Regular article

Use of a continuous-flow bioreactor to evaluate nitrate reduction rate of *Halomonas desiderata* in cementitious environment relevant to nuclear waste deep repository

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ABSTRACT

The redox level of repositories can influence the mobility of the waste components stored in them (i.e. radionuclides) and thus the related safety assessments. Microbial activity is known to impact the control of redox reactions, the mechanisms and kinetics of which must be evaluated. This study investigates the denitrification rates of a model bacterium *Halomonas desiderata* (*Hd*) in cementitious environment with alkaline and anoxic conditions comparable to those found in radioactive waste repository cells. The growth and the total oxidized nitrogen (TON) reduction rates of *Hd* was determined in a continuous bioreactor with several feeding solutions with or without solid cement paste. Temporary nitrite accumulation and reduced denitrification rates are correlated with diminished bacterial growth. When the system was fed by optimal culture medium supplemented with acetate and nitrate, the TON reduction rates varied between 0.082 mM TON/h and 0.063 mM TON/h, depending on whether solid cement paste was present in the reactor or not. When the culture medium was replaced with pure cement leachate, the reaction rates increased to 0.137 mM TON/h with solid cement paste and dropped to 0.023 mM TON/h without. In these conditions at pH 10, solid cement paste had no negative influence on *Hd* activity.

1. Introduction

Part of the French long-lived intermediate-level radioactive waste (LLILW) results from the reprocessing of nuclear fuel by the PUREX (Plutonium Uranium Redox EXtraction) recycling procedure. This procedure involves the dissolution of the fuel rods in a hot nitric acid medium followed by selective solvent extraction [1]. During the process, the wastes are significantly enriched with nitrate. In a final process, the waste is stabilized, notably in a bitumen matrix, and stored in cylindrical steel containers (primary packages). For final storage, it is proposed that the primary packages should be grouped in reinforced concrete packages and placed inside concrete waste cells. A project is underway in France to build such waste cells at a depth of around 500 m within a clay rock formation (the Callovo-Oxfordian mudstone formation), which constitutes a geological barrier [2–4]. After repository closure, water will start to resaturate the cell, and leaching of the concrete (hydroxide and cations such as Ca^{2+}, Na^+, K^+, etc.) and waste components (oxyanions including nitrate, and organic matter) will occur [5]. Hydrogen gas will form as a consequence of water radiolysis, anaerobic steel corrosion and possibly fermentative organic matter degradation. The presence of nitrate will promote oxidizing conditions in the repository, which may increase the mobility of some redox sensitive radionuclides (Se, U, Tc, Pu, Np, etc.) [6]. Nitrate can be reduced by biotic and abiotic processes in the presence of electron donors, such as organic matter or dihydrogen [7–13]. Microorganisms are assumed to be principally introduced into the repository systems by ventilation and other human activities during both the construction and operational phases [14,15]. The activity of denitrifying bacteria could induce nitrate reduction into nitrogen gas (N_2) via several intermediate species: nitrite (NO_2^-), nitric oxide (NO) and nitrous oxide (N_2O). Because of the presence of bacteria, nitrate concentrations

should drop in the repository system, allowing a return to the initial reducing conditions and thus potentially leading to less mobile reduced forms of radionuclides (RN).

Nonetheless, the main question behind the scenario considered is whether bacterial denitrifying activity is really possible under the drastic pH conditions imposed by the large quantity of cementitious materials present in the concrete cell. The infiltration water in repository systems is considered a liquid media restrictive for bacterial development because of pH likely above 12 [6]. Indeed, the literature on microbial denitrification mainly reports on experimental or environmental systems working at pH conditions between 6.0 and 8.5 [16–18]. In alkaline systems, nitrate reactivity has been shown to be affected by the higher pH, and nitrite accumulation occurs mostly as a consequence of bacterial growth inhibition [10,19].

However, recent work has shown that a bacterial strain or consortia are capable of nitrate reduction at high pH [8,9,20,21]. For example, *Halomonas desiderata (Hd),* an alkaliphilic, halotolerant and denitrifying bacterium from the Gammaproteobacteria class, can reduce nitrate at pH between 9.0 and 12.0 in cement dominated environments using acetate as electron donor, in both static (batch reactor) and dynamic conditions (continuous feeding) [8,9,22]. *Hd* is also described to form biofilms on the surface of solid cementitious material [8,9]. The biofilm formation on solid support could be a key strategy for the biomass to survive in hostile environments [23]. Rafrafi et al. [9] have already shown that *Hd* was able to develop in cement leachate supplemented only by acetate and nitrate. The limiting pH for nitrate reduction by *Hd* in cement leachate was clearly identified [9]. The cement leachate was used as a model aqueous solution representing the alkaline cementitious aqueous environment. The cement leachate is a poor nutritive medium for bacteria, containing only calcium, sodium and magnesium as major salts [9,24,25]. The new objective of our work is now to evaluate the impact of the cement leachate as well as the impact of the presence of solid cementitious surface on the nitrate reduction rate of *Hd.* The specific experimental set-up implemented by Rafrafi et al. [9] was reused in the present work to determine denitrification rates by *Hd* in a multiphase system (presence or absence of cement matrices) for different feeding conditions. In a first experiment, the optimal culture medium for *Hd* cultivation described by Alquier et al. [8] was used. Then this culture medium was progressively replaced by a pure cement leachate supplemented with acetate and nitrate. All the experiments were carried out at pH 10, close to the optimal pH already defined for the growth of *Hd* [21].

2. Materials and methods

The experimental set-up implemented consisted of a bioreactor connected to an exposure chamber (containing cement pastes) in an open circuit (see section 2.4) fed by solutions with various proportions of cement leachate and culture medium (see section 2.5).

2.1. 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, Ø = 27 mm). Cement paste specimen were kept in hermetic bags (to avoid any hydric exchanges with the exterior and to protect them against carbonation) for 28 days after demoulding. Then, they were cut into slices (h ≈ 10 mm) and sanded to create a surface roughness favourable to the development of a bacterial biofilm.

Table 1
Chemical composition of the cement leachate (average).

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
Chemical composition of the culture medium.

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. Cement leachate

Cement leachates were prepared by immersing CEM V/A 42.5 cement paste slices in 1L of deionized water (solid/liquid volume ratio: 1.03% – solid surface/liquid volume around 60 cm^2/L) for 3 days under stirring. The average chemical composition of the cement leachate is given in Table 1.

