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

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## 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

47 the Callovo-Oxfordian clay rock host formation. The concrete surrounding the waste packages  
48 serves as the engineered barrier and the host rock as the geological barrier (Andra, 2005;  
49 Gaucher et al., 2004; Vinsot et al., 2008). After closure of the repository cell, the progressive  
50 resaturation is expected to induce progressive leaching of cement-based materials (waste  
51 packages, cell lining etc.), leading to release of hydroxide anions and cations, such as  $\text{Ca}^{2+}$ ,  $\text{Na}^+$   
52 and  $\text{K}^+$ . After corrosion and failure of the primary steel waste containers, substances present  
53 with the stabilizing bituminous matrix (oxyanions, dissolved organic matter, radionuclides  
54 etc.) will slowly start to diffuse out (Sercombe et al., 2006; Walczak et al., 2001). The release  
55 of soluble oxyanions such as nitrate, incorporated in the nuclear waste during recycling  
56 procedures (Nikitenko et al., 2010), will promote oxidizing conditions in the vicinity of the  
57 waste if nitrate reduction occurs. This may promote the mobility of some redox sensitive  
58 radionuclides (Se, U, Tc, Pu, Np, etc.) (Albrecht et al., 2013). Mostly biotic processes could  
59 catalyse the reduction of nitrate in the interstitial aqueous phase of the near field. Under  
60 abiotic conditions, fresh steel surfaces may catalyse some nitrate reduction and produce  
61 ammonium ions but the related processes are limited to situations where fresh steel surfaces  
62 are present and when temperatures are beyond those expected in a waste cell (50°C) (Rafrafi  
63 et al., 2015; Truche et al., 2013). Nitrate reduction rates have been measured at around  $10^{-3}$   
64 mM/h under laboratory conditions (pH > 10, anaerobic conditions, cementitious environment,  
65 etc.) (Rafrafi et al., 2015; Truche et al., 2013). In the presence of electron donors, such as  
66 organic matter or dihydrogen, microorganisms are able to catalyse the reduction of nitrate to  
67 nitrogen gas ( $\text{N}_2$ ) via several denitrification intermediate chemical species; nitrite ( $\text{NO}_2^-$ ), nitric  
68 oxide (NO), and nitrous oxide ( $\text{N}_2\text{O}$ ) (Jones et al., 2008; Mateju et al., 1992; Parmentier et al.,  
69 2014). At temperatures below 40 °C, denitrifying bacteria catalyse nitrate reduction at a faster  
70 rate than the steel surface catalysed reduction at alkaline pH (Rafrafi et al., 2015; Truche et  
71 al., 2013). A sedimentary microcosm sampled from a site contaminated by high pH legacy lime  
72 works was able to reduce 15 mM nitrate solution at pH between 9 and 10 in less than a week  
73 (0.089 mM  $\text{NO}_3^-$ -N/h) and at pH between 9 and 11 in less than two weeks (0.045 mM  $\text{NO}_3^-$ -N  
74 /h) (Rizoulis et al., 2012). In order to simulate a more realistic cementitious environment,  
75 Rafrafi et al. (Rafrafi et al., 2017) replaced the optimal culture medium commonly applied for  
76 bacterial growth by a minimal medium composed only of cement leachate supplemented by  
77 acetate and nitrate. *Halomonas desiderata*, an alkaliphilic bacterial strain, could grow and  
78 reduce nitrate and nitrite in an experimental set-up continuously supplied with this medium.  
79 Under these conditions, the nitrate reduction rate catalysed by *H. desiderata* was around 0.08  
80 mM/h at pH 10 (Rafrafi et al., 2017), i.e. similar to the rate obtained by Rizoulis et al. (Rizoulis  
81 et al., 2012) with a microcosm. Introducing hardened cement paste in Rafrafi et al.'s  
82 experimental set-up (Rafrafi et al., 2017, 2015) highlighted the formation of a biofilm several  
83 tens of microns thick on the surface of cementitious material. This biofilm allowed active  
84 biomass to be maintained in a flow through reactor. At pH 12, nitrate reduction (0.24 mM/h)  
85 was detected only in the reactor with colonised solid cement pastes (Rafrafi et al., 2015).  
86 In comparison with a microbial monoculture, microbial consortia can sustain more complex  
87 metabolic reactions, they can survive in environments subject to larger fluctuations (pH,  
88 substrate concentration, temperature, etc.) (Brenner et al., 2008). The associated microbial  
89 diversity is certainly more representative of a community likely to grow in a deep repository  
90 compared to a single species such as *H. desiderata*.  
91 The focus of this study is to evaluate the rate of nitrate and nitrite reduction by a naturally  
92 microcosms occurring sediment collected from a former lime works site (Rizoulis et al., 2012),  
93 under alkaline pH conditions comparable to a cementitious waste cell environment. To

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

102

## 103 **2. Materials and methods**

104

### 105 **2.1. Cementitious materials**

106

107 The cement paste specimens (CEM V/A 42.5; Calcia's Airvault factory) were made with a  
108 water/cement ratio of 0.32. The CEM V/A cement is a standardized cement containing clinker  
109 and blast furnace slag with fly ash addition (Olmeda et al., 2017).

