Nitrate and nitrite reduction at high pH in a cementitious environment by a microbial microcosm

Nadège Durban, Yan Rafrafi, Athanasios Rizoulis, Achim Albrecht, Jean-Charles Robinet, Jonathan Lloyd, Alexandra Bertron, Benjamin Erable

Nitrate and nitrite reduction at high pH in a cementitious environment by a microbial microcosm

Authors: Durban Nadège [a,b,*], Rafrafi Yan [a,b], Rizoulis Athanasios [c], Albrecht Achim [d], Robinet Jean-Charles [d], Lloyd Jonathan R. [c], Bertron Alexandra [a], Erable Benjamin [b]

[a] LMDC, Université de Toulouse, INSA/UPS Génie Civil, 135 Avenue de Rangueil, 31077 Toulouse cedex 04 France

[b] Laboratoire de Génie Chimique, Université de Toulouse, CNRS, INPT, UPS, Toulouse, France

[c] Research Centre for Radwaste and Decommissioning and the Williamson Research Centre for Molecular Environmental Science, School of Earth and Environmental Sciences, The University of Manchester, Oxford Road, Manchester M13 9PL, UK

[d] Andra, 1-7, rue Jean-Monet, 62298 Châtenay-Malabry, France

* Corresponding author: nadege.durban@ensiacet.fr

Key-words: *alkaline denitrification, biofilm, hardened cement paste, nuclear waste management*

Abstract

The possible release of oxyanions, such as nitrate, from radioactive waste repositories may influence redox-conditions of the near field environment and thus promote mobility of some redox sensitive radionuclides. The fate of dissolved oxyanions will be significantly conditioned by microbial activities, if present in the aqueous interstitial phase of a waste cell. This study investigates microbial nitrate reduction in a cementitious environment. A consortium of microorganisms was used, an inoculum prepared with sediments collected from a former lime works site, characterized by a pH of porewater of 11-12. The biomass was acclimated to cement leachate supplemented with nitrate, acetate and yeast extract. According to experiments performed in closed and in dynamic systems, the microbial consortium was adapted to reduce nitrate and nitrite in a cementitious, anaerobic environment (pH 11, with and without hardened cement paste and leachate). Although, nitrite accumulation was observed in close system and temporally in dynamic system. The rate of nitrate reduction was between 0.12 and 0.75 mM/h with incoming nitrate concentrations between 6 and 48 mM, respectively. Sessile microorganisms in biofilm present on the hardened cement paste and the large diversity helped maintain microbial activity under all of the conditions simulating cementitious environments.

1. Introduction

Nitrate bearing intermediate-level long-lived (ILW-LL) radioactive wastes is proposed to be stored in concrete packages inside a repository at a depth of approximately 500 metres within

the Callovo-Oxfordian clay rock host formation. The concrete surrounding the waste packages serves as the engineered barrier and the host rock as the geological barrier(Andra, 2005; Gaucher et al., 2004; Vinsot et al., 2008). After closure of the repository cell, the progressive resaturation is expected to induce progressive leaching of cement-based materials (waste packages, cell lining etc.), leading to release of hydroxide anions and cations, such as Ca^{2+}, Na^{+} and K^{+}. After corrosion and failure of the primary steel waste containers, substances present with the stabilizing bituminous matrix (oxyanions, dissoleved organic matter, radionuclides etc.) will slowly start to diffuse out (Sercombe et al., 2006; Walczak et al., 2001). The release of soluble oxyanions such as nitrate, incorporated in the nuclear waste during recycling procedures (Nikitenko et al., 2010), will promote oxidizing conditions in the vicinity of the waste if nitrate reduction occurs. This may promote the mobility of some redox sensitive radionuclides (Se, U, Tc, Pu, Np, etc.) (Albrecht et al., 2013). Mostly biotic processes could catalyse the reduction of nitrate in the interstitial aqueous phase of the near field. Under abiotic conditions, fresh steel surfaces may catalyse some nitrate reduction and produce ammonium ions but the related processes are limited to situations were fresh steel surfaces are present and when temperatures are beyond those expected in a waste cell (50°C) (Rafrafi et al., 2015; Truche et al., 2013). Nitrate reduction rates have been measured at around 10^{-3} mM/h under laboratory conditions (pH > 10, anaerobic conditions, cementitious environment, etc.) (Rafrafi et al., 2015; Truche et al., 2013). In the presence of electron donors, such as organic matter or dihydrogen, microorganisms are able to catalyse the reduction of nitrate to nitrogen gas (N_2) via several denitrification intermediate chemical species; nitrite (NO_2^-), nitric oxide (NO), and nitrous oxide (N_2O) (Jones et al., 2008; Mateju et al., 1992; Parmentier et al., 2014). At temperatures below 40 °C, denitrifying bacteria catalyse nitrate reduction at a faster rate than the steel surface catalysed reduction at alkaline pH (Rafrafi et al., 2015; Truche et al., 2013). A sedimentary microcosm sampled from a site contaminated by high pH legacy lime works was able to reduce 15 mM nitrate solution at pH between 9 and 10 in less than a week (0.089 mM NO_3^--N/h) and at pH between 9 and 11 in less than two weeks (0.045 mM NO_3^--N /h) (Rizoulis et al., 2012). In order to simulate a more realistic cementitious environment, Rafrafi et al. (Rafrafi et al., 2017) replaced the optimal culture medium commonly applied for bacterial growth by a minimal medium composed only of cement leachate supplemented by acetate and nitrate. *Halomonas desiderata,* an alkaliphilic bacterial strain, could grow and reduce nitrate and nitrite in an experimental set-up continuously supplied with this medium. Under these conditions, the nitrate reduction rate catalysed by *H. desiderata* was around 0.08 mM/h at pH 10 (Rafrafi et al., 2017), i.e. similar to the rate obtained by Rizoulis et al. (Rizoulis et al., 2012) with a microcosm. Introducing hardened cement paste in Rafrafi et al.'s experimental set-up (Rafrafi et al., 2017, 2015) highlighted the formation of a biofilm several tens of microns thick on the surface of cementitious material. This biofilm allowed active biomass to be maintained in a flow through reactor. At pH 12, nitrate reduction (0.24 mM/h) was detected only in the reactor with colonised solid cement pastes (Rafrafi et al., 2015).

In comparison with a microbial monoculture, microbial consortia can sustain more complex metabolic reactions, they can survive in environments subject to larger fluctuations (pH, substrate concentration, temperature, etc.) (Brenner et al., 2008). The associated microbial diversity is certainly more representative of a community likely to grow in a deep repository compared to a single species such as *H. desiderata*.

The focus of this study is to evaluate the rate of nitrate and nitrite reduction by a naturally microcosms occurring sediment collected from a former lime works site (Rizoulis et al., 2012), under alkaline pH conditions comparable to a cementitious waste cell environment. To

approach even further a concrete-dominated environment, the optimal culture medium described by Rizoulis et al. (Rizoulis et al., 2012) was replaced by a medium simulating a cementitious environment (Alquier et al., 2014; Rafrafi et al., 2017), i.e. cement leachate supplemented by nitrate as electron acceptor and acetate as carbon source and electron donor, with or without yeast extract. Furthermore, cement paste specimen were introduced in order to investigate the interactions between biomass and cement surfaces; especially the possibility of biofilm formation. Finally, the multiphase system (with or without hardened cement paste specimen) was studied both under batch and dynamic conditions.

2. Materials and methods

2.1. Cementitious materials

The cement paste specimens (CEM V/A 42.5; Calcia's Airvault factory) were made with a water/cement ratio of 0.32. The CEM V/A cement is a standardized cement containing clinker and blast furnace slag with fly ash addition (Olmeda et al., 2017).
The cement paste specimens were cast in cylindrical moulds (50 mm high and 27 mm in diameter) and were kept in sealed bags (to avoid any hydric exchanges with the exterior, and thus favour cement hydration reactions and to protect them against carbonation) for 28 days after demoulding. Then, they were cut into slices (h ≈ 10 - 20 mm) and sanded with silicon carbide polishing disks (P120 - ≈ 127 µm - Presi®) to impose a surface roughness favourable to bacterial cell attachment.

2.2. Standard medium

The standard medium used as the feed solution was made of cement leachate 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) at pH 11. The cement leachate was prepared by immersing cement paste specimens in 1 L of demineralized water (solid/liquid volume ratio: 1.03) for 3 days under continuous stirring. The average chemical composition of the cement leachate is given in Table 1.

2.3. Sedimentary microcosm

2.3.1. Sediment sampling

Sediment samples were collected at a depth of ≈ 20 cm from the surface of an area contaminated by a legacy lime works site at Harpur Hill, Buxton, UK. These sediments generally have a pH around 11-12 and contain high calcium and silicate concentrations (Rizoulis et al., 2012).

2.3.2. Enrichment culture preparation

Nitrate-reducing microorganisms were enriched in anaerobic cultures (prepared under N_2 atmosphere, in crimp-sealed 100 mL sterile bottles) that were set up using 1 g of the Buxton sediment, mixed with 50 mL of standard medium (cement leachate with 8.3 mM sodium acetate and 5.9 mM sodium nitrate) and 0.1% (w/v) yeast extract, from a 5% (w/v) sterile stock solution. The anaerobic cultures were incubated at 25°C in the dark, and acetate and nitrate

concentrations were monitored regularly. Once the nitrate was reduced to nitrite (within 7 days; data not shown), 5 mL of the culture was transferred to a new bottle, which contained 45 mL of standard medium and 0.1% yeast extract. Once the nitrate in the subculture was also reduced to nitrite, 5 mL of the subculture was transferred to a new bottle that contained 45 mL of standard medium and 0.1% yeast extract. In a similar way, enrichment cultures without added yeast extract were prepared and subcultured twice (after nitrate reduction was observed). The second subcultures from the yeast-amended and the non-yeast amended anaerobic cultures were used as the enrichment cultures for subsequent batch bioreactor experiments below.

