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**To link to this article** : DOI:10.1016/j.ibiod.2015.03.013  
URL : <http://dx.doi.org/10.1016/j.ibiod.2015.03.013>

**To cite this version :**

Rafrafi, Yan and Ranaivomanana, Harifidy and Bertron, Alexandra and Albrecht, Achim and Erable, Benjamin *Surface and bacterial reduction of nitrate at alkaline pH: Conditions comparable to a nuclear waste repository*. (2015) *International Biodeterioration & Biodegradation*, vol. 101. pp. 12-22. ISSN 0964-8305

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# Surface and bacterial reduction of nitrate at alkaline pH: Conditions comparable to a nuclear waste repository

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## A B S T R A C T

This study investigates the reactivity of nitrates in abiotic and biotic conditions at alkaline pH in the context of a repository for long-lived intermediate-level radioactive waste. The work, carried out under environmental conditions comparable to those prevailing in the repository: alkaline pH, no oxygen, solid materials (cementitious material, steel), aims to identify the by-products of the nitrate reduction and to evaluate reaction kinetics of these reactions with and without the presence of denitrifying microorganisms. This paper demonstrates that, even at the high pH characteristic of nuclear waste repositories, nitrate reduction may be a likely scenario, with biotic catalysis in the presence of microorganisms and surface catalysis in the presence of steel and/or corrosion products.

## Keywords:

Alkaline conditions  
Nitrate reduction  
Concrete cells  
Nuclear waste repositories  
*Halomonas desiderata*  
Biofilms

## Introduction

Before being placed in a repository, a significant fraction of long-lived intermediate-level radioactive waste (LLILW) is first stabilized in a bitumen matrix and is further cast in cylindrical steel containers (primary packages). These containers are then placed in reinforced concrete packages (secondary package) that will eventually be stored in concrete cells (engineered barrier) built in a host rock (Callovo-Oxfordian or COx) constituting the geological barrier (Andra, 2005). After several thousand years, driven by the combined effects of water resaturation and dissolution, bitumen and concrete are likely to release chemical species, particularly soluble salts including, hydroxides, nitrates, organic matter (organic acids, phenols, ...), and gas (i.e. H<sub>2</sub>) (Walczak et al., 2001).

The reactivity of nitrates (NO<sub>3</sub><sup>-</sup>) released from bitumen matrices inside nuclear waste repository cells is not well identified or understood, with or without microbial activity. However, the fate of nitrates has direct consequences on the repository safety (Albrecht et al., 2013) as nitrates can create or stabilize existing oxidizing

conditions, which promote the mobility of some radionuclides (Se, U, Tc, Pu, Np, ...). Nevertheless, in the presence of electron donors (organic matter, H<sub>2</sub>) different redox reactions will probably lead to nitrate reduction and return the system to a reducing state. This reduction of nitrate can occur via solid surface catalysis provided by different types of reactive steel present in the repository (Devlin et al., 2000; Truche et al., 2013a,b) and/or via biological catalysis through denitrifying bacterial metabolism (Alquier et al., 2014). Several electron donors are present in the repository environment (forms of waste, in the containers or in the host rock, e.g. organic acids released by bitumen matrices, H<sub>2</sub>, zerovalent metals etc.). Nitrate reduction can lead to the formation of nitrite (NO<sub>2</sub><sup>-</sup>), nitrogenous intermediate species (NO and N<sub>2</sub>O), gaseous nitrogen (N<sub>2</sub>) and/or ammonium (NH<sub>4</sub><sup>+</sup>). The reaction pathways and kinetics are not well known, particularly in the alkaline conditions imposed by the surrounding concrete.

The aim of this study is to gain deeper understanding of nitrate reduction mechanisms in alkaline conditions and to distinguish abiotic (in the presence of steel without bacteria) from biotic (related to denitrifying microorganisms) reaction kinetics and rates. Experiments were carried out under the following conditions: alkaline pH, no oxygen, and presence of solid materials

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(cement paste, steel). Abiotic experiments, exploring steel surface catalysis, were performed in closed experimental systems (batch) at pH 11–11.5. Hydrogen was sometimes added as a possible electron donor. Biotic experiments were performed under dynamic conditions (continuous medium supply), at pH 10 to 12, in the presence of denitrifying bacterial cells using organic matter (acetic acid) as an electron donor. A specific experimental set-up was developed to consider the microbial development and interactions between the various components of the system (i.e. microorganisms, solid cement paste, and cement paste and bitumen leachates) separately.

## Materials and methods

### Experiments in abiotic systems

#### Materials

**Cementitious materials.** CEM V/A 42.5 cement pastes (Airvault Calcia factory) were made with a water/cement ratio of 0.32. They were cast in cylindrical moulds ( $h = 50$  mm,  $\phi = 27$  mm). Cement paste specimens were kept in hermetic bags (to avoid any hydric exchanges with the exterior and to protect them against carbonation) for 28 days after demoulding. They were then used in the experiments.

Cement paste leachates were prepared by immersing a cylinder of cement paste in 1 l of deionized water (solid/liquid volume ratio: 1.03%). After 3 days under stirring, the final pH of the leaching solution was around 11.5. The average chemical composition of the cement leachate is given in Table 1.

**Metallic materials.** Three types of metallic materials were considered: Fe(0), carbon steel (XC48) and stainless steel (316L) in the form of:

- i. cylinders for Fe(0) ( $h = 200$  mm,  $\phi = 9.5$  mm, obtained from Goodfellow<sup>®</sup>),
- ii. chips for XC48 (the chips were obtained from the milling of crude XC48 and were degreased with acetone before the tests)
- iii. powders: Fe(0) and stainless steel powders, with average granulometry of 6–8  $\mu\text{m}$  and 3  $\mu\text{m}$  respectively, were provided by Goodfellow<sup>®</sup>. XC48 powder was obtained by grinding with a disc mill. The average diameter of particles was 300  $\mu\text{m}$ .

The metallic materials were carefully protected from the air in order to avoid initial corrosion before the beginning of the experiments.

**Nitrate solutions.** 32 mM nitrate solution was prepared using  $\text{NaNO}_3$  salt.

**H<sub>2</sub> gas.** A proton exchange membrane type water electrolyzer (Paxitech) was used to supply some flux of gaseous H<sub>2</sub> into aqueous solution.

#### Experimental device and procedure of the abiotic tests

The experiments were carried out at  $T = 20$  °C in batch conditions using a Pyrex reactor fitted with a tap for liquid sampling, a

gas inlet to fix the composition of the gaseous atmosphere of the system (argon or nitrogen), an inlet for H<sub>2</sub> and an entry port for a pH probe (for more details, see Bertron et al., 2013a, 2014).

The metallic materials (7 g) were placed on a polyethylene (square) mesh grid and the cement matrix was suspended in the solution with a PTFE thread. The reactor was then filled with 1 l of the 32 mM nitrate solution or with cement leachate solution containing 32 mM of nitrate.

The cement paste was introduced in the system just before the reactor was closed. The system was then flushed with N<sub>2</sub> or Ar gas flow for 5 min to fix anoxic conditions. A magnetic stirrer was used during the tests in order to homogenize the composition of the aqueous solution (150  $\mu\text{m}$ ). The solid (cement paste)-to-liquid volume ratio was 1.03%.

#### List of experiments

Five series of abiotic tests were performed (identified as N1 to N5) (Table 2). A control series was first carried out without any metallic material (N1) to evaluate the possibility of nitrate adsorption on the cement matrix surface or on the reactor glass surface. The aim of series N2, N3 and N5 was to evaluate nitrate reduction in presence of XC48 C-steel, 316L stainless steel and Fe(0) respectively, in the form of cylinders, chips and/or powder. In series N4, both carbon steel and stainless steel were added. Some experiments were performed either in the presence of cement paste (CP in Table 1) or with the cement leachate solution (CL). The aim was to evaluate the influence of the buffering capacity of the cement paste on the reduction of nitrates under these alkaline conditions (the buffer effect of cement leachate being much lower). The possible competition between metallic materials and H<sub>2</sub> as potential electron donors was also studied. The reactor was also filled with either nitrogen (N<sub>2</sub>) or argon (Ar) in order to evaluate the possible effect of the nature of the gaseous atmosphere in the reactor.

