonas denitrificans DSM 18045	Kim et al., 2007	Seawater	Complete (N_2)	7 < pH < 10 pH_{opt} = 8 to 9 5 °C < T < 50 °C T_{opt} 25–35 °C	/	Halotolerant 8–10% NaCl
Halomonas alcaliphila DSM 16354	Romano et al., 2006	Salty pool (Italy)	Incomplete (NO_2^-)	7.5 < pH < 10 pH_{opt} = 9 5 °C < T < 50 °C T_{opt} 37 °C	Anaerobic	Halotolerant 10% NaCl
Halomonas campisalis DSM 15413	Mormile et al., 1999 Peyton et al., 2001	Salty soil	Complete (N_2)	6 < pH < 12 pH_{opt} = 9.5 4 °C < T < 50 °C T_{opt} 30 °C	Facultatively anaerobic	Halotolerant
Halomonas desiderata DSM 9502	Berendes et al., 1996	Municipal water treatment plant	Complete (N_2)	9 < pH_{opt} < 11 T_{opt} 30 °C	Facultatively anaerobic	Halotolerant

organic substances (naphthalene, alcohols, linear carboxylic acids, aromatics and glycols) and salts ($NaNO_3$, Na_2SO_4, etc.) (Kagawa et al., 2000; Walczak, 2000; Nakayama et al., 2003; Marien et al., 2008). On the basis of this literature data, bitumen release was simulated by aqueous solutions made of acetate and nitrates. Acetate was considered (i) since it is easily assimilated by bacteria and (ii) because of its low interaction with cement phase (Bertron et al., 2005a, 2005b, 2007; Larreur-Cayol et al., 2011).

Experiments on the leaching of concrete solid matrices were then conducted as described by Bertron et al. (2013) using a model aqueous solution theoretically simulating a bitumen leachate (0.50 mM acetate/0.33 mM nitrates). Several successive batches corresponding to 24 h of contact between a solid matrix of cement and the model solution were used. Changes in the pH and the concentrations of cations and anions (acetate, nitrate, nitrite, OH^-, K^+, Ca^{2+}, $NH4^+$) were followed.

The variations of pH were similar over the five 24 h-cycles: from the initial value of 4.0, pH increased rapidly during the first 6 h of leaching, to about 9.5, then slowed down and reached 10.6 after 24 h (Fig. 2). Alkaline conditions were thus very rapidly imposed in the leaching medium. The increase in pH was very probably due to the release of hydroxide ions by the cement paste matrix because of dissolution of the cementitious phase.

Concentrations of K^+ and Ca^{2+} varied in the same way. During the first leaching cycle, variations followed those of pH and concentrations reached $[Ca^{2+}] \approx 0.25$ mmol/L and $[K^+] \approx 0.15$ mmol/L. On leaching days 2–5, concentrations at the end of each cycle decreased progressively to reach $[Ca^{2+}] \approx 0.25$ mmol/L and $[K^+] \approx 0.09$ mmol/L at the end of the 5th leaching cycle. These variations are typical of the leaching of a cementitious matrix.

Concentrations of nitrates were stable with to time (data not presented) and were equivalent to the initial quantity (32.3 mM). Neither nitrites ($NO2^-$) nor ammonium ($NH4^+$), produced by abiotic reduction of nitrates under anoxic conditions, were detected. The concentration of acetate was almost constant, at the initial value of 0.50 mM.

4. *H. desiderata*: an alkalophilic nitrate reducing bacterium

Microbial denitrification, in the particular context of the disposal of radioactive waste as defined in Fig. 1, has so far been a matter of mere speculation. No denitrifying microorganism has yet been isolated from samples in situ. But, theoretically, many microorganisms are known to produce such denitrification under conditions of high pH (in the range of pH 9–11). The first step was therefore to identify a model microorganism capable of (i) growing in alkaline conditions, (ii) using nitrate as electron acceptor and catalyzing its reduction to the step of nitrogen gas, and (iii) oxidizing simple organic substrates (electron donors) such as carboxylic acids with short aliphatic chains (acetate, butyrate, oxalate, etc.).