110 The cement paste specimens were cast in cylindrical moulds (50 mm high and 27 mm in  
111 diameter) and were kept in sealed bags (to avoid any hydric exchanges with the exterior, and  
112 thus favour cement hydration reactions and to protect them against carbonation) for 28 days  
113 after demoulding. Then, they were cut into slices ( $h \approx 10 - 20$  mm) and sanded with silicon  
114 carbide polishing disks (P120 -  $\approx 127 \mu\text{m}$  - Presi®) to impose a surface roughness favourable to  
115 bacterial cell attachment.

116

### 117 **2.2. Standard medium**

118 The standard medium used as the feed solution was made of cement leachate supplemented  
119 with 8.3 mM sodium acetate (as the organic carbon source and electron donor) and 5.9 mM  
120 sodium nitrate (as the electron acceptor) at pH 11. The cement leachate was prepared by  
121 immersing cement paste specimens in 1 L of demineralized water (solid/liquid volume ratio:  
122 1.03) for 3 days under continuous stirring. The average chemical composition of the cement  
123 leachate is given in Table 1.

124

### 125 **2.3. Sedimentary microcosm**

#### 126 *2.3.1. Sediment sampling*

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

131

#### 132 *2.3.2. Enrichment culture preparation*

133 Nitrate-reducing microorganisms were enriched in anaerobic cultures (prepared under  $\text{N}_2$   
134 atmosphere, in crimp-sealed 100 mL sterile bottles) that were set up using 1 g of the Buxton  
135 sediment, mixed with 50 mL of standard medium (cement leachate with 8.3 mM sodium  
136 acetate and 5.9 mM sodium nitrate) and 0.1% (w/v) yeast extract, from a 5% (w/v) sterile stock  
137 solution. The anaerobic cultures were incubated at 25°C in the dark, and acetate and nitrate

138 concentrations were monitored regularly. Once the nitrate was reduced to nitrite (within 7  
139 days; data not shown), 5 mL of the culture was transferred to a new bottle, which contained  
140 45 mL of standard medium and 0.1% yeast extract. Once the nitrate in the subculture was also  
141 reduced to nitrite, 5 mL of the subculture was transferred to a new bottle that contained 45  
142 mL of standard medium and 0.1% yeast extract. In a similar way, enrichment cultures without  
143 added yeast extract were prepared and subcultured twice (after nitrate reduction was  
144 observed). The second subcultures from the yeast-amended and the non-yeast amended  
145 anaerobic cultures were used as the enrichment cultures for subsequent batch bioreactor  
146 experiments below.

147

## 148 **2.4. Experimental set-up and conditions**

149

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

161

### 162 *2.4.1. Optimisation of the medium simulating a cementitious environment in the* 163 *batch bioreactor*

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

171

### 172 *2.4.2. Investigation of colonisation of the hardened cement paste specimen in the* 173 *batch bioreactor*

174

175 Successive batch tests were performed in two 500 mL bioreactors containing 2 (reactor  
176 R.B\_YE\_2CP) or 4 cement paste specimens (reactor R.B\_YE\_4CP), which provided a surface  
177 available for microbial colonisation of around 40 cm<sup>2</sup> and 80 cm<sup>2</sup>, respectively. At the  
178 beginning of the experiment, the reactors containing 500 mL of standard medium,  
179 supplemented with yeast extract were inoculated with 0.5 mL of microbial culture from  
180 reactor R.A\_YE (see section 2.4.1). Then, the two reactors were deoxygenated by bubbling N<sub>2</sub>  
181 through the medium for 15 min and the temperature was set at 30°C, without any pH  
182 regulation or stirring being performed. After 7 days of culture, the cement specimens were  
183 removed, rinsed with cement leachate and finally introduced into a new reactor with fresh

184 standard medium. This operation was reiterated twice, raising the total number of batch tests  
185 to 4. The duration of each test was 7 days (Figure 1).  
186

### 187 *2.4.3. Experiment under continuous supply*

188  
189 At the end of the batch test (see section 2.4.1), reactor R.A\_YE was connected to the feed tank  
190 and to the exposure chamber in order to perform this experiment under continuous supply  
191 using the same set-up design as the one described in by Rafrafi et al. (2017, 2015)( (Figure  
192 A.1). Fresh standard medium was continuously added to the bioreactor by means of a  
193 peristaltic pump having a constant flow rate of 0.66 mL/min (Model 7554-85, 7-200 rpm, Easy  
194 Load L/S head for tube 13-18 7518-00 model). The feed tank contained standard medium  
195 supplemented with 0.1% of yeast extract. The bioreactor contained 2 L of microbial culture  
196 enriched with the sedimentary microcosm. Three solid slices of cement paste (thickness = 10  
197 mm) were introduced in the 1 L working volume exposure chamber. The surface available for  
198 microbial colonisation was around 60 cm<sup>2</sup>. The hydraulic retention time (HRT) was set at 50.5 h  
199 in the bioreactor and 25.25 h in the exposure chamber. Both the bioreactor and the exposure  
200 chamber were thermostatically controlled at 30°C, constantly mixed with mechanical stirring  
201 and flushed with continuous N<sub>2</sub> bubbling (anaerobic conditions).  
202