All experiments lasted 21 days, except for one case in the N2 series (N2-2, Table 2), which was extended to 3 months to allow longer term observations.

#### Experiments in biotic systems

Biotic experiments were performed under a continuous flow of aqueous solution containing nitrate and acetate (dynamic mode). An experimental set-up was developed (i) to control/maintain the growth of bacteria catalyzing nitrate reduction and (ii) to observe interactions between bacteria and solid cement pastes.

#### Experimental set-up

The experimental device (Fig. 1) was composed of: (A) a feeding tank, (B) a bioreactor containing 2 l of actively growing microbial culture, and (C) an exposure chamber with a working volume of 1 l containing solid cement paste slices. This exposure chamber was fed by the outlet of the bioreactor.

Cement matrices, prepared as described in Section 2.1.1, were cut into slices ( $h \approx 10$  mm) and sanded to impose a surface roughness favourable to the development of a bacterial biofilm. Three slices were then placed in the exposure chamber (Fig. 1).

#### Experimental conditions

Each individual experiment started with a 7-day bioreactor batch period before operating in a continuous mode. 7 days was the time required to reach maximum biomass with *Hd* in a closed system (Alquier et al., 2014). At this time, the exposure chamber containing three cement paste slices ( $\phi = 2.7$  cm,  $h = 1.2$  cm) was connected to the bioreactor. The hydraulic retention time (HRT) was fixed at 50.5 h inside the bioreactor; consequently 25.25 h in

**Table 1**  
Chemical composition of the cement leachate.

Concentration (mM)						pH
Ca	K	Na	Si	Al	Fe	
0.38	2.85	0.63	0.38	0.15	<0.1	11.58

**Table 2**  
Experiments performed in abiotic conditions: N (nitrates), CP (cement paste), CL (cement leachate). Concentration of nitrates: 0.32 M, steel or Fe(0) contents: 7 g l<sup>-1</sup>, cement paste/liquid volume ratio: 1.03%.

Test reference	Cement	Metal or metallic solid/materials	Gas phase	Duration (days)
N1-1	CP		Ar	21
N1-2	CP		N <sub>2</sub>	21
N2-1	CP	XC48 (chips)	Ar	21
N2-2	CP	XC48 (chips)	Ar	90
N2-3	CL	XC48 (chips)	Ar	21
N2-4	CP	XC48 (powder)	Ar	21
N2-5	CP	XC48 (chips)	N <sub>2</sub>	21
N3-1	CP	316L (powder)	Ar	21
N3-2	CP	316L (powder)	Ar + H <sub>2</sub>	21
N4-1	CP	316L (powder) +XC 48 (chips)	Ar	21
N5-1	CP	Fe(0) (cylinder)	Ar	21
N5-2	CP	Fe(0) (powder)	Ar	21

the exposure chamber. Fresh medium was continuously added to the bioreactor, using a peristaltic pump, and the outlet of the bioreactor was used to feed the exposure chamber. The bioreactor and the exposure chamber were thermostated at 37 °C. Both bioreactor and exposure chamber were stirred at 300 rpm and were continuously sparged with N<sub>2</sub> gas to limit the presence of oxygen, which could compete with nitrate in accepting electrons.

Solution samples were collected regularly in (1) the feeding tank, (2) the bioreactor and (3) the exposure chamber. An unfiltered sample was used for measurements of pH and optical density (OD) at 600 nm. A filtered sample (through a 0.2 µm pore size filter, cellulose acetate and polyethersulfone based filters from Minisart) was used for HPIC analysis of NO<sub>3</sub><sup>-</sup>, NO<sub>2</sub><sup>-</sup> and acetate concentrations.

#### Alkalophilic denitrifying bacterial model strain

*Halomonas desiderata* (*Hd*) has already been described as a bacterial model for studying nitrate reduction at high pH (González-Domenech et al., 2010; Alquier et al., 2014). It is a facultative aero-anaerobic bacterium capable of (i) growing in alkaline conditions, (ii) using nitrate as electron acceptor and catalyzing its reduction to nitrogen gas, and (iii) oxidizing organic substrates (electron donors) such as acetate. *H. desiderata* DSM 9502 was obtained from the strain collection of DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Germany).

#### Aqueous minimal media

Feeding solution for growing *Hd* was composed of cement leachate (prepared as described in Section 2.1.1) supplemented with 500 mg l<sup>-1</sup> of acetate and 500 mg l<sup>-1</sup> of potassium nitrate

(8.3 mM acetate and 5.9 mM nitrate). No additional boosting solutions such as yeast, often used in microcosm experiments (Rizoulis et al., 2012), were used.

The pH of the feeding solution was adjusted to 10, 11 or 12 using 4 M NaOH.

#### Analytical techniques

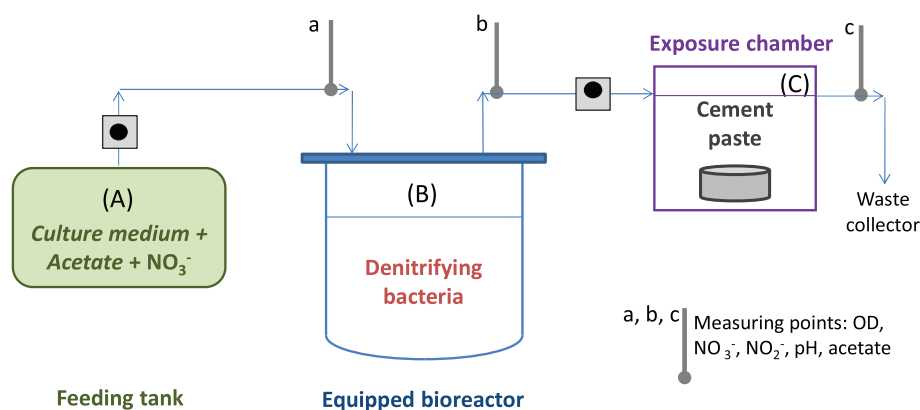
##### Bacterial growth

The bacterial growth was monitored by measuring Optical Density at 600 nm (JENWAY 7315 spectrophotometer). For measuring optical density at 600 nm, the solution feeding the bioreactor (cement leachate) is systematically used as a blank. Optical density values are quite low (<0.25) but according to those already observed with the same type of bacterial culture without the presence of cement solid matrix (Alquier et al., 2013). These optical density values can only give a trend in the relative amount of biomass in experimental systems. Nothing can ensure that mineral particles from the cement pastes can pollute the measurements.

##### Chemical analysis (Ca<sup>2+</sup>, K<sup>+</sup>, acetate, nitrate, nitrite, ammonium)

Concentrations of Ca<sup>2+</sup>, K<sup>+</sup>, Na<sup>+</sup>, acetate, NO<sub>3</sub><sup>-</sup>, and NO<sub>2</sub><sup>-</sup> were measured by High Performance Ionic Chromatography (Equipment: Dionex ICS-2000 and ICS-3000) using analytical methods detailed in Alquier et al. (2014) and Bertron et al. (2014).

The concentration of dissolved ammonium (N-NH<sub>4</sub><sup>+</sup> and N-NH<sub>3</sub>(aq)) was measured with Fisher Scientific photometric test kits in a concentration range from 5.5 × 10<sup>-4</sup> mM to 0.144 mM.



