Nitrate and nitrite reduction activity of activated sludge microcosm in a highly alkaline environment with solid cementitious material

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ABSTRACT

Denitrification is a major biological process contributing to nitrate and nitrite reduction. However, this process remains poorly understood at alkaline pH although such conditions can be encountered in natural (e.g. soda lakes) or industrial environments (e.g. geological waste repositories with cementitious materials). To investigate the nitrate reduction (NR) rate for pH > 9.5 in a cementitious environment, several batch reactors were implemented, with cement leachate or with hardened cement paste (HCP).

In the experiments carried out with cement leachate, NR dropped from 0.72 mM/h at pH 9.5 to 0.17 mM/h at pH > 11, while the concentration of nitrite increased. The NR was inhibited at pH close to 12, as was the nitrite reduction at pH above 11. In the reactor containing HCP, the NR rate was 0.75 mM/h at pH close to 10. Calcite precipitated on the HCP surface. Epifluorescence microscopy observations coupled with DNA labelling suggested the presence of microorganisms attached to the HCP surface. This was confirmed by biological growth coupled with NR activity after the transfer of the HCP into a new medium, considered to be sterile. The bacterial community analysis showed that the highly selective culture conditions led to the selection of two species: *Halomonas* sp. and a species known for its versatile metabolism and ability to form biofilms, i.e. *Thauera* sp.

1. Introduction

Nitrate is one of the most common pollutants in aquatic and terrestrial ecosystems (Ashok and Hait, 2015). Nitrate contamination is increasing dramatically with the extent of anthropogenic activities and induces various deleterious effects on ecosystems: eutrophication, toxic algal blooms, habitat deterioration in aquifers, etc. In natural environments (soils, sea floor sediments or aquifers), the nitrate can be reduced by microbial activity. This microbial process of denitrification plays an important role not only in the ecology of ecosystems but also in industrial activities (wastewater treatment plants) for water pollution control. The action of microorganisms induces the reduction of nitrate to dinitrogen gas via several intermediates: nitrite (NO_2^-), nitric oxide (NO), and nitrous oxide (N_2O) (Mateju et al., 1992; Jones et al., 2008; Ashok and Hait, 2015). According to the literature on microbial nitrate reduction, the optimal pH for the reaction is often between 6.0 and 9.0 (Hiscock et al., 1991; Glass and Silverstein, 1998; Ashok and Hait, 2015). However, microbially driven denitrification has also been documented in alkaline environments (pH > 9) such as in natural soda lakes (Shapovalova et al., 2008), in industrial wastewater treatment plants (Park et al., 2005; Dhamole et al., 2008), in cement based deep geological repositories (Rizoulis et al., 2012; Alquier et al., 2014; Durban et al., 2018) or in cementitious environments for self healing applications (Erşan et al., 2015b; Ersan et al., 2016; Algaifi et al., 2018). Nonetheless, microbial nitrate reduction at high pH remains poorly understood, especially in systems containing cementitious material. It is important to define the conditions under which partial or complete nitrate biological reduction is possible in a cementitious environment for specific applications such as the construction of deep geological repositories for radioactive waste storage (Albrecht et al., 2013). In fact, the release of soluble oxyanion such as nitrate will promote oxidizing conditions in the vicinity of the waste and promote the mobility of some redox sensitive radionuclides (Nikitenko et al., 2010; Albrecht et al., 2013). The works of Alquier et al. (2014) carried out in a batch reactor

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with a pure strain, *Halomonas desiderata*, revealed an inhibition of de nitrification at pH around 11. However, in the presence of cementitious material (hardened cement paste), the denitrifying activity was main tained at pH between 10 and 12. The strain *H. desiderata* was able to colonise the cement surface and to form a biofilm despite a local pH above 10.0. Microbial colonisation of concrete surfaces has also been observed in environment with a pH generally bellow 9.0 such as anaerobic digestion reactors (Voegel et al., 2016) or in sewage treat ment and conveyance systems (Grengg et al., 2015, 2018; Peyre Lavigne et al., 2016; Voegel et al., 2016; C. Voegel et al., 2019). Voegel et al.'s study (2016) highlighted deterioration mechanisms (calcium leaching and carbonation) of cementitious material in an anaerobic digester, which were amplified by aggressive chemical conditions in duced by the presence of a biofilm on the surface. The interactions between cementitious material and attached microorganisms are com plex. On the one hand, microbial activities in the vicinity of the surfaces could increase the deterioration of the material (Magniont et al., 2011). On the other hand, the presence of a support promotes biofilm forma tion, which contributes to more efficient degradation of pollutants and helps to maintain microbial activities (Qureshi et al., 2005) apparently even on high pH surfaces such as those of cementitious materials (Bertron, 2014; Voegel et al., 2016; Rafrafi et al., 2017). Regarding denitrification processes, the cementitious material support could pro mote formation of a biofilm and improve the denitrification rate or, conversely, the alkaline pH induced by the cementitious material could inhibit the nitrate reduction. Likewise, a denitrification activity near the cementitious surface could accelerate the material deterioration due to the proximity of a biological activity, or have no significant impact, or induce the formation of a protective layer (De Muynck et al., 2008). Although the cementitious environment is a harsh environment to mi croorganisms, i.e. very high pH (pH up to 13) and small pore sizes (< 0.1 μm) (Wang et al., 2012; Erşan et al., 2015b), recent work highlighted the ability of bacteria, and in particular denitrifying bac teria, to induce precipitation of $CaCO_3$ in cementitious materials which contributes to their self healing (Jonkers, 2007; Van Tittelboom and De Belie, 2013; Ersan et al., 2016). The interaction between denitrifying bacteria and cementitious materials must be clarified because un certainties remain on the durability of the concrete structure exposed to this biological activity.

Therefore, the objective of this work was to perform experiments to (i) determine the pH limit at which heterotrophic microbial deni trification processes persist in a cementitious environment, (ii) evaluate the impact of alkaline pH on nitrate and nitrite reduction rates and (iii) investigate the possible impact of the microbial activity on the surface of the cementitious material (microbial colonisation, material dete rioration, etc.).

2. Materials and methods

2.1. Cementitious materials

The cement paste specimens (CEM V/A ROMBAS) with a water/ cement ratio of 0.40 were made following the recommendations of French standard NF EN 196 3A1, which details the cement paste mixing. CEM V/A cement is a standardised cement containing clinker and blast furnace slag with fly ash addition (EN 197 1). The cement paste specimens were cast in hermetic cylindrical moulds (50 mm high and 50 mm in diameter) and were stored in a chamber with relative humidity above 98% at 22 °C for at least 28 days (hardening phase) until needed. Before being used, they were cut into slices (h ≈ 7 mm) and then into four quarters to obtain coupons with a projected surface area of 16 cm^2. The surfaces were treated with silicon carbide polishing disks (P120 ≈ 127 μm Presi®) to impose a homogeneous surface roughness favourable to bacterial cell attachment.

2.2. Cement leachate

The cement leachate was used for the experiment without hardened cement paste. It was prepared by immersing four coupons in 1 L of demineralized water (solid surface/liquid volume ratio: 64 cm^2/L) for 3 days under continuous stirring. The average chemical characteristics of the cement leachate, analysed by ICP OES (Inductively Coupled Plasma Optical Emission Spectrometry), are given in Table 1 of the supplementary data.

2.3. Microbial consortium

The source of the microorganisms used as the microbial consortium in this study was activated sludge collected from the aerobic tank of the wastewater treatment plant in Castanet, France.

The mixed liquor suspended solid (MLSS) concentrations were be tween 7.0 and 8.2 g/L. The mixed liquor was centrifuged in order to concentrate the microorganisms and to limit the soluble organic matter input: 250 mL of mixed liquor was centrifuged at 4600 g for 15 min at 6 °C. A part of the pellet was stored at 20 °C for microbial population analysis. The reactors were inoculated with 2 g of centrifuged sludge pellet.

2.4. Experimental set up and conditions

Fed batch reactors, some containing hardened cement paste and some not, were run. The inoculum from activated sludge was accli mated to the denitrification condition in an alkaline environment (pH > 9) with cement leachate or leached cement paste. To control the experimental conditions, nitrate and/or acetate were added and, for the experiments without cement paste, the pH was adjusted during the experimental follow up. After each of these actions, the systems were deaerated by N_2 bubbling for 15 min. Glass bottles of 250 mL, with a rubber septum and a screw cap with an aperture, were used to limit reactor openings. The reactors were stored in the dark at 30 °C in a temperature controlled chamber.

2.4.1. Experiments with cement leachate

Two fed batch reactors with cement leachate, Lx.1 and Lx.2, were prepared in duplicate. The cement leachate was supplemented with 8.3 mM sodium acetate (as the organic carbon source and electron donor) and 5.9 mM sodium nitrate (as the electron acceptor and ni trogen source). Both reactors were inoculated with 2 g of activated sludge pellet. The leachate pH (11.3) was adjusted to be close to 9.0 with hydrochloric acid (HCl 1M) at the beginning of the experiments. The pH was then gradually increased using sodium hydroxide (NaOH 1 M) as indicated in Fig. 1a.

2.4.2. Experiments with hardened cement paste specimens

Two pairs of fed batch bioreactors, named CEMV A and CEMV B, each containing a single cement paste specimen, were implemented (see all the experimental steps in Figure A.1 of the supplementary data). Each cement paste specimen was kept in 240 mL of distilled water for six days to enhance its leaching (progressive release of cations and hydroxide anions from the cement paste to the liquid phase). The re actors were deaerated by bubbling N_2 for 15 min and stored in a thermostatic chamber (30 °C) (Step 1, Figure A.1). After six days of leaching, the liquid medium of the reactors was inoculated with 2 g of activated sludge pellet and supplemented with 8.3 mM of sodium acetate and 5.9 mM of sodium nitrate without pH adjustments (Step 2, Figure A.1). The reactors were deaerated by N_2 bubbling during 15 min and then stored at 30 °C. No pH adjustments were made during the experiments. Nitrate and acetate were supplemented occasionally in the reactor when chemical analyses failed to detect the target compounds (see 2.5 section).