### 203 *2.4.4. Nitrate concentration increase in standard medium (continuous supply)*

204  
205 During the first 27 days (≈ 650 hours), the experimental set-up described was fed with the  
206 standard medium supplemented with 0.1% of yeast extract (section 2.2). The surface available  
207 for microbial colonisation was progressively raised from 100 cm<sup>2</sup> to 339 cm<sup>2</sup> in the exposure  
208 chamber. The approach and results of this experiment are available in the supplementary  
209 data. Next, the concentration of nitrate was progressively increased from 6.0 mM to 11.8 mM  
210 after 792 hours of culture, and then to 48 mM from day 55 (1314 hours of culture) until the  
211 end of the experiment; i.e. 2200 hours. The experimental conditions are reported in Table A.2  
212 in supplementary data and the results are discussed below.  
213

## 214 **2.5. Analytical techniques**

### 215 *2.5.1. Monitoring of the various devices*

216 For each experiment, regular sampling was performed for immediate measurement of pH  
217 (6500pH/ ion meter, Eutech Instruments) and bacterial growth by measuring the optical  
218 density (OD) at 600 nm (JENWAY 7315 spectrophotometer) (Alquier et al., 2014; Rafrafi et al.,  
219 2015). The feed solution was systematically used as a blank. Two millilitres of sample were  
220 collected and filtered (through a 0.2 µm - Minisart PES, Fisher Scientific) for analyses of ionic  
221 species (nitrate, nitrite, acetate, etc.). Sterile syringes were used for sampling. The sampling  
222 point for dynamics tests is specified in Figure A.1.  
223

### 224 *2.5.2. Chemical analysis*

225 The concentrations of Ca<sup>2+</sup>, K<sup>+</sup>, Na<sup>+</sup>, CH<sub>3</sub>COO<sup>-</sup>, NO<sub>3</sub><sup>-</sup> and NO<sub>2</sub><sup>-</sup> were quantified by High  
226 Performance Ion Chromatography (Dionex ICS-2000 and ICS-3000) using analytical methods  
227 detailed by Alquier et al. (Alquier et al., 2014) and Bertron et al. (Bertron et al., 2014).

### 228 2.5.3. SEM analysis

229 Biofilms grown on the surface of the cement paste specimen were observed by SEM-FEG (JEOL  
230 7100F TTLS, 5kW). Before SEM observations with secondary electron (SEM-SE), biofilms on  
231 the cement paste specimens were chemically fixed and then dehydrated according to the  
232 procedure described by Voegel et al. (Voegel et al., 2016, 2015).

233 The aim of the fixation step was to preserve the integrity of the microbial cells and the biofilm  
234 3D architecture during water extraction. The chemical fixation method required several steps:  
235 (i) 20 minutes of immersion in aldehyde fixation solution composed of 2 volumes of  
236 glutaraldehyde (4%), 1 volume of phosphate buffer (pH 7.4, 0.4 M) and 1 volume of distilled  
237 water, (ii) two times 15 minutes of immersion in a cleaning solution made of 1 volume of  
238 phosphate buffer (pH 7.4, 0.4 M), 2 volumes of sucrose solution (0.4 M) and 1 volume of  
239 distilled water.

240 The aim of the chemical dehydration was to replace water by volatile solvents, such as acetone  
241 and hexamethyldisilazane (HMDS). The chemical dehydration was progressively carried out by  
242 successive immersion of the specimens in solutions of; acetone and water (50%-50%, 5 min),  
243 acetone and water (70%-30%, 5 min), acetone (30 min), acetone and HMDS (50%-50%, 10  
244 min) and, finally, HMDS (100%) until complete evaporation. After fixation and dehydration,  
245 the specimens were coated with a thin layer of gold before SEM observations.  
246

## 247 3. Results and discussion

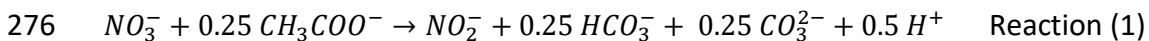
### 248 3.1. Microcosm activity in closed bioreactors

249 The ability of the microcosm to catalyse nitrate reduction at pH 11 in a medium prepared from  
250 cement leachate was first evaluated in closed bioreactors (batch test) with acetate only  
251 (reactor R.A) or with acetate and yeast extract (reactor R.A\_YE) as electron donors. Anaerobic  
252 microbial growth and nitrate reduction were detected in reactor R.A\_YE only (Figure 1.A).  
253 Approximately 90% of nitrate had been reduced to nitrite while 10% had been reduced further  
254 (likely to nitrogen gas) at the end of the 50 hours of the batch test (Figure 2. b). Nitrite  
255 accumulation in a batch test has already been reported in previous works performed at  
256 alkaline pH (Glass and Silverstein, 1998), (i) using a pure culture of *Halomonas desiderata*  
257 (Alquier et al., 2014) or (ii) using the same microbial consortium enriched with sediments  
258 polluted by lime works (Bassil et al., 2015; Rizoulis et al., 2012). The reduction rates of nitrite  
259 and nitrate were greatly impacted by the pH, the nitrite reduction being impacted on more  
260 strongly than the nitrate (Cao et al., 2013; Thomsen et al., 1994). One possible explanation  
261 could be the difference in the locations of bacterial enzymes. The bacterial enzymes involved  
262 in nitrate reduction can be anchored to the cytoplasmic face of the membrane (NAR) or  
263 present in the periplasmic compartment (NAP), whereas nitrite reductase (NIR) are found only  
264 in the periplasmic compartment (Richardson et al., 2009; Richardson\* et al., 2001). Enzymes  
265 outside the cytoplasmic membrane, such as nitrite reductase, are more sensitive to the  
266 environmental conditions (including pH) than the enzymes inside, and are most competitive  
267 for pH close to neutrality (Berks et al., 1994; Richardson et al., 2009).  
268