2.4. Experimental set-up and conditions

Several experiments were performed in a batch bioreactor in order (i) to optimize the culture medium for the simulated cementitious environment and (ii) to investigate the possible colonisation of the hardened cement paste surface by the biomass. The experimental conditions are described in the following subsection and summarised in Figure 1. Then, two experiments were performed in dynamic systems according to the set-up described by Rafrafi et al. (2017)(Figure A.1 in supplementary data), with a bioreactor and an exposed chamber containing cement paste specimens. The standard medium optimised in batch mode was used in a first experiment. Then, after several cement paste specimen additions in the exposed chamber (results available in supplementary data), the nitrate concentration was increased in the feed solution. The conditions of these two experiments are detailed in the following subsection.

2.4.1. Optimisation of the medium simulating a cementitious environment in the batch bioreactor

Two 2 L reactor experiments were run in batch conditions over a period of three days in anaerobic condition (bubbling N_2 through the medium for 15 min) and thermostatically controlled at 30°C. The first reactor (R.A) was inoculated with 50 mL of an enrichment prepared without yeast extract supplementation (see section 2.3.2). The second one (R.A_YE) was inoculated with 50 mL of the enrichment with yeast extract supplementation (0.1% of YE). The standard medium previously described was used to support growth in the two reactors and 0.1% of yeast extract was added in R.A_YE.

2.4.2. Investigation of colonisation of the hardened cement paste specimen in the batch bioreactor

Successive batch tests were performed in two 500 mL bioreactors containing 2 (reactor R.B_YE_2CP) or 4 cement paste specimens (reactor R.B_YE_4CP), which provided a surface available for microbial colonisation of around 40 cm^2 and 80 cm^2, respectively. At the beginning of the experiment, the reactors containing 500 mL of standard medium, supplemented with yeast extract were inoculated with 0.5 mL of microbial culture from reactor R.A_YE (see section 2.4.1). Then, the two reactors were deoxygenated by bubbling N_2 through the medium for 15 min and the temperature was set at 30°C, without any pH regulation or stirring being performed. After 7 days of culture, the cement specimens were removed, rinsed with cement leachate and finally introduced into a new reactor with fresh

standard medium. This operation was reiterated twice, raising the total number of batch tests to 4. The duration of each test was 7 days (Figure 1).

2.4.3. Experiment under continuous supply

At the end of the batch test (see section 2.4.1), reactor R.A_YE was connected to the feed tank and to the exposure chamber in order to perform this experiment under continuous supply using the same set-up design as the one described in by Rafrafi et al. (2017, 2015)((Figure A.1). Fresh standard medium was continuously added to the bioreactor by means of a peristaltic pump having a constant flow rate of 0.66 mL/min (Model 7554-85, 7-200 rpm, Easy Load L/S head for tube 13-18 7518-00 model). The feed tank contained standard medium supplemented with 0.1% of yeast extract. The bioreactor contained 2 L of microbial culture enriched with the sedimentary microcosm. Three solid slices of cement paste (thickness = 10 mm) were introduced in the 1 L working volume exposure chamber. The surface available for microbial colonisation was around 60 cm^2. The hydraulic retention time (HRT) was set at 50.5 h in the bioreactor and 25.25 h in the exposure chamber. Both the bioreactor and the exposure chamber were thermostatically controlled at 30°C, constantly mixed with mechanical stirring and flushed with continuous N_2 bubbling (anaerobic conditions).

2.4.4. Nitrate concentration increase in standard medium (continuous supply)

During the first 27 days (≈ 650 hours), the experimental set-up described was fed with the standard medium supplemented with 0.1% of yeast extract (section 2.2). The surface available for microbial colonisation was progressively raised from 100 cm^2 to 339 cm^2 in the exposure chamber. The approach and results of this experiment are available in the supplementary data. Next, the concentration of nitrate was progressively increased from 6.0 mM to 11.8 mM after 792 hours of culture, and then to 48 mM from day 55 (1314 hours of culture) until the end of the experiment; i.e. 2200 hours. The experimental conditions are reported in Table A.2 in supplementary data and the results are discussed below.

2.5. Analytical techniques

2.5.1. Monitoring of the various devices

For each experiment, regular sampling was performed for immediate measurement of pH (6500pH/ ion meter, Eutech Instruments) and bacterial growth by measuring the optical density (OD) at 600 nm (JENWAY 7315 spectrophotometer) (Alquier et al., 2014; Rafrafi et al., 2015). The feed solution was systematically used as a blank. Two millilitres of sample were collected and filtered (through a 0.2 µm - Minisart PES, Fisher Scientific) for analyses of ionic species (nitrate, nitrite, acetate, etc.). Sterile syringes were used for sampling. The sampling point for dynamics tests is specified in Figure A.1.

2.5.2. Chemical analysis

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. (Alquier et al., 2014) and Bertron et al. (Bertron et al., 2014).

2.5.3. SEM analysis

Biofilms grown on the surface of the cement paste specimen were observed by SEM-FEG (JEOL 7100F TTLS, 5kW). Before SEM observations with secondary electron (SEM-SE), biofilms on the cement paste specimens were chemically fixed and then dehydrated according to the procedure described by Voegel et al. (Voegel et al., 2016, 2015).

The aim of the fixation step was to preserve the integrity of the microbial cells and the biofilm 3D architecture during water extraction. The chemical fixation method required several steps: (i) 20 minutes of immersion in aldehyde fixation solution composed of 2 volumes of glutaraldehyde (4%), 1 volume of phosphate buffer (pH 7.4, 0.4 M) and 1 volume of distilled water, (ii) two times 15 minutes of immersion in a cleaning solution made of 1 volume of phosphate buffer (pH 7.4, 0.4 M), 2 volumes of sucrose solution (0.4 M) and 1 volume of distilled water.

The aim of the chemical dehydration was to replace water by volatile solvents, such as acetone and hexamethyldisilazane (HDMS). The chemical dehydration was progressively carried out by successive immersion of the specimens in solutions of; acetone and water (50%-50%, 5 min), acetone and water (70%-30%, 5 min), acetone (30 min), acetone and HDMS (50%-50%, 10 min) and, finally, HMDS (100%) until complete evaporation. After fixation and dehydration, the specimens were coated with a thin layer of gold before SEM observations.

3. Results and discussion

3.1. Microcosm activity in closed bioreactors

The ability of the microcosm to catalyse nitrate reduction at pH 11 in a medium prepared from cement leachate was first evaluated in closed bioreactors (batch test) with acetate only (reactor R.A) or with acetate and yeast extract (reactor R.A_YE) as electron donors. Anaerobic microbial growth and nitrate reduction were detected in reactor R.A_YE only (Figure 1.A). Approximately 90% of nitrate had been reduced to nitrite while 10% had been reduced further (likely to nitrogen gas) at the end of the 50 hours of the batch test (Figure 2. b). Nitrite accumulation in a batch test has already been reported in previous works performed at alkaline pH (Glass and Silverstein, 1998), (i) using a pure culture of *Halomonas desiderata* (Alquier et al., 2014) or (ii) using the same microbial consortium enriched with sediments polluted by lime works (Bassil et al., 2015; Rizoulis et al., 2012). The reduction rates of nitrite and nitrate were greatly impacted by the pH, the nitrite reduction being impacted on more strongly than the nitrate (Cao et al., 2013; Thomsen et al., 1994). One possible explanation could be the difference in the locations of bacterial enzymes. The bacterial enzymes involved in nitrate reduction can be anchored to the cytoplasmic face of the membrane (NAR) or present in the periplasmic compartment (NAP), whereas nitrite reductase (NIR) are found only in the periplasmic compartment (Richardson et al., 2009; Richardson* et al., 2001). Enzymes outside the cytoplasmic membrane, such as nitrite reductase, are more sensitive to the environmental conditions (including pH) than the enzymes inside, and are most competitive for pH close to neutrality (Berks et al., 1994; Richardson et al., 2009).

The onset of the microbial denitrifying activity was accompanied by a pH decrease from 11.0 to 8.5 for reactor R.A_YE (Figure 2.a). This is in accordance with both reaction 1 (valid for pH 10.3, i.e., at equivalent concentrations of HCO_3^- and CO_3^{2-}) and reaction 2 (valid for pH around 8, i.e. when HCO_3^- is the preponderant species), which shows that the reduction of nitrate into

nitrite theoretically leads to the production of protons (H^+) (equivalents of acidity) (Glass and Silverstein, 1998; Mateju et al., 1992).

$$NO_3^- + 0.25\ CH_3COO^- \rightarrow NO_2^- + 0.25\ HCO_3^- + 0.25\ CO_3^{2-} + 0.5\ H^+ \quad \text{Reaction (1)}$$

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

After 70 hours of batch testing, the acetate concentration had increased from 9.3 to 10.7 mM (data not presented) in the bioreactor R.A_YE containing the yeast extract. The production of acetate probably resulted from microbial hydrolysis and fermentation of amino-acids, peptides and carbohydrates present in the yeast extract (Mosser et al., 2012).

This batch experiment indicated that heterotrophic indigenous microbial communities enriched from the sedimentary microcosm could catalyse nitrate reduction at an initial pH of 11.0 in a medium prepared from cement leachate. However, nitrate reduction could only take place in the presence of yeast extract as organic substrate. The microbial nitrate reduction rate was about 0.128 mM N-NO_3^-/h, which was in the same range as the reduction rate reported by Rizoulis et al. (Rizoulis et al., 2012) (15 mM of nitrate reduced in 2 weeks, i.e. 0.045 mM N-NO_3^-/h) using a microcosm inoculated with similar sediments.