**Fig. 1.** Schematic representation of the experimental device used for the study of interactions between microorganisms and solid materials during continuous microbial denitrification. Arrows indicate the direction of the advective flow in the system. (B) and (C): temperature = 37 °C; stirring = 300 rpm.

### X-ray diffraction

Carbon (XC48) and stainless (316L) steel chips or powders were analyzed by XRD before and/or after the tests using a Siemens D5000 apparatus fitted with a Co cathode with operating conditions as follows: anode voltage 40 kV, current intensity 30 mA.

### Microscopic observations

**SEM observation.** Cement matrices exposed to biotic tests were post-treated before SEM observation as described in (Alquier et al., 2014). Steel specimens exposed to abiotic tests were also observed with SEM without any specific preliminary treatment. The SEM observation was performed on a “low vacuum” JEOL JSM 6380L scanning electron microscope (operated at 60 Pa, 15 kV) equipped with an EDX detector (RONTEC Xflash 3001).

**Epifluorescence observation.** Observations were made by epifluorescence microscopy after specific labelling of bacterial cells with YO-PRO®.

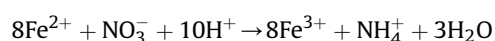
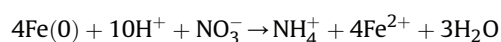
## Results and discussion

### Abiotic experiments

The concentrations of nitrogen compounds ( $\text{NO}_3^-$ ,  $\text{NH}_4^+$  and  $\text{NO}_2^-$ ) of the five series of experiments are reported in Table 3. Fig. 2 shows the evolution of ammonium concentration in the aqueous medium for the different tests carried out with carbon steel (XC48) chips or powder (N2 series).

For all experiments with cement matrix (i.e. except for N2-3 with cement leachate) an increase of the pH value from about 7 to 11.0–11.5 was observed within 2 h, due to the release of hydroxide ions from the cement paste. A constant pH was then recorded in the solutions until the end of the experiments from  $t = 2$  h to  $t = 21$  or 90 days depending on the experiment.

For the experiment with the cement leachate, a progressive decrease of the pH was noted (from 11.7 at the beginning of the test, to 11 at the end of the 21-day experiment). The progressive decrease in pH is unlikely related to the reduction of nitrates. The reactions of reduction of nitrates in the presence of steel rather suggest an increase of pH in the solution:



The decrease of pH may be due to the adsorption and/or precipitation of the cementitious species and phases at the surface of the steel cheeps. It was indeed observed a decrease in the calcium

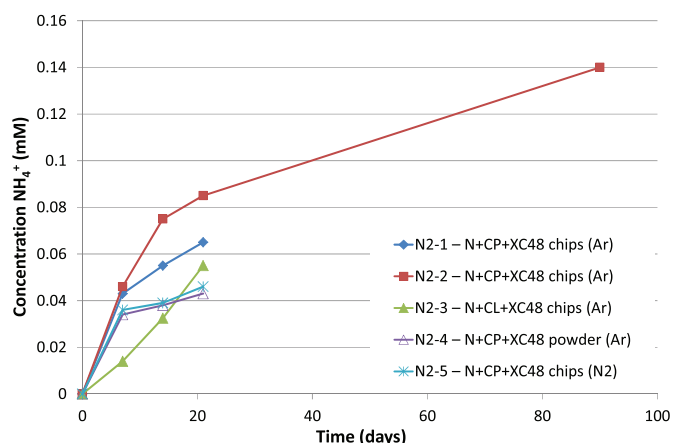


Fig. 2. Evolution of ammonium concentration in the solutions of abiotic experiments with XC48. N: nitrates, CP: cement paste, CL: cement leachate.

concentration in the solution according to time (data not shown) and calcium was detected at the surface of the steel with EDS analysis when steel chips were observed with SEM. Soluble complex species formed through the leaching of the cement paste (such as  $\text{Ca}(\text{OH})^+_{\text{aq}}$ , or  $\text{Al}(\text{OH})^+_{\text{aq}}$ ) may have been adsorbed at the surface and may have caused the drop of pH in the surrounding solution. The same type of phenomenon may have occurred in the presence of cement solids but the abundant release of  $\text{OH}^-$  and  $\text{Ca}^{2+}$  by the cement paste likely made them undetectable through  $\text{Ca}^{2+}$  and pH measurements.

### Series N1 (reference tests: nitrates + cement matrix, without steel)

No decrease of nitrate concentration was observed in the system, indicating that nitrate was neither significantly adsorbed on the solid surfaces (cement matrix and reactor) nor abiotically reacted in the 21 days of the experiment (Table 3).

### Series N2 with carbon steel XC48 (nitrates + cement matrix or leachate + XC48-chips or powder)

Significant reduction of nitrates was only observed for the long-term experiment (90 days, N2-2; Table 3) in the conditions explored (pH 11–11.5,  $\text{NO}_3^-$  32.3 mM, XC38 7  $\text{g l}^{-1}$ ), indicating that the kinetics of reactions were very low. The concentration of nitrates in solution was 24.8 mM at the end of the experiment, which represents an average reaction rate of nitrate reduction of  $3.4 \times 10^{-3} \text{ mM h}^{-1}$  on the overall test duration. For the other experiments, the decrease of nitrate concentration was very low. In most cases, this decrease was lower or equivalent to the standard

Table 3

Concentration of nitrogen compounds for the 5 series of experiments carried out in abiotic conditions (N: nitrate, CP: cement paste, CL: cement leachate, n.m.: not measured, DL detection limit). Conditions of the experiments: concentration of nitrates: 32 mM, steel or Fe(0) contents: 7  $\text{g l}^{-1}$ , cement paste/liquid volume ratio: 1.03%.

Test ref.	Components of the system	Gas phase	Duration of test (h)	$[\text{NO}_3^-]$ initial (mM)	$[\text{NO}_3^-]$ final (mM)	$[\text{NH}_4^+]$ final (mM)	$[\text{NO}_2^-]$ final (mM)
N1-1	N + CP	Ar	21	32.17	33.04	n.m.	<DL
N1-2	N + CP	N <sub>2</sub>	21	32.65	33.13	n.m.	<DL
N2-1	N + CP + XC48 (chips)	Ar	21	32.57	32.37	0.06	<DL
N2-2	N + CP + XC48 (chips)	Ar	90	32.24	24.80	0.14	<DL
N2-3	N + CL + XC48 (chips)	Ar	21	33.31	33.08	0.06	<DL
N2-4	N + CP + XC48 (powder)	Ar	21	32.75	32.78	0.04	<DL
N2-5	N + CP + XC48 (chips)	N <sub>2</sub>	21	32.67	32.11	0.05	<DL
N3-1	N + CP + 316L (powder)	Ar	21	33.61	33.08	<DL	<DL
N3-2	N + CP + 316L (powder)+H <sub>2</sub>	Ar	21	34.15	33.46	<DL	<DL
N4-1	N + CP + 316L (powder) + XC 48 (chips)	Ar	21	32.92	32.69	0.3	<DL
N5-1	N + CP + Fe(0) (cylinder)	Ar	21	33.49	33.35	0.02	<DL
N5-2	N + CP + Fe(0) (powder)	Ar	21	33.49	32.33	0.52	0.25

deviation of the nitrate concentration measured in the solutions at the initial state:  $0.52 \text{ mmol l}^{-1}$  for a nominal concentration of  $32.00 \text{ mmol l}^{-1}$  (variation coefficient of 1.17%).

For all the experiments of the N2 series,  $\text{NH}_4^+$  concentration increased throughout the tests (Fig. 2) but remained low ( $\leq 0.14 \text{ mM}$ ). Actually, at pH 11.5, ammonia is the major chemical species of the acid-base couple  $\text{NH}_4^+/\text{NH}_3$  since its pKa is 9.2. Consequently, quantified N- $\text{NH}_4^+$  included  $\text{NH}_4^+$  ions and dissolved gaseous  $\text{NH}_3$ . Ammonia in the gas phase of the reactor was not quantified. This and/or the sorption of  $\text{NH}_4$  onto solid surfaces could be the reason why the N molar balance was not verified for all experiments in series N2.