At the end of the experiments, the cement paste specimen of one of

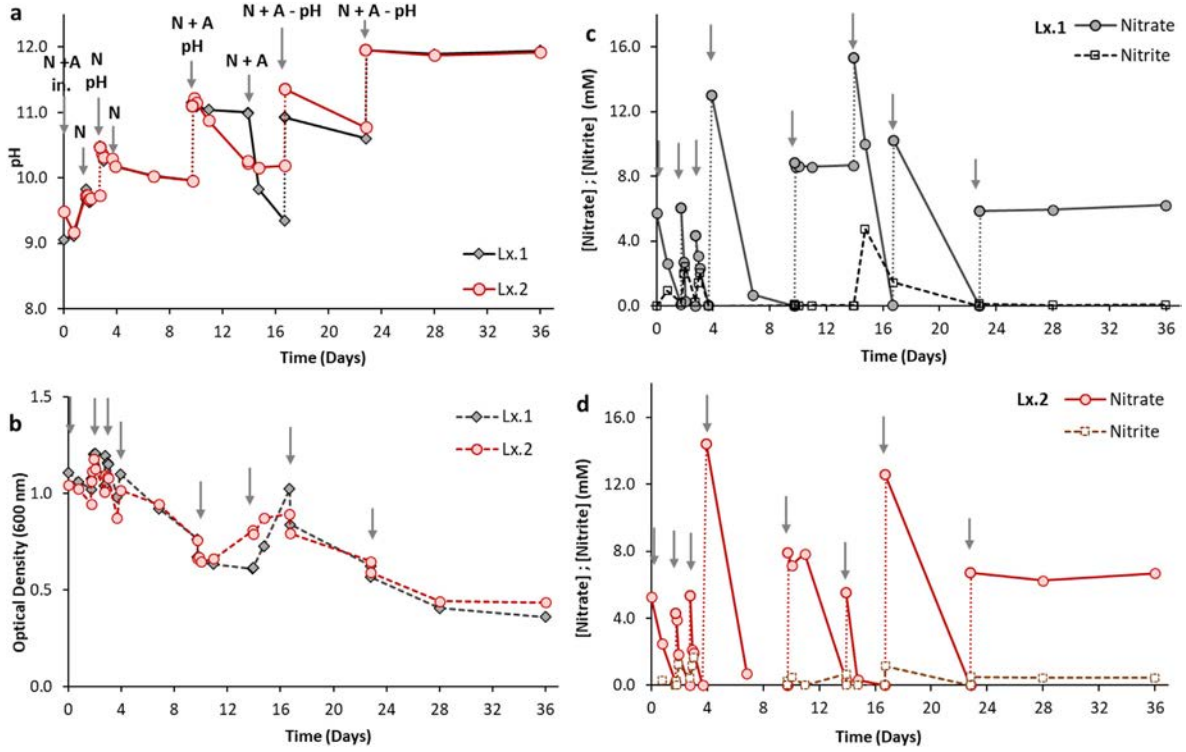

Fig. 1. Evolution of pH (a), bacterial growth (b), and concentrations of nitrate and nitrite (c–d) for the experimentations with cement leachate only i.e. the reactor Lx.1 (black) and the reactor Lx.2 (red) – The arrows indicate the actions performed during the experiment: **in.** inoculation, **N** nitrate addition, **N + A** nitrate and acetate addition, pH pH adjustment. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

the duplicated reactors was observed with microscopic techniques (fluorescence microscopy, scanning electron microscopy (SEM)) and analysed by X ray diffraction (XRD). The cement paste specimen was collected from the second reactor and rinsed with cement leachate to detach the microorganisms weakly bound to the surface. The cement paste was finally introduced into a new reactor containing cement leachate considered to be sterile in order to validate the possible pre sence of microorganisms settled on the cement surface. The cement leachate was supplemented with 8.3 mM of sodium acetate and 5.9 mM of sodium nitrate and the pH was adjusted to 10 (Step 3.a, Figure A.1). Supplementary acetate and nitrate addition were carried out based on the concentrations obtained during the controls, without pH adjust ment.

In the case of reactor pair CEMV B, the liquid phase was reused to explore the NR rate for pH above 10 (Step 3.b, Figure A.1). Further acetate and nitrate additions were performed occasionally and the pH was progressively increased from 10 to 12. In the case of reactor CEMV B.2, the acetate and nitrate concentration and the pH were modified in order to investigate the impact of the pH on the nitrate reduction and on the nitrite accumulation phenomena. The pH was decreased from 11.5 to 10.0 using acetic acid (1 M pH 5.1) setting the final acetate concentration at 16 mM. The initial nitrate concentration was thus doubled with respect to the other reactor, increasing the initial nitrate concentration to 11.5 mM.

2.5. Analytical techniques

Regular sampling was performed for immediate measurement of pH (6500 pH/ion meter, Eutech Instruments) and optical density (OD) at 600 nm (JENWAY 7315 spectrophotometer) (Rafrafi et al., 2015). Two millilitres of sample were collected and filtered (through a 0.2 µm Minisart® PES, Sartorius) for analyses of ionic species.

To quickly estimate the nitrate and acetate consumption as well as the possible nitrite production and COD (chemical oxygen demand), nitrate and nitrite cuvette tests were performed occasionally using Hach® commercial kits LCK 514, LCK 340, LCK 341. The amounts of nitrate and/or acetate added were adjusted according to the results obtained with the cuvette test. Then, the concentrations of Ca^{2+}, K^{+}, Na^{+}, CH_3COO^{-}, NO_3^{-} and NO_2^{-} were quantified by High Performance Ion Chromatography (Dionex ICS 2000 and ICS 3000) using analytical methods detailed by Alquier et al. (2014) and Bertron et al. (2014). The nitrate reduction (NR) rates were determined as: $[X]_{consumed}/\Delta t$ where $[X]_{consumed} = [X]_{initial.time} - [X]_{final.time}$ where [X] is corresponding to either the nitrate or acetate concentration. The in itial time corresponded either to the addition of the substrates (nitrate or acetate) if the nitrate reduction was observed immediately, or at the end of the lag time. The final time was the second to last point before complete consumption of the nitrate or acetate.

2.6. Cement paste specimen study

2.6.1. Fluorescence microscopy

At the end of the experimental monitoring, the cement paste cou pons were rinsed abundantly with commercial phosphate buffer saline solution (PBS, 10 mM, pH = 7.4; Invitrogen, USA). Afterwards, they were successively immersed in 1.36% paraformaldehyde solution (4% diluted with PBS; Thermo Scientific, USA) for 10 min (Chang et al., 2003) to fix the structure of the microbial cells, and then in 10 mM PBS for 5 min to rinse the sample. For the labelling step, the surfaces of the coupons were immersed in a solution of PBS and SYTO® 9 (Invitrogen, USA) for 10 min with a final stain concentration of 10 µM. The coupons were finally rinsed with PBS in order to remove the excess stain before microscopic observations.

2.6.2. Scanning electron microscopy (SEM)

Biofilms bound to the cement paste surface were observed by SEM (JEOL 6380 LV coupled with XFlash 6/30 Bruker EDS detector). Before SEM observations, biofilms on the cement paste specimens were

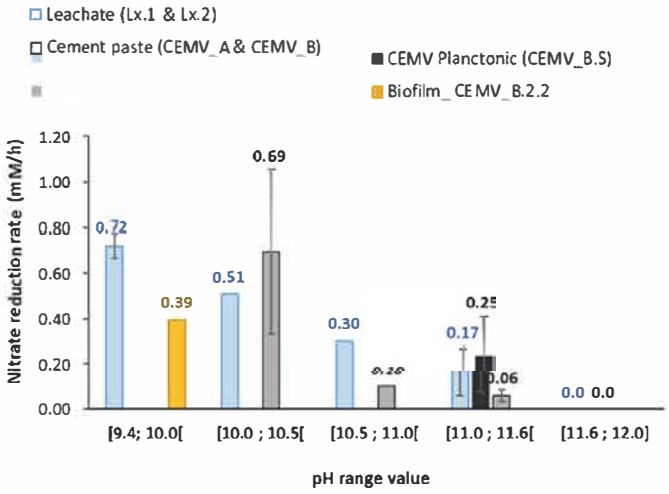

Fig. 2. Nitrate reduction (NR) rates versus the initial pH for the experiments with cement leachate (Lx.1, Lx.2 - ■), with cement paste incubated 30 and 55 days (CEMV A, CEMV B - ■), after the transfer of the colonised cement paste into a sterile liquid medium (CEMV B.2.2 - ■), and in the planktonic phase after withdrawal of the cement paste (CEMV B.1.S, CEMV B.2.S - ■).

chemically fixed and then dehydrated according to the procedure described by Voegel et al. (2016).

2.6.3. X ray diffraction analysis (XRD)

The surface mineralogical composition was characterised by X Ray Diffraction (XRD, BRUKER D8 Advance, Cu cathode, 40 kV, 40 mA) with 15 min acquisition time and variable gap opening. The top surface of the specimen, which was directly exposed to the medium, was analysed first. Then, the outer layer was carefully abraded (P120 ≈ 127 µm Presi®) to a depth of 100 200 µm before the next analysis. The procedure was repeated until the precipitates had been completely removed. Material was abraded from the back surface of the specimen to a depth 2 3 mm in order to eliminate the altered zone and to analyse the unaffected specimen core.

2.7. Bacterial community analysis

For the experiment with or without hardened cement paste, only the bacterial community from the supernatant was investigated. Biofilm present on the cement paste surface was collected but the quality and quantity of DNA after extraction was not sufficient for sequencing. This notably led to step 3.a of the protocol described in section 2.4.2.

The samples analysed were (i) the supernatant at the end of the first step of the experiment (see Fig. A1 in supplementary data) (reactor CEMV B.2) and (ii) that at the end of the second step (Reactor CEMV B2.2), (iii) the supernatant of the reactor Lx.1 and Lx.2 at day 23 (pH 10.7) and (iv) two samples of activated sludge collected in March 2016 (sample name: In.1) and October 2017 (sample name: In.2) at Castanet WWTP.