A literature review of the denitrifying microbial species highlighted 6 bacterial strains capable of working in the conditions defined (Table 3). The majority of denitrifying strains identified operate in a pH range between 6 and 10. Only 2 are known to reduce nitrate at more alkaline pH (up to 11): *Halomonas campisalis* DSM 15413 and *H. desiderata* DSM 9502. The first one, *H. campisalis*, is obligatory halophilic, i.e. it can develop only in salty (NaCl) environments, which is outside our field of study. Our choice of model strain thus turned to *H. desiderata* DSM 9502.

According to Berendes et al. (1996), *H. desiderata* DSM 9502 is a rod shaped cell 0.4–0.6 μm wide × 1.0–2.6 μm long, which is Gram-negative and motile by peritrichous flagella. Growth occurs under aerobic conditions but may be facultatively anaerobic in the presence of nitrate. It is obligately alkaliphilic (optimum pH 9.7) and possibly halotolerant (growth occurring between 0 and 18% NaCl). Growth is possible in the temperature range 10–45 °C.

5. Denitrifying activity of *H. desiderata (Hd)* DSM 9502 in alkaline model media

The growth kinetics of *Hd* and its heterotrophic denitrifying activity were first evaluated in a synthetic growth medium approaching the environmental conditions already actually

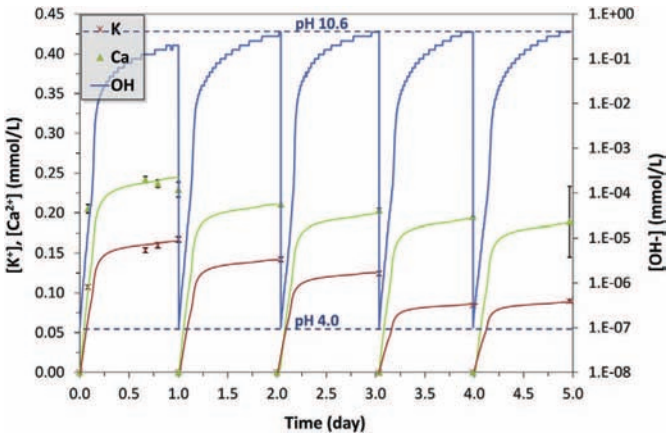

Fig. 3. Evolution of concentrations of K^+, Ca^{2+} and OH^- in 0.50 mM acetate/0.32 mM nitrates leaching solution determined by HPIC analysis. The solution was renewed daily. Solid area/liquid volume ≈ 50 cm^2/L. Concentration of OH^- was calculated from the pH values.

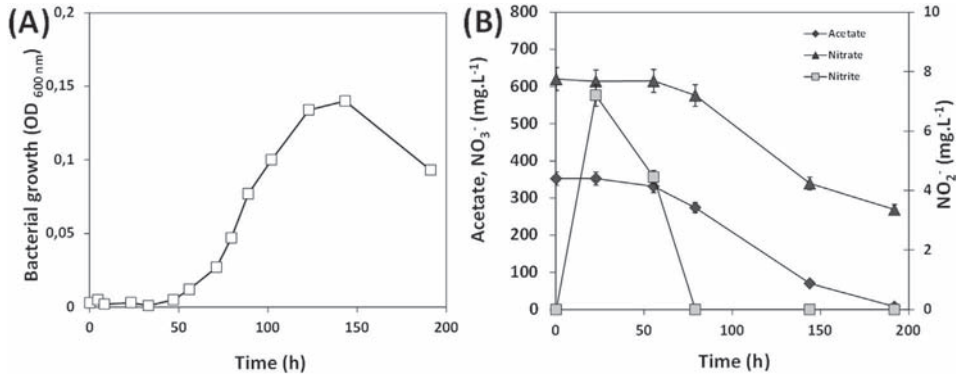

Fig. 4. Bacterial growth of *Halomonas desiderata* (A) and analytical monitoring of species in solution during the bacterial denitrification reaction (B) in the presence of acetate (350 mg/L) and an excess of nitrate (620 mg/L) at pH 9.7. Note, an OD of 0.15 at 600 nm corresponds to an average of 5.5×10^7 cells/mL and a quantity of biomass equivalent to 230 mg of bacteria per liter (given here in dry weight).