2.3. Bacterial culture

Halomonas desiderata DSM 9502 obtained from the strain collection of DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Germany) [22] was chosen as the model bacterium. It is a heterotrophic bacterium capable of using acetate as an electron donor and nitrate/nitrite as an electron acceptor in alkaline pH conditions (9 < pH < 12) [8,9]. Culture medium DSM 9502 was prepared from the solutions described in Table 2 as detailed by Alquier et al. [8].

2.4. Overview of the experimental set-up

The experimental set-up was designed to evaluate the microbial denitrifying activity, notably the reduction rate for nitrate and nitrite in the presence of acetate in a multiphase system, while partly decoupling the microbial growth from the interactions between the various components of the system (microorganisms, cement leachate, solid cement paste) (Fig. 1).

The bioreactor, with a working volume of 2L, was the entrance component of the system. It was used to adapt the microbial culture to the batch reactor prior to the dynamic experiment. It was also used to explore the influence of device-related parameters (e.g. hydraulic retention time, temperature, etc.) on denitrification (Fig. 1). When the valve was open, the bioreactor was connected to a feeding solution tank ensuring a continuous supply at a constant flow rate of 0.66 mL/min via a peristaltic pump (Model 7554-85, 7–200 rpm, head Easy Load L/S for tube 13–18 7518-00 model). An exposure chamber having a working volume of 1L and containing 3 solid slices of cement pastes was connected downstream of the bioreactor (Fig. 1). Here, the aim was to evaluate the effect of solid cementitious matrices on the bacterial activity.

2.5. Experimental conditions, composition of feeding solutions

The bioreactor was inoculated with a preculture of *Hd* (2 mL of active growing culture, optical density around of 0.3 at 600 nm). Each experiment started with a 7-day batch culture period in the bioreactor, until an OD value around 0.1 was reached. 7 days was the time required to reach maximum biomass of *Hd* in a closed system [8]. Subsequently, the bioreactor was fed continuously with a
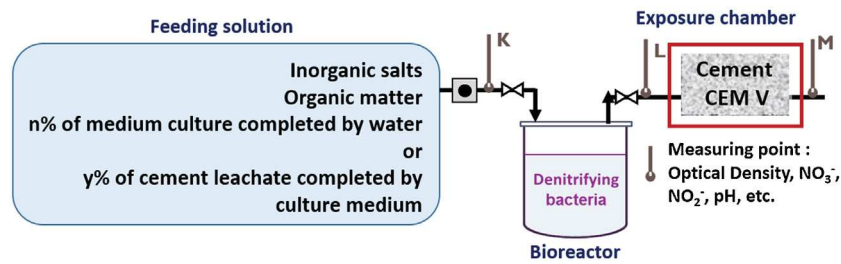
Fig. 1. Schematic representation of the experimental set-up to study the impact of the feeding solution on the microbial activity with or without cement paste.

fresh medium and the exposure chamber was linked to the bioreactor.

Four feeding solutions (marked S1 to S4 – Table 3), were tested in order to evaluate their impact on denitrifying activity in the bioreactor and then in the exposure chamber. They consisted of mixtures of culture medium, water and cement leachate (Table 1) in different proportions. These solutions were then supplemented by sodium acetate ($8.3 \, mM \, NaC_2H_3O_2$) as the only electron donor, and sodium nitrate ($5.9 \, mM \, NaNO_3$) as the only electron acceptor (Table 3). The pH of the feeding solution was adjusted to 10 using NaOH (4 M).

The hydraulic retention time (HRT) was set at 50.5 h in the bioreactor, and thus 25.25 h in the exposure chamber. The bioreactor and the exposure chamber were thermostated at 37 °C, continuously deoxygenated by N_2 bubbling, and stirred (300 rpm).

Samples of solution were collected regularly at the outlet of the feeding tank (point K, Fig. 1), of the bioreactor (point L, Fig. 1) and of the chamber (point M, Fig. 1). Unfiltered samples were used for immediate measurements of optical density (OD) at 600 nm and pH. Two millilitre samples were filtered through a 0.2 μm pore size filter (Minisart PES, Fisher Scientific) for ionic species analyses (nitrate, nitrite and acetate).

2.6. Analytical techniques

2.6.1. Bacterial growth

The bacterial growth was measured using optical density (OD) at 600 nm (JENWAY 7315 spectrophotometer). This method enables the quantity of biomass suspended in the medium to be measured directly. Both living cells and dead cells are counted by this method. The feeding solution was systematically used as a blank. Turbidity measurements to monitor bacterial growth were applicable when the liquid medium was not cloudy. Overestimation in the presence of cementitious particles could not be excluded.

2.6.2. Chemical analyses

Concentrations of $Ca^{2+}, K^+, Na^+, CH_3COO^-, NO_3^-$ and NO_2^- were measured by High Performance Ion Chromatography (Dionex ICS-2000 and ICS-3000) using analytical methods detailed by Alquier et al. [8] and Bertron et al. [26].

3. Results

3.1. Reference experiment: 100% culture medium in the feeding tank (solution S1)

Based on batch experiments, Alquier et al. [8] described *H. desiderata* (*Hd*) as a model denitrifying bacterium capable of efficiently reducing nitrate in the presence of concrete (alkaline conditions). The main objective of the reference experiment presented here was to gain access to the growth rates and the denitrification rates under the optimal experimental conditions for *Hd* growth.
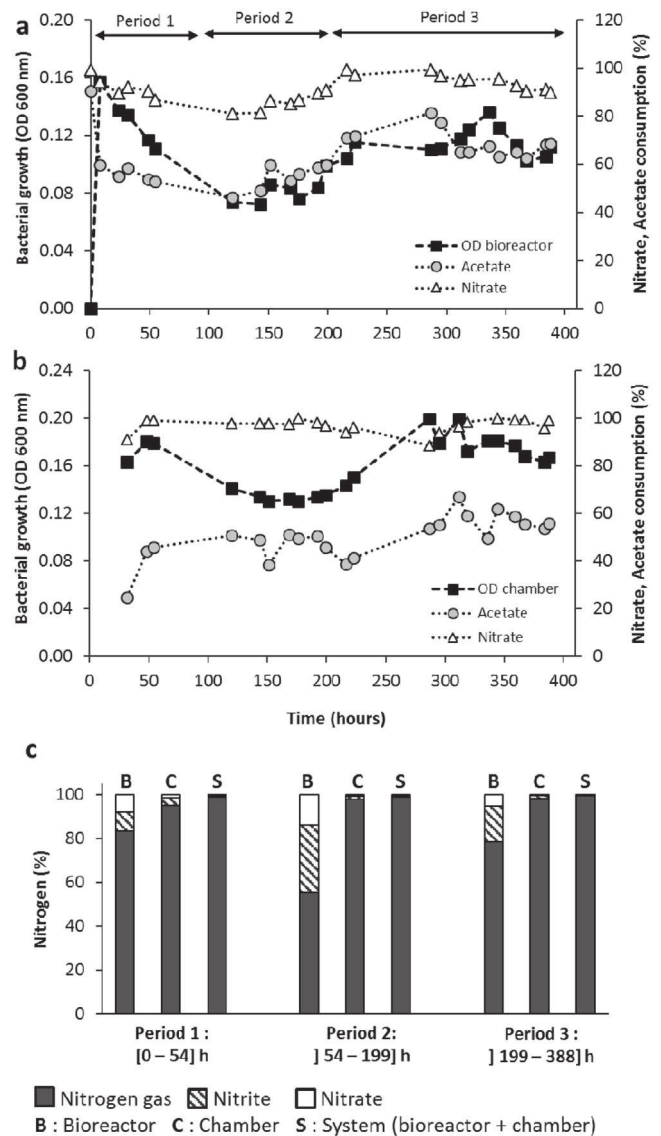
Fig. 2. Bacterial growth and evolution of acetate and nitrate consumption rates in the bioreactor (a) and in the exposure chamber (b) during the continuous culture of *Hd* with 100% of optimal culture medium (solution S1) at pH 10. Nitrogen mass balance in the bioreactor, in the chamber and in the global system (i.e. bioreactor + chamber) (c).