Two 50 mL samples of activated sludge were centrifuged at 4600 × *g* for 15 min at 6 °C. For the other samples, the supernatant was collected in the reactor and the cells were concentrated by centrifugation.

The DNA extraction was performed with the DNeasy PowerBiofilm QIAGEN kit according to the manufacturer's recommendations. Full pellets (0.01 and 0.03 g) were used for the reactors Lx.1, Lx.2 and CEM B.2 and about 0.20 pellet for the two activated sludge samples and the reactor CEMV B2.2. The DNA concentrations were checked with absorbance at 260 nm, and possible contamination by protein and humic acid were tested with absorbance at 280 and 230 nm, respectively. Then, the DNA samples were sent to the Research and Testing Laboratory (RTL, Texas, USA) where the DNA samples were amplified by PCR and sequenced with the bacterial primers 28F (5′ GAG TTT GAT YMT GGC TC 3′) and 519R (5′ GWA TTA CCG CGG CKG CTG 3′) according to RTL protocols (www.researchandtesting.com). Subsequent data analyses of the DNA quality, DNA sequence alignment, clustering in operational taxonomic units (Edgar, 2013) and the assignment were also performed by RTL according to their protocol.

3. Results

3.1. Nitrate reduction in cement leachate medium

Duplicates Lx.1 and Lx.2 were inoculated with activated sludge and supplemented with acetate and nitrate. As specified in Fig. 1, eight supplementary nitrate additions and five acetate additions were performed during the experiment (Fig. 1). The pH was progressively increased from 9.3 to 12.0 with sodium hydroxide (NaOH 1 M) 1M) (Fig. 1a). It should be noted that the acetate, added regularly to avoid any carbon and electron donor limitation, was never fully consumed by the microorganisms (see Fig. A.2 in supplementary data).

According to results in Fig. 1b in the absence of nitrate and acetate limitation bacterial growth was stable for the first 6 days, the OD (optical density) was close to 1.1 with some fluctuations (peaks at 1.15 + 0.05) a few hours (3 4 h) after nitrate addition. The decrease in the optical density (minimal value of 0.76) observed between days 4 and 10 resulted from a period of nitrate and acetate limitation. After day 10, a bacterial growth was observed although the pH was between 10.0 and 11.0 for Lx.2. For pH > 11.3 in reactor Lx.2, i.e. after 17 days, the optical density values decreased progressively certainly because of a slow die off. The same evolution was observed in reactor Lx.1: a bacterial growth was observed after day 14 followed by a decline after day 17 correlated to a pH close to 11.0. At the end of the experiment, when the pH was set to 12.0, the OD value was below 0.4 in both reactors. Finally, for pH > 11 the bacterial growth would be slowed down or even inhibited.

At pH between 9.4 and 10.0, i.e. between days 2 and 3, microorganisms from activated sludge reduced approximately 6 mM of nitrate in less than 8 h. For pH between 9 and 10.5, the acetate oxidation rate was 0.23 mM/h on average and the ratio of consumed acetate to nitrate reduced ($R_{C/N} = \Delta[C_2H_3O_2^-]/\Delta[NO_3^-]$) was 0.72 on average on both reactors. The corresponding NR rate was 0.72 mM/h. The NR rate decreased from 0.51 mM/h to 0.17 mM/h on average on both reactors when the pH increased from 10 to 11 respectively (Fig. 2), and no NR was observed for pH > 11.8 (Fig. 2).

The pH influenced the nitrate reduction rate and the biological activity impacted the pH by inducing a pH decrease. In particular, the pH in the Lx.1 assays decreased by 0.9 units between days 10 and 17. Generally, nitrite appeared temporarily a few hours after nitrate addition (Fig. 1). The nitrite concentration was always < 1.5 mM except at day 15, when a nitrite peak of 4.7 mM was reached in reactor Lx.1 due to the rapid reduction of a significant amount of nitrate (5.3 mM in less than 19h) (Fig. 1c).

3.2. Nitrate reduction in cement leachate medium with addition of cement paste specimen

3.2.1. Case study 1: NR evaluation over 30 days

The duplicates CEMV A.1 and CEMV A.2 were inoculated with activated sludge and supplemented with acetate and nitrate as indicated in section 2.4.2 (see Fig. A.1 in supplementary data). As specified in Fig. 3 and in Fig. A.1, two supplementary nitrate additions and a single acetate addition were performed (Fig. 3). The acetate was never fully consumed by the microorganisms; regular analyses were performed with commercial COD kits to check this and, if necessary, additions were made (see Fig. A.3 in supplementary data).

The nitrate reduction to nitrite was observed at initial pH close to 11.5 after a lag time of 4 days, and then the nitrite concentration increases in reactors. No ammonium was observed in the reactor. The heterotrophic denitratation was complete and no denitritation was

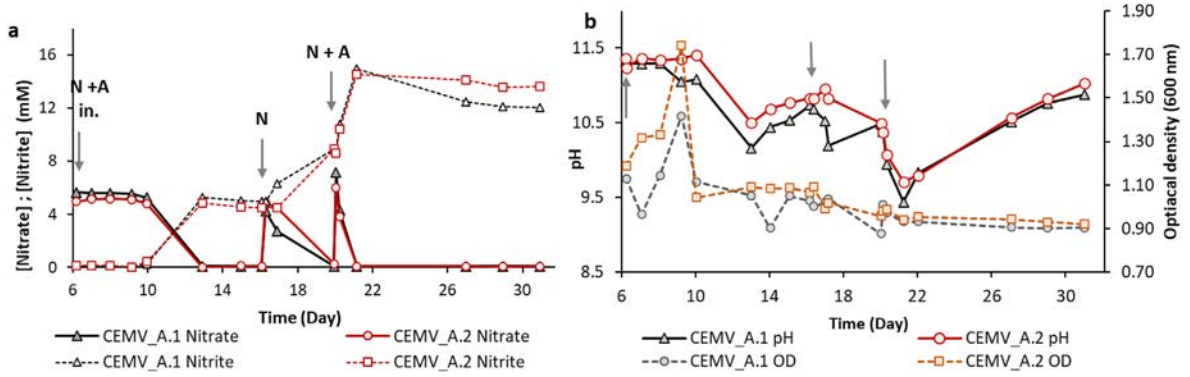

Fig. 3. Evolution of concentrations of nitrate (——) and nitrite (·····) (a), the pH (——) and the bacterial growth (- - - -) (b) in reactors CEMV A (cement paste immersed in a medium supplemented by acetate and nitrate after six days of leaching and inoculated with activated sludge). The arrows indicate the actions performed during the experiment: **in.** inoculation, **N** nitrate addition, **N + A** nitrate and acetate addition.

observed. . The nitrate reduction was associated with a pH decrease from 11.4 to 10.2 (Fig. 3a). After complete nitrate consumption, i.e. between days 13 and 16, the pH increased and reached approximately 11.0. In less than 20 h after nitrate addition, the pH decreased again in conjunction with the nitrate reduction.

The NR rate after the first nitrate addition was about 0.070 mM/h for an initial pH of 11.4 in both reactors. After the second nitrate ad dition, the maximum NR rate was 0.10 mM/h in reactor CEMV A.1, and 0.056 mM/h in reactor CEMV A.2, for an initial pH around 10.8 in both reactors. The NR was initiated later in CEMV A.2 than in CEMV A.1. After the third nitrate addition, the maximum NR rates were close to 0.52 mM/h for an initial pH of around 10 in both reactors.

Nitrite accumulated in both reactors throughout the experiments and reached a maximum concentration close to 14 mM at day 21. During the last ten days, less than 3 mM of nitrite were reduced.

The microorganisms did not grow significantly once the biological NR activities had taken place: the optical density increased before the start of the NR activity and remained stable throughout the experi mental monitoring. The acetate concentration remained constant (Fig. A.3 in supplementary data). The heterotrophic denitrification without acetate oxidation involves that another carbon source and another electron donor were used such as hydrolysed organic matter from the activated sludge inoculum or by endogenous respiration, i.e. the bio mass undergoes cell decay leading to residual dead cells (Laspidou and Rittmann, 2002). The nitrate reduction into nitrite without acetate oxidation has been observed in previous work in the presence of cement paste (Rafrafi et al., 2017; Durban et al., 2018).

3.2.2. Case study 2: NR evaluation over 55 days

In the reactor CEMV B.1, the pH decreased progressively from 11.6 (start of the experiment) to 10 at day 18 and remained stable until the end of the experiment, on day 55 (Fig. 4a). In the reactor CEMV B.2, the pH did not change and remained close to 10 throughout the experi ment. Nitrate was reduced to nitrite after a lag time of 3 days (from day 6 to day 9) (Fig. 4b). Then, nitrite was completely metabolized to below detection limit in less than 3 days without ammonium production. The heterotrophic denitrification, at least the steps of denitratation and denitritation, was complete. After the second nitrate addition (day 13), nitrate was immediately reduced, without any lag time. The NR rates calculated after nitrate addition, on days 19, 23 and 53, were 0.57 mM/ h, 0.79 mH/h and 0.97 mM/h, respectively. Nitrite did not accumulate in these reactors.

In the reactor starting at pH 11.6 (CEMV B.1), the lag time for ni trate reduction was at least 4 days (Fig. 2d). Nitrate was reduced to nitrite, the concentration of which increased progressively during the first 19 days. After the third substrate addition (day 19), nitrite was completely reduced without ammonium production. The heterotrophic denitrification, at least the steps of denitratation and denitritation, was complete after a 15 days lag time. The NR rate was around 0.07 mM/h for an initial pH close to 11.5 and between 0.59 and 1.38 mM/h for pH of about 10.2.

For reactors CEMV B.1 and CEMV B.2, the NR may have been limited on day 19 because of acetate limitation in the medium (Fig. 4c). Outside this period, acetate was not completely consumed by the mi croorganisms. The acetate oxidation rate was 0.82 mM/h on average and the ratio $R_{C/N}$ was 0.99 on average, for an average pH of 10. The acetate consumption was higher with the cement paste presence than with the cement leachate alone.