3.2. Activity of microbial communities adhering to the surface of hardened cement paste in closed bioreactors

To investigate the possible attachment and development of denitrifying microorganisms on the surface of the cement paste specimen, successive fed-batch tests were performed in two bioreactors (R.B_YE_2CP and R.B_YE_4CP) filled with standard medium supplemented with yeast extract. Two cement paste specimens were introduced into reactor R.B_YE_2CP and four into reactor R.B_YE_4CP (see section 2.4.2 for details). At the end of the first batch period, the cement specimens were carefully rinsed with cement leachate, and were then introduced into a new bioreactor containing fresh standard medium (uninoculated cement leachate supplemented by acetate and nitrate). The only source of denitrifying microorganisms in the new bioreactors was the microbial population that had been able to attach to and proliferate on the surfaces or in the porosity of the hardened cement pastes.

According to the results presented in Figure 3.a and Figure 3.c, the increase of OD values simultaneously with a decrease in the nitrate concentration observed for batches 2, 3 and 4, clearly demonstrates a development of denitrifying populations in both bioreactors, R.B_YE_2CP and R.B_YE_4CP. The sedimentary microorganisms initially present in the inoculum colonised the cement pastes specimen during the first batch. Then, after each liquid fraction renewal in the bioreactors, microorganisms adhering to the cement surface acted as a source for the growth of new planktonic microorganisms. The renewal of the feed solution may have stimulated bacterial detachment by chemotaxis because nutrients were more available in the bulk than inside the biofilm (Morgan et al., 2006).

During the first batch test with 2 specimens of CEM V (batch 1 – reactor R.B_YE_2CP), the pH increased slightly, from 11.0 to 11.7, and then progressively decreased to 9.9 (Figure 3.b).

With 4 CEM V specimens, the maximum alkaline pH reached 12.1 at 72 h, and then stabilised around 11.8. The initial fast pH increase was due to the release of hydroxide ions from the cement paste specimen (higher with 4 CEM V than with 2 CEM V specimens). The acidification observed afterwards was caused by the microbial denitrifying activity, especially in reactor R.B_YE_2CP with 2 CEM V, where the bacterial growth was higher than in the reactor with 4 cement specimens (Figure 3.a). For batches 2 to 4, the pH evolution was quite similar in bioreactors R.B_YE_2CP and R.B_YE_4CP regardless of the quantity of solid cement paste specimen. The pH rose slightly (0.2-0.3 pH unit) before decreasing sharply.

Except for the first batch test, complete nitrate reduction was achieved in 110 hours of culture on average. A strong nitrite accumulation was observed for the first two batch tests in R.B_YE_2CP, then the concentration of nitrite decreased more and more rapidly. For the last batch test, nitrite no longer accumulated. Nitrite accumulation was also observed in reactor R.B_YE_4CP and the time required to reduce the accumulated nitrite was longer than in reactor R.B_YE_2CP. In the presence of 2 specimens of CEM V, the microbial inoculum required an adaptation period equivalent to 3 batch tests to perform complete reduction of 6.5 mM nitrate. The adaptation time was obviously higher in the presence of 4 CEM V specimens. Nitrate reduction rates are reported in Table 2. Nitrate reduction rates with 2 or 4 hardened cement pastes were globally similar after biomass acclimatization, i.e. in the last three batch tests. The number of cement paste specimens did not affect the maximum nitrate reduction rates directly but did influence the biomass acclimatization. Cement pastes specimen released higher quantities of ions, affecting the time required by bacteria to adapt their metabolism to changing environmental conditions. The presence of larger quantities of cement paste specimen limited the reduction of nitrate beyond the nitrite step.

3.3. Microbial activity under dynamic conditions (continuous supply)

The results in a closed system at pH 11.0 clearly demonstrated (i) the ability of the microcosm to reduce nitrate in a cement leachate based mineral medium, and (ii) the ability of the sedimentary bacterial consortium to colonise the surface of the cement paste specimen. Nonetheless, the microbial denitrification process catalysed by the sedimentary microcosm was mainly interrupted after the first step of nitrate reduction, which resulted in nitrite accumulation in the closed bioreactors. Nitrite accumulation in closed reactors (batch systems) was also reported by Alquier et al. (Alquier et al., 2014) with *Halomonas desiderata* cultivated in mineral synthetic medium for pH between 9.0 and 10.5, with and without a solid cementitious matrix. In the study by Rafrafi et al. (Rafrafi et al., 2015), the transition to a continuous supply of standard medium allowed *Halomonas desiderata* to achieve both nitrate and nitrite reduction, after an adaptation period equivalent to three hydraulic retention times in the bioreactor. In the present study, the ability of the sedimentary microcosm to reduce total oxidized nitrogen (TON), i.e. nitrate and nitrite, was investigated under continuous supply of the standard medium supplemented with yeast extract.

3.3.1. Continuous supply of standard medium

At the end of the batch test described in subsection 3.1, i.e. after 70 hours of batch culture, reactor R.A_YE was connected to the exposure chamber and fed continuously with the

standard medium supplemented with 0.1% of yeast extract (see section 2.2) at a constant flow rate of 0.66 mL/min (see subsection 2.4.3).

The optical density (OD) increased quickly during the first few hours. Throughout the experiment, the values were higher in the two reactors of the continuous set-up (bioreactor and exposure chamber) (Figure 4.a) than in the batch reactor (0.24 and 0.33 vs. 0.15 in batch). After 250 hours of culture, the OD values decreased progressively in the exposure chamber and reached values similar to those observed in the bioreactor (0.28 on average), indicating that no more additional planktonic growth occurred in the exposure chamber. The presence of a microbial biofilm on the cement paste surface was confirmed by SEM-SE observation. Several morphologies of microorganisms were observed: cocci, single bacilli and bacilli in chains (Figure 5). The ability of microorganisms to colonize cement paste has been reported previously for *H. desiderata* (Rafrafi et al., 2015), in alkaline conditions and also in a biogas digester (Voegel et al., 2015).

The overall acetate concentration during the test period was approximately 8.9 mM in the feed solution. However, the acetate concentration was higher in the bioreactor and in the exposure chamber than in the feed solution during the first 370 hours (periods 1 and 2 - Figure 3.c), starting at a concentration of 11.4 mM and progressively decreasing. Acetate was produced in the bioreactor most likely by hydrolysis of the yeast extract. During period 3 ([390; 600] hours), the concentration in the bioreactor was similar to the concentration in the feeding system, i.e. 9.0 mM on average. This result does not mean that there was no acetate consumption for TON reduction but it shows that the acetate produced by hydrolysis of the yeast extract was used to reduce nitrate. The quantity of acetate produced from hydrolysis of the yeast extract in the bioreactor and the quantity consumed by oxidation cannot be accurately determined.

The instantaneous nitrate, nitrite and TON reduction rate was estimated (Rafrafi et al., 2017) and the nitrogen mass balance was calculated over the system studied (bioreactor, chamber or overall system i.e. including bioreactor and chamber) for three time periods differentiated according to the nitrate and nitrite concentrations (Figure 3.b, Figure A.2 in supplementary data).

Nitrate was entirely reduced to nitrite in the early hours of the experiment in the bioreactor but nitrite accumulated strongly, reaching a maximum concentration of 7.0 mM in the bioreactor after 30 hours of culture and in the exposure chamber after 52 hours (Figure 3.b – Figure A.2). The nitrite reduction started later and nitrite was almost entirely reduced (likely to nitrogen gas) after 100 h and 270 h in the exposure chamber and in the bioreactor, respectively (Figure 3.b – Figure A.2). After approximately 300 hours, the TON, i.e. nitrate and nitrite, had been entirely consumed in the bioreactor. The absence of electron acceptors in the exposure chamber at the end of period 2 can partly explain the decline in the growth of planktonic bacteria observed in the exposure chamber. The acetate concentration decrease in the exposure chamber (roughly 20% of the acetate, i.e. 1.9 mM of acetate on average) revealed low bacterial activity without TON as electron acceptor. It can be hypothesised that the predominant reaction pathway, which took place in the exposure chamber after denitrification, is a reaction similar to acetoclastic methanogenesis (acetate biodegradation into methane) (Sorokin et al., 2015). As the methane production was not tracked, this hypothesis cannot be verified. Nonetheless, alternative microbial pathways probably occurred with eventually a shift within the microbial population. Although this was not investigated in

this study, it can be assumed that specific phenomena could occur inside the biofilm, such as syntrophic interactions between bacteria.

Although the pH was set at 11 in the feed solution, the pH in the bioreactor and in the exposure chamber systematically reached values around 9.3 $\pm$ 0.1 (Figure 4.d). Theoretically, at normal temperature and pressure, the predominant chemical form of inorganic carbon (due to bacterial activity) in water is HCO_3^- for a pH around 8.0 (Andersen, 2002). According to Reaction 3, for a pH around 8.0, the complete denitrification process, without nitrite accumulation, consumes protons (H^+), and thus promotes an increase of the pH. For pH values close to the pKa for carbon dioxide, i.e. 10.3 with equal concentrations of HCO_3^- and CO_3^{2-}, the complete reduction of nitrate into nitrogen gas liberates protons (Reaction 4), and thus promotes a pH decrease.

$$NO_3^- + 0.625\ CH_3COO^- + 0.375\ H^+ \rightarrow 0.5\ N_2 + 1.25\ HCO_3^- + 0.5\ H_2O$$ Reaction (3)

$$NO_3^- + 0.625\ CH_3COO^- \rightarrow 0.5\ N_2 + 0.625\ HCO_3^- + 0.625\ CO_3^{2-} + 0.5\ H_2O + 0.25\ H^+$$ Reaction (4)

The carbonate equilibrium was thus strongly involved in the pH regulation. Other works dealing with granular sludge have highlighted the opposite, with a pH increase from 7.0 in the reactor influent to 9.3 in the effluent (Li et al., 2015, 2014). This behaviour is called “self-alkalinisation” because of the consumption of hydroxide ions by the denitrification process. For pH values above 10.3, the denitrification process produces hydroxide ions. In contrast, granular sludge “self-acidification” or “self-de-alkalinisation” can be evoked.