Fig. 2.a and Fig. 2.b show the evolution of the *Hd* growth with time and the evolution of the nitrate and acetate consumption rates ($[X]_{t \, consumed}/[X]_{t \, input}$ where $[X]_{t \, consumed} = [X]_{t \, input} - [X]_{t \, outlet}$) in the bioreactor and in the exposure chamber, respectively. The evolution of the nitrate, nitrite and acetate concentrations of the feeding solution and in the outlet of the bioreactor and the expo-

Table 3
Composition and pH of the feeding solutions.

Solution name	Composition in percentage (%)			Additional component	pH
	Culture medium	Water	Cement leachate		
S1	100	0	0		
S2	10	90	0	8.3 mM	10 (adjusted by
S3	10	0	90	$NaC_2H_3O_2 + 5.9$ mM	addition of NaOH)
S4	0	0	100	$NaNO_3$	

sure chamber are available in the supplementary material, Figure A.1.

The bacterial growth and both nitrate and acetate consumption rates followed similar trends. For this experiment, three time periods have been defined according to the trend of nitrate reduction rate in the bioreactor. For each time period, the nitrogen mass balance was calculated to highlight the preponderant nitrogen species (nitrate, nitrite, or nitrogen gas, i.e. NO or N_2O or N_2) in the bioreactor, the chamber and the overall system (including bioreactor and chamber) (Fig. 2.c).

In the bioreactor, during the first time period ([0,54 h], Fig. 2.a), OD and both nitrate reduction and acetate oxidation efficiencies decreased, reaching the low values of 0.07, 81% and 46%, respectively, found at the beginning of period 2 ([54, 199 h], Fig. 2.a). This coincided with progressive production of nitrite in the bioreactor (Fig. 2.c) ([NO_2^-] ≈ 1.8 mM on average, Figure A.1). Then, OD and both nitrate and acetate consumption rates in the bioreactor increased progressively to 0.10, 91% and 60%, respectively, and remained fairly stable during period 3 (] 199,388 h], Fig. 2.a).

The same trend was observed for the OD values in the exposure chamber over the three time periods, whereas no significant evolution was observed for nitrate and acetate consumption rates. The average nitrate consumption rate was 97% over the complete experiment. Neither significant nitrite production, nor inhibition or decrease of the microbial activity were observed. At the chamber outlet, after around 40 h, the proportion of nitrite relative to the total incoming nitrogen was always below 3% (Fig. 2.c) and the concentration was below 0.07 mM (Figure A.1). The fluctuation of the biomass growth in the exposure chamber resulted mainly from the fluctuations already observed in the bioreactor rather than from variations of growth in the exposure chamber.

For this reference experiment, the minimal nitrate consumption rate was above 80% in the bioreactor and 95% in the chamber (Fig. 2.c). In our design of experiment, it was assumed that the nitrogen gas produced was continuously evacuated from the aqueous phase (feeding solution) by the N_2 gas bubbling. Since the amount of nitrite measured was negligible compared to the nitrate consumed, the denitrification process could be considered as complete, with the gaseous stage being reached. The instantaneous nitrate reduction rate (RedR) (mM h^{-1}) was calculated as in Eq. (1).

$$RedR = ([NO_3^-]_{t\_in} - [NO_3^-]_{t\_out})/HRT \qquad (1)$$

where $[NO_3^-]_{in}$ (mM) and $[NO_3^-]_{out}$ (mM) are the concentrations of nitrate at the inlet and outlet, respectively, of the system under consideration (bioreactor, chamber or overall system) and HRT is the hydraulic retention time in hours. The same approach was applied to estimate the instantaneous total oxidized nitrogen (nitrate and nitrite – TON) reduction rate, and instantaneous acetate oxidation rate (OxR). The average of the OxR and RedR obtained by this calculation are reported in Table 4.

The reference experiment gave access to the nitrate reduction rate catalysed by Hd cells at pH 10 under optimal conditions of growth. The next step was to switch from a growth culture medium optimal for Hd to a simpler culture medium (less optimal) in order to approach the environment existing in packages of a radioactive waste repository.

3.2. 10% of optimal culture medium and 90% of sterilized water in the feeding tank (solution S2)

In the second step of the experiment, the feeding solution medium was made of an optimal culture medium diluted by replacing 90% with sterilized water. The aim was to assess Hd activity in minimal medium (low concentration of vitamins and minerals). Nitrogen mass balances were performed under four interesting time periods defined in accordance with the denitrification rate observed in the bioreactor for the period 1 and 2 and with the denitrification rate in the exposure chamber for the periods 3 and 4.

After a preliminary batch period of 7 days in the bioreactor containing the feeding solution with 10% culture medium, the OD value was 0.10. Immediately after the start of the continuous supply, the biomass dropped drastically, OD values reaching values below 0.04 after the first 72 h (time periods 1 and 2 in Fig. 3.a) and then remained globally low during the rest of the experiment (to 340 h). The same trends were observed for the nitrate and acetate consumption rates, which decreased from 50% and 30%, respectively, to below about 20% for both of them after 96 h of dynamic supply (period 2–]99, 200] h – Fig. 3.a – evolution of concentrations is available in Figure A.2). It would seem that the growth rate of the strain was severely reduced. Consequently, the bacterial cells were progressively washed out of the bioreactor and the exposure chamber, leading to lower acetate and nitrate consumption rates and no nitrite production.