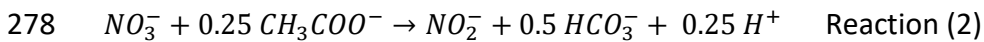
269 The onset of the microbial denitrifying activity was accompanied by a pH decrease from 11.0  
270 to 8.5 for reactor R.A\_YE (Figure 2.a). This is in accordance with both reaction 1 (valid for pH  
271 10.3, i.e., at equivalent concentrations of  $\text{HCO}_3^-$  and  $\text{CO}_3^{2-}$ ) and reaction 2 (valid for pH around  
272 8, i.e. when  $\text{HCO}_3^-$  is the preponderant species), which shows that the reduction of nitrate into

273 nitrite theoretically leads to the production of protons (H<sup>+</sup>) (equivalents of acidity) (Glass and  
274 Silverstein, 1998; Mateju et al., 1992).

275



277



279

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

284

285 This batch experiment indicated that heterotrophic indigenous microbial communities  
286 enriched from the sedimentary microcosm could catalyse nitrate reduction at an initial pH of  
287 11.0 in a medium prepared from cement leachate. However, nitrate reduction could only take  
288 place in the presence of yeast extract as organic substrate. The microbial nitrate reduction  
289 rate was about 0.128 mM N-NO<sub>3</sub><sup>-</sup>/h, which was in the same range as the reduction rate  
290 reported by Rizoulis et al. (Rizoulis et al., 2012) (15 mM of nitrate reduced in 2 weeks, i.e.  
291 0.045 mM N-NO<sub>3</sub><sup>-</sup>/h) using a microcosm inoculated with similar sediments.

292

293

### 294 **3.2. Activity of microbial communities adhering to the surface of hardened cement paste** 295 **in closed bioreactors**

296 To investigate the possible attachment and development of denitrifying microorganisms on  
297 the surface of the cement paste specimen, successive fed-batch tests were performed in two  
298 bioreactors (R.B\_YE\_2CP and R.B\_YE\_4CP) filled with standard medium supplemented with  
299 yeast extract. Two cement paste specimens were introduced into reactor R.B\_YE\_2CP and  
300 four into reactor R.B\_YE\_4CP (see section 2.4.2 for details). At the end of the first batch period,  
301 the cement specimens were carefully rinsed with cement leachate, and were then introduced  
302 into a new bioreactor containing fresh standard medium (uninoculated cement leachate  
303 supplemented by acetate and nitrate). The only source of denitrifying microorganisms in the  
304 new bioreactors was the microbial population that had been able to attach to and proliferate  
305 on the surfaces or in the porosity of the hardened cement pastes.

306

307 According to the results presented in Figure 3.a and Figure 3.c, the increase of OD values  
308 simultaneously with a decrease in the nitrate concentration observed for batches 2, 3 and 4,  
309 clearly demonstrates a development of denitrifying populations in both bioreactors,  
310 R.B\_YE\_2CP and R.B\_YE\_4CP. The sedimentary microorganisms initially present in the  
311 inoculum colonised the cement pastes specimen during the first batch. Then, after each liquid  
312 fraction renewal in the bioreactors, microorganisms adhering to the cement surface acted as  
313 a source for the growth of new planktonic microorganisms. The renewal of the feed solution  
314 may have stimulated bacterial detachment by chemotaxis because nutrients were more  
315 available in the bulk than inside the biofilm (Morgan et al., 2006).

316

317 During the first batch test with 2 specimens of CEM V (batch 1 – reactor R.B\_YE\_2CP), the pH  
318 increased slightly, from 11.0 to 11.7, and then progressively decreased to 9.9 (Figure 3.b).



319 With 4 CEM V specimens, the maximum alkaline pH reached 12.1 at 72 h, and then stabilised  
320 around 11.8. The initial fast pH increase was due to the release of hydroxide ions from the  
321 cement paste specimen (higher with 4 CEM V than with 2 CEM V specimens). The acidification  
322 observed afterwards was caused by the microbial denitrifying activity, especially in reactor  
323 R.B\_YE\_2CP with 2 CEM V, where the bacterial growth was higher than in the reactor with 4  
324 cement specimens (Figure 3.a). For batches 2 to 4, the pH evolution was quite similar in  
325 bioreactors R.B\_YE\_2CP and R.B\_YE\_4CP regardless of the quantity of solid cement paste  
326 specimen. The pH rose slightly (0.2-0.3 pH unit) before decreasing sharply.