The experimental set-up and the feed solution composition used in this work were similar to those in the experimental approach implemented by Rafrafi et al. (Rafrafi et al., 2017, 2015) with *Halomonas desiderata*. The nitrate reduction rates obtained experimentally, and shown in Figure 4, are reproduced in Table 3 in order to compare the performance of the consortium isolated from Buxton sediment with that of *H. desiderata*. When data from the bioreactor and exposure chamber were combined, the global nitrate reduction rate was comparable for *Halomonas desiderata* and for microbial consortium in this study, i.e. 0.076 mM NO_3^-/h and 0.083 mM NO_3^-/h, respectively. Nonetheless, the nitrate reduction rate was twice as high in the bioreactor with the consortium (0.124 mM NO_3^-/h) as with *H. desiderata* (0.066 NO_3^-/h). In the work of Rafrafi et al. (Rafrafi et al., 2017, 2015), the partial nitrate reduction by *H. desiderata* in the bioreactor was coupled with strong nitrite accumulation and the TON were then reduced in the exposure chamber. In the case of the experiment with the consortium, TON was rapidly reduced in the bioreactor and the biological activity in the exposure chamber was restricted by the low quantity of residual nitrite (below 1 mM). The sedimentary consortium seems to be better suited to TON reduction and seems to be less affected by the nitrite accumulation (no growth inhibition correlated with nitrite accumulation). The presence of yeast extract certainly promoted the biological activity of the consortium by providing additional compounds, such as peptides/polypeptides, free amino acids and vitamins (Mosser et al., 2012). Moreover, the multi-species interaction that can occur in a consortium is also likely to promote the TON reduction (Brenner et al., 2008; Nozhevnikova et al., 2015; Yang et al., 2011).

3.3.2. Nitrate concentration increase

Before evaluating the impact of an increase in nitrate concentration, another experiment was performed in order to evaluate the impact of an increased number of cement paste slices in the exposure chamber. A new bioreactor was inoculated with the sedimentary consortium (see section 2.3.2). After a 3-day batch period, the bioreactor was continuously supplied with the standard medium supplemented with 0.1% of yeast extract. The surface area of cement paste specimen exposed to the medium was progressively raised from 100 cm² to 339 cm² over 27 days of experiment (see additional experiment in supplementary data and Table A.1). Nitrate and nitrite were completely consumed in the bioreactor in 24 and 48 hours, respectively (see Figure A.3 in supplementary data). Therefore, it was not possible to evaluate the interaction between the cement paste specimen and the microorganisms, nor to investigate the performance of the biofilm for TON reduction (growth, resistance to environmental stress, etc.). Thus, the experiment is not described in this paper but is available as supplementary data. The nitrate reduction rate displayed in Table A.2 confirms the results previously described in section 3.3.1. Following this first experiment, the nitrate concentration was progressively raised from 6.0 mM, to 12 mM, and finally to 48 mM in the feed solution to avoid nitrate limitation inside the exposure chamber (Table A.1). The nitrogen mass balance was calculated over the system studied (bioreactor, chamber or overall system, i.e. including bioreactor and chamber) for three time periods, differentiated according to the nitrate concentration of the feed solution.

The OD values were 0.33 $\pm$ 0.061 in the bioreactor, and 0.60 $\pm$ 0.10, in the exposure chamber (fairly scattered values) over the three periods of the experiment (Figure 6.a). Microbial growth was not impacted by the increase in nitrate concentration. In the bioreactor, the pH was similar to previously reported, stable pH values of 9.7 $\pm$ 0.2. In the exposure chamber, the pH increased progressively from 9.2 during period 1, to reach 10.1 at the end of period 3 (Figure A.3.a). The pH was influenced by several chemical equilibria. Those involved in the chamber appeared complex and were likely to depend on:

(i) The carbonate equilibrium linked to the carbon dioxide produced by the biological activity, depending on whether the denitrification process was complete or not.

(ii) The leaching of cement paste specimen, the amount of which was 6 times higher than in the experiment supplied with standard medium, inducing significant release of hydroxide ion and of some cations such as calcium and alkalis (Bertron et al., 2014). Calcium could precipitate with hydrogen carbonate to form calcium carbonate, modifying the carbonate equilibrium.

The first nitrate concentration rise from 5.9 to 12 mM (period 2 – [693 ; 1290]) did not alter the TON reduction rate (Figure 6.b and Figure A.3.b). The sedimentary microcosm completely reduced 12 mM of nitrate in the bioreactor without nitrite accumulation. The TON reduction rate was 0.23 mM/h (Table 4). With twice as much nitrate in the feed solution, the nitrate and the TON reduction rate doubled. Nitrate reduction into nitrogen gas was complete, even though, based on the nitrogen mass balance, some nitrite loss, lower than 0.03 mM NO_2^-, was detected in the outlet of the chamber during period 2 (Figure 6.d). The second nitrate concentration rise, from 12 to 48 mM (period 3) induced a significant increase of the nitrate reduction rate in the bioreactor and in the exposure chamber (Table 4). However, the denitrification process slowed drastically after the first step, leading to a strong nitrite

accumulation (Figure 6.b and Figure A.3.b). The denitrification process was certainly limited by the complete oxidation of acetate in the bioreactor after approximately 1400 hours (58 days) of culture (≈170 h after the last nitrate concentration increase) (Figure 6.c). Although the electron donor source (acetate) was limited in the exposure chamber during period 3, on average 4.2 mM of nitrate was reduced to nitrite. Other sources of electron donor could include:

(i) Yeast extract hydrolysis products: the yeast extract components may not have been fully hydrolysed and/or oxidised in the bioreactor;

(ii) From the biofilm; via Extracellular Polymeric Substances (EPS), the biofilm could sequester dissolved and particulate nutrients, which can then be utilised as a nutrient and energy source during starvation periods (Flemming and Wingender, 2010);

(iii) From endogenous respiration: the biomass undergoes cell decay leading to residual dead cells. These products and the hydrolysis of some biodegradable components of EPS are utilised by active biomass as recycled electron-donor substrates and/or carbon sources (Laspidou and Rittmann, 2002).

In this experiment, performed with a pH between 9 and 10 inside the reactors, the nitrate concentration increase (from 6 to 46 mM) did not inhibit the sedimentary consortium denitrifying activity or its growth. The reduction of higher nitrate concentration (from 121 to 586 mM N-NO_3^-) at pH values of 9 and 10.5 has been reported in the literature and the nitrate reduction rates were evaluated at between 43 and 120 mM N-NO^{3-}/h (Dhamole et al., 2008; Glass and Silverstein, 1999). An increase of nitrate concentration (at least for nitrate concentrations close to 500 mM) does not necessary inhibit the growth and the microbial activity if the community has been previously acclimated. According to the literature, when nitrate reduction begins, a nitrite accumulation could appear, which is then reduced in a few hours (Dhamole et al., 2008, 2007; Glass and Silverstein, 1999; Nair et al., 2008). For example, Dhamol et al. (Dhamole et al., 2008) reported a nitrite peak of 33 mM for an initial nitrate concentration around 61 mM. Nitrite was then reduced in less than 1 hour.

3.4. Denitrification under cementitious environment

The experimental system consisted of a feed solution continuously supplying a bioreactor with a cement leachate medium supplemented by nitrate, acetate, yeast extract and with or without cement paste specimen. Although the pH in the feed solution was as high as 11, the pH remained broadly stable at around of 9.3 in the reactor. The pH has been regulated mostly by biological mechanisms and carbonate chemical equilibria. Nitrite accumulation was observed in closed systems (batch reactor) and periodically in dynamic mode (continuous supply). Nitrite accumulation partly depends on the adaptation time required by the bacteria. In dynamic mode, the nitrate reduction was complete; the system reached a steady state in less than 24 hours of bioreactor operation. The microbial inoculum colonised the cement paste sample and eventually formed a biofilm on its surface. This biofilm, certainly composed by several microorganisms, probably helped sustaining microbial activity even when one of the reagents (carbon source, electron donor or acceptor) became limiting. The total oxidised nitrogen reduction rate of the consortium was twice as fast (e.g. 0.124 mM/h in the bioreactor) as that obtained with a single strain (*Halomonas desiderata*: 0.066 mM/h) under

similar experimental conditions (Rafrafi et al., 2017). Nonetheless, the kinetics obtained with this consortium is open to discussion because of the use of a yeast extract, which promotes bacterial growth. Further ongoing works are exploring the possibility of microbial nitrate reduction without yeast extract in the culture medium and also the biodegradability of other carbon sources and/or electron donors. These results highlighted the interest to consider a consortium with several microorganisms in comparison with a single species to investigate the possible biological reduction of oxyanion (as nitrate) and the oxidation of the organic matter (acetate) in cementitious environment. It also seemed important to consider possible multi-species colonisation and formation of a biofilm on the cement paste surface. The biological reaction as denitrification could be promoted by synergetic microbial interactions. Quantifying such activities at high pH under conditions relevant to the disposal of cementitious radioactive waste is an important first step in understanding the impact of microbial processes on the biogeochemistry of priority radionuclides, especially those that are redox active and prone to oxidation (and changes in solubility) under denitrifying conditions (Newsome et al., 2014; Rafrafi et al., 2015; Rizoulis et al., 2012).

4. Conclusion

This study showed the ability of a microbial consortium, collected from sediments contaminated by high pH residues from a lime production plant, to reduce nitrate in a cementitious environment with and without hardened cement paste. The maximal total oxidised nitrogen reduction rate by the consortium was 0.124 mM/h.

Acknowledgments

This research project was financially supported by the French national radioactive waste management agency (Andra) and by the University of Toulouse. The authors are grateful to S. Becker for improving the English language. Athanasios Rizoulis and Jonathan R. Lloyd acknowledge support from the NERC BIGRAD consortium (NERC grant NE/H007768/1) and the Euratom research and training programme 2014-2018 under Grant Agreement no. 861880.