In the exposure chamber during the first time period ([0,32 h] – Fig. 3.b), OD values, and also both nitrate and acetate consumption rates dropped drastically (to values around 0.08, 35% and 24%, respectively) and remained low during period 2 ([32, 96] – Fig. 3.b – evolution of concentrations is available in Figure A.2). Solid cement paste slices were added into the exposure chamber after 132 h of culture to evaluate their impact on Hd's growth rate and denitrification rates. Less than 35 h after this addition, OD values and nitrate and acetate consumption rates had increased significantly, to 0.16, 56% and 50% respectively (time period 3]96, 200] h – Fig. 3.b). However, the positive effect of the cement paste addition on Hd's growth and denitrification activity was only temporary. At the beginning of time period 4 ([200, 336] h – Fig. 3.b), i.e. approximately 84 h after the cement paste was immersed, the nitrate and acetate consumption rates decreased very quickly, followed 30 h later by the OD values, which fell to below 0.1. The progressive loss of bacterial metabolic activity was thus observed first and then the bacterial cells were washed out of the reactors.

Fig. 3.c clearly shows that the denitrification process was progressively interrupted in the bioreactor. Especially from time period 3, less than 10% of the nitrate concentration entering the bioreactor was reduced. The same trends of nitrate reduction occurred in the exposure chamber except in time period 3, where a production of 50% of nitrogen gas was noted. This sudden and short-term increase of nitrate reduction rate was observed several hours after the addition of the cement paste in the exposure chamber. The release of soluble species induced by cement paste leaching seemed to boost the activity of the denitrifying strain Hd with solution 2. But after a few tens of hours (time period 4), some key elements

Table 4
Average values of acetate OxR, nitrite RedR and nitrate RedR for the four compositions of feeding solutions.

| Solution name		S1	S2	S3	S4
Solution composition		100% CM[a]	10% CM[a] + water	10% CM[a] + cement leachate	100% cement leachate
Nitrate (mM h^{-1})	Bioreactor	0.103	0.023	0.093	0.066
	Chamber	0.020	0.054	0.033	0.099
	System	0.077	0.033	0.071	0.076
TON (mM h^{-1})	Bioreactor	0.082	0.012	0.079	0.024
	Chamber	0.063	0.056	0.044	0.137
	System	0.076	0.027	0.067	0.062
Acetate (mM h^{-1})	Bioreactor	0.104	0.033	0.090	0.057
	Chamber	0.064	0.057	0.054	0.084
	System	0.092	0.041	0.078	0.047

[a] CM: Culture medium.
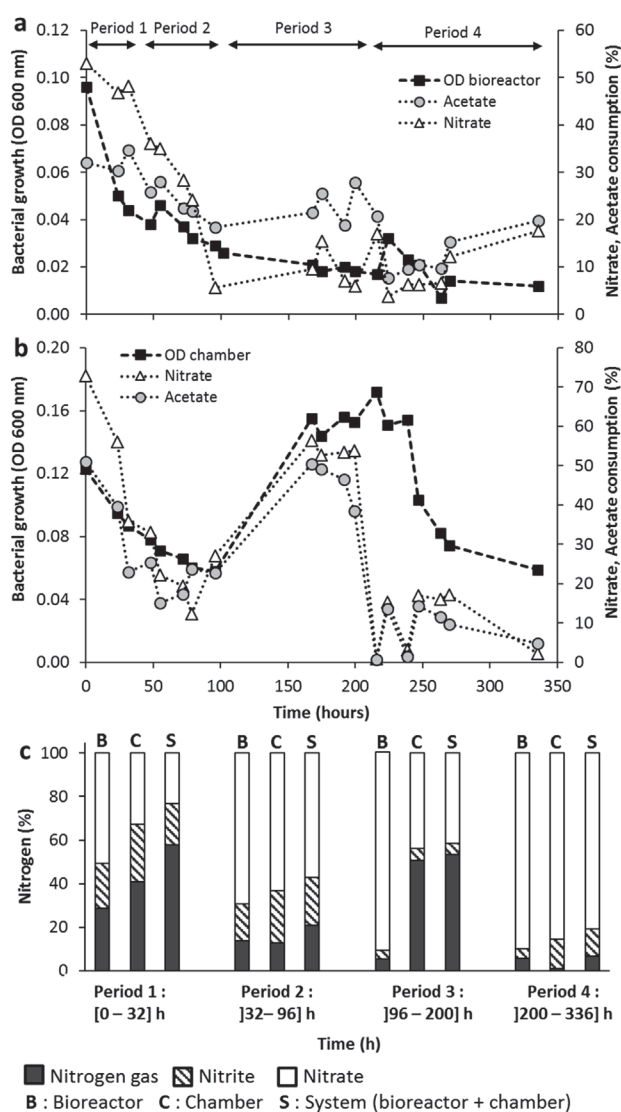
Fig. 3. Bacterial growth and evolution of acetate and nitrate consumption rates in the bioreactor (a) and in the exposure chamber (b) during the continuous culture of Hd with 10% of optimal culture medium completed by 90% of water (solution S2) at pH 10. Nitrogen mass balance on the bioreactor, the chamber and the global system (i.e. bioreactor + chamber) (c) over four interesting periods selected according to the particular trend of the OD values and the consumption rates in the exposure chamber.

required for sustainable *Hd* growth leading to nitrate reduction, and which had been released from the cement paste by leaching, seemed to be missing in the 10% culture medium. The overall nitrate reduction rate was estimated from Eq. (1). The average RedR defined for these experiments were very low: 0.023 mM N–NO$_3^-$/h and 0.012 mM TON (TON includes NO$_2^-$ and NO$_3^-$) in the bioreactor (Table 4), 0.054 mM N–NO$_3^-$/h and 0.064 mM TON in the exposure chamber (Table 4), or 0.036 mM N–NO$_3^-$/h and 0.034 mM TON if the period when the cement pastes had a positive impact was excluded. The use of only 10% of optimal culture medium clearly affected *Hd* growth and nitrate reduction. The nitrate reduction rate was divided by 5 in the bioreactor compared to the reduction rate obtained with 100% of optimal culture medium.