At the end of the test, macroscopic signs of corrosion were observed at the surface of the XC48 steel chips and powder (brown colour). Corrosion of the steel was confirmed by SEM observations (Fig. 3) and by XRD analyses showing precipitation of amorphous phases possibly attributable to iron hydroxide (Fig. 4). In this case, steel would have acted as an electron donor for nitrate reduction.

A study by Truche et al. (2013a, unpublished report) showed that nitrates were not reduced in the presence of C-steel in hyper-alkaline conditions (pH > 12) or with  $\text{NO}_3^-$   $1 \text{ mM}$ , steel  $20 \text{ g l}^{-1}$  and  $20 < T < 50 \text{ }^\circ\text{C}$ . In this experiment, little or no sign of steel corrosion was noted by the authors. This difference in results observed between the pH value near 11 (our study) and pH greater than 12 (Truche et al., 2013a) confirmed the reactivity of nitrate in the presence of C-steel was highly dependent on the pH. The possible antagonist effects of the iron hydroxide precipitation should also be noted: on the one hand, it can be responsible for lowered kinetics (by diffusion barrier) and, on the other hand, it can stimulate a surface catalysis process. A longer duration test ( $\geq 1$  year) would then be relevant.

The presence of solid cement matrix in the system did not influence the reactions of nitrate since similar results were obtained in the presence of both solid cement and cement leachate (N2-1 and N2-3 series). Finally, the evolution of the reactive system

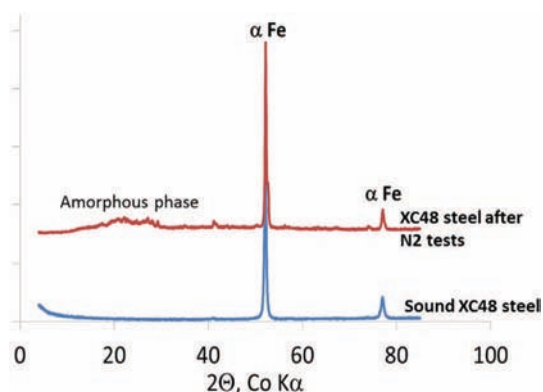


Fig. 4. X-ray traces of XC48 steel before (sound steel) and after series N2 (apparition of an amorphous phase).

seemed to be independent of the nature of the gas phase (N2-1(Ar) and N2-2 (N<sub>2</sub>), Table 1).

*Series N3 with 316L (nitrates + cement matrix + 316L powder + H<sub>2</sub> or not)*

Neither reduction of nitrate nor ammonium formation were observed during the experiments. Truche et al. (2013a, unpublished results) highlighted a possible reduction of nitrate in the presence of 316L stainless steel at pH > 12 at ambient temperature ( $20 \text{ }^\circ\text{C}$ ) but in the presence of hydrogen, and in the following conditions:  $p(\text{H}_2) = 7 \text{ bars}$ , steel  $20 \text{ g l}^{-1}$  and nitrates  $1 \text{ mM}$ . The sorption of hydrogen at the surface of stainless steel was, in this case, a key phenomenon in the reduction of nitrates. In the case of these experiments at pH > 12, the authors noted signs of corrosion, when stainless steel acted as an electron donor for nitrate reduction. For our experiment, insufficient presence of H<sub>2</sub> in the experiment (i.e. sorbed on the surface of stainless steel) may explain why no

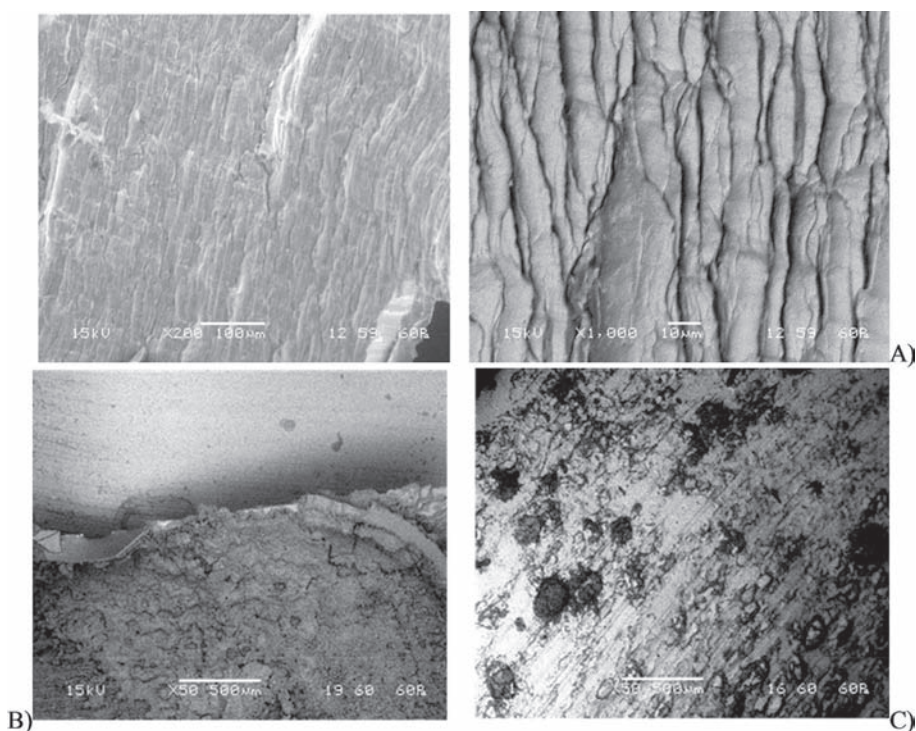


Fig. 3. SEM observation of XC48 steel, before (A) and after series N2 carried out in presence of Ar (B) or N<sub>2</sub> (C) gas.

reduction of nitrate occurred. The solubility of  $H_2$  in water at 20 °C and at atmospheric pressure is only 0.8 mM. Moreover, results were almost identical in the presence or absence of  $H_2$  injection in the system (Series N3-2).

*Series N4 with stainless (316L) and carbon (XC48) steel (nitrates + cement matrix + 316L-powder + XC48-chips)*

As for series N2 (XC48 only), a very low nitrate reduction was observed in series N4. However, the amount of ammonium formed was greater than what was obtained for the N2 series (0.3 mM) (Table 2). In this case,  $H_2$  produced by the corrosion of carbon steel might have sorbed at the surface of stainless steel, which could have helped to make the stainless steel surface more reactive.

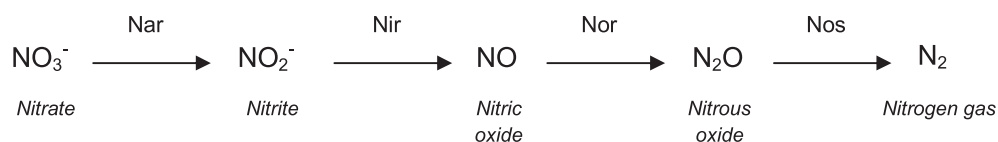
*Series N5 with Fe(0) (nitrates + cement matrix + Fe(0)-cylinder or powder)*

Greater quantities of ammonium were observed for series N5-2 (with powder). Nitrite was also detected (0.25 mM). The use of Fe(0), coupled with the large reactive surface area of the powder shape, could lead to a significant reduction of nitrates as suggested by Hwang et al. (2011) and Tang et al. (2012).