evaluated at the interface of bituminous and cement matrices. Acetate was used as the electron donor for *Hd* at various ratios from limiting to excess. Then, the influence of pH was investigated on the ability of *Hd* both to multiply (biomass growth) and to reduce nitrate (kinetics of denitrification).

5.1. Bacterial growth and denitrification kinetics at optimal pH (pH 9.7)

Fig. 4A shows that the growth of *Hd* in the presence of acetate and nitrate started after a lag phase of about 48 h. This latency was the time required for the bacterial cells to adapt to new environmental constraints, particularly by switching their metabolism from oxygen respiration (aerobic pathway) to nitrate respiration (anaerobic pathway) here. *Hd* has a respiratory metabolism, and oxygen is the terminal electron acceptor. However, this strain can use nitrate as an alternative electron acceptor and can carry out oxygen-repressible denitrification. Denitrification may appear only under (semi) anaerobic conditions, i.e. an oxygen-free environment as can occur in the waste cell after closure.

During the first 48 h, the concentrations of acetate and nitrate did not evolve significantly (Fig. 4B) but traces of nitrite did indicate nitrate reduction, thus some microbial activity. The presence of nitrite was temporary, with a maximum of 1.3% of the initial concentration of nitrate (8 mg/L or 0.14 mM). As no surface catalysis was expected in our system, the transitory presence of nitrite indicated enzymatic conversion of nitrate to nitrite via nitrate reductase (Nar), synthesized constitutively in *Hd* or induced by the presence of nitrates (Zhao et al., 2012). Nitrite cannot accumulate when nitrite reduction kinetics becomes faster that nitrate reduction kinetics, as seemed to be the case when bacterial growth started to become measurable (time $>$ 50 h).

During the exponential growth phase, observed from 70 to 120 h (Fig. 4A), the generation time or "doubling time" (g), i.e. the average time required for the bacterial cells of the culture to double in number, was 0.36 h. During this time, the consumption profiles of the acetate and nitrate were quite similar. Consumption rates were respectively 3.13 and 3.65 mg/L/h (corresponding to 0.058 and 0.063 mM/L/h) for acetate and nitrate, i.e. an acetate/nitrate molar ratio of 0.90.

Considering only the stoichiometric reaction of acetate oxidation and denitrification (complete nitrate reduction leading to the formation of N_2 gas):

$$5CH_3COO^- + 8NO_3^- + 13\ H^+ \rightarrow 4N_2 + 10CO_2 + 14H_2O \quad (1)$$

The theoretical acetate/nitrate molar ratio in this case is only 0.63, which would lead to the denitrification of 496 mg/L of nitrate (8.55 mM) as nitrogen gas in the presence of 300 mg/L of acetate (5.17 mM). However, during the exponential growth phase of bacteria, cell multiplication must be taken into account: the bacteria have basic nutritional needs (C, H, O, N mainly S and P in defined quantities). Carbon is the main constituent of particular cellular material and represents 50% of the dry weight of a cell (empirical formula of biomass represented by $C_5H_7O_2N$). Acetate is the sole carbon source in the culture medium, so a significant fraction should be used for cell growth and maintenance. Taking biomass production into account, the complete denitrification reaction forming nitrogen gas is as follows (Mateju et al., 1992):

$$0.82CH_3COOH + NO_3 \rightarrow 0.07C_5H_7O_2N + HCO_3 + 0.47N_2 + 0.30CO_2 + 0.90H_2O \quad (2)$$