3.3. 10% of culture medium and 90% of cement leachate in the feeding tank (solution S3)

Hd culture in the presence of low concentrations of optimal culture medium (10%) showed a lower denitrifying activity overall, both in the bioreactor and in the exposure chamber. However, for a short time following the addition of solid cement pastes in the exposure chamber, the denitrifying activity of *Hd* was improved under the low concentration of optimal culture medium (10%). In order to examine the impact of the leachate on *Hd* activity, distilled water was replaced by cement leachate. Three times periods were defined according to the trend of the nitrate reduction rate measured in the bioreactor.

Following the start-up of the constant supply of culture medium in the bioreactor, i.e. during time period 1 ([0,69] – Fig. 4.a), the OD values decreased from 0.14 to 0.06, with parallel generation of a decrease of 10% of the reduction rate of nitrate. Then, during time period 2 (] 69, 236] h – Fig. 4.a – evolution of concentrations is available in Figure A.3), the biomass growth remained quite stable whereas the removal efficiencies of nitrate and acetate continued to decrease – to 49% and 28%, respectively. The decrease of nitrate reduction efficiency was accompanied by nitrite production in the bioreactor, as in the reference experiment (Fig. 4.c; Fig. 2.c). Then, both acetate oxidation and nitrate reduction rates increased during period 3 ([236, 386] h – Fig. 4.a). One hundred percent of the nitrate was consumed whereas the OD values increased slightly to reach around 0.08. Finally, the global output flow of TON (nitrate and nitrite) from the bioreactor was below 1.1 mM except during the transient period 2, during which it reached around 3.4 mM, in particular because of nitrite accumulation.

In the exposure chamber, during the three distinct time periods (Fig. 4.b – evolution of concentrations is available in Figure A.3), the nitrate reduction efficiency was above 90%. The acetate consumption rate was 20% lower at the beginning of time period 1 and then increased following the input of a higher quantity of electron acceptor (nitrite and nitrate), notably during period 2 (Fig. 4). The acetate oxidation rate was limited by TON availability in the expo-
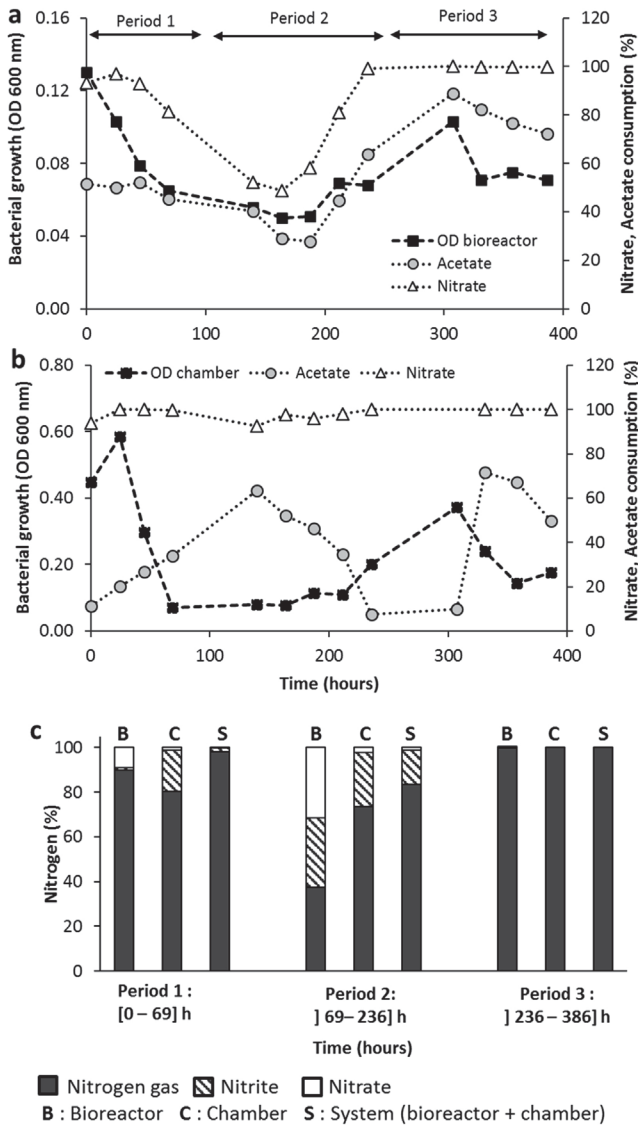
Fig. 4. Bacterial growth and evolution of acetate and nitrate removal efficiencies in the bioreactor (a) and in the exposure chamber (b) during the continuous culture of *Hd* with 10% of optimal culture medium completed by 90% of cement leachate (solution S3) at pH 10. Nitrogen mass balance on the bioreactor, the chamber and the system (i.e. bioreactor + chamber) (c).
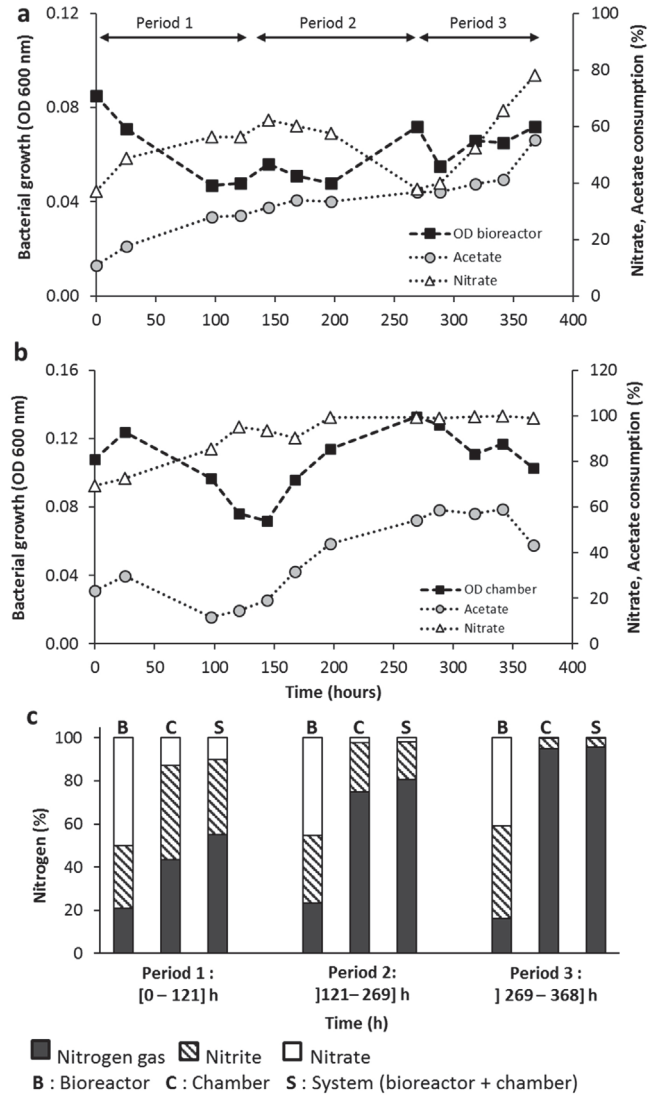