*Biotic experiments: influence of the pH on the denitrifying activity of H. desiderata (Hd) in a minimum medium*

In a nuclear waste cell where solid concrete is present, very alkaline conditions (pH greater than 13) can develop (Bernier, 1992; Bertron et al., 2013b). In batch, with a rich growth liquid medium, *Hd* was shown to be able to reduce nitrate into  $N_2$  gas at an optimal pH between 9 and 10 (Berendes et al., 1996) but its denitrifying activity was also possible at higher pH, especially in presence of cementitious matrices (Alquier et al., 2013, 2014). These authors also described the ability of *Hd* to grow not only in planktonic form but also as a biofilm on the surface of concrete. These observations needed to be explored further in a system with a continuous flux of aqueous medium, with a limited concentration of nutrients and a pH higher than 11 (Bertron et al., 2013b). With this in mind, the bacterial growth of *Hd* and its denitrifying activity were studied in a bioreactor under continuous feeding with a cement leachate solution supplemented with 8.3 mM of acetate and 5.9 mM of nitrate. Three different pH values – 10, 11, and 12- were tested. In addition, interactions between *Hd* and solid samples of cement paste were investigated by attaching an exposure chamber after the bioreactor (Fig. 1).

For all series of experimental tests, bacterial growth and concentrations of acetate,  $NO_3^-$ , and  $NO_2^-$  were monitored in the bioreactor and in the exposure chamber over time (Figs. 5–7). They are discussed in the following sections. Ammonium concentrations were negligible throughout the different tests performed with *Hd* (lower than the detection limit) confirming that the process of dissimilatory nitrate reduction to ammonium (DNRA) did not occur as already discussed in a previous study with *Hd* (Alquier et al., 2014).



*Denitrifying activity of H. desiderata at pH 10 in cement leachate*

At pH 10, after a batch period of a week to activate bacterial growth of *Hd* cells ( $T_0$  in the Figure), the optical density of the

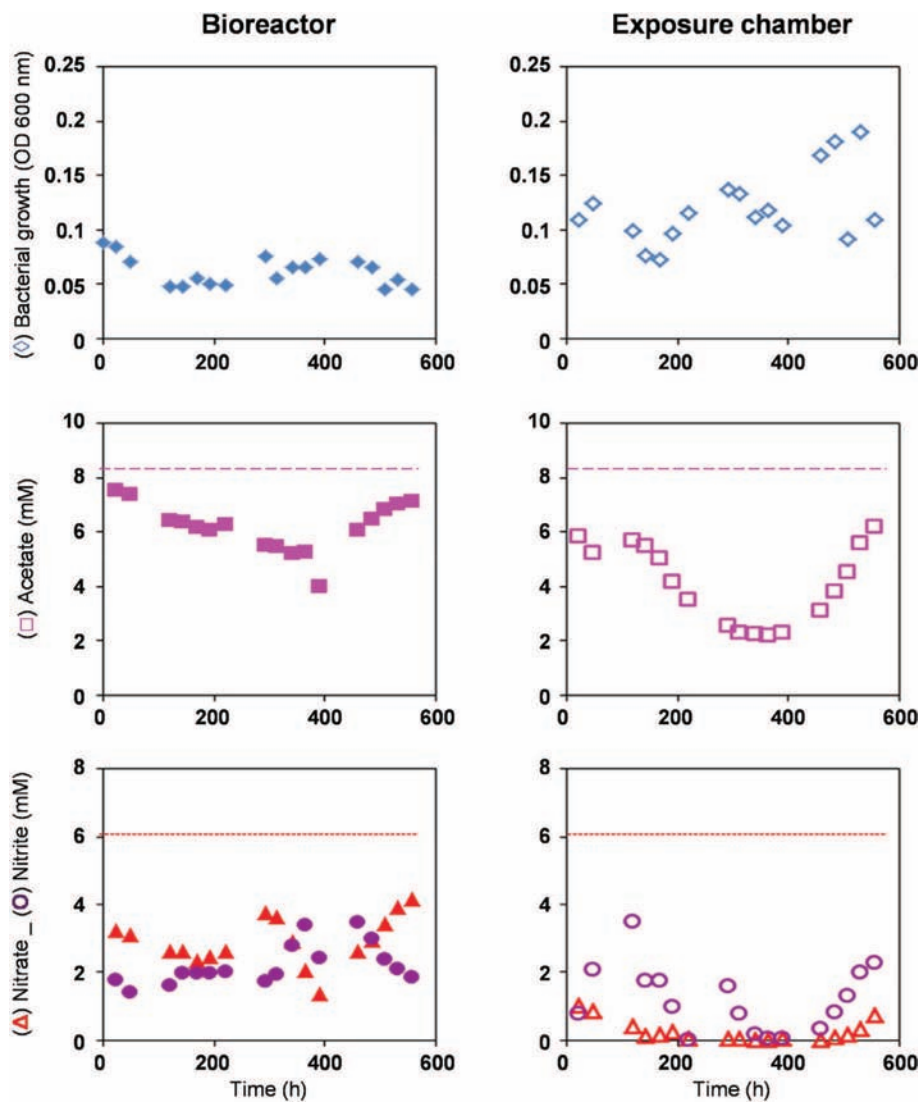
bacterial culture ( $OD_{600nm}$ ) in the bioreactor was 0.090. Switching to the continuous renewal of the aqueous volume by fresh medium (Fig. 5), in so-called continuous mode, led to a decline of the  $OD_{600nm}$  values in the bioreactor during the first hundred hours. After this period, the mean value of  $OD_{600nm}$  was  $0.05 \pm 0.01$  from 150 to 600 h. This value, typical of the rate of basic bacterial growth, was half those observed in the same dynamic conditions at pH 10 with a rich culture medium especially optimized for the growth of *Hd*, and one third those observed by Alquier et al. (2014) in a previous work carried out in batch, with the same rich culture medium. Nevertheless, the possible growth of *Hd* at pH 10 under dynamic feeding with an aqueous medium as poor as a cement leachate supplemented with acetate and nitrate was thus demonstrated.

At the outlet of the chamber, the average value of the  $OD_{600nm}$  was  $0.12 \pm 0.04$ , i.e. the passage in the exposure chamber increased the  $OD_{600nm}$  by a factor of two. This may have been due to the increase of the global hydraulic retention time, which rose from 50.5 h at the outlet of the bioreactor to 75.75 h at the outlet of the exposure chamber. However, the doubling of  $OD_{600nm}$  did not completely match the increase in hydraulic residence time by a factor of 1.5. The presence of the cement paste slices in the exposure chamber was a possible explanation for the beneficial effect on the growth of *Hd*. The favourable impact of solid cement matrix has already been observed in batch by Alquier et al. (2014). The authors put forward two hypotheses, which remain valid in the context of our study, to explain the role of the solid cement paste:

- The cement matrix can release minerals (such as trace elements) essential to the growth of the bacterial cells, or the synthesis of the proteins. The concentration of soluble essential elements, usually limiting bacterial growth, was then more significant and thus promoted the growth of cells
- The cement matrix is a solid support to which *Hd* can attach, so as to proliferate and then form a three-dimensional bacterial organization called a biofilm. The continuous growth of cells in the biofilm phenotype is accompanied by a constant release of the attached cells, and can impact the overall value of the optical density.

The evolution of the concentrations of acetate, nitrate and nitrite over time is in agreement with the results observed from the evolution of the  $OD_{600nm}$ . Twenty-four percent of the acetate and 45% of the nitrates present in the medium supplied were consumed inside the bioreactor, with average acetate and nitrate concentrations at the outlet of the bioreactor of 6.8 and 3.3 mM respectively. Thus, the percentage of nitrate consumed in the bioreactor was much lower than those of the experiments conducted under the same conditions but with a rich synthetic medium. Schematically, the overall pathway of denitrification can be summarized as follows:

The ultimate product of the reduction is nitrogen gas. The overall mechanism successively involves four specific enzymes: nitrate reductase (Nar), nitrite reductase (Nir), nitric oxide



**Fig. 5.** Bacterial growth and evolution of acetate, nitrate and nitrite concentrations in the bioreactor (on the left) and in the exposure chamber (on the right) during the continuous culture of *Hd* with a minimum medium at pH 10. The dashed lines represent the concentrations of acetate (electron acceptor) and nitrate (electron donor) in the inlet.