Step 3.a: Characterisation of NR activity of the microorganisms from to the cement paste surface

The NR activity of the microorganisms attached to the cement paste surface was evaluated after 55 days of incubation. The cement coupon from reactor CEMV B.2 was rinsed with cement leachate and the coupon was introduced into a new bioreactor (CEMV B2.2) containing sterile cement leachate supplemented by acetate and nitrate (section 2.3.2 and Step 3.a on Fig. A.1 in supplementary data). Three supple mentary nitrate and acetate additions were performed (reported on Fig. 6 and Fig. A.1).

The optical density increased progressively and reached the max imal value of 0.65 at the end of the experiment (Fig. 6a). Micro organisms attached to the cement paste colonized progressively the supernatant. The pH was quite stable at 9.9 + 0.06 throughout the experiment (Fig. 6b). After the experiment had been started, the nitrate was progressively reduced and this reduction was coupled with an acetate oxidation without strong nitrite accumulation at a stable pH of 10. The presence of microorganisms able to perform denitrification on the hardened cement paste was confirmed. The maximal rates of nitrate reduction and the acetate oxidation were obtained during the last substrate addition. The corresponding values were respectively 0.39 mM NO_3^-/h and 0.17 mM $C_2H_4O_2$/h with an $R_{C/N}$ close to 0.56.

Step 3.b: Characterisation of NR activity of the planktonic micro organisms after removal cement paste

The liquid phase of reactors CEMV B.1 and CEMV B.2 was used to explore the NR rate for pH above 10.5 without cement paste (step 3.b, Fig. A.1). The corresponding reactors were CEMV B.1.S and CEMV B.2.S. The results are shown in Fig. 7. Three supplementary ni trate and acetate additions were performed and the pH was progres sively increased from 10.5 to 12.0 (reported in Fig. 7 and Fig. A.1).

For pH > 11.0, the bacterial growth decreased: the optical density decreased continuously from 0.7 to 0.2 (Fig. 7a b). The nitrate was reduced at pH values close to 11.5 but the nitrite was not reduced, and accumulated in the two reactors. Nitrite was reduced only in reactor

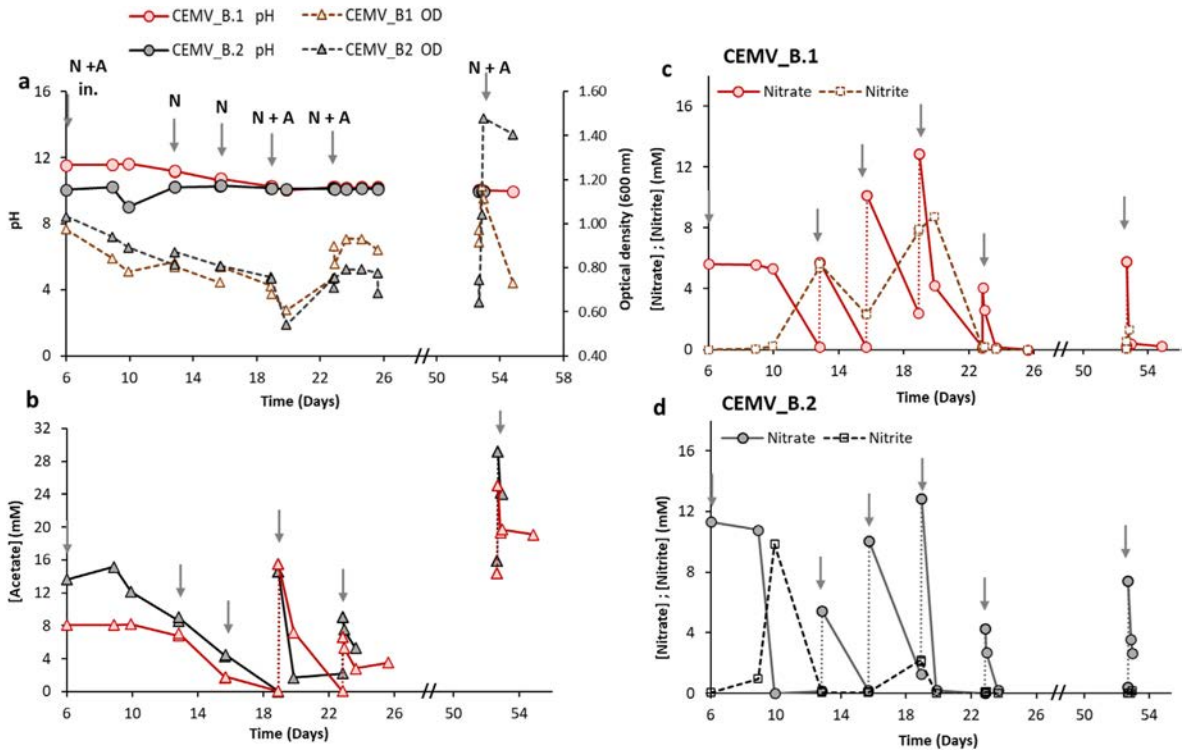

Fig. 4. Evolution of the pH and bacterial growth (a), acetate concentration (b), nitrate and nitrite concentration in the reactor CEMV B.1 (c) and reactor CEMV B.2 (d) (cement paste immersed in a medium supplemented by acetate and nitrate after six days of leaching and inoculated with activated sludge). The arrows indicate the actions performed during the experiment: **in.** inoculation, **N** nitrate addition, **N + A** nitrate and acetate addition.

CEMV B.2.S, where the pH decreased progressively to reach values below 11.0, whereas the pH in the reactor CEMV B.1.S decreased but remained above 11.0. The nitrate and nitrite reduction in reactor CEMV B2.S was coupled with significant acetate oxidation (though slow: 0.04 mM/h), which was not observed in reactor CEMV B.1.S. For pH > 11.6, no nitrate or nitrite reduction was observed. For pH close to 11.0, the NR rate was 0.40 mM/h on average. The NR rate decreased to an average of 0.096 mM/h when the pH reached values close to 11.5 (CEMV planktonic spot, Fig. 2). Globally, higher NR rates were achieved (0.25 mM/h) by these planktonic microorganisms (exposed to a medium containing solid cement paste for almost two months) than the microorganisms exposed to a progressive pH increase in a cement leachate medium (reactors Lx1 and Lx.2) (Fig. 2).

3.2.3. Interaction between cement paste surface and the microorganisms

During the experiments with hardened cement paste, whitish precipitates appeared on the surface of all cement paste specimens (Fig. 5a). Using scanning electron microscopy (SEM) coupled to Energy Dispersive X Ray (EDX) analyses, spots of newly formed Ca bearing precipitates were regularly identified on the surface (Fig. 5b and c). These spots were quite heterogeneous in size with an average diameter of a few hundred micrometres. Some spots had typical morphologies (notably scalenohedral morphology), suggesting crystallized mineral precipitates (Fig. 5c).

According to the mineralogical characterisation by XRD (Fig. A.4 in supplementary data), the white precipitates were predominantly calcite (in accordance with the scalenohedral morphology) (Fig. 5a). Vaterite, a metastable calcium carbonate polymorph, was also detected on the CEMV B.1 surface. Calcite is the thermodynamically stable phase of calcium carbonates but other allotropic forms, such as vaterite, are commonly detected on cement surfaces (Plummer and Busenberg, 1982; Goñi et al., 2002). Diffractograms of the specimen core obtained at 2 3 mm depth presented typical peaks of anhydrous (di and tri calcium silicates, denisovite, mullite) and hydrated (ettringite, portlandite) (Figure A.4) cementitious phases, and quartz (Bertron et al., 2014). The comparison between the core and the cement paste surface after the removal of the calcite precipitates by abrasion showed that portlandite and ettringite had disappeared from the outer layer of the specimen (dissolution of these phases in contact with the medium (Fig. A.4)).

During the phase of cement paste leaching (see phase 1, Fig. A.5 in supplementary data) and at the end of the microbial acclimation phase, i.e. the lag time of 3 4 days observed before nitrate reduction (Phase 2, Fig. A.5), the calcium concentration increased in the liquid medium, reaching concentrations close to 1.5 mM in all the reactors (Fig. A.5). Once the microbial activity was initiated, i.e. when an NR was observed (Phase 3, Fig. A.5), the Ca concentration in the liquid phase decreased and was below 0.05 mM at the end of the experiments, in accordance with the secondary formation of a solid Ca bearing phase (here, cal cite).

The specific labelling of the microorganism by Syto® 9 (nucleic acid stain) confirmed the presence of microorganisms attached to the surface of the cement paste specimen (Fig. 5d and e). Apart from the calcite, which auto fluoresces (dull green on Fig. 5d), the attached micro organisms formed clusters of about ten microns that were dispersed on the cement paste surface extracted from reactor CEMV A.1 (bright green spots on Fig. 5d). Isolated microbial clusters were also observed on the cement paste surface from reactor CEMV B.1 (Fig. 5e), together with a heterogeneous microbial layer having a surface area of at least 0.01 mm^2.