The acetate/nitrate ratio determined experimentally (0.90) was then quite close to the theoretical ratio (0.82) when bacterial growth of *Hd* was considered and confirmed the idea that almost all the nitrate was converted to the step of nitrogen gas, without any nitrite accumulation. Control experiment performed in the absence of nitrate in the medium under anaerobic conditions did not lead to any bacterial growth, proving that no trace of oxygen was used as an electron acceptor by *Halomonas* cells. Also, no nitrate reduction either to nitrogen gas or to ammonium was verified when *H. desiderata* was not inoculated. Obviously, the ammonium concentration (measured with a colorimetric kit) was negligible throughout the reaction time ($<$0.5 mg/L) with or without bacterial the presence of bacterial cells. In conclusion, denitrification by microbial respiration of nitrogen oxides catalyzed by the *Hd* strain was the only possible and feasible means of nitrate reduction under the conditions of this study.

Here, at a pH of 9.7, 200 h were required to reduce about 300 mg/L of nitrate. In comparison, at a pH close to neutral, a denitrifying strain such as *Pseudomonas stutzeri* is capable of reducing the same amount of nitrate in only 10 h (Van Niel et al., 1952; Alquier et al., 2012).

5.2. Influence of the pH

The pH at the interface between the solid matrices of bitumen and concrete was evaluated as being more than pH 10.6 - previously determined in experiments on leaching/release from solid matrices of CEM V cement paste (Bertron et al., 2013). To come as close as possible to the real conditions of the disposal facility, the bacterial growth of *Hd*, and consequently its denitrifying activity, were studied for 3 different pH values around 10.6: pH 9, pH 10 and pH 11.