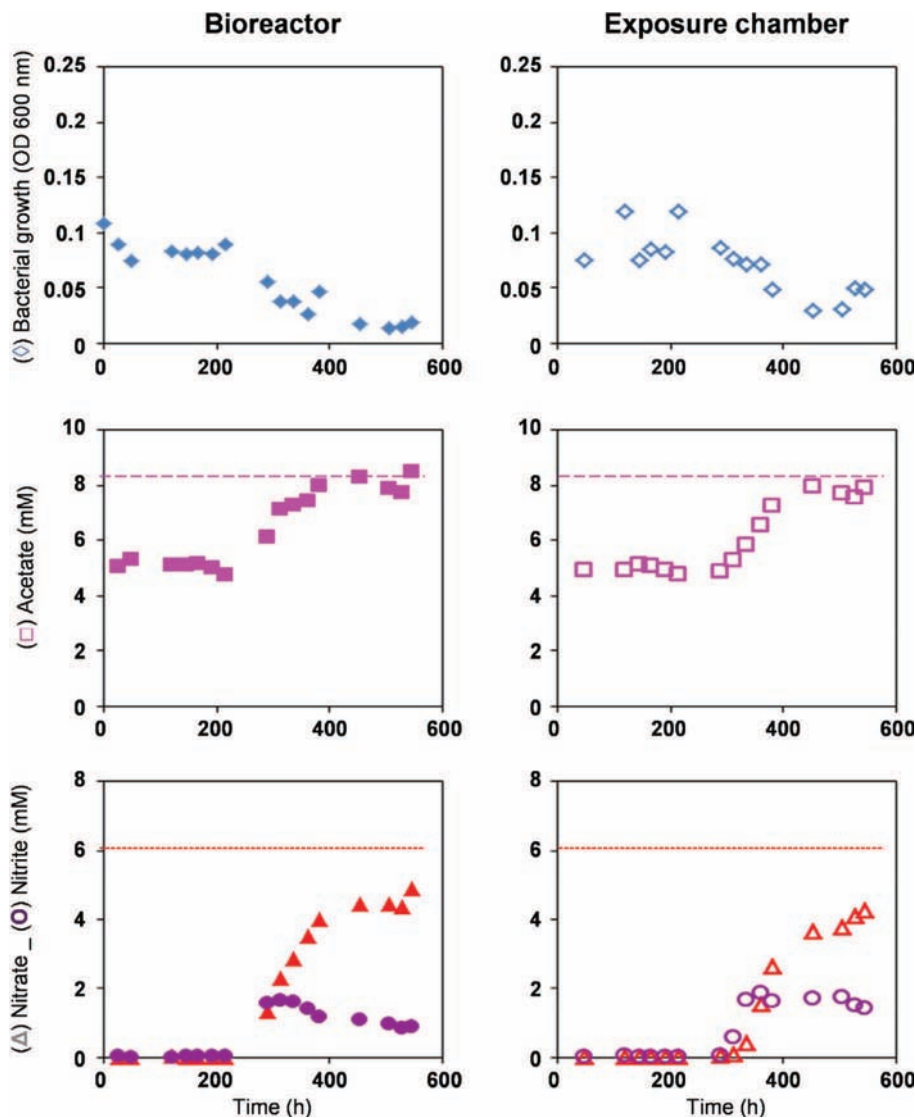
reductase (Nor) and nitrous oxide reductase (Nos) (Ye et al., 1994; Averill, 1996). These enzymes generally contain a metal cofactor like molybdenum for Nar, copper for Nir or Nos, and iron for Nor (Averill, 1996).

In our experiment at pH 10 in the bioreactor, part of the reduction of nitrate (70%) was inhibited at the nitrite step with an average concentration of nitrite in the bioreactor of 1.96 mM. Denitrification continued in the exposure chamber with average outflow concentrations of 5.04 and 0.62 mM, respectively for acetate and nitrate. But, as for the bioreactor, part of the nitrate was only reduced to nitrite. Theoretically *Hd* has the complete cascade of enzymes necessary for the successive steps of reduction of nitrate to  $N_2$  (Berendes et al., 1996). Nitrite is commonly thought to have a broad inhibitory effect on bacterial metabolism (Ma et al., 2010). Its accumulation indicates that the conditions were not optimal for *Hd*. For example, the lack of trace elements such as copper could prevent the formation of the nitrite reductase and thus stop the reaction at the nitrite step. It is also possible that the pH was not optimal for the denitrifying activity of *Hd*. Previous studies on wastewater treatment have shown that the pH directly affects the bacterial growth and denitrifying enzyme activities

(Campos and Flotats, 2003; Magrí et al., 2007). In these studies, with pH higher than 8, nitrite reduction rates were inhibited, causing nitrite accumulation during the denitrification process. However, this phenomenon appears to depend on the species studied and does not seem to be applicable to our study with *Hd* since many authors describe optimal denitrification as well as optimal growth for *Hd* between pH 9 and 11 (Berendes et al., 1996; Alquier et al., 2014). Shapovalova et al. (2008) have gone further by determining the optimum pH for three of the four reductases involved in the denitrification pathway of *Halomonas* strain AGD3. These authors determined that nitrite-reductase was the most alkali-dependent of the three reductases with an optimum pH of 10.6–11.0. The nitrous oxide reductase was also alkali-dependent, with an optimum pH of 10, and the nitrate reductase was only moderately alkaliphilic with an optimum pH of 9. In any case, the system never reached equilibrium. The variability of electron donors and acceptors clearly indicates the inherent instability of the system, and probably a continuous need for *Hd* to adapt.

Investigations on the pH limit of the denitrifying activity of *Hd* continued with the continuous culture of *Hd* in a minimum media (cement leachate with acetate and nitrate) at pH 11.