3.3. Analysis of the microbial communities

The proportions of gene sequences allocated to different bacterial phyla was similar in the two inoculums (AS.1 and AS.2, Fig. A.6) despite having been collected on WWTPs at 19 month intervals. The major phyla composing the microbial community in the inoccula were Proteobacteria (≈20%), Actinobacteria (≈13%), Chloroflexi (≈7.5%),

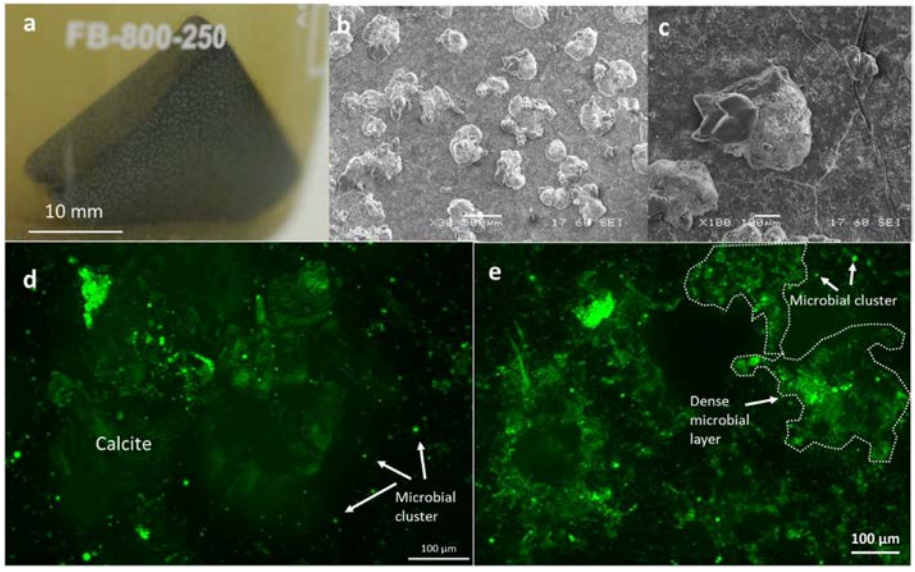

Fig. 5. White precipitates on cement paste surface (a), SEM observations in SE mode (b–c) Fluorescence microscopy observations with Syto9 labelling, (d–e) after 30 days of cement paste specimen immersion in the CEMV A.1 reactor (final pH ≈ 10.7), (e) after 55 days of cement paste specimen immersion in the CEMV B.1 reactor (final pH ≈ 10.0).

Planctomycetes (≈7%), Firmicutes (≈5%) and Bacteriodetes (≈3%) (Fig. A.6).

The microbial communities from reactors with cement leachate (Lx.1 and Lx.2, after 23 days), reactor with cement paste (CEMV B.2, 55 days, only the supernatant), and after 32 days in the reactor where a colonised cement paste had been introduced in sterile medium (CEMV B.2.2, theoretically bacteria from biofilm adherent on cement paste surface immerged in reactor CEMV B.2) were very similar. The phylum Proteobacteria dominated all the samples, with a relative abundance of at least 80% (Fig. A.6.a). The second dominant phylum (≈15%) was Firmicutes. The microbial community was principally dominated by one or two Proteobacteria species: *Halomonas* sp. for reactors Lx.1 and Lx.2 (pH 10.6 at the time of sampling) and *Thauera* sp. for the reactors in which hardened cement pastes were present (CEMV B.2 and CEMV B.2.2) (Fig. A.6.b).

4. Discussion

4.1. Interaction between pH and the denitrifying microorganisms

In the reactors where cement paste was incubated 30 days (i.e. CEMV A, *reactors containing one cement paste, inoculated with activated sludge after 6 days of leaching and whose medium has been supplemented with acetate and nitrate*), the pH decreased with the nitrate reduction. It was only when nitrate was totally depleted that the pH increased again, most likely because of hydroxide released by the cement paste (Bertron et al., 2014). The pH decrease coupled with nitrate reduction was also observed in reactors Lx (reactors without cement paste, only cement

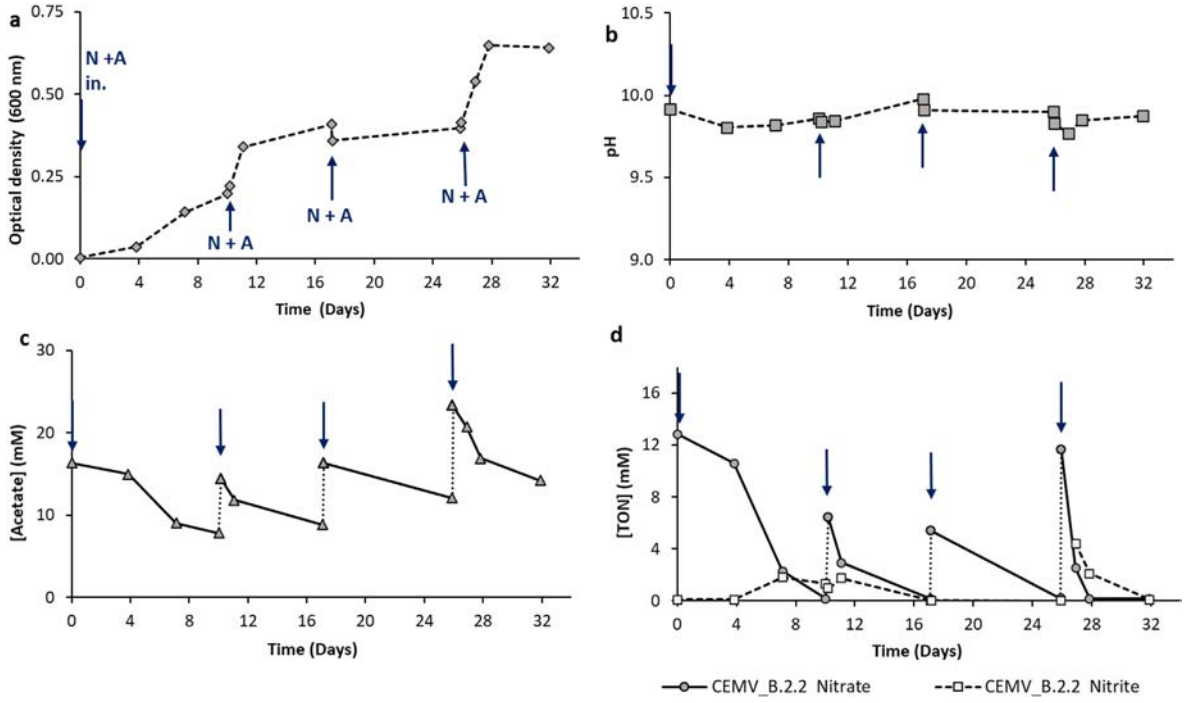

Fig. 6. Evolution of the bacterial growth (a), the pH (b), the acetate concentration (c) and the nitrate and nitrite concentrations (d) in the reactor CEMV B.2.2, with cement paste issued from reactor CEMV B.2 and then exposed to fresh sterile medium - The arrows indicate the actions performed during the experiment: **in.** inoculation, **N + A** nitrate and acetate addition.

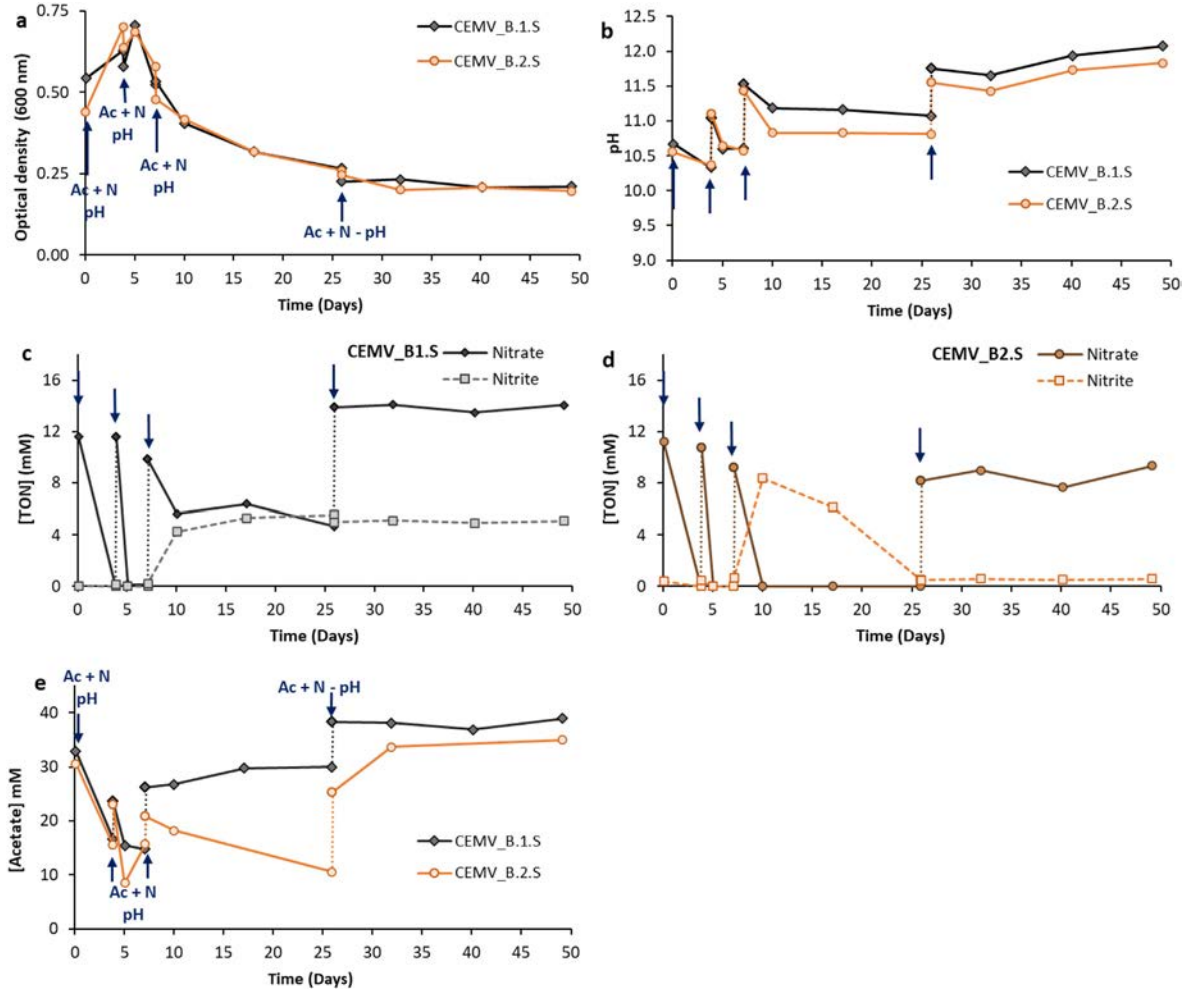

Fig. 7. Evolution of bacterial growth (a), the pH (b), nitrate and nitrite concentrations for the supernatant collected at the end of the second set of experimentations with cement paste immerged during 55 days i.e. the reactor CEMV B1.S (c) and the reactor CEMV B2.S (d), the acetate concentration (e) for the reactor CEMV B1.S (black) and the reactor CEMV B2.S (orange) without cement paste - **N** nitrate addition, **N + A** nitrate and acetate addition, pH pH adjustment. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

leachate supplemented with acetate and nitrate and inoculated with activated sludge). The nitrate reduction to nitrite can produce a drop of pH by producing protons (reaction 2), while nitrite reduction would tend to consume protons (reaction 3). Nitrate reduction leads to a pH decrease (reaction 1) due to "self acidification" phenomenon controlled by carbonate equilibrium (Rafrafi et al., 2017; Durban et al., 2018; Albina et al., 2019):

$$NO_3^- + 0.625\ CH_3COO^- \rightarrow 0.5\ N_2 + 0.625\ HCO_3^- + 0.625\ CO_3^{2-} + 0.5\ H_2O + 0.25\ H^+ \quad (1)$$