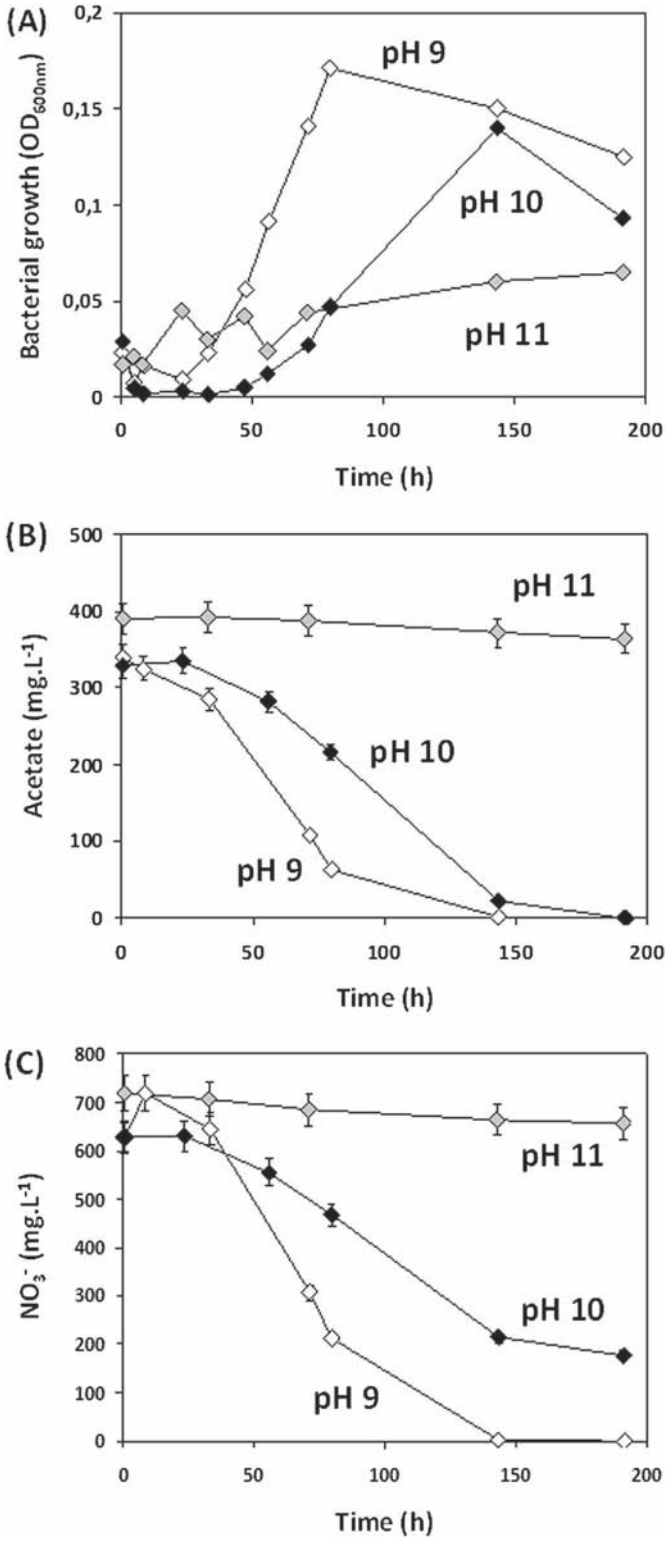

Fig. 5. Influence of pH on bacterial growth of *Halomonas desiderata* (A) and on the evolution of the acetate (B) and nitrate (C) concentrations during the bacterial denitrification reaction.

The growth of the *Hd* strain started after a lag phase that depended on the pH (Fig. 5A). The more alkaline the pH was, the longer was the latency period (24 h at pH 9—72 h at pH 11). Similarly, the kinetics of the microbial growth was strongly influenced by the pH. The more alkaline the pH was, the slower was the growth kinetics of *Hd*. There was a factor of almost 2 between the growth kinetics of *Hd* at pH 9 and at pH 10 (to be exact, the difference was 44%). Although it was inhibited at pH 11, the growth of *Hd* seemed possible in such extreme conditions of low pH as a small increase in OD at 600 nm was observed over the 200 h of the study.