327  
328 Except for the first batch test, complete nitrate reduction was achieved in 110 hours of culture  
329 on average. A strong nitrite accumulation was observed for the first two batch tests in  
330 R.B\_YE\_2CP, then the concentration of nitrite decreased more and more rapidly. For the last  
331 batch test, nitrite no longer accumulated. Nitrite accumulation was also observed in reactor  
332 R.B\_YE\_4CP and the time required to reduce the accumulated nitrite was longer than in  
333 reactor R.B\_YE\_2CP. In the presence of 2 specimens of CEM V, the microbial inoculum  
334 required an adaptation period equivalent to 3 batch tests to perform complete reduction of  
335 6.5 mM nitrate. The adaptation time was obviously higher in the presence of 4 CEM V  
336 specimens. Nitrate reduction rates are reported in Table 2. Nitrate reduction rates with 2 or 4  
337 hardened cement pastes were globally similar after biomass acclimatization, i.e. in the last  
338 three batch tests. The number of cement paste specimens did not affect the maximum nitrate  
339 reduction rates directly but did influence the biomass acclimatization. Cement pastes  
340 specimen released higher quantities of ions, affecting the time required by bacteria to adapt  
341 their metabolism to changing environmental conditions. The presence of larger quantities of  
342 cement paste specimen limited the reduction of nitrate beyond the nitrite step.

### 343 **3.3. Microbial activity under dynamic conditions (continuous supply)**

344  
345 The results in a closed system at pH 11.0 clearly demonstrated (i) the ability of the microcosm  
346 to reduce nitrate in a cement leachate based mineral medium, and (ii) the ability of the  
347 sedimentary bacterial consortium to colonise the surface of the cement paste specimen.  
348 Nonetheless, the microbial denitrification process catalysed by the sedimentary microcosm  
349 was mainly interrupted after the first step of nitrate reduction, which resulted in nitrite  
350 accumulation in the closed bioreactors. Nitrite accumulation in closed reactors (batch  
351 systems) was also reported by Alquier et al. (Alquier et al., 2014) with *Halomonas desiderata*  
352 cultivated in mineral synthetic medium for pH between 9.0 and 10.5, with and without a solid  
353 cementitious matrix. In the study by Rafrafi et al. (Rafrafi et al., 2015), the transition to a  
354 continuous supply of standard medium allowed *Halomonas desiderata* to achieve both nitrate  
355 and nitrite reduction, after an adaptation period equivalent to three hydraulic retention times  
356 in the bioreactor. In the present study, the ability of the sedimentary microcosm to reduce  
357 total oxidized nitrogen (TON), i.e. nitrate and nitrite, was investigated under continuous supply  
358 of the standard medium supplemented with yeast extract.

#### 359 360 *3.3.1. Continuous supply of standard medium*

361  
362 At the end of the batch test described in subsection 3.1, i.e. after 70 hours of batch culture,  
363 reactor R.A\_YE was connected to the exposure chamber and fed continuously with the

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

366  
367 The optical density (OD) increased quickly during the first few hours. Throughout the  
368 experiment, the values were higher in the two reactors of the continuous set-up (bioreactor  
369 and exposure chamber) (Figure 4.a) than in the batch reactor (0.24 and 0.33 vs. 0.15 in batch).  
370 After 250 hours of culture, the OD values decreased progressively in the exposure chamber  
371 and reached values similar to those observed in the bioreactor (0.28 on average), indicating  
372 that no more additional planktonic growth occurred in the exposure chamber. The presence  
373 of a microbial biofilm on the cement paste surface was confirmed by SEM-SE observation.  
374 Several morphologies of microorganisms were observed: cocci, single bacilli and bacilli in  
375 chains (Figure 5). The ability of microorganisms to colonize cement paste has been reported  
376 previously for *H. desiderata* (Rafrafi et al., 2015), in alkaline conditions and also in a biogas  
377 digester (Voegel et al., 2015).

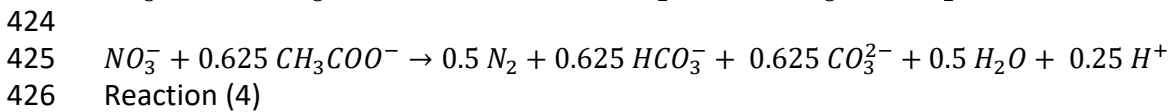
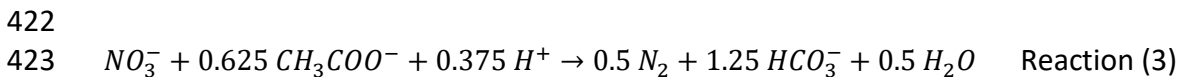
378 The overall acetate concentration during the test period was approximately 8.9 mM in the  
379 feed solution. However, the acetate concentration was higher in the bioreactor and in the  
380 exposure chamber than in the feed solution during the first 370 hours (periods 1 and 2 -  
381 Figure 3.c), starting at a concentration of 11.4 mM and progressively decreasing. Acetate was  
382 produced in the bioreactor most likely by hydrolysis of the yeast extract. During period 3 ([390;  
383 600] hours), the concentration in the bioreactor was similar to the concentration in the  
384 feeding system, i.e. 9.0 mM on average. This result does not mean that there was no acetate  
385 consumption for TON reduction but it shows that the acetate produced by hydrolysis of the  
386 yeast extract was used to reduce nitrate. The quantity of acetate produced from hydrolysis of  
387 the yeast extract in the bioreactor and the quantity consumed by oxidation cannot be  
388 accurately determined.