Conflicts of Interest

The authors declare no conflict of interest.

References

Albrecht, A., Bertron, A., Libert, M., 2013. Microbial Catalysis of Redox Reactions in concrete cells of nuclear waste repositories: a review and introduction, in: Springer (Ed.), Cement-Based Materiels for Nuclear Waste Storage. pp. 147–159.

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. Manage. 132, 32–41. https://doi.org/10.1016/j.jenvman.2013.10.013

Andersen, C.B., 2002. UNDERSTANDING CARBONATE EQUILIBRIA BY MEASURING ALKALINITY IN EXPERIMENTAL AND NATURAL SYSTEMS. J. Geosci. Educ. 50, 389–403. https://doi.org/10.5408/1089-9995-50.4.389

595 Andra, 2005. Dossier 2005 : Argile, Tome Architecture et Gestion du stockage géologique
596 (Rapport Andra No. C.RP.ADP.04.0001B).
597 Bassil, N.M., Bryan, N., Lloyd, J.R., 2015. Microbial degradation of isosaccharinic acid at high
598 pH. Isme J. 9, 310–320. https://doi.org/10.1038/ismej.2014.125
599 Berks, B.C., Richardson, D.J., Robinson, C., Reilly, A., Aplin, R.T., Ferguson, S.J., 1994.
600 Purification and characterization of the periplasmic nitrate reductase from
601 Thiosphaera pantotropha. Eur. J. Biochem. 220, 117–124.
602 https://doi.org/10.1111/j.1432-1033.1994.tb18605.x
603 Bertron, A., Jacquemet, N., Erable, B., Sablayrolles, C., Escadeillas, G., Albrecht, A., 2014.
604 Reactivity of nitrate and organic acids at the concrete-bitumen interface of a nuclear
605 waste repository cell. Nucl. Eng. Des. 268, 51–57.
606 https://doi.org/10.1016/j.nucengdes.2013.11.085
607 Brenner, K., You, L., Arnold, F.H., 2008. Engineering microbial consortia: a new frontier in
608 synthetic biology. Trends Biotechnol. 26, 483–489.
609 https://doi.org/10.1016/j.tibtech.2008.05.004
610 Cao, X., Qian, D., Meng, X., 2013. Effects of pH on nitrite accumulation during wastewater
611 denitrification. Environ. Technol. 34, 45–51.
612 https://doi.org/10.1080/09593330.2012.679700
613 Dhamole, P.B., Nair, R.R., D'Souza, S.F., Lele, S.S., 2008. Denitrification of Highly Alkaline
614 Nitrate Waste Using Adapted Sludge. Appl. Biochem. Biotechnol. 151, 433–440.
615 https://doi.org/10.1007/s12010-008-8211-6
616 Dhamole, P.B., Nair, R.R., D'Souza, S.F., Lele, S.S., 2007. Denitrification of high strength nitrate
617 waste. Bioresour. Technol. 98, 247–252.
618 https://doi.org/10.1016/j.biortech.2006.01.019
619 Flemming, H.C., Wingender, J., 2010. The biofilm matrix. Nat. Rev. Microbiol. 8, 623–633.
620 https://doi.org/10.1038/nrmicro2415
621 Gaucher, E., Robelin, C., Matray, J.., Négrel, G., Gros, Y., Heitz, J.., Vinsot, A., Rebours, H.,
622 Cassagnabère, A., Bouchet, A., 2004. ANDRA underground research laboratory:
623 interpretation of the mineralogical and geochemical data acquired in the Callovian–
624 Oxfordian formation by investigative drilling. Phys. Chem. Earth Parts ABC 29, 55–77.
625 https://doi.org/10.1016/j.pce.2003.11.006
626 Glass, C., Silverstein, J., 1999. Denitrification of high-nitrate, high-salinity wastewater. Water
627 Res. 33, 223–229. https://doi.org/10.1016/s0043-1354(98)00177-8
628 Glass, C., Silverstein, J., 1998. Denitrification kinetics of high nitrate concentration water: pH
629 effect on inhibition and nitrite accumulation. Water Res. 32, 831–839.
630 https://doi.org/10.1016/s0043-1354(97)00260-1
631 Jones, C.M., Stres, B., Rosenquist, M., Hallin, S., 2008. Phylogenetic analysis of nitrite, nitric
632 oxide, and nitrous oxide respiratory enzymes reveal a complex evolutionary history for
633 denitrification. Mol. Biol. Evol. 25, 1955–1966.
634 https://doi.org/10.1093/molbev/msn146
635 Laspidou, C.S., Rittmann, B.E., 2002. A unified theory for extracellular polymeric substances,
636 soluble microbial products, and active and inert biomass. Water Res. 36, 2711–2720.
637 https://doi.org/10.1016/S0043-1354(01)00413-4
638 Li, W., Li, C., Lin, X., Liu, Y., Abbas, G., Zheng, P., 2015. Effects of operation mode on self-
639 alkalization of high-load denitrifying reactor. Bioresour. Technol. 187, 282–287.
640 https://doi.org/10.1016/j.biortech.2015.03.063

641 Li, W., Zheng, P., Guo, J., Ji, J., Zhang, M., Zhang, Z., Zhan, E., Abbas, G., 2014. Characteristics
642 of self-alkalization in high-rate denitrifying automatic circulation (DAC) reactor fed
643 with methanol and sodium acetate. Bioresour. Technol. 154, 44–50.
644 https://doi.org/10.1016/j.biortech.2013.11.097
645 Mateju, V., Cizinska, S., Krejci, J., Janoch, T., 1992. Biological water denitrification - A review.
646 Enzyme Microb. Technol. 14, 170–183. https://doi.org/10.1016/0141-0229(92)90062-
647 s
648 Morgan, R., Kohn, S., Hwang, S.-H., Hassett, D.J., Sauer, K., 2006. Bd1A, a chemotaxis regulator
649 essential for biofilm dispersion in Pseudomonas aeruginosa. J. Bacteriol. 188, 7335–
650 7343. https://doi.org/10.1128/JB.00599-06
651 Mosser, M., Kapel, R., Aymes, A., Bonanno, L.M., Olmos, E., Chevalot, I., Marc, I., Marc, A.,
652 2012. Chromatographic fractionation of yeast extract: A strategy to identify
653 physicochemical properties of compounds promoting CHO cell culture. Process
654 Biochem. 47, 1178–1185. https://doi.org/10.1016/j.procbio.2012.04.015
655 Nair, R.R., Dhamole, P.B., Lele, S.S., D'Souza, S.F., 2008. Biotreatment of High Strength Nitrate
656 Waste Using Immobilized Preadapted Sludge. Appl. Biochem. Biotechnol. 151, 193–
657 200. https://doi.org/10.1007/s12010-008-8170-y
658 Newsome, L., Morris, K., Lloyd, J.R., 2014. The biogeochemistry and bioremediation of
659 uranium and other priority radionuclides. Chem. Geol. 363, 164–184.
660 https://doi.org/10.1016/j.chemgeo.2013.10.034
661 Nikitenko, S.I., Venault, L., Pflieger, R., Chave, T., Bisel, I., Moisy, P., 2010. Potential
662 applications of sonochemistry in spent nuclear fuel reprocessing: A short review.
663 Ultrason. Sonochem. 17, 1033–1040.
664 Nozhevnikova, A.N., Botchkova, E.A., Plakunov, V.K., 2015. Multi-species biofilms in ecology,
665 medicine, and biotechnology. Microbiology 84, 731–750.
666 https://doi.org/10.1134/S0026261715060107
667 Olmeda, J., Henocq, P., Giffaut, E., Grivé, M., 2017. Modelling of chemical degradation of
668 blended cement-based materials by leaching cycles with Callovo-Oxfordian porewater.
669 Phys. Chem. Earth Parts ABC, Mechanisms and Modelling of Waste-Cement and
670 Cement-Host Rock Interactions 99, 110–120.
671 https://doi.org/10.1016/j.pce.2017.05.008
672 Parmentier, M., Ollivier, P., Joulian, C., Albrecht, A., Hadi, J., Greneche, J.M., Pauwels, H., 2014.
673 Enhanced heterotrophic denitrification in clay media: The role of mineral electron
674 donors. Chem. Geol. 390, 87–99. https://doi.org/10.1016/j.chemgeo.2014.10.014
675 Rafrafi, Y., Durban, N., Bertron, A., Albrecht, A., Robinet, J.-C., Erable, B., 2017. Use of a
676 continuous-flow bioreactor to evaluate nitrate reduction rate of Halomonas
677 desiderata in cementitious environment relevant to nuclear waste deep repository.
678 Biochem. Eng. J. 125, 161–170. https://doi.org/10.1016/j.bej.2017.05.016
679 Rafrafi, Y., Ranaivomanana, H., Bertron, A., Albrecht, A., Erable, B., 2015. Surface and bacterial
680 reduction of nitrate at alkaline pH: Conditions comparable to a nuclear waste
681 repository. Int. Biodeterior. Biodegrad. 101, 12–22.
682 https://doi.org/10.1016/j.ibiod.2015.03.013
683 Richardson, D., Felgate, H., Watmough, N., Thomson, A., Baggs, E., 2009. Mitigating release of
684 the potent greenhouse gas N2O from the nitrogen cycle – could enzymic regulation
685 hold the key? Trends Biotechnol. 27, 388–397.
686 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

Sercombe, J., Gwinner, B., Tiffreau, C., Simondi-Teisseire, B., Adenot, F., 2006. Modelling of bituminized radioactive waste leaching. Part I: Constitutive equations. J. Nucl. Mater. 349, 96–106. https://doi.org/10.1016/j.jnucmat.2005.10.014

Sorokin, D.Y., Abbas, B., Geleijnse, M., Pimenov, N.V., Sukhacheva, M.V., van Loosdrecht, M.C.M., 2015. Methanogenesis at extremely haloalkaline conditions in the soda lakes of Kulunda Steppe (Altai, Russia). Fems Microbiol. Ecol. 91, fiv016. https://doi.org/10.1093/femsec/fiv016

Thomsen, J., Geest, T., Cox, R., 1994. Mass-Spectrometric Studies of the Effect of Ph on the Accumulation of Intermediates in Denitrification by Paracoccus-Denitrificans. Appl. Environ. Microbiol. 60, 536–541.