The two reactions pathways involved in nitrate reduction are:

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

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

All the NR rate data obtained from the different experiments are plotted in Fig. 2 according to the corresponding initial pH, i.e. the pH value after nitrate addition. The NR rates reached with cement leachate (i.e. between 0.17 and 0.72 mM/h for reactors Lx.1 and Lx.2) were significantly higher, i.e. by a factor 10, than those reported by Alquier et al. (2014) in batch experiment at pH 9.7 with *Halomonas desiserata* (i.e. 0.063 mM/h) or by Rafrafi et al. (2017) in continuous experiments at pH 10 with *Halomonas desiderata* (i.e. 0.076 mM/h). However, they were of the same range as the NR rates reported by Durban et al. (2018) at pH close to 9.5 with a consortium from a former lime works sedi ments in particular for the higher nitrate concentration tested (i.e 0.74 mM/h, initial nitrate concentration of 48 mM). Nitrate reduction by bacteria directly incorporated in the cementitious material was ob served at pH 9.5 by Ersan et al. (2016) and reached similar nitrate reduction rate, i.e. 0.10 mM/h (10 mM NO_3 N consumed in approx imatively 4 days). The NR was not significantly impacted by the cement paste addition (Fig. 2). Although, the NR rates obtained in reactors with cement pastes incubated 55 days (CEMV B) increased over time due to the microorganisms becoming progressively more efficient. The max imal NR rate reached was 0.97 mM/H at pH close to 10.

The compilation in Fig. 2 clearly shows the impact of the pH on the nitrate reduction. The NR rate was greatly decreased at pH higher than 11.0, and for pH $>$ 11.8, the NR was close to zero. Microbial growth was inhibited at pH $>$ 11.0, as was nitrite reduction. The upper pH limit for bacterial growth and/or activities is around 12 (Rizoulis et al., 2012; Smith et al., 2016). Bacteria can maintain a cytoplasmic pH compatible with their metabolism via several pH regulation mechan isms such as a "cation proton antiporter" (Krulwich et al., 2011; Smith et al., 2016). The enzymatic complex that catalyses the reduction of nitrate is anchored in the cytoplasmic face of the cytoplasmic mem brane (NAR). The enzyme complex (NIR) responsible for nitrite re duction is located in the periplasmic compartment (Richardson et al.,

2001, 2009). The nitrite reduction could be more impacted by the pH than the nitrate reduction, due to the lesser ability to regulate pH in the periplasmic space. Additionally, the activity of the enzyme complex NIR decreased with the pH increase from 7.2 to 9.2 according to Zhang et al. (2020). Nitrite accumulation was observed at pH close to 11. Nitrite accumulation could be problematic for the denitrifying process because low nitrite concentration (tens of mM) accumulated in the medium could inhibit or slow down bacteria activity (Rizoulis et al., 2012; Albina et al., 2019). According to the results obtained with reactors containing cement pastes incubated 55 days (CEMV B), if the pH decreases to value below 11, nitrite could be reduced after a lag time of 3 days. The nitrite reduction inhibition by a pH above or close to 11 could be reversed with pH decrease after a lag time.

The pH does not seem to be the only factor that can affect nitrite reduction. Effectively, no nitrite reduction was observed in reactors CEMV A (for pH value between 9.5 and 11.5) whereas the nitrite was progressively reduced in reactor CEMV B.2 (for pH value between 10 and 11.6), i.e. than 5% of nitrite reduced after 7 days of culture (day 13), more than 60% between 16 and 24 days of cultivation and finally 100% after 25 days of cultivation. A latency period of at least 15 days was required before nitrite reduction occurred in the experimental conditions. The genes from NIR complex are induced by nitrogen oxide, the reaction product of nitrite reduction (Baumann et al., 1996; Philippot, 2002; Bergaust et al., 2008). The fact that the synthesis of nitrite reductase is driven by its reaction product would slow down the synthesis of the enzymes involved and could partly explain the accumulation of nitrite. Nitrite inhibition can possibly be reversed when the pH decreases (< 11), and after a lag time (some twenty days) and/or after a certain amount of nitrate has been reduced.

It can be assumed that, for pH > 11: (i) the chemical equilibria (carbonate) were no longer favourable to the reduction of nitrite (no proton available in the medium), which was therefore chemically inhibited, and/or (ii) nitrite reduction was biologically inhibited to allow at least a nitrate reduction activity to be maintained as a metabolic strategy for bacteria or due to the localization of enzyme complexes (cytoplasmic for NAR involved in nitrate reduction and periplasmic for NIR involved in nitrite reduction) (Richardson et al., 2001, 2009; Albina et al., 2019).

4.2. Microbial community

The six phyla composing the microbial community in the inoccula (Proteobacteria, Actinobacteria, Chloroflexi, Planctomycetes, Firmicutes and Bacteriodetes (Fig. A.6) are characteristic of the WWTP bacterial community, with the predominant phylum Protebacteria, mainly involved in nutrient and organic matter removal (Lu et al., 2014; Cydzik Kwiatkowska and Zielinska, 2016). The microbial communities were also investigated in one of the reactors with cement paste incubated 55 days (CEMV B.2) with leached cement paste, and reactors Lx.1 and Lx.2 with cement leachate. Although they were inoculated with the same activated sludge sample, the two Lx reactors and the reactor CEMV B.2 were not dominated by the same species probably because of the presence of cement paste in the reactor CEMV B.2.

Halomonas sp. was the dominant species for the reactors run with cement leachate (Lx.1 and Lx.2 Figure A.6), was not detected in the activated sludge used for inoculation. According to the DNA sequence alignment corresponding to the DNA sequence of *Halomonas* sp. with the Blast database (Zhang et al., 2000), the species involved was most probably *Halomonas desiderata* (data base: RefSeq, Query Cover: 100%, E value: 0.0, Percent Identity 98.6%). During previous work, the pure *Halomonas desiderata* strain, an alkaliphilic denitrifying bacterium isolated from municipal sewage works (Berendes et al., 1996), was successfully used to evaluate the denitrification process in a cementitious environment (cement leachate at pH above 9.0 with or without hardened cement paste) (Alquier et al., 2014; Rafrafi et al., 2015, 2017). The works of Rafrafi et al. (2015) highlighted nitrate reduction by *H. desiderata* for a pH between 11 and 11.5 in continuously fed bioreactors. According to the NR rate reported in section 4.1, even if the microbial community of the reactors Lx.1 and Lx.2 were dominated at 80% by *H. desiderata*, the NR rates reached by a microbial consortium were higher than NR rate reached with a pure culture of *H. desiderata* in cementitious environment (Alquier et al., 2014; Rafrafi et al., 2017).

For the reactors containing hardened cement paste, in the bulk (CEMV B.2) and from the cement paste surface (CEMV B.2.2), the dominant species was *Thauera* sp. This species was also detected in the activated sludge used as inoculum with an abundance of less than 0.2%. *Thauera* sp. is commonly found in microbial communities from WWTPs as the predominant nitrate reducer (Cydzik Kwiatkowska and Zielinska, 2016; Zielińska et al., 2016). This bacterium is not necessarily known to be resistant to alkaline pH but it is known to have a versatile metabolism (Mao et al., 2013; Li et al., 2018). With a low relative abundance (7%), *Halomonas* sp. was also identified in the supernatant from reactor with cement paste incubated 55 days (CEMV B.2) but not in the supernatant after the cement paste transfer in a new medium to identified microorganism attached to cement paste (reactor CEMV B.2.2), where *Thauera* sp. developed preferentially on the cement paste surface. Members of *Thauera* sp. are known to form biofilms. Some members of *Thauera* sp., such as *T. aminoaromatica*, are considered as major producers of extracellular polymeric substances (EPS) and may play an important role in biofilm formation (Allen et al., 2004; Cydzik Kwiatkowska and Zielińska, 2018).

To sum up, culture conditions were very selective, and two species dominated the reactors: a species known to resist alkaline pH, *Halomonas* sp., presumably *H. desiderata*, and a species known for its versatile metabolism and ability to form biofilms, *Thauera* sp.

4.3. Interactions of microorganisms with hardened cement paste

The presence of bacteria attached to the cement paste surface was highlighted by epifluorescence and confirmed by experiments in which the cement paste was transferred to a sterile medium (subsection 3.2.4). After 30 days of culture, dispersed microbial clusters were observed on the cement paste surface. In a second experiment and after 55 days of culture, in addition to microbial clusters, denser and more extensive biofilm like microbial layers were observed. The difference of colonisation between the two cement pastes may have been due to the different immersion times (30 days) or to a different microbial strain. *Thauera* sp. was presumably the dominant species constituting the biofilm on the cement paste in reactors with cement paste incubated 55 days (CEMV B) but no population analysis was performed for the first experimental set, i.e. reactors with cement paste incubated 30 days (CEMV A).

The colonisation of hardened cement paste by a pure strain or a microbial consortium has already been reported in the literature (Alquier et al., 2014; Rafrafi et al., 2015; Voegel et al., 2016). According to Voegel et al. (2016), surface conditioning of the cementitious material is a key step for biofilm formation. The bioreceptivity of the material was probably improved by the initial deterioration. The disappearance of portlandite and ettringite from the outer layer of the specimen observed by XRD (Figure A.4 section 3.23) certainly resulted from the dissolution of these phases in contact with the medium and, as Voegel et al. (2016, 2019) suggested in their works, it could be amplified by the production of metabolites aggressive to the cement paste, by the planktonic microorganisms.