Fig. 5. Bacterial growth and evolution of acetate and nitrate consumption in the bioreactor (a) and in the exposure chamber (b) during the continuous culture of *Hd* with 100% of cement leachate (solution S4) at pH 10. Nitrogen mass balance on the bioreactor, the chamber and the system (i.e. bioreactor + chamber) (c).

sure chamber. In spite of difficulties in accurately monitoring *Hd* growth by measuring the OD in the exposure chamber (the measurements may have been affected by the presence of non-soluble inorganic particles coming from the cement paste leaching), the OD values were found to lie between 0.08 and 0.59 (Fig. 4.b), generally higher than the values obtained during the control experiment (Fig. 2.b).

According to Fig. 4.c, it can be estimated that more than 80% of nitrate was reduced to nitrogen gas except during the peak of nitrite accumulation observed in both the bioreactor and the exposure chamber (from 69 h to 236 h). The OxR and RedR obtained for this experiment are reported in Table 3. The use of solution S3 (10% of optimal culture medium and 90% of cement leachate) was favourable to the cultivation of *Hd*. The cement leachate seemed to provide some elements essential for *Hd* growth, probably elements that were present in limiting concentrations in the 10% optimal culture medium.

3.4. 100% cement leachate in the feeding tank (solution S4)

The experiment with 10% of optimal culture medium completed with 90% of cement leachate highlighted the positive impact of the cement leachate on *Hd* growth and its heterotrophic denitrifying activity. However, in the scenario scheduled for the LLIL waste repository, the aqueous environment should be similar to a cement leachate. Therefore, to consider conditions that were closer to reality, only cement leachate was used as a culture medium for *Hd* in the last step of this study. Three interesting times periods were defined according to the trend of nitrate reduction rate in the bioreactor.

In the bioreactor, during the first time period ([0,121] h – Fig. 5.a), the OD values first decreased from 0.09 to 0.05, remained quite stable during the second time period (] 121, 269] h) and then increased slightly to 0.07 during the 3rd period ([269, 368] h – Fig. 5.a). The nitrate reduction rate fluctuated between 37% and 62% during time periods 1 and 2 and then slowly increased to 78% during time period 3 (Fig. 5.a – evolution of concentrations is available in Figure A.4). At the same time, the acetate oxidation rate increased progressively (around 0.2% per hour during the whole experiment) (Fig. 5.a). In contrast with the previous experiment, the evolution

Table 5
Average pH in the bioreactor and in the exposure chamber.

Solution name	Average pH (standard deviation)		
	Feeding solution	Bioreactor	Chamber
S1	10.2 (0.03)	10.1 (0.03)	10.1 (0.03)
S2	10.1 (0.03)	10.0 (0.13)	10.0 (0.13)
S3	10.2 (0.23)	10.1 (0.10)	10.1 (0.06)
S4	10.1 (0.07)	**9.7 (0.30)**	10.2 (0.12)

of OD and the nitrate and acetate oxidation rates did not follow the same trend in the bioreactor. The biomass quantity decreased when the nitrate reduction rates increased and *vice versa*. An average of 35% of nitrate was reduced into nitrite and not into nitrogen gas during the experiment time (Fig. 5.c.). The fraction of each nitrogen component studied was globally stable during the experiment. The proportions were approximately: 20% of nitrogen gas, 35% of nitrite and 45% of nitrate. Additionally, in contrast with the previous experiments, the pH was not stable (Table 5): it decreased from 10.2 to the minimal value of 9.2 after 293 h of culture and increased to 9.6 during time period 3.

In contrast with the bioreactor pH, that in the exposure chamber remained quite stable (Table 5). The nitrate reduction rate increased progressively during time periods 1 and 2 and reached values above 95% at the end of the 2nd time period, i.e. after 221 h of continuous culture (Fig. 5.c – evolution of concentrations is available in Figure A.4). Nonetheless, the percentage of nitrite produced compared to the nitrite and nitrate input was 45%, 22% and 4% on average for periods 1, 2 and 3, respectively (Fig. 5.c). The nitrite proportion at the outlet of the chamber was higher than for previous positive experiments supplied with solutions S1 and S3. Denitrification could be considered as almost complete (at least 85% of nitrate and nitrite was reduced to nitrogen gas) only after 312 h of *Hd* cultivation with a dynamic supply of cement leachate (Fig. 5.b and Fig. 5.c).

Both the OD values and the acetate oxidation rate decreased in the exposure chamber during time period 1. The acetate oxidation rate reached the minimal value of 12% after 97 h of culture in continuous supply, while the nitrite production was the highest (3.5 mM) at the chamber outlet. OD reached its minimal value at 0.07 after 144 h of culture, i.e. roughly 50 h after the acetate oxidation inhibition. This was probably because the *Hd* growth was only inhibited before the bacterium was washed away and was not directly affected by the toxic impact of nitrite. Then, the bacterial activity inhibition seemed to dissipate: the OD increased to 0.13 and the acetate oxidation rate increased to 54% during the second time period (Fig. 5.b). Nonetheless, a decrease of biomass was observed at the end of time period 3, which was coupled with a slowdown of acetate oxidation rates and an increase of the nitrite concentration at the outlet of the chamber (about 1.8 mM on average).

In summary, under a constant supply of cement leachate supplemented by acetate and nitrate, after approximately 300 h of culture, the percentage of nitrate reduced to nitrogen gas was below 20% in the bioreactor and above 95% in the exposure chamber. The OxR and RedR obtained for this experiment are reported in Table 4. The reduction of nitrate by *Hd* was possible in an aqueous medium as poor as cement leachate (pH 10 and culture medium containing only cement leachate) [1].