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

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

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

412  
413 Although the pH was set at 11 in the feed solution, the pH in the bioreactor and in the  
414 exposure chamber systematically reached values around  $9.3 \pm 0.1$  (Figure 4.d). Theoretically,  
415 at normal temperature and pressure, the predominant chemical form of inorganic carbon  
416 (due to bacterial activity) in water is  $\text{HCO}_3^-$  for a pH around 8.0 (Andersen, 2002). According to  
417 Reaction 3, for a pH around 8.0, the complete denitrification process, without nitrite  
418 accumulation, consumes protons ( $\text{H}^+$ ), and thus promotes an increase of the pH. For pH values  
419 close to the pKa for carbon dioxide, i.e. 10.3 with equal concentrations of  $\text{HCO}_3^-$  and  $\text{CO}_3^{2-}$ , the  
420 complete reduction of nitrate into nitrogen gas liberates protons (Reaction 4), and thus  
421 promotes a pH decrease.



427  
428 The carbonate equilibrium was thus strongly involved in the pH regulation. Other works  
429 dealing with granular sludge have highlighted the opposite, with a pH increase from 7.0 in the  
430 reactor influent to 9.3 in the effluent (Li et al., 2015, 2014). This behaviour is called “self-  
431 alkalisation” because of the consumption of hydroxide ions by the denitrification process.  
432 For pH values above 10.3, the denitrification process produces hydroxide ions. In contrast,  
433 granular sludge “self-acidification” or “self-de-alkalisation” can be evoked.

434  
435 The experimental set-up and the feed solution composition used in this work were similar to  
436 those in the experimental approach implemented by Rafrafi et al. (Rafrafi et al., 2017, 2015)  
437 with *Halomonas desiderata*. The nitrate reduction rates obtained experimentally, and shown  
438 in Figure 4, are reproduced in Table 3 in order to compare the performance of the consortium  
439 isolated from Buxton sediment with that of *H. desiderata*. When data from the bioreactor and  
440 exposure chamber were combined, the global nitrate reduction rate was comparable for  
441 *Halomonas desiderata* and for microbial consortium in this study, i.e.  $0.076 \text{ mM NO}_3^-/\text{h}$  and  
442  $0.083 \text{ mM NO}_3^-/\text{h}$ , respectively. Nonetheless, the nitrate reduction rate was twice as high in  
443 the bioreactor with the consortium ( $0.124 \text{ mM NO}_3^-/\text{h}$ ) as with *H. desiderata* ( $0.066 \text{ NO}_3^-/\text{h}$ ).  
444 In the work of Rafrafi et al. (Rafrafi et al., 2017, 2015), the partial nitrate reduction by  
445 *H. desiderata* in the bioreactor was coupled with strong nitrite accumulation and the TON  
446 were then reduced in the exposure chamber. In the case of the experiment with the  
447 consortium, TON was rapidly reduced in the bioreactor and the biological activity in the  
448 exposure chamber was restricted by the low quantity of residual nitrite (below 1 mM). The  
449 sedimentary consortium seems to be better suited to TON reduction and seems to be less  
450 affected by the nitrite accumulation (no growth inhibition correlated with nitrite  
451 accumulation). The presence of yeast extract certainly promoted the biological activity of the  
452 consortium by providing additional compounds, such as peptides/polypeptides, free amino  
453 acids and vitamins (Mosser et al., 2012). Moreover, the multi-species interaction that can  
454 occur in a consortium is also likely to promote the TON reduction (Brenner et al., 2008;  
455 Nozhevnikova et al., 2015; Yang et al., 2011).

456

457

### 3.3.2. Nitrate concentration increase

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

477

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

485

- 486 (i) The carbonate equilibrium linked to the carbon dioxide produced by the biological  
487 activity, depending on whether the denitrification process was complete or not.
- 488 (ii) The leaching of cement paste specimen, the amount of which was 6 times higher  
489 than in the experiment supplied with standard medium, inducing significant  
490 release of hydroxide ion and of some cations such as calcium and alkalis (Bertron  
491 et al., 2014). Calcium could precipitate with hydrogen carbonate to form calcium  
492 carbonate, modifying the carbonate equilibrium.

493

494 The first nitrate concentration rise from 5.9 to 12 mM (period 2 – [693 ; 1290]) did not alter  
495 the TON reduction rate (Figure 6.b and Figure A.3.b). The sedimentary microcosm completely  
496 reduced 12 mM of nitrate in the bioreactor without nitrite accumulation. The TON reduction  
497 rate was 0.23 mM/h (Table 4). With twice as much nitrate in the feed solution, the nitrate and  
498 the TON reduction rate doubled. Nitrate reduction into nitrogen gas was complete, even  
499 though, based on the nitrogen mass balance, some nitrite loss, lower than 0.03 mM NO<sub>2</sub><sup>-</sup>, was  
500 detected in the outlet of the chamber during period 2 (Figure 6.d). The second nitrate  
501 concentration rise, from 12 to 48 mM (period 3) induced a significant increase of the nitrate  
502 reduction rate in the bioreactor and in the exposure chamber (Table 4). However, the  
503 denitrification process slowed drastically after the first step, leading to a strong nitrite

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

- 510 (i) Yeast extract hydrolysis products: the yeast extract components may not have  
511 been fully hydrolysed and/or oxidised in the bioreactor;
- 512 (ii) From the biofilm; via Extracellular Polymeric Substances (EPS), the biofilm could  
513 sequester dissolved and particulate nutrients, which can then be utilised as a  
514 nutrient and energy source during starvation periods (Flemming and  
515 Wingender, 2010);
- 516 (iii) From endogenous respiration: the biomass undergoes cell decay leading to  
517 residual dead cells. These products and the hydrolysis of some biodegradable  
518 components of EPS are utilised by active biomass as recycled electron-donor  
519 substrates and/or carbon sources (Laspidou and Rittmann, 2002).