Calcite or, to a lesser extent, vaterite precipitation on the cement paste surface was probably due to the reaction between calcium released by the cement paste and carbonates resulting from microbial oxidation of acetate in the liquid phase or close to the cement paste surface (by planktonic and/or adhered population). This phenomenon is relatively similar to the formation of calcium carbonate ($CaCO_3$) by microorganisms, studied in Microbial Induced Calcium carbonate Precipitation (MICP) (van Paassen et al., 2010; Erşan et al., 2015a) and

also the biodeposition treatment of a calcite layer described by De Muynck et al. (2008). According to this study, the calcite layer on the surface has the potential to improve the resistance of cementitious material to the degradation processes as the calcite layer induces a decrease in the permeation properties of cementitious material and thus an increased resistance to carbonation, chloride migration, and freezing and thawing (De Muynck et al., 2008).

Furthermore, the presence of calcium and the production of dissolved inorganic carbon by microorganisms are not the only two parameters necessary for $CaCO_3$ precipitation. According to Erşan et al. (2018), alkalinity and the presence of nucleation sites are two other parameters essential to MICP. The microorganisms may play a significant role in the control of the medium alkalinity, i.e. their microbial activities may promote alkalinity (self alkalinisation) and thus influence the carbonate balance to CO_3^{2-}. Moreover, according to Erşan et al. (2018), microorganisms can also serve as nucleation sites by concentrating calcium ion (Ca^{2+}) around their negatively charged cell membrane. Microorganisms and, more specifically, *Thauera* sp. in the case of our works, appear to play an important role in $CaCO_3$ precipitation.

4.4. Two scenarios can be assumed

(i) The alteration of the cementitious surface induced by the reaction medium makes bacterial adhesion possible. The biological activity near the surface contributes to trapping calcium leached as calcite precipitates on the surface of the cementitious material.
(ii) Before biofilm establishment, the microorganisms induce surface modification (formation of conditioning film by protein or polysaccharide production and/or surface leaching induced by metabolite production) of the material independently from the classical leaching phenomena induced by the medium. Then the biofilm activities will contribute to trap the released calcium.

5. Conclusion

The nitrate reduction in cementitious environments is possible up to pH around 11.5. For higher pH values, NR is inhibited. Nitrite reduction appears to be inhibited for values above 11, as does the microbial growth.

In the presence of hardened cement paste, the reaction between CO_2 emission via oxidation of organic matter (acetate) by heterotrophic bacteria and the release of calcium by the cement paste contributed to the calcite precipitation on the material surface. The presence of microorganisms attached to the cement surface, such as *Thauera* sp., could promote this phenomenon and make it an interesting candidates for different applications such as self healing.

Declaration of competing interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ibiod.2020.104971.

References

Albina, P., Durban, N., Bertron, A., Albrecht, A., Robinet, J.-C., Erable, B., 2019. Influence of hydrogen electron donor, alkaline pH, and high nitrate concentrations on microbial denitrification: a review. Int. J. Mol. Sci. 20, 5163. https://doi.org/10.3390/ijms20205163.

Albrecht, A., Bertron, A., Libert, M., 2013. Microbial catalysis of redox reactions in concrete cells of nuclear waste repositories: a review and introduction. In: Bart, F., Cau-di-Coumes, C., Frizon, F., Lorente, S. (Eds.), Cement-Based Materials for Nuclear Waste Storage. Springer New York, New York, NY, pp. 147–159. https://doi.org/10.1007/978-1-4614-3445-0 14.

Algaifi, H.A.A., Bakar, S.A., Sam, A.R.M., Abidin, A.R.Z., 2018. Crack-healing in cementitious material to improve the durability of structures: Review. MATEC Web Conf. 250, 03005. https://doi.org/10.1051/matecconf/201825003005.

Allen, M.S., Welch, K.T., Prebyl, B.S., Baker, D.C., Meyers, A.J., Sayler, G.S., 2004. Analysis and glycosyl composition of the exopolysaccharide isolated from the floc-forming wastewater bacterium Thauera sp. MZ1T Environ. Microbiol. 6, 780–790. https://doi.org/10.1111/j.1462-2920.2004.00615.x.

Alquier, M., Kassim, C., Bertron, A., Sablayrolles, C., Rafrafi, Y., Albrecht, A., Erable, B., 2014. Halomonas desiderata as a bacterial model to predict the possible biological nitrate reduction in concrete cells of nuclear waste disposals. J. Environ. Manag. 132, 32–41. https://doi.org/10.1016/j.jenvman.2013.10.013.

Ashok, V., Hait, S., 2015. Remediation of nitrate-contaminated water by solid-phase denitrification process-a review. Environ. Sci. Pollut. Res. 22, 8075–8093. https://doi.org/10.1007/s11356-015-4334-9.

Baumann, B., Snozzi, M., Zehnder, A.J., Meer, J.R.V.D., 1996. Dynamics of denitrification activity of Paracoccus denitrificans in continuous culture during aerobic-anaerobic changes. J. Bacteriol. 178, 4367–4374. https://doi.org/10.1128/jb.178.15.4367-4374.1996.

Berendes, F., Gottschalk, G., Heine-Dobbernack, E., Moore, E.R.B., Tindall, B.J., 1996. Halomonas desiderata sp. nov, a new alkaliphilic, halotolerant and denitrifying bacterium isolated from a municipal sewage works. Syst. Appl. Microbiol. 19, 158–167. https://doi.org/10.1016/S0723-2020(96)80041-5.

Bergaust, L., Shapleigh, J., Frostegard, A., Bakken, L., 2008. Transcription and activities of NO(x) reductases in Agrobacterium tumefaciens: the influence of nitrate, nitrite and oxygen availability. Environ. Microbiol. 10, 3070–3081. https://doi.org/10.1111/j.1462-2920.2007.01557.x.

Bertron, A., 2014. Understanding interactions between cementitious materials and microorganisms: a key to sustainable and safe concrete structures in various contexts. Mater. Struct. 47, 1787–1806. https://doi.org/10.1617/s11527-014-0433-1.

Bertron, A., Jacquemet, N., Erable, B., Sablayrolles, C., Escadeillas, G., Albrecht, A., 2014. Reactivity of nitrate and organic acids at the concrete-bitumen interface of a nuclear waste repository cell. Nucl. Eng. Des. 268, 51–57. https://doi.org/10.1016/j.nucengdes.2013.11.085.

Chang, Y.C., Le Puil, M., Biggerstaff, J., Randall, A.A., Schulte, A., Taylor, J.S., 2003. Direct estimation of biofilm density on different pipe material coupons using a specific DNA-probe. Mol. Cell. Probes 17, 237–243. https://doi.org/10.1016/j.mcp.2003.07.004.

Cydzik-Kwiatkowska, A., Zielińska, M., 2018. Microbial composition of biofilm treating wastewater rich in bisphenol A. J. Environ. Sci. Health Part A 53, 385–392. https://doi.org/10.1080/10934529.2017.1404326.

Cydzik-Kwiatkowska, A., Zielinska, M., 2016. Bacterial communities in full-scale wastewater treatment systems. World J. Microbiol. Biotechnol. 32, 66. https://doi.org/10.1007/s11274-016-2012-9.

De Muynck, W., Debrouwer, D., De Belie, N., Verstraete, W., 2008. Bacterial carbonate precipitation improves the durability of cementitious materials. Cement Concr. Res. 38, 1005–1014. https://doi.org/10.1016/j.cemconres.2008.03.005.

Dhamole, P.B., Nair, R.R., D'Souza, S.F., Lele, S.S., 2008. Denitrification of highly alkaline nitrate waste using adapted sludge. Appl. Biochem. Biotechnol. 151, 433–440. https://doi.org/10.1007/s12010-008-8211-6.

Durban, N., Rafrafi, Y., Rizoulis, A., Albrecht, A., Robinet, J.-C., Lloyd, J.R., Bertron, A., Erable, B., 2018. Nitrate and nitrite reduction at high pH in a cementitious environment by a microbial microcosm. Int. Biodeterior. Biodegrad. 134, 93–102. https://doi.org/10.1016/j.ibiod.2018.08.009.

Edgar, R.C., 2013. UPARSE: highly accurate OTU sequences from microbial amplicon reads. Nat. Methods 10, 996–998. https://doi.org/10.1038/nmeth.2604.

Erşan, Y.Ç., Belie, N. de, Boon, N., 2015a. Microbially induced CaCO3 precipitation through denitrification: an optimization study in minimal nutrient environment. Biochem. Eng. J. 101, 108–118. https://doi.org/10.1016/j.bej.2015.05.006.

Ersan, Y.C., Boon, N., De Belie, N., 2018. Granules with activated compact denitrifying core (ACDC) for self-healing concrete with corrosion protection functionality. In: Presented at the Final Conference of RILEM TC 253-MCI: Microorganisms-Cementitious Materials Interactions. RILEM Publications, pp. 475–484.

Erşan, Y.Ç., Da Silva, F.B., Boon, N., Verstraete, W., De Belie, N., 2015b. Screening of bacteria and concrete compatible protection materials. Construct. Build. Mater. 88, 196–203. https://doi.org/10.1016/j.conbuildmat.2015.04.027.

Ersan, Y.C., Verbruggen, H., De Graeve, I., Verstraete, W., De Belie, N., Boon, N., 2016. Nitrate reducing CaCO3 precipitating bacteria survive in mortar and inhibit steel corrosion. Cement Concr. Res. 83, 19–30. https://doi.org/10.1016/j.cemconres.2016.01.009.

Glass, C., Silverstein, J., 1998. Denitrification kinetics of high nitrate concentration water: pH effect on inhibition and nitrite accumulation. Water Res. 32, 831–839. https://doi.org/10.1016/s0043-1354(97)00260-1.

Goñi, S., Gaztañaga, M.T., Guerrero, A., 2002. Role of cement type on carbonation attack. J. Mater. Res. 17, 1834–1842. https://doi.org/10.1557/JMR.2002.0271.