4. Discussion

4.1. Adaptation of H. desiderata *from optimal growth medium to cement leachate*

Decreasing the percentage of optimal culture medium from 100% to 10% by dilution with water (solution S2) or by cement leachate (solution S3, with 90% of cement leachate) had an impact on the *Hd* nitrate reduction rate. With solution S2, the nitrate reduction yield decreased and stayed below 20% in the bioreactor during the experiment whereas, with solution S3, the nitrate reduction was almost 100% after 240 h of culture in the bioreactor. The difference between results obtained with S2 and S3 could be explained by the mineral elements present in cement leachate. Table 1 shows that the major components of cement leachate were, by decreasing order of concentration: potassium, sodium, calcium, silicon, aluminium and iron. Given that *Hd* is a halotolerant microorganism and that its alkaliphilic growth depends strongly on the availability of ions [21], it can be assumed that trace elements present in cement leachate can have an important influence on *Hd* activity.

This was supported by the results obtained for the solution S4, containing 100% of cement leachate. Even if the denitrification rates were lower, the culture of *Hd* with cement leachate alone was clearly observed and the nitrate reduction efficiency reached almost 100% after 200 h of culture in the exposure chamber.

4.2. Cement paste impact on H. desiderata *culture and denitrifying activity*

During the experiment with the 10% optimal culture medium (solution S2), a transient increase of biomass growth and of both nitrate and acetate removal efficiencies was observed a few hours after the addition of cement paste to the exposure chamber. This positive effect of the presence of cement paste on *Hd* denitrifying activity was thus time-limited.
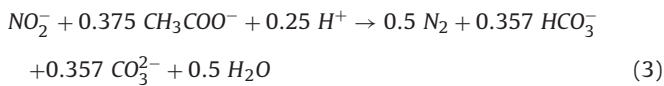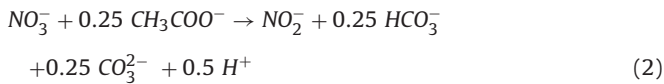
Cementitious matrices release salts, notably cations such as calcium, sodium and magnesium, when in contact with aqueous media and, in particular, those under consideration in this study [6,27,28]. The high and/or fast release of some minerals (Na^+, Ca^{2+}, K^+, etc.) could stimulate *Hd* activity during approximately 30 h (period 3 – Fig. 3.b). The effect of adding solid cement paste was limited in time, probably because (i) the release kinetics of soluble species from the cement matrices progressively decreased and (ii) the reserve of bioavailable elements leached from cement paste was completely consumed after 30 h.

In the case of the experiment supplied with solution S4 (100% cement leachate)), the nitrate reduction was not complete in the bioreactor but the growth conditions in the exposure chamber enabled nitrate and nitrite reduction with better performances, i.e. 0.137 mM TON/h (Table 4). Nitrite and nitrate reduction started more quickly in the chamber. These differences between chamber and bioreactor may have resulted from the cement paste presence in the exposure chamber. In addition to the release by cement matrices of minerals that could have a positive impact on *Hd* activity, the cement pastes may have served as a solid support for the proliferation of *Hd* biofilms. *Hd* biofilm development could not be quantified by OD measurement because only free planktonic bacteria in the liquid culture medium are analysed by this analytical method. Nevertheless, microscopic observation of cement paste surfaces by epifluorescence microscopy (after staining cement surfaces with Syto® 9 as a nucleic acid marker – data not shown) confirmed the ability of *Hd* to colonise cement pastes. This confirmed the results previously published by Alquier et al. [8] and Rafrafi et al. [9]. More specifically, Rafrafi et al. [9]. reported the presence of a biofilm of *Hd* on CEM V cement paste under conditions equivalent to those explored in the present paper (similar chemical composition of the feeding solution S4) but at pH 12. According to Alquier et al. [8], *Hd*'s biofilm could play an important role in the denitrification process. Inside the biofilm, conditions could be locally more favourable for bacterial growth and activity as the extracellular polymeric substance that constitutes the biofilm helps to sequester dissolved and particulate nutrients from the water phase, which can be then utilised as nutrients and energy

sources [23]. Moreover, the bacterial phenotype can vary depending on whether bacteria are planktonic or organised in a biofilm; the gene expression can vary to adjust the physiology of the bacteria [23,29]. Thus, the gene expression of nitrate and/or nitrite reductase could be better adapted to the specific conditions of *Hd* growth. Biofilm formation is a key factor for survival in hostile environments as it helps the biomass to develop specific abilities and tolerance (to low substrate, unfavourable pH, toxins . . .) [23].

4.3. Stability of the pH

The pH stayed rather stable in the bioreactor and in the exposure chamber for the experiment series, except for the experiment without culture medium (solution S4) (Table 5). The culture medium contributed to the pH stabilisation. For this experiment, the pH decreased from 10.2 to 9.2 after 293 h of culture in the bioreactor (unlike solutions S1, S2 and S3, solution S4 was not buffered by carbonate). However, the pH stayed fairly stable in the exposure chamber, possibly due to the presence of solid cement pastes that contributed to pH stabilisation. For neutral pH, the reduction of nitrate into nitrite generates H+, which is generally reused in the nitrite reduction reaction. Thus, the complete denitrification contributed to a net consumption of 0.317 H+ (equivalence of acidity) for each mole of nitrate reduced to nitrogen gas, so the pH could rise during the denitrification [10]. Whereas, for pH around 10.2 (pH of the feeding solution), the first step of denitrification (reduction of nitrate to nitrite) generates protons (Eq. (2)), which are partially consumed when nitrite is reduced in accordance with Eq. (3) [10,11].

$$NO_3^- + 0.25\ CH_3COO^- \rightarrow NO_2^- + 0.25\ HCO_3^-$$
$$+0.25\ CO_3^{2-} + 0.5\ H^+ \tag{2}$$

$$NO_2^- + 0.375\ CH_3COO^- + 0.25\ H^+ \rightarrow 0.5\ N_2 + 0.357\ HCO_3^-$$
$$+0.357\ CO_3^{2-} + 0.5\ H_2O \tag{3}$$

At pH around 10.2, the reaction of CO_2 with H_2O produces 0.25 equivalent of acidity, which makes the pH decrease. And, whatever the pH value, if the nitrite denitrifying activity is inhibited, H+ accumulates and the pH decreases if the medium is not buffered. Thus, the pH has an important effect on the denitrification rate [10], but the denitrification reaction can also affect the pH stability, especially for a non-buffered medium.

4.4. Nitrite accumulation

An increase in the concentration of nitrite produced during nitrate reduction by *Hd* was observed in the bioreactor for experiments with feeding solutions containing culture medium alone (S1), 10% of culture medium diluted by cement leachate (S3) and cement leachate alone (S4) supplemented by acetate and nitrate. The nitrite accumulation was transient (S1 and S3) or permanent (S4) depending on the stability of the pH (around 10). The maximal nitrite concentrations never exceeded 2.3 mM. The increase of nitrite concentration was systematically accompanied by a decrease in the growth of *Hd* strain and its denitrifying activity.