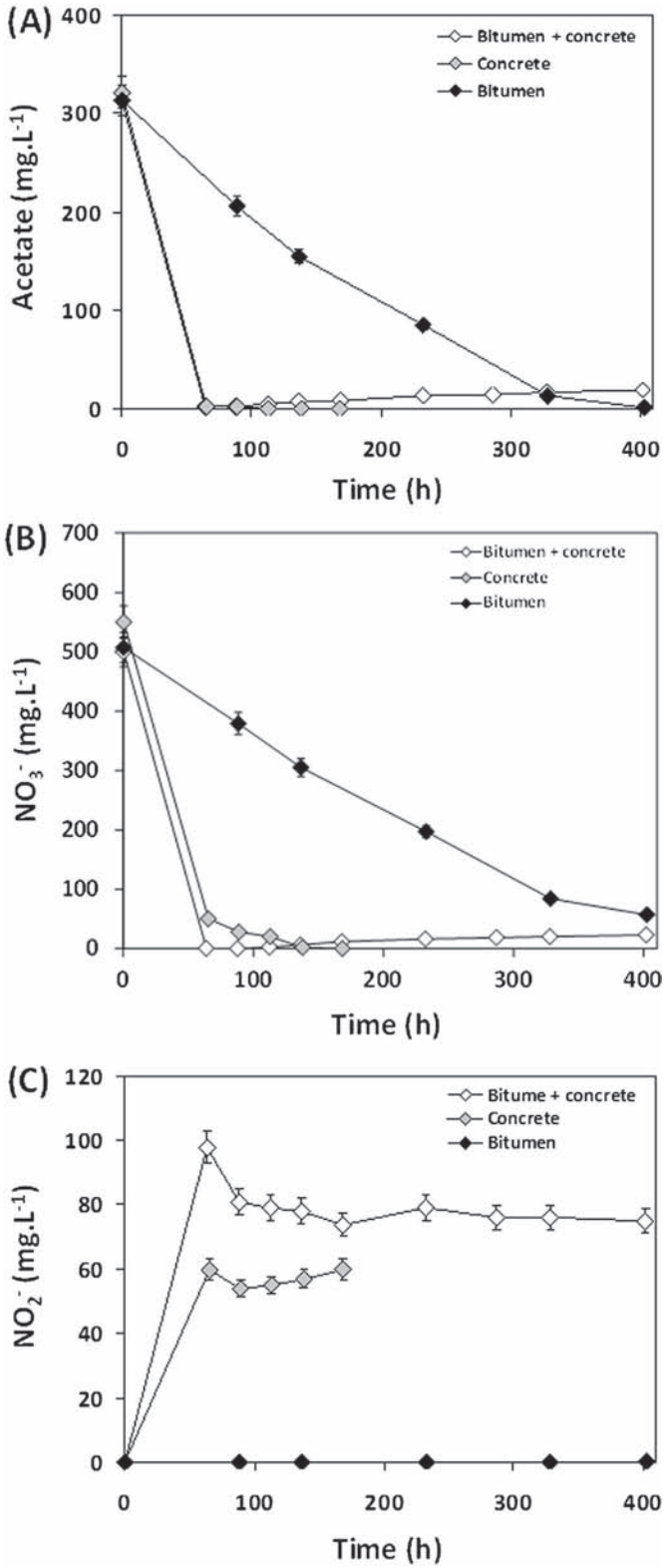

Fig. 6. Evolution of acetate (A), nitrates (B) and nitrite (C) concentrations during the bacterial growth of *H. desiderata* in the presence of solid matrices of bitumen and/or cement.

Also, low consumptions of substrates (both acetate and nitrate) evaluated at 2.5 mg/L/h for acetate and 3.6 mg/L/h for nitrate (or 0.046 mM/L/h and 0.062 mM/L/h), could be observed at pH 11 (Fig. 5B and C).

Comparable research related to the effect of pH on denitrification has focused on denitrification rates in soils. Several studies have established that denitrification rates tend to decrease at low soil pH values (Muller et al., 1980; Parkin et al., 1985; Simek et al.,

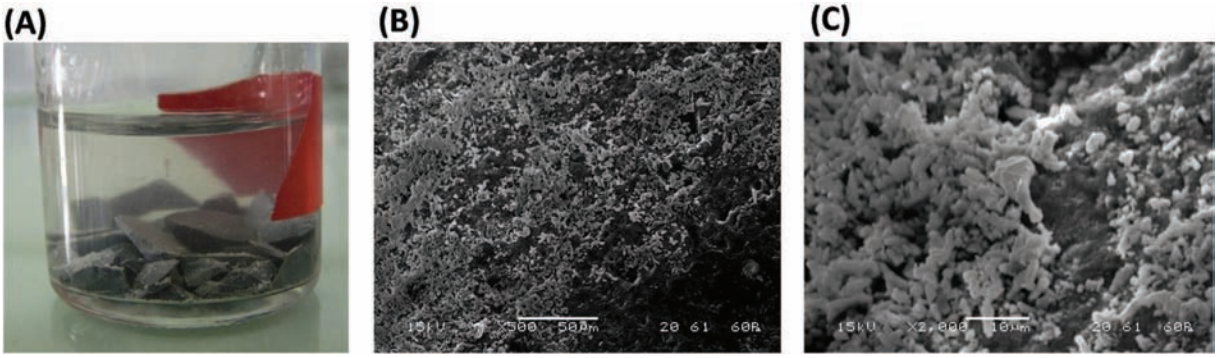

Fig. 7. Solid cement matrix exposed for 400 h in a culture model of alkalophilic bacteria and denitrifying: *Halomonas desiderata* (*Hd*). (A) Macroscopic observation of bacterial deposition, (B) and (C) low vacuum SEM observation of the surface of cement paste colonized by *Hd*.