520  
521 In this experiment, performed with a pH between 9 and 10 inside the reactors, the nitrate  
522 concentration increase (from 6 to 46 mM) did not inhibit the sedimentary consortium  
523 denitrifying activity or its growth. The reduction of higher nitrate concentration (from 121 to  
524 586 mM  $\text{N-NO}_3^-$ ) at pH values of 9 and 10.5 has been reported in the literature and the nitrate  
525 reduction rates were evaluated at between 43 and 120 mM  $\text{N-NO}_3^-/\text{h}$  (Dhamole et al., 2008;  
526 Glass and Silverstein, 1999). An increase of nitrate concentration (at least for nitrate  
527 concentrations close to 500 mM) does not necessary inhibit the growth and the microbial  
528 activity if the community has been previously acclimated. According to the literature, when  
529 nitrate reduction begins, a nitrite accumulation could appear, which is then reduced in a few  
530 hours (Dhamole et al., 2008, 2007; Glass and Silverstein, 1999; Nair et al., 2008). For example,  
531 Dhamol et al. (Dhamole et al., 2008) reported a nitrite peak of 33 mM for an initial nitrate  
532 concentration around 61 mM. Nitrite was then reduced in less than 1 hour.

533

#### 534 **3.4. Denitrification under cementitious environment**

535

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

550 similar experimental conditions (Rafrafi et al., 2017). Nonetheless, the kinetics obtained with  
551 this consortium is open to discussion because of the use of a yeast extract, which promotes  
552 bacterial growth. Further ongoing works are exploring the possibility of microbial nitrate  
553 reduction without yeast extract in the culture medium and also the biodegradability of other  
554 carbon sources and/or electron donors. These results highlighted the interest to consider a  
555 consortium with several microorganisms in comparison with a single species to investigate the  
556 possible biological reduction of oxyanion (as nitrate) and the oxidation of the organic matter  
557 (acetate) in cementitious environment. It also seemed important to consider possible multi-  
558 species colonisation and formation of a biofilm on the cement paste surface. The biological  
559 reaction as denitrification could be promoted by synergetic microbial interactions.  
560 Quantifying such activities at high pH under conditions relevant to the disposal of  
561 cementitious radioactive waste is an important first step in understanding the impact of  
562 microbial processes on the biogeochemistry of priority radionuclides, especially those that are  
563 redox active and prone to oxidation (and changes in solubility) under denitrifying conditions  
564 (Newsome et al., 2014; Rafrafi et al., 2015; Rizoulis et al., 2012).  
565

#### 566 **4. Conclusion**

567  
568 This study showed the ability of a microbial consortium, collected from sediments  
569 contaminated by high pH residues from a lime production plant, to reduce nitrate in a  
570 cementitious environment with and without hardened cement paste. The maximal total  
571 oxidised nitrogen reduction rate by the consortium was 0.124 mM/h.  
572

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579

#### 580 **Conflicts of Interest**

581 The authors declare no conflict of interest.  
582

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734 **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 |

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

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|         | 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              |

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740 **Table 3:** Average nitrate reduction rates and average OD values for bacterial growth

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| Cement paste Surface area (cm <sup>2</sup> )  | [NO <sub>3</sub> <sup>-</sup> ] <sub>inlet</sub> (mM) | Nitrate consumption rate (mM/h) |                  |               | TON consumption rate (mM/h) |                  |               |
|---|---|---------------------------------|------------------|---------------|-----------------------------|------------------|---------------|
|   |   | Bioreactor                      | Exposure chamber | System        | Bioreactor                  | Exposure chamber | System        |
| <b>Sedimentary consortium culture in continuous bioreactor (this study)</b>                   |   |                                 |                  |               |                             |                  |               |
| 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 |
| <b>Experiment performed by Rafrafi et al. (Rafrafi et al., 2017) with an initial pH of 10</b> |   |                                 |                  |               |                             |                  |               |
| 60  | 5.9   | 0.066                           | 0.099            | 0.076         | 0.024                       | 0.137            | 0.062         |
| <b>Experiment performed by Rafrafi et al. (Rafrafi et al., 2015) with an initial pH of 11</b> |   |                                 |                  |               |                             |                  |               |
| 60  | 5.9   | 0.080                           | 0.018            | -             | -                           | -                | -             |

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**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 (cm <sup>2</sup> ) | [NO <sub>3</sub> <sup>-</sup> ] <sub>input</sub> (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 <sup>-3</sup> ± 0.2 10 <sup>-3</sup> | 0.079 ± 0.002 | 0.12 ± 0.003              | 0.16 10 <sup>-3</sup> ± 0.1 10 <sup>-3</sup> | 0.079 ± 0.002 |
|   | 12  | 0.23 ± 0.018                  | 3.0 10 <sup>-3</sup> ± 0.005                 | 0.16 ± 0.012  | 0.23 ± 0.018              | 4.4 10 <sup>-3</sup> ± 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   |

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**Exp. 1 : Optimisation of the medium simulating cementitious environment in batch bioreactor**