Grengg, C., Mittermayr, F., Baldermann, A., Boettcher, M.E., Leis, A., Koraimann, G., Grunert, P., Dietzel, M., 2015. Microbiologically induced concrete corrosion: a case study from a combined sewer network. Cement Concr. Res. 77, 16–25. https://doi.org/10.1016/j.cemconres.2015.06.011.

Grengg, C., Mittermayr, F., Ukrainczyk, N., Koraimann, G., Kienesberger, S., Dietzel, M., 2018. Advances in concrete materials for sewer systems affected by microbial induced concrete corrosion: a review. Water Res. 134, 341–352. https://doi.org/10.1016/j.watres.2018.01.043.

Hiscock, K.M., Lloyd, J.W., Lerner, D.N., 1991. Review of natural and artificial denitrification of groundwater. Water Res. 25, 1099–1111. https://doi.org/10.1016/0043-1354(91)90203-3.

Jones, C.M., Stres, B., Rosenquist, M., Hallin, S., 2008. Phylogenetic analysis of nitrite, nitric oxide, and nitrous oxide respiratory enzymes reveal a complex evolutionary history for denitrification. Mol. Biol. Evol. 25, 1955–1966. https://doi.org/10.1093/molbev/msn146.

Jonkers, H.M., 2007. Self healing concrete: a biological approach. In: van der Zwaag, S. (Ed.), Self Healing Materials: an Alternative Approach to 20 Centuries of Materials Science, Springer Series in Materials Science. Springer Netherlands, Dordrecht, pp. 195–204. https://doi.org/10.1007/978-1-4020-6250-6 9.

Krulwich, T.A., Sachs, G., Padan, E., 2011. Molecular aspects of bacterial pH sensing and homeostasis. Nat. Rev. Microbiol. 9, 330–343. https://doi.org/10.1038/nrmicro2549.

Laspidou, C.S., Rittmann, B.E., 2002. A unified theory for extracellular polymeric substances, soluble microbial products, and active and inert biomass. Water Res. 36, 2711–2720. https://doi.org/10.1016/S0043-1354(01)00413-4.

Li, E., Jin, X., Lu, S., 2018. Microbial communities in biological denitrification system using methanol as carbon source for treatment of reverse osmosis concentrate from coking wastewater. J. Water Reuse Desalination 8, 360–371. https://doi.org/10.2166/wrd.2017.024.

Lu, H., Chandran, K., Stensel, D., 2014. Microbial ecology of denitrification in biological wastewater treatment. Water Res. 64, 237–254. https://doi.org/10.1016/j.waters.2014.06.42.

Magniont, C., Coutand, M., Bertron, A., Cameleyre, X., Lafforgue, C., Beaufort, S., Escadeillas, G., 2011. A new test method to assess the bacterial deterioration of cementitious materials. Cement Concr. Res. 41, 429–438. https://doi.org/10.1016/j.cemconres.2011.01.014.

Mao, Y., Xia, Y., Zhang, T., 2013. Characterization of Thauera-dominated hydrogen-oxidizing autotrophic denitrifying microbial communities by using high-throughput sequencing. Bioresour. Technol. 128, 703–710. https://doi.org/10.1016/j.biortech.2012.10.106.

Mateju, V., Cizinska, S., Krejci, J., Janoch, T., 1992. Biological water denitrification - a review. Enzym. Microb. Technol. 14, 170–183. https://doi.org/10.1016/0141-0229(92)90062-s.

Nikitenko, S.I., Venault, L., Pflieger, R., Chave, T., Bisel, I., Moisy, P., 2010. Potential applications of sonochemistry in spent nuclear fuel reprocessing: a short review. Ultrason. Sonochem. 17, 1033–1040.

Park, H.I., Choi, Y.-J., Pak, D., 2005. Autohydrogenotrophic denitrifying microbial community in a Glass beads biofilm reactor. Biotechnol. Lett. 27, 949–953. https://doi.org/10.1007/s10529-005-7654-x.

Peyre Lavigne, M., Bertron, A., Botanch, C., Auer, L., Hernandez-Raquet, G., Cockx, A., Foussard, J.-N., Escadeillas, G., Paul, E., 2016. Innovative approach to simulating the biodeterioration of industrial cementitious products in sewer environment. Part II: validation on CAC and BFSC linings. Cement Concr. Res. 79, 409–418. https://doi.org/10.1016/j.cemconres.2015.10.002.

Philippot, L., 2002. Denitrifying genes in bacterial and Archaeal genomes. Biochim. Biophys. Acta Gene Struct. Expr. 1577, 355–376. https://doi.org/10.1016/s0167-4781(02)00420-7.

Plummer, L.N., Busenberg, E., 1982. The solubilities of calcite, aragonite and vaterite in CO2-H2O solutions between 0 and 90°C, and an evaluation of the aqueous model for the system CaCO3-CO2-H2O. Geochem. Cosmochim. Acta 46, 1011–1040. https://doi.org/10.1016/0016-7037(82)90056-4.

Qureshi, N., Annous, B.A., Ezeji, T.C., Karcher, P., Maddox, I.S., 2005. Biofilm reactors for industrial bioconversion processes: employing potential of enhanced reaction rates. Microb. Cell Factories 4, 24. https://doi.org/10.1186/1475-2859-4-24.

Rafrafi, Y., Durban, N., Bertron, A., Albrecht, A., Robinet, J.-C., Erable, B., 2017. Use of a continuous-flow bioreactor to evaluate nitrate reduction rate of Halomonas desiderata in cementitious environment relevant to nuclear waste deep repository. Biochem. Eng. J. 125, 161–170. https://doi.org/10.1016/j.bej.2017.05.016.

Rafrafi, Y., Ranaivomanana, H., Bertron, A., Albrecht, A., Erable, B., 2015. Surface and bacterial reduction of nitrate at alkaline pH: conditions comparable to a nuclear waste repository. Int. Biodeterior. Biodegrad. 101, 12–22. https://doi.org/10.1016/j.ibiod.2015.03.013.

Richardson, D., Felgate, H., Watmough, N., Thomson, A., Baggs, E., 2009. Mitigating release of the potent greenhouse gas N2O from the nitrogen cycle – could enzymic regulation hold the key? Trends Biotechnol. 27, 388–397. https://doi.org/10.1016/j.tibtech.2009.03.009.

Richardson, D.J., Berks, B.C., Russell, D.A., Spiro, S., Taylor, C.J., 2001. Functional, biochemical and genetic diversity of prokaryotic nitrate reductases: Cell. Mol. Life Sci. 58, 165–178. https://doi.org/10.1007/PL00000845.

Rizoulis, A., Steele, H.M., Morris, K., Lloyd, J.R., 2012. The potential impact of anaerobic microbial metabolism during the geological disposal of intermediate-level waste. Mineral. Mag. 76, 3261–3270. https://doi.org/10.1180/minmag.2012.076.8.39.

Shapovalova, A.A., Khijniak, T.V., Tourova, T.P., Muyzer, G., Sorokin, D.Y., 2008. Heterotrophic denitrification at extremely high salt and pH by haloalkaliphilic Gammaproteobacteria from hypersaline soda lakes. Extremophiles 12, 619–625. https://doi.org/10.1007/s00792-008-0166-6.

Smith, S.L., Rizoulis, A., West, J.M., Lloyd, J.R., 2016. The microbial ecology of a hyperalkaline spring, and impacts of an alkali-tolerant community during sandstone batch and column experiments representative of a geological disposal facility for intermediate-level radioactive waste. Geomicrobiol. J. 33, 455–467. https://doi.org/10.1080/01490451.2015.1049677.

van Paassen, L.A., Daza, C.M., Staal, M., Sorokin, D.Y., van der Zon, W., van Loosdrecht, MarkC.M., 2010. Potential soil reinforcement by biological denitrification. Ecol. Eng. Special Issue: BioGeoCivil Eng. 36, 168–175. https://doi.org/10.1016/j.ecoleng.2009.03.026.

Van Tittelboom, K., De Belie, N., 2013. Self-healing in cementitious materials—a review. Materials 6, 2182–2217. https://doi.org/10.3390/ma6062182.

Voegel, C., Bertron, A., Erable, B., 2016. Mechanisms of cementitious material deterioration in biogas digester. Sci. Total Environ. 571, 892–901. https://doi.org/10.1016/j.scitotenv.2016.07.072.

Voegel, C., Durban, N., Bertron, A., Landon, Y., Erable, B., 2019. Evaluation of microbial proliferation on cementitious materials exposed to biogas systems. Environ. Technol. https://doi.org/10.1080/09593330.2019.1567610.

Voegel, Giroudon, M., Bertron, A., Patapy, C., Matthieu, P.L., Verdier, T., Erable, B., 2019. Cementitious materials in biogas systems: biodeterioration mechanisms and kinetics in CEM I and CAC based materials. Cement Concr. Res. 124https://doi.org/10.1016/j.cemconres.2019.105815. UNSP 105815.

Wang, J.Y., Belie, N.D., Verstraete, W., 2012. Diatomaceous earth as a protective vehicle for bacteria applied for self-healing concrete. J. Ind. Microbiol. Biotechnol. 39, 567–577. https://doi.org/10.1007/s10295-011-1037-1.

Zhang, Z., Schwartz, S., Wagner, L., Miller, W., 2000. A greedy algorithm for aligning DNA sequences. J. Comput. Biol. J. Comput. Mol. Cell Biol. 7, 203–214. https://doi.org/10.1089/10665270050081478.

Zhang, Z., Zhang, Y., Chen, Y., 2020. Recent advances in partial denitrification in biological nitrogen removal: from enrichment to application. Bioresour. Technol. 298https://doi.org/10.1016/j.biortech.2019.122444. 122444.

Zielińska, M., Rusanowska, P., Jarząbek, J., Nielsen, J.L., 2016. Community dynamics of denitrifying bacteria in full-scale wastewater treatment plants. Environ. Technol. 37, 2358–2367. https://doi.org/10.1080/09593330.2016.1150350.