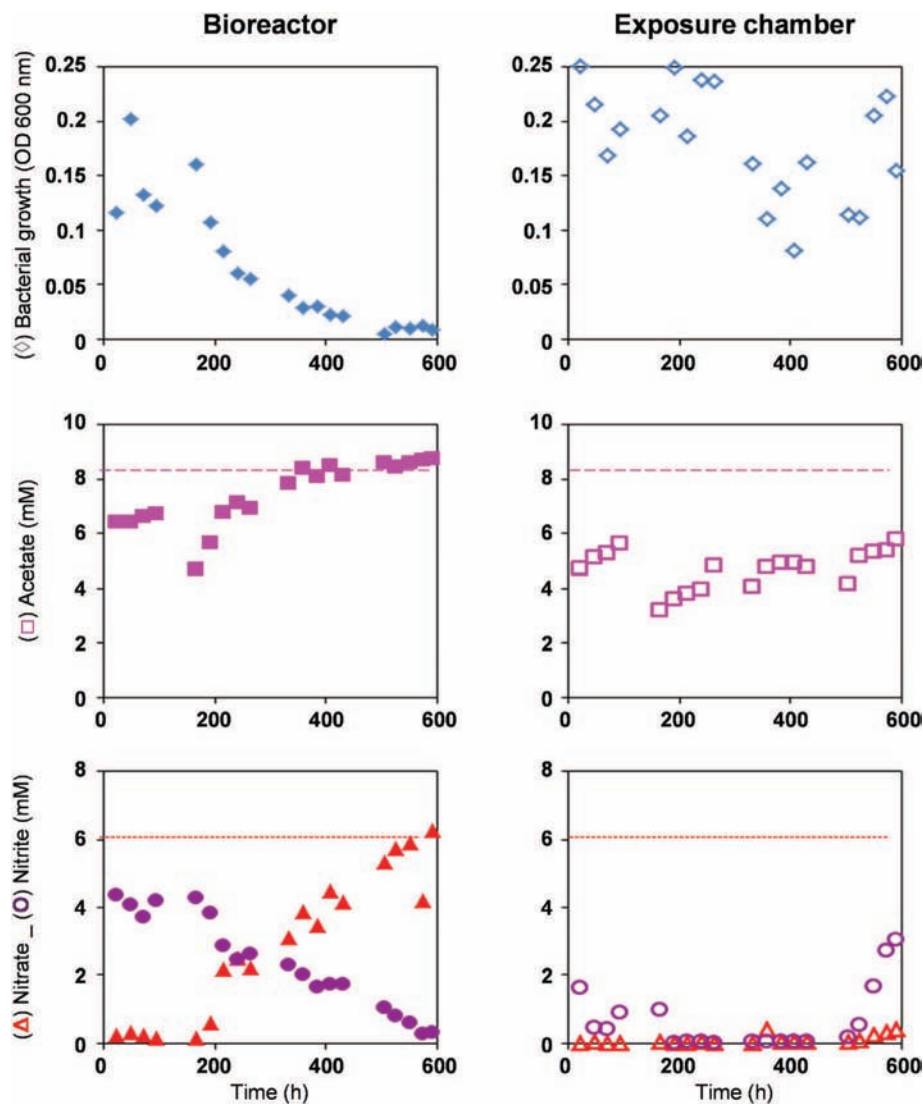
**Fig. 6.** Bacterial growth and evolution of acetate, nitrate and nitrite concentrations in the bioreactor (on the left) and in the exposure chamber (on the right) during the continuous culture of *Hd* with a minimum medium at pH 11. The dashed lines represent the concentrations of acetate (electron acceptor) and nitrate (electron donor) in the inlet.

*Denitrifying activity of H. desiderata at pH 11 in cement leachate (minimum medium)*

**At pH 11** (Fig. 6), two different periods were observed as a function of time

- During the first 300 h of reaction,  $OD_{600nm}$  measured in the bioreactor was double those observed at pH 10 while those measured in the exposure chamber did not change much. They were respectively  $0.08 \pm 0.005$  and  $0.09 \pm 0.02$ . During this period, the concentrations of acetate and nitrate in the bioreactor were respectively 5.060 and 0.004 mM, corresponding to a consumption of 41% of the acetate and nearly all the nitrates (99.9%) originally present in the supply medium. In this case, no accumulation of nitrites was measured. This suggests that the optimum pH of the nitrite reductase of *Hd* is located around 11 in the chemical conditions of the experiment, similarly to the findings of Shapovalova et al. (2008) for *Halomonas* strain AGD3. The absence of nitrate and/or nitrite in the outlet of the bioreactor prevents further growth of *Hd* in the exposure chamber and explains why acetate concentration did not change after the passage through the exposure chamber.

- From  $t = 300$  h–600 h, a progressive decrease of the  $OD_{600nm}$  was observed. At the end of the experiment, it reached a value close to 0.015 in the bioreactor and 0.03 in the exposure chamber. This phenomenon can be explained by the growth rate of *Hd*, which became lower than the dilution rate imposed by the continuous mode. This caused dilution of *Hd* and its wash-out from the bioreactor and the exposure chamber. This decrease of  $OD_{600nm}$  was accompanied by an increase in acetate and nitrate concentrations in the two compartments of the experimental device. Only 10% of acetate and 17% of nitrates present in the supply medium reacted in the bioreactor. These conversion rates increased very slightly in the exposure chamber with 12% of acetate and 28% of nitrate consumed. Contrary to what was observed during the first 300 h of culture, the nitrate reduction stopped mainly at the nitrite step, with final nitrite concentrations of 0.86 mM in the bioreactor and 1.49 mM in the exposure chamber. Taken together, these results suggest that the chemical conditions were too restrictive for the continuous culture of *Hd*. The pH of the supply medium was too high and/or lack of minerals and trace elements reduced the growth and the activity of *Hd*.



**Fig. 7.** Bacterial growth and evolution of acetate, nitrate and nitrite concentrations in the bioreactor (on the left) and in the exposure chamber (on the right) during the continuous culture of *Hd* with a minimum medium at pH 12. The dashed lines represent the concentrations of acetate (electron acceptor) and nitrate (electron donor) in the inlet.

The phenomenon of wash-out observed after 300 h of culture has not yet been clearly explained.

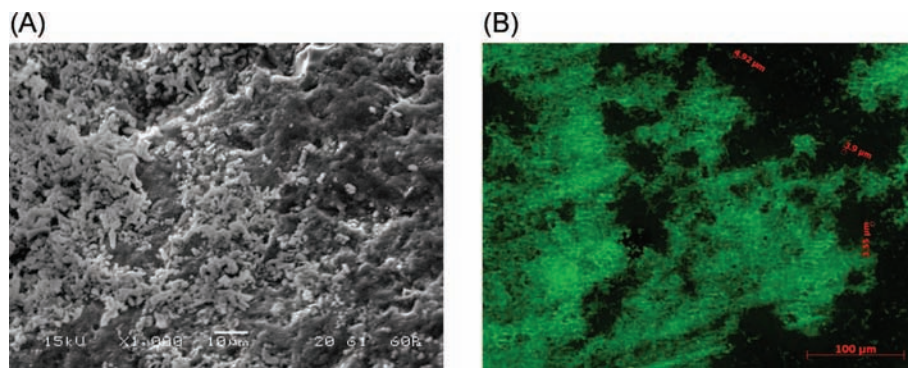
#### Denitrifying activity of *H. desiderata* at pH 12 in cement leachate

The continuous culture of *Hd* at pH 12 showed behaviour comparable to that observed at pH 11. Two distinct periods of growth and reactivity can be clearly distinguished (Fig. 7).

- During the first 170 h, the  $OD_{600nm}$  in the bioreactor and in the exposure chamber were stable and quite high (respectively  $0.14 \pm 0.03$  and  $0.20 \pm 0.03$ ), compared to those previously observed at pH 10 and 11, in view of the high pH (12) of the supply medium. During this period, the concentrations of acetate and nitrate in the bioreactor were 6.17 and 0.21 mM respectively, corresponding to a consumption of 27% of the acetate and 96% of the nitrates initially present in the supply medium. Nitrate reduction mostly stopped at the nitrite step. The nitrite concentration was 4.1 mM, corresponding to 73% of the nitrate consumed in the bioreactor. Contrary to what was observed at pH 11, at pH 12, the presence of acetate, nitrate and

nitrite in the outlet of the bioreactor allowed *Hd* to grow again in the exposure chamber. Thus, for at least 500 h of operation, the nitrate and nitrite concentrations at the outlet of the exposure chamber were close to zero.