Truche, L., Berger, G., Albrecht, A., Domergue, L., 2013. Abiotic nitrate reduction induced by carbon steel and hydrogen: Implications for environmental processes in waste repositories. Appl. Geochem. 28, 155–163. https://doi.org/10.1016/j.apgeochem.2012.10.010

Vinsot, A., Mettler, S., Wechner, S., 2008. In situ characterization of the Callovo-Oxfordian pore water composition. Phys. Chem. Earth Parts ABC 33, S75–S86. https://doi.org/10.1016/j.pce.2008.10.048

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., Bertron, A., Erable, B., 2015. Biodeterioration of cementitious materials in biogas digester. Matér. Tech. 103, 202. https://doi.org/10.1051/mattech/2015023

Walczak, I., Libert, M.F., Camaro, S., Blanchard, J.M., 2001. Quantitative and qualitative analysis of hydrosoluble organic matter in bitumen leachates. Agronomie 21, 247–257.

Yang, L., Liu, Y., Wu, H., Hóiby, N., Molin, S., Song, Z., 2011. Current understanding of multi-species biofilms. Int. J. Oral Sci. 3, 74–81. https://doi.org/10.4248/IJOS11027

Table 1: Average chemical composition of the cement leachate

Concentration (mM)						pH
Ca	K	Na	Si	Al	Fe	
2.61	1.03	0.15	0.20	0.05	<0.10	≈11

Table 2: Nitrate reduction rates determined for the four successive batch periods

	Nitrate reduction rates (mM/h)	
	4 Cement specimens	2 Cement specimens
Batch 1	0.004	0.035
Batch 2	0.041	0.043
Batch 3	0.038	0.036
Batch 4	0.045	0.046

Table 3: Average nitrate reduction rates and average OD values for bacterial growth assessment in the experiments under dynamic supply with the standard medium

Cement paste Surface area *(cm²)*	$[NO_3^-]_{inlet}$ *(mM)*	Nitrate consumption rate *(mM/h)*			TON consumption rate *(mM/h)*		
		Bioreactor	Exposure chamber	System	Bioreactor	Exposure chamber	System
Sedimentary consortium culture in continuous bioreactor (this study)							
60	6.2	0.124 ± *0.003*	0.000 ± *0.001*	0.083 ± *0.002*	0.085 ± *0.048*	0.025 ± *0.049*	0.091 ± *0.038*
Experiment performed by Rafrafi et al. (Rafrafi et al., 2017) **with an initial pH of 10**							
60	5.9	0.066	0.099	0.076	0.024	0.137	0.062
Experiment performed by Rafrafi et al. (Rafrafi et al., 2015) **with an initial pH of 11**							
60	5.9	0.080	0.018	-	-	-	-

Table 4: Average nitrate and TON reduction rates for the experiments under dynamic conditions with nitrate concentration increase in the feed solution (acetate concentration kept constant at 8.5 mM)

Cement paste surface *(cm²)*	$[NO_3^-]_{input}$ *(mM)*	Nitrate reduction rate *(mM/h)*			TON reduction rate *(mM/h)*		
		Bioreactor	Exposure chamber	System	Bioreactor	Exposure chamber	System
339	6	0.12 ± *0.003*	$0.89\ 10^{-3}$ ± *0.2* 10^{-3}	0.079 ± *0.002*	0.12 ± *0.003*	$0.16\ 10^{-3}$ ± *0.1* 10^{-3}	0.079 ± *0.002*
	12	0.23 ± *0.018*	$3.0\ 10^{-3}$ ± *0.005*	0.16 ± *0.012*	0.23 ± *0.018*	$4.4\ 10^{-3}$ ± *0.005*	0.15 ± *0.003*
	48	0.74 ± *0.089*	0.17 ± *0.12*	0.52 ± *0.14*	0.44 ± *0.19*	0.22 ± *0.15*	0.42 ± *0.18*

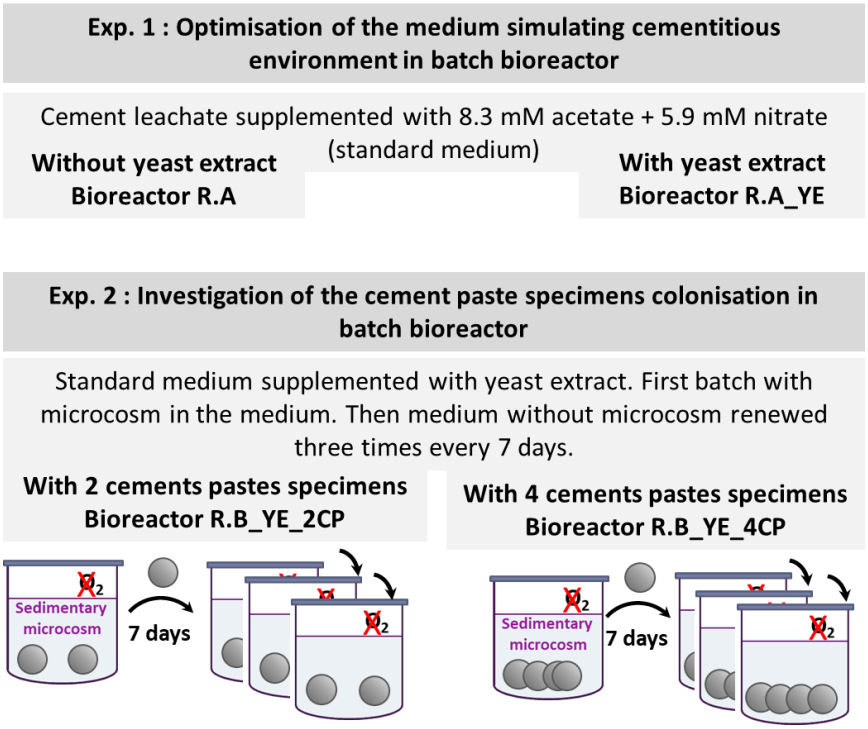

Figure 1: Scheme of the experiments performed in batch bioreactor

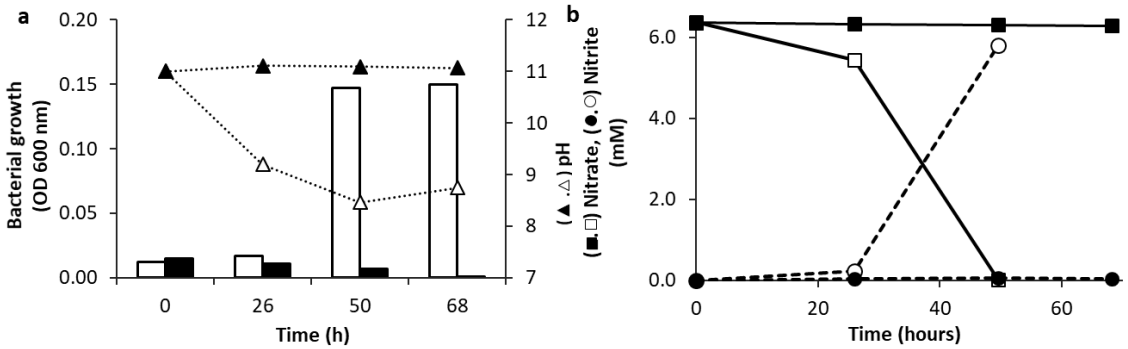

Figure 2: Bacterial growth and changes in pH **(a)**, nitrate and nitrite concentrations **(b)** with (reactor R.A_YE: white) and without (reactor R.A: black) yeast extract during batch tests in closed bioreactors.

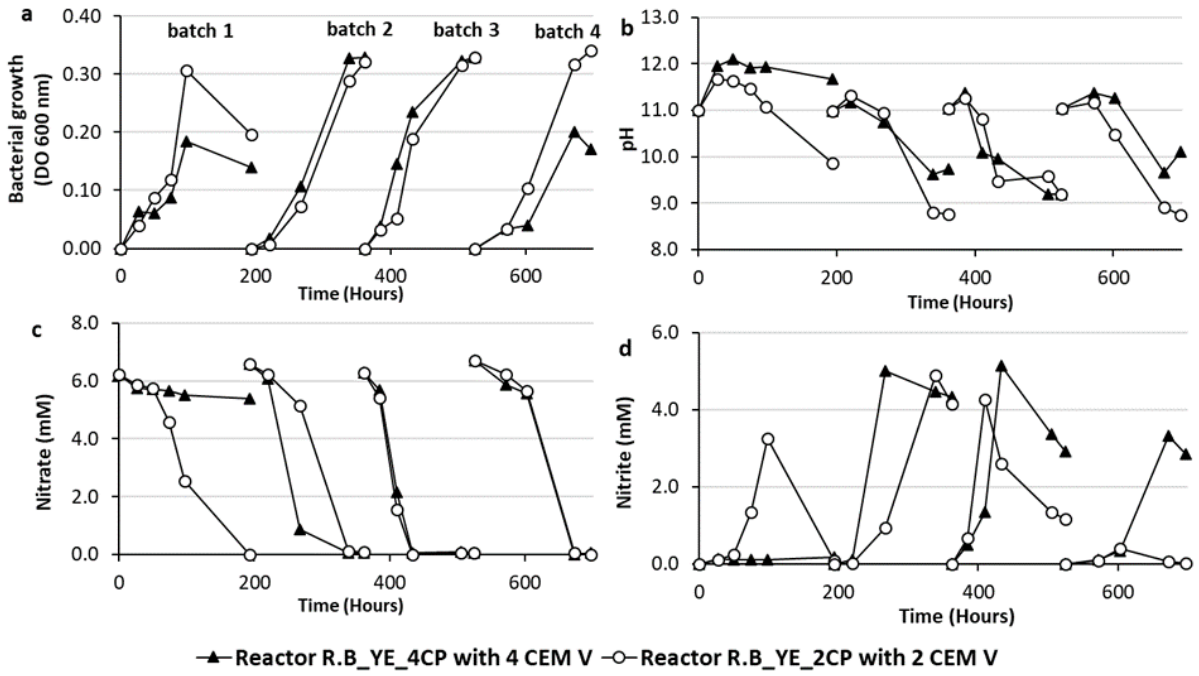

Figure 3: Microbial growth (OD 600nm) **(a)**, pH **(b)**, nitrate concentration **(c)**, and nitrite concentration **(d)**, in fed-batch bioreactors initially inoculated with a sedimentary microbial consortium and containing 2 or 4 solid specimens of CEM V cement paste