Cement leachate supplemented with 8.3 mM acetate + 5.9 mM nitrate (standard medium)

**Without yeast extract**  
Bioreactor R.A

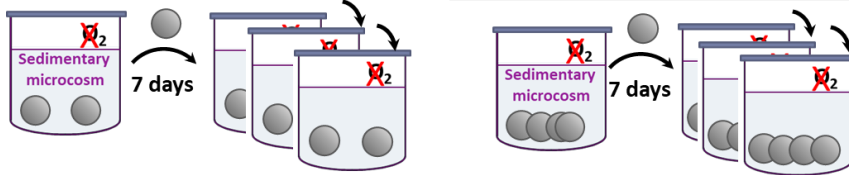
**With yeast extract**  
Bioreactor R.A\_YE

**Exp. 2 : Investigation of the cement paste specimens colonisation in batch bioreactor**

Standard medium supplemented with yeast extract. First batch with microcosm in the medium. Then medium without microcosm renewed three times every 7 days.

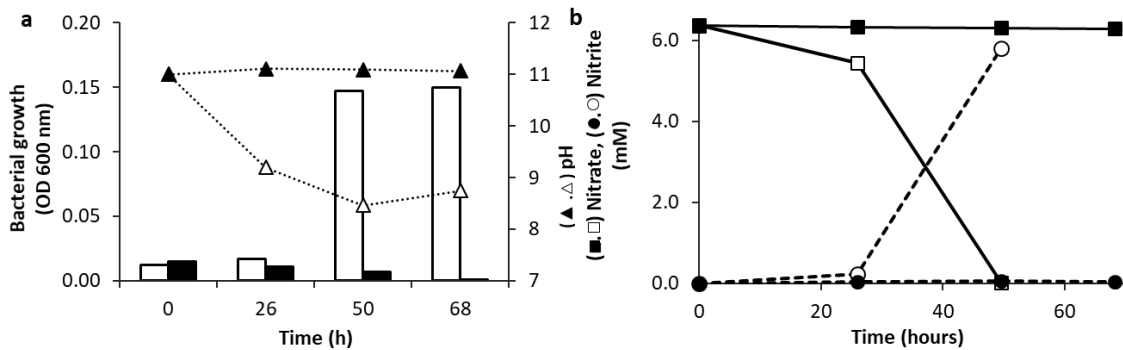
**With 2 cements pastes specimens**  
Bioreactor R.B\_YE\_2CP

**With 4 cements pastes specimens**  
Bioreactor R.B\_YE\_4CP



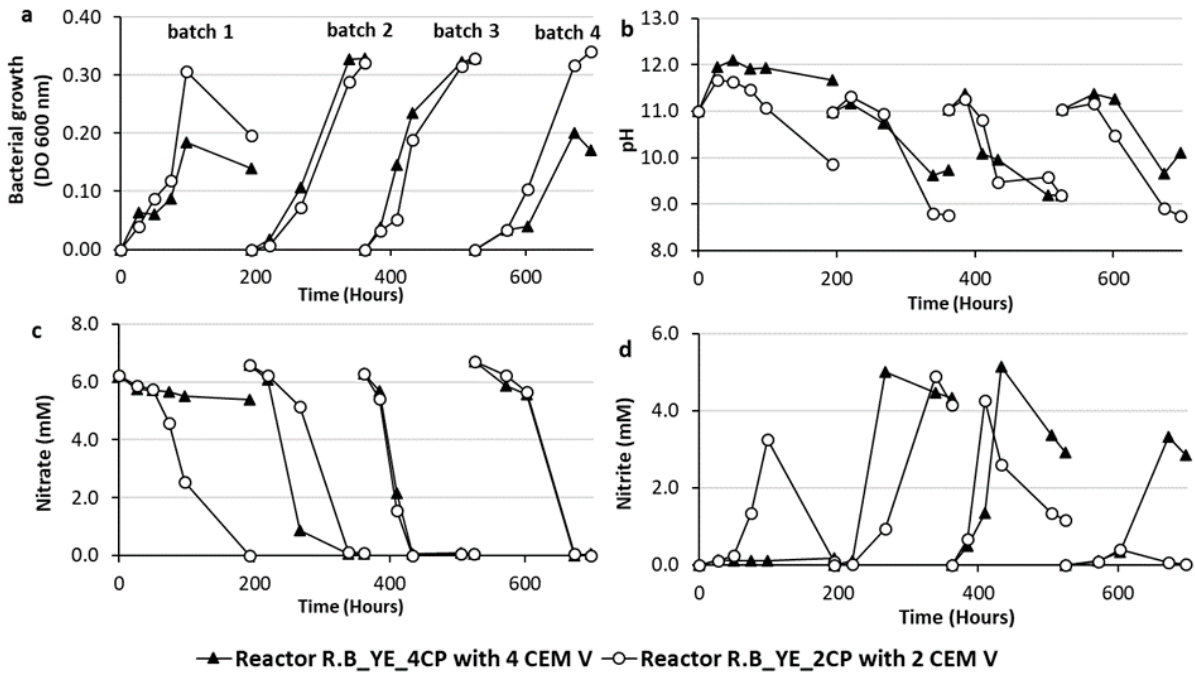
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**Figure 1:** Scheme of the experiments performed in batch bioreactor