2002). In contrast, the kinetics of heterotrophic bacterial denitrification slows markedly at alkaline pH. Increasing the pH directly affects the bacterial growth and enzymatic activities (Campos and Flotats, 2003), including denitrifying enzymes. Nitrite reduction rates are then inhibited by the presence of nitrate, which stimulates nitrite accumulation during the denitrification process.

6. Denitrifying activity of *H. desiderata* DSM 9502 in the presence of both bitumen and concrete solid matrices

The growth of the alkalophilic denitrifying strain (*Hd*) and its kinetics of denitrification were investigated in the presence of solid matrices of both cement and bitumen, which are usually present in the storage facilities. The influence of solid matrices in the reaction medium was tested, first individually (cement or bitumen added alone), then simultaneously (combination of two types of solid matrices).

6.1. Denitrification kinetics

Adding the solid matrix of concrete into the reaction medium rapidly induced the appearance of inorganic suspended materials. It was consequently not possible to follow the growth of bacteria using optical density in the samples where the medium was in contact with solid concrete. Measurements of denitrification kinetics (and associated mechanisms) were based primarily on changes in the concentrations of acetate and nitrate. However, the precipitates observed in the presence of cementitious matrix did not affect the assay methods used.

In the presence of cementitious matrix alone and in the presence of both bituminous and cement matrices, the oxidation of the acetate and the reduction of the nitrate took place simultaneously and these two substrates were completely consumed in less than 100 h (Fig. 6A and B). The reaction rates of denitrification were about 7.7 mg/L/h (0.13 mM/h), i.e. twice as fast as in the classical experiment in mineral culture medium at pH 9.7 (Fig. 5C). The corresponding rate of acetate oxidation was 4.6 mg/L/h (0.085 mM/h), giving an acetate/nitrate molar ratio of 0.6. This molar ratio corresponded to complete nitrate reduction leading to the formation of N_2 gas without any bacterial growth. But nitrite accumulation in the range of 15% of its initial concentration was also highlighted in the presence of concrete (Fig. 6C). The reduction reaction of nitrate to nitrite involves only two electrons and therefore the corresponding acetate/nitrate molar ratio is only 0.25 in this case. It is suspected that the apparent molar ratio of 0.6 is actually a mix of the complete denitrification to nitrogen gas (N_2) coupled with bacterial growth and microbial reduction of nitrate to the step of nitrite caused by the high alkalization of the bacterial cells' environment.

$$CH_3COO^- + 4NO_3^- + 5H_2O + 8H^+ \rightarrow 2CO_2 + 4NO_2^- + 7H_3O^+ \quad (3)$$

The presence of solid cement matrix has a positive effect on the kinetics of denitrification. Unfortunately, we were unable to determine whether faster kinetics were explained by a higher microbial growth rate or by a stimulation of the enzymatic denitrifying activity. As expected, 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, and 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 (see Fig. 3). However, we had seen previously that, with only the mineral culture medium, both the growth of *Hd* in the classical form of a cell suspension, and the denitrifying activity were inhibited starting from pH 11. However, the sole presence of the solid matrix of cement completely changed the behaviour of *Hd* in a range of pH as alkaline as $10 < pH < 12$. Several hypotheses can be advanced to explain how *Hd* is able to maintain its denitrifying activity in an environment with progressively increasing alkalinity:

- The cement matrix releases minerals essential to the growth of Hd, such as vitamins and metallic elements. Growth rates are much faster in this case and the impact on the kinetics of denitrification is justified by the significantly greater amount of biomass.
- The cement matrix is a support conducive to the proliferation of Hd with a "biofilm" phenotype. This means the bacterial cells in contact with the solid matrix organize themselves to create a three-dimensional architecture in which the biological microenvironment is regulated and can differ markedly from the planktonic phase (Costerton et al., 1994). Bacterial cells inside the biofilm consequently have distinct physiological requirements and so distinct properties (or activities).