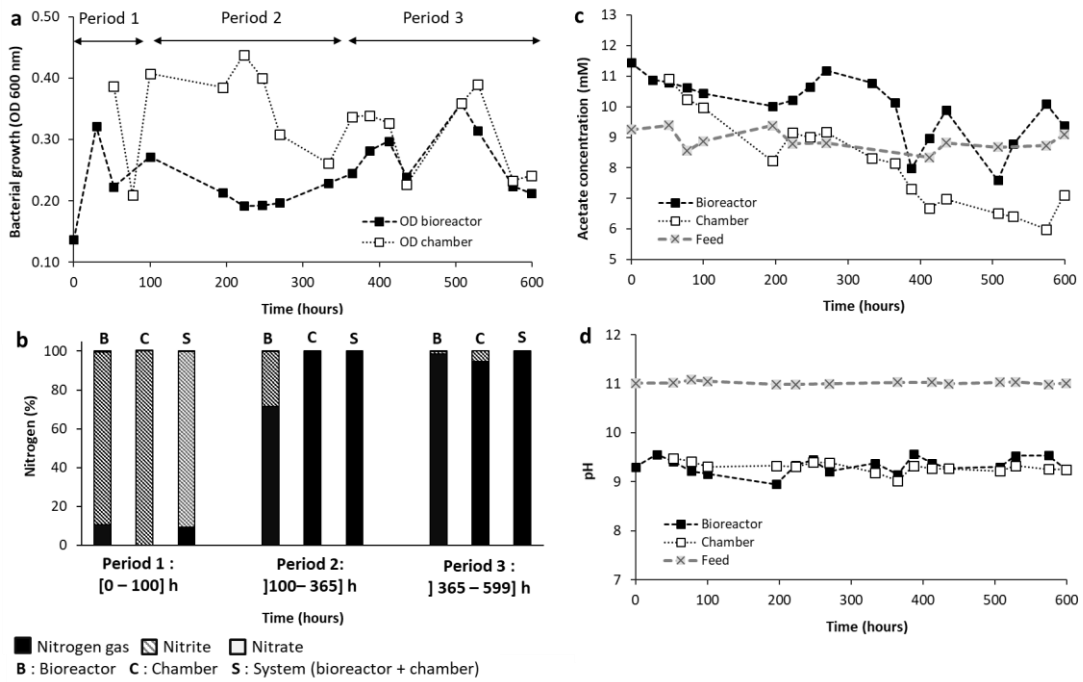

Figure 4: Monitoring of sedimentary microcosm culture under dynamic conditions – **(a)** bacterial growth in the bioreactor and in the exposure chamber, **(b)** nitrogen mass balance in the bioreactor, the chamber and on the global system (i.e. bioreactor + chamber), **(c)** acetate concentrations and **(d)** pH value in the feed solution (▨), the bioreactor (■) and the exposure chamber (□)

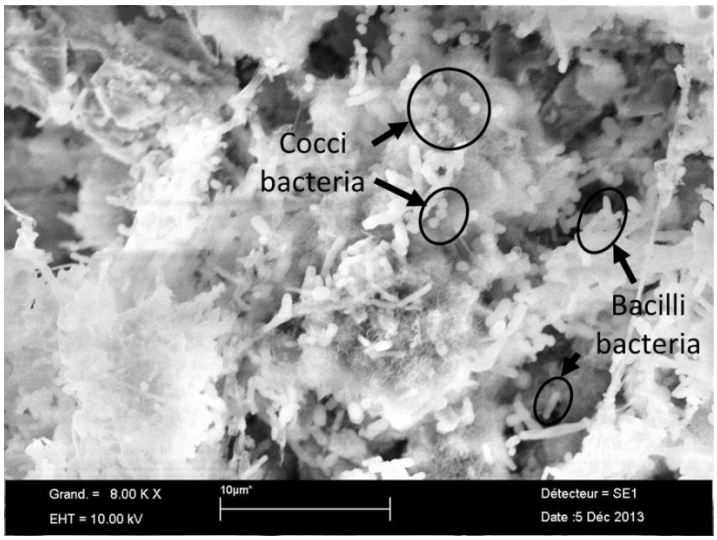

Figure 5: SEM observation of cement paste surface specimen after 700 hours of presence in the culture of the exposure chamber supplied by the bioreactor effluent

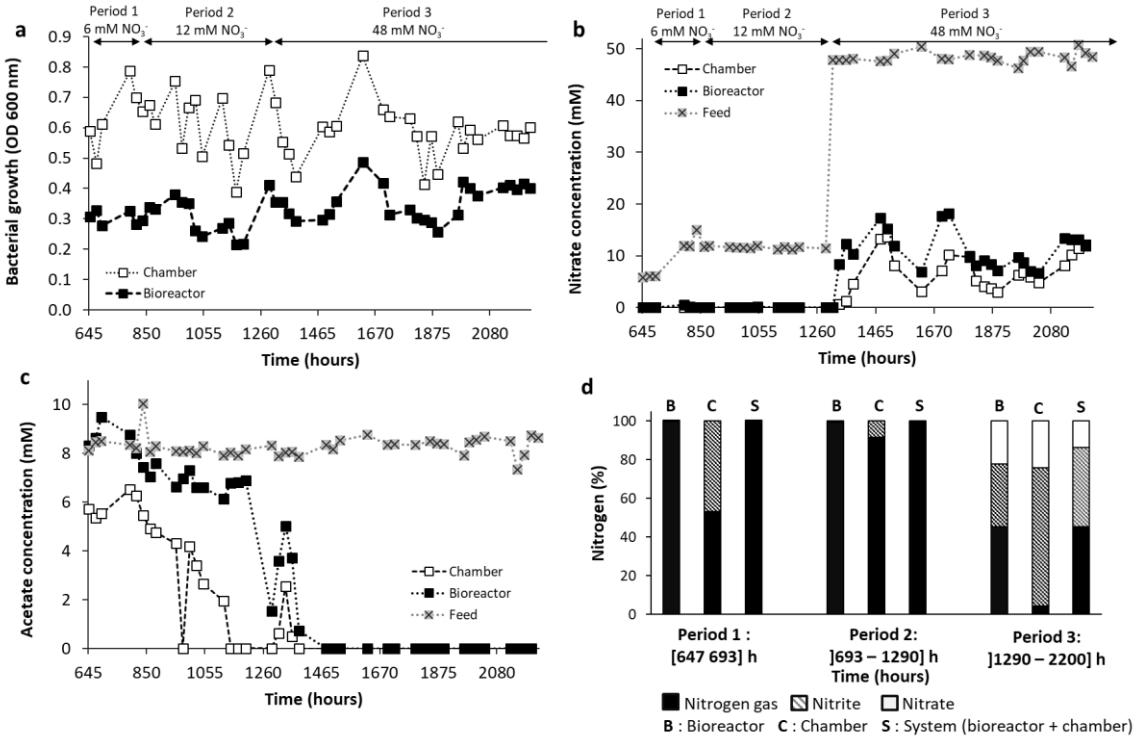

Figure 6: Monitoring of the microcosm culture under continuous supply of standard medium with nitrate concentration increasing from 6.0 to 48 mM – **(a)** bacterial growth, **(b)** nitrate concentration, **(c)** acetate concentration; in the feed solution (▨), the bioreactor (■) and the exposure chamber (□) - **(d)** nitrogen mass balance: in the bioreactor, the chamber and the complete system (i.e. bioreactor + chamber)

Supplementary data

Table A.1: Experimental conditions and medium composition with means and standard deviation for the nitrate and acetate concentrations

Culture medium	Starting time *(hours)*	Duration *(hours)*	$[NO_3^-]$ *(mM)*	$[C_2H_3O_2^-]$ *(mM)*	[acetate]/ [Nitrate] Feed	Cement paste surface area *(cm^2)*
Cement leachate with adjusted pH (11) and 0.1% of yeast extract	648	144	5.99 ± 0.13	8.27 ± 040	1.40 ± 0.03	339 cm^2
	792	522	11.84 ± 0.90		0.70 ± 0.01	
	1314	888	48.30 ± 1.06		0.17 ± 0.01	

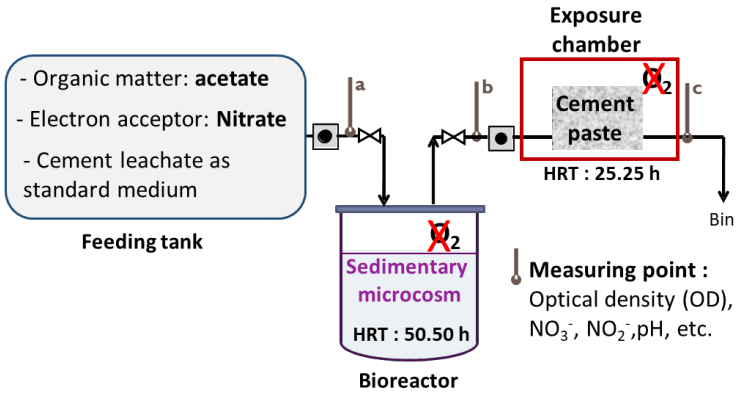

Figure A.1: Scheme of the experimental set-up designed to study the impact of the cement leachate on the Buxton sediment's microbial activity, with or without hardened cement paste specimen, under continuous supply (adapted from Rafrafi et al. [9]).