Transient nitrite accumulation associated with an inhibition of the bacterial growth and activity has already been observed in previous works [10,19,30]. A nitrite concentration of about 23 mmol/L has been reported to inhibit or have a weak toxic impact on the specific enzyme function and on bacterial growth [19,31]. The protonated species of nitrite, HNO_2, has been reported as a strong inhibitor of the denitrification process [31]. Nonetheless, the HNO_2

effects were mainly observed for pH below 8 [10,31–33]. According to Eqs. (4) and (5) presented by Anthonisen at al. [34], the HNO_2 concentration ($[HNO_2]$) that is pH dependent is almost negligible at pH 10 with low nitrite concentration ($[NO_2^-]$) (2.3 mmol/L maximum), i.e. below 2.10^{-9} mM/L.

$$[HNO_2] = \frac{[NO_2^-]}{K_a + 10^{pH}} \tag{4}$$

where K_a is the ionisation constant of the nitrous acid equilibrium equation, which depends on temperature as follows:

$$K_a = e^{\left(-\frac{2300}{273 + T°C}\right)} \tag{5}$$

In an alkaline environment, the transient nitrite accumulation could correspond to the time necessary for the biomass to adjust to a change of the system, i.e. the transition from a batch system to a continuous system. Some time could be necessary for *Hd* cells to activate transcription of the genes encoding for nitrite reductase (NIR), the expression of which is notably induced by the nitrogen oxide [35,36]. The time required to reach the steady state is theoretically equivalent to 3 or 4 cycles of reactor volume renewal, i.e. 3 or 4 times the HRT, thus approximately 150–200 h for the bioreactor in this experiment. This corresponds to the time required for almost complete denitrification (>90%) in the experiment with 100% of optimal culture medium (solution S1) and with 10% of optimal culture medium completed by cement leachate (solution S3).

For the experiment with only cement leachate as the culture medium (solution S4), more time may be required to reach complete denitrification. Moreover, the pH variability from 10 to 9.2 could promote the permanent accumulation of nitrite in a concentration range of 2.3 ± 0.7 mM. Many factors, and especially the pH, may influence the nitrite and nitrate reduction rates and the effects of pH on the reduction rates of nitrite and nitrate can be different [7,37]. Denitrifying enzymes are relatively sensitive to pH values and there is a specific pH for each enzyme [38]. The bacterial enzymes involved in the reduction of nitrate are very different in their composition and location in the bacterial cells from those involved in the reduction of nitrite [39]. Thus, for the experiment with only cement leachate as the culture medium, the accumulation of nitrite was caused by the difference between the nitrate and nitrite reduction rates and the variability of the pH (Δ pH of 0.8 unit).

4.5. Nitrate and nitrite reduction rate by H. desiderata

Most studies on the determination of denitrification rates concern the domain of wastewater treatment or remediation, i.e. of polluted soil. In such research areas, the classical optimal pH for the denitrification process is close to neutrality, i.e. between 6 and 8.5 [16–18]. According to the result obtained by Cao et al. [7], the nitrate reduction rate decreased from 0.63 to 0.12 mg N/g MLVSS/min (equivalent to 5.29 and 1.01 mM/h with a Mixed Liquor Volatile Suspended Solids (MLVSS) concentration of 1.960 g/L), an increase of the pH value from 6.5 to 9.2. Nitrate reduction under alkaline conditions (pH above 9) is possible [8,9,20] but the RedR are lower than those obtained at neutral pH. Results reported in this study support this finding. The nitrate reduction rates in continuous supply with 100% of optimal culture medium, i.e. 0.103 mM NO_3^--N/h (Table 4), was similar to those obtained by Alquier et al. [8] and Rizoulis et al. [20] in a batch reactor with a pH about 10. Alquier et al. [8] obtained 0.13 mM NO_3^--N/h for the nitrate reduction by *Hd* with cement pastes in the reactor and Rizoulis et al. [20] observed the reduction of 15 mM nitrate in one week, i.e. 0.089 mM NO_3^--N/h for nitrate reduction by a sedimentary microcosm. The denitrifying activity of *Hd* was not substantially impacted by the dynamic supply of opti-

mal culture medium supplemented by acetate and nitrate; the HRT of 50.50 h was well suitable. When the optimal culture medium were replaced by 100% of cement leachate without HRT adjustment, the nitrate reduction rate decreased and reached the value of 0.066 mM NO_3^--N/h in the bioreactor. This rate was consistent to the *Hd* nitrate reduction rate defined by Rafrafi et al. [9], i.e. 0.056 mM NO_3^--N/h in the bioreactor, under similar experimental set-up supplied continuously with cement leachate supplemented with nitrate and acetate at pH 10. In the case of denitrification at alkaline pH and especially with poor culture medium (100% cement leachate), high HRT was required. *Hd* needed a few hours to adapt to the environmental conditions and to complete the total reduction of nitrate and nitrite. In this study, the denitrification was considered as complete (above 90%) after approximately 220 h in the bioreactor (HRT of 50.5 h) for the experiment with solutions S1 and S3 and after 200 h – but only at the outlet of the exposure chamber – (HRT of 75.75 h) for the experiment with cement leachate (solution S4). The nitrogen mass balance calculated for all the experiments clearly showed the significant nitrite production due to incomplete denitrification. This production could be transitional (experiment S1 and S3) or constant during the experiment (experiment S4).

5. Conclusion

The experimental set-up allowed the impact of cement leachate and solid cement paste on the denitrification rates of *H. desiderata* at pH 10 to be determined. We have highlighted that cement leachate (poor nutrient substrate) can provide some elements (certainly a complex combination – not identified) that enable *H. desiderata* growth and the reduction of nitrate and nitrite. Nonetheless, at the beginning of the experiment, nitrate reduction was faster than nitrite reduction. A temporary nitrite accumulation did occur which slowed down denitrification rates and bacterial growth.

According to these results and previous works, the cement paste in the exposure chamber can be colonised by a biofilm of *H. desiderata* that can help to improve the denitrification rates, especially when the growing conditions become unfavourable in the reaction chamber. Further ongoing works are exploring the possibility of microbial nitrate reduction by a natural consortium of bacteria and also the biodegradability of other carbon sources and/or electron donors.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bej.2017.05.016.

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