In the absence of cement matrix to buffer alkaline pH, the pH of the medium in the presence of only the bituminous matrix tends to become less alkaline (final pH close to 8.5 after 400 min). As a result, the denitrification kinetics are significantly slowed: values of about 0.95 mg nitrate/L/H were found instead of the 3.65 mg/L/h classically observed at the optimal pH of 9.7.

6.2. Observation of the surface of solid matrices

In the bioreactors, the visual aspect of the bitumen surface did not seem to change with time, and was still black, opaque, and

glassy after 400 h of contact with the bacterial cell suspension. In contrast, a white deposit was clearly visible on the surface of the concrete matrix (Fig. 7A). Bituminous and cementitious matrices exposed to bacterial cultures of *Hd* for 400 h were carefully observed by SEM in order to search for possible bacterial deposits (or real biofilms) at the micro-scale. The SEM observation of matrices of bitumen confirmed that they were completely free of bacteria, probably because the bitumen surface is hydrophobic and acidic and because it is slightly soft at room temperature. As expected, SEM observations confirmed the presence of bacteria on the surface of concrete samples (Fig. 7B and C). Several bacterial carpets were observed. They were not very thick – up to 10 μm, which corresponds to a stack of 7 or 8 bacteria. Complementary observations by epifluorescence microscopy after staining the concrete surface with specific fluorescent markers of bacterial nucleic acids also confirmed that these observed deposits were of biological origin (data not presented).

Additional experiments directly using the matrices of colonized concrete as a source of microorganisms to inoculate bioreactors clearly showed that the adherent cells were alive and that the denitrifying activity itself was also very active. It was difficult to make precise measurements of the amount of biomass actually present on the matrices and to determine the specific activity of he attached denitrifying microorganisms. Additional tests based on measurements of ATP should allow us to obtain a more precise idea of the residual microbial activity of the fixed biomass. The available data on bacterial colonization and biofilm formation on concrete are limited because it was long believed that the high alkalinity of concrete (pH $>$ 12) completely inhibited colonization of the concrete surface by bacteria. However, by considering that the carbonation of concrete is accompanied by a decrease in surface pH (pH $\approx$ 9.5), several types of microorganisms have now been identified from the surface of cementitious materials, especially microorganisms implicated in the bioalteration of concretes, such as sulfur-oxidizing bacteria, whose metabolism leads to the formation of acids (Roux et al., 2007; Jensen et al., 2011). More generally, it has been known since the early 2010s that the concrete biofilms can hold very different phylogenetic community structures, such as archaea, fungi, and several bacterial groups (Proteobacteria and Bacteroidetes are among the most dominant bacterial groups), which are also found in most environmental biofilms (Iker et al., 2010). However, nothing is yet known of the physiology of these organisms when they are attached to the surface of concrete.

7. Conclusion

The release of hydroxide ions by the cementitious matrix quickly changes the pH of a solution containing small amounts of acetate (0.5 mM) to highly alkaline values (pH $\approx$ 10.6). No significant reactions (adsorption, complexing, reduction…) have been found between the cement matrix and the nitrates on the one hand, and acetate on the other. *H. desiderata* DSM 9502 was able to grow in such extreme pH conditions using acetate as the electron donor and nitrate as electron acceptor. Its optimal pH was between 9.0 and 10.0 in mineral growth liquid medium, but its denitrifying activity was also possible at higher pH, especially in presence of concrete. *H. desiderata* has the ability to grow not only in planktonic form (i.e. in suspension) in the liquid phase but also as a biofilm on the surface of the concrete, where the local pH would be much higher than pH 10.0. The denitrifying activity was still active inside the biofilms of *H. desiderata* on concrete. *H. desiderata* was not, a priori, able to colonize the surface of the solid bitumen, probably because the local pH was more acidic and the surface was highly hydrophobic.

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