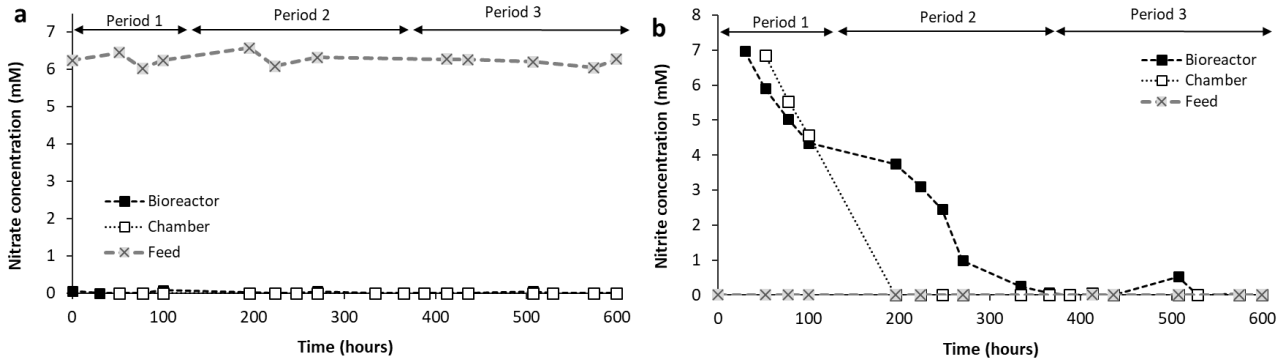

Figure A.2: Microcosm culture under continuous supply and standard condition - **(a)** nitrate and **(b)** nitrite concentrations in the feed solution (⊠), the bioreactor (■) and the exposure chamber (□)

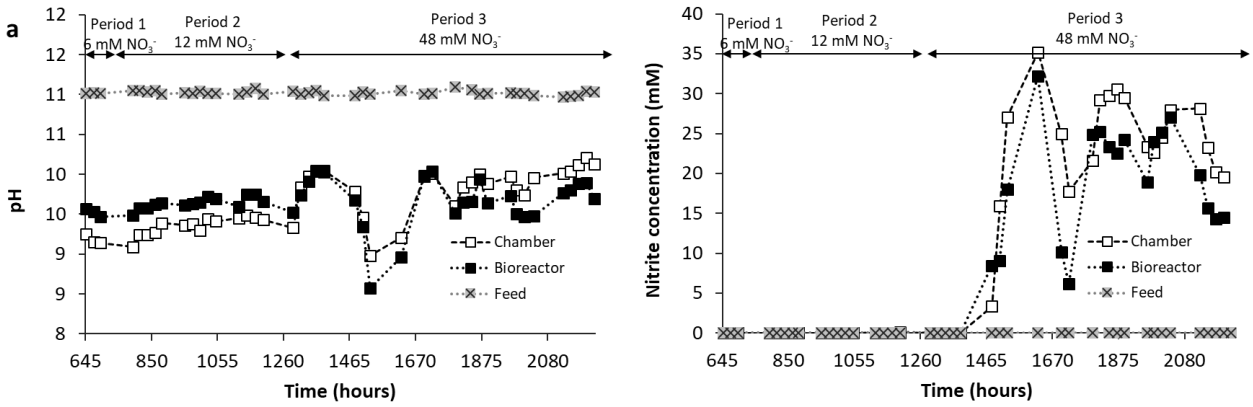

Figure A.3: Microcosm culture under continuous supply with nitrate concentration increasing in the feed solution, nitrate concentration specified in graphics - **(a)** pH - **(b)** nitrite concentration in the feed solution (⊠), the bioreactor (■) and the exposure chamber (□).

Additional experiments

After 3 days in batch operation, the bioreactor was continuously supplied with fresh standard medium according to the same protocol as the one described in section 2.4.3. The bioreactor was inoculated with the enrichment obtained from standard medium incubation (section 2.3.2). Five slices (h= 10 mm), 2 slices (h= 20 mm) and then 10 slides (h=10 mm) of cement paste were added into the exposure chamber after approximately 1 day, 14 days, and 20 days of culture, respectively. The surface available for microbial colonisation increased progressively from 100 cm² with five cement pastes, to 156 cm^2 and to 356 cm² at the end of the experiment (Table A.1).

Table A.4: Summary of experimental conditions and medium composition with means and standard deviation for the nitrate and acetate concentration

	Time *(days)*	**[NO_3^-] *(mM)***	**[$C_2H_3O_2H$] *(mM)***	**[acetate]/N Feed**	**Cement paste surface**	**Culture medium**
CEM V increase under continuous supply	1	5.9 ± 0.1	8.4 ± 0.25	1.4 ± 0.03	100 cm^2	Cement leachate with adjusted pH (11) and 0.1% of yeast extract
	14				156 cm^2	
	20				339 cm^2	

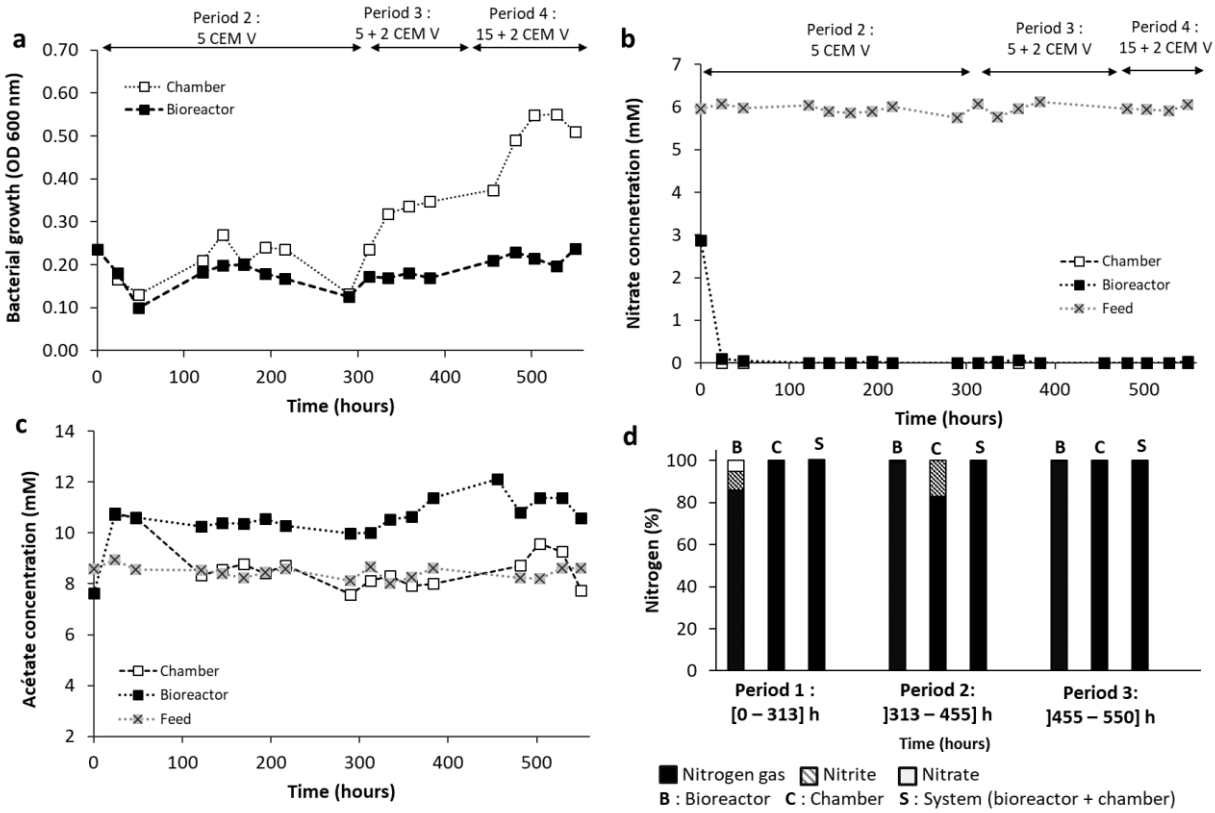

Figure A.5: Continuous culture of sedimentary microcosm at pH 11 with cement paste addition in the exposure chamber - **(a)** bacterial growth in the bioreactor and exposure chamber, **(b)** nitrate concentration, **(c)** acetate concentration in the feed solution (▨), the bioreactor (■) and the exposure chamber (□) - **(d)** nitrogen mass balance on the bioreactor, the chamber and on the system (i.e. bioreactor + chamber)

Table A.6: The average nitrate reduction rate and the average values of OD for bacterial growth assessment under dynamic conditions with increasing number of cement paste specimens in the exposure chamber (acetate concentration kept constant at 8.5 mM)

CEM V paste *surface area* (cm^2)	$[NO_3^-]_{inlet}$ *(mM)*	Nitrate consumption rate *(mM/h)*			Bacterial growth *(DO 600 nm)*	
		Bioreactor	Exposure chamber	System	Bioreactor	Exposure chamber
Experiment with cement paste addition						
100	6.0	0.11 ± *0.018*	0.001 ± *0.001*	0.079 ± *0.001*	0.17 ± *0,04*	0.22 ± *0,05*
156		0.12 ± *0.004*	0.001 ± *0.001*	0.078 ± *0.002*	0.20 ± *0,02*	0.39 ± *0,02*
356		0.12 ± *0.001*	0.001 ± *0.001*	0.079 ± *0.001*	0.22 ± *0,02*	0.52 ± *0,03*