- In a second phase, after 170 h of culture, a decrease of the  $OD_{600nm}$  to the value of 0.01 was gradually observed, corresponding to a wash-out of the bioreactor. At the same time, while the biomass present in the bioreactor ceased to progress and started to be severely washed out, the concentrations of nitrate and acetate were back to values close to the concentrations of the supply medium. In parallel, nitrite concentrations decreased to near-zero values, confirming that there was no more microbial activity in the bioreactor. Despite the halt in *Hd* growth in the bioreactor, the reaction of denitrification continued to operate in the exposure chamber as the final concentrations of acetate and nitrate were respectively 4.6 and 0.1 mM. Furthermore, for at least the first 500 h, nitrate reduction continued beyond the nitrite step. The preservation of the denitrifying activity in the exposure chamber was probably due to the proliferation of *Hd* as a "biofilm" phenotype on the surface of the cement pastes. This organization of bacterial cells in an



**Fig. 8.** Microscopic observation of the surface of solid matrices colonized by *Hd* (at pH 12, after 600 h of exposure): (A) SEM and (B) epifluorescence.

attached three-dimensional architecture allows them to avoid wash-out and possibly to resist difficult chemical conditions (Costerton et al., 1994; Davies et al., 1998; Mah and O'Toole, 2001). Bacteria living in a biofilm phenotype usually have significantly different properties from planktonic bacteria (i.e. in suspension) of the same species, as the dense and protected environment of the "film" allows them to cooperate and interact in various ways (Flemming and Wingender, 2010). Biofilm formation is a key factor for survival in diverse environments in the sense that it provides a protected mode of growth that allows cells to survive in hostile environments (Hall-Stoodley et al., 2004). For example McNeill and Hamilton (2003) showed that biofilm formation, in contrast with planktonic cells, affords protection from acid exposure in *Streptococcus mutans*. The same phenomenon can be observed in our study under alkaline conditions of exposure. While the "biofilm" phenotype was active at least until pH 12, the planktonic phenotype was inhibited at pH 12 (data not shown). However, after 500 h of culture, nitrites were found in the exposure chamber outlet, meaning that experimental conditions had started to become too restrictive even for fixed bacterial biomass.

#### Observation of the surface of solid matrices

SEM observations of the cement paste surfaces at the end of the culture of *Hd* at pH 12 confirmed the presence of a microbial biofilm of *H. desiderata* on solid surfaces in the form of a thin bacterial mat, not exceeding 3 or 4 stacked layers of bacteria, i.e. less than 10  $\mu\text{m}$  thick (Fig. 8A). Observation by epifluorescence microscopy after staining of the concrete surface with specific fluorescent markers of bacterial nucleic acids also confirmed that these observed deposits were of biological origin (Fig. 8B). The organization of bacteria in a biofilm probably allowed the denitrifying activity of *H. desiderata* at pH 12 to be maintained in the exposure chamber whereas it was no longer active in the bioreactor. Contrary to what was observed in

the experiment at pH 12, at pH 11 the fixed biomass on cement pastes of the exposure chamber did not allow the complete maintenance of denitrification activity. This was probably due to the absence of nitrate and/or nitrite in the inlet to the chamber during the first 290 h of culture, which probably prevented the development of a significant biofilm on the cement pastes. In a previous work, Alquier et al. (2013) showed that immobilized biomass could play an important role in the denitrification process because of its denitrifying activity comparable to that of planktonic bacteria (i.e. in suspension).

#### Bacterial growth and kinetics of denitrification

For each experiment, the average values of optical density and kinetics of denitrification in the bioreactor and in the exposure chamber were calculated when the microbial growth was stabilized (Table 4).

Our biotic experiments with a limited nutrient medium highlighted the influence of pH on the denitrification rate and, in addition, showed the important role of cement as a potential nutrient source. At pH 10, results indicated that the denitrifying activity of *Hd* could be maintained for at least 600 h. Under this condition, although the growth of *Hd* was halved compared to those observed in the same conditions with an optimal synthetic growth medium (0.05 compared to 0.11), the kinetics of denitrification were slower but remained of the same order (0.056 against 0.075  $\text{mM h}^{-1}$ ). At pH 11, the average values of  $\text{OD}_{600\text{nm}}$  and the kinetics of denitrification (0.080  $\text{mM h}^{-1}$ ) suggest a possible bacterial cells multiplication (growth) and a still active denitrifying activity of *Hd*. As shown previously, the bioreactor started to be washed out after 290 h of continuous culture. The absence of nitrate and/or nitrite at the exposure chamber input limited the formation of biofilm on the cement pastes and consequently the preservation of the denitrifying activity within the exposure chamber. At pH 12, the environmental conditions were again difficult for *H. desiderata* (high pH and lack of trace nutrients) and

**Table 4**

Summary table of main results in the biotic experiment for the different pH tested.

pH	Stage	OD 600 nm	Acetate consumed (%)	Nitrates consumed (%)	Denitrification rate ( $\text{mM h}^{-1}$ )
pH 10	Bioreactor	0.05	24% <sup>a</sup>	45% <sup>a</sup>	0.056
	Exposure chamber	0.12	25% <sup>a</sup>	81% <sup>a</sup>	0.055
	Total		43% <sup>b</sup>	90% <sup>b</sup>	
pH 11	Bioreactor	0.06	26% <sup>a</sup>	68% <sup>a</sup>	0.080
	Exposure chamber	0.07	10% <sup>a</sup>	30% <sup>a</sup>	0.018
	Total		33% <sup>b</sup>	81% <sup>b</sup>	
pH 12	Bioreactor	0.10	—	—	—
	Exposure chamber	0.20	50% <sup>a</sup>	100% <sup>a</sup>	0.237
	Total		50% <sup>b</sup>	100% <sup>b</sup>	

<sup>a</sup> % of the Amount entering the bioreactor or the exposure chamber.

<sup>b</sup> % of the Total amount of nitrate or acetate.

after 170 h of continuous culture, the planktonic bacteria started to be washed out. But, in contrast to what was observed at pH 11, nitrate reduction still operated in the exposure chamber at reaction rates exceeding  $0.237 \text{ mM h}^{-1}$  due to the biofilm present on cement paste surfaces. This shows the potential importance of the fixed biomass.

All the rates of biologically induced nitrate reduction in our biotic experiments were much higher than those obtained in the abiotic part of the study in which, in the presence of XC48 steel, the average rate of nitrate reduction did not exceed  $3.4 \times 10^{-3} \text{ mM h}^{-1}$  on the overall test duration.

It should be noted that the low denitrification rates remain significant considering the several thousand years of life of a nuclear waste repository. Other studies carried out in a comparable context showed that microbial communities or pure strains such as *Pseudomonas stutzeri* could reduce millimolar nitrate concentrations with denitrification rates up to  $0.8 \text{ mM h}^{-1}$  (Spain and Krumholz, 2011; Masuda et al., 2013; Bertron et al., 2013a). These studies were carried out in a pH range of 6–8 and with a rich culture medium. Our study was carried out at pH values between 10 and 12 without the use of boosting media. They are therefore more easily applicable to the prediction of reaction rates under conditions of deep geological nuclear waste repositories.

## Conclusion

Experiments carried out under abiotic conditions in the presence of carbon and stainless steel showed that the reduction of nitrates was possible at pH 11–11.5 but the reaction rates remained low and the final product obtained from the reduction of the nitrate was ammonium. In contrast, in the presence of *H. desiderata*, a heterotrophic denitrifying bacterium, the nitrate was reduced into nitrite or even more reduced species (nitrogenous intermediates NO and  $\text{N}_2\text{O}$ , or gaseous nitrogen  $\text{N}_2$ ). This biotic reduction of nitrate was possible at pH up to 12 and in a nutrients-limited environment (leachate of concrete completed with acetate and nitrate, without boosting additives such as yeast extract). Maximal kinetics of denitrification of  $0.237 \text{ mM h}^{-1}$  was demonstrated at pH 12 when the bacterial cells of *Hd* were organized in a biofilm on the surface of the cement paste.

Our work demonstrates that, under conditions of pH 12, comparable to the pH that will likely occur in a nuclear waste repository cell, nitrate reduction is possible in the presence of alkalophilic microorganisms. This study highlighted the importance of biologically catalyzed reactions and the need to consider them in evaluating the fate of nitrates in radioactive waste repositories. Further work is ongoing on the possibility of microbial nitrate reduction in the presence of hydrogen gas, an electron-donor which will be released during anoxic corrosion of steel or radiolysis.

## Acknowledgements

The authors would like to thank Andra (Agence Nationale pour la Gestion des Déchets Radioactifs/French National Radioactive Waste Management Agency) for its financial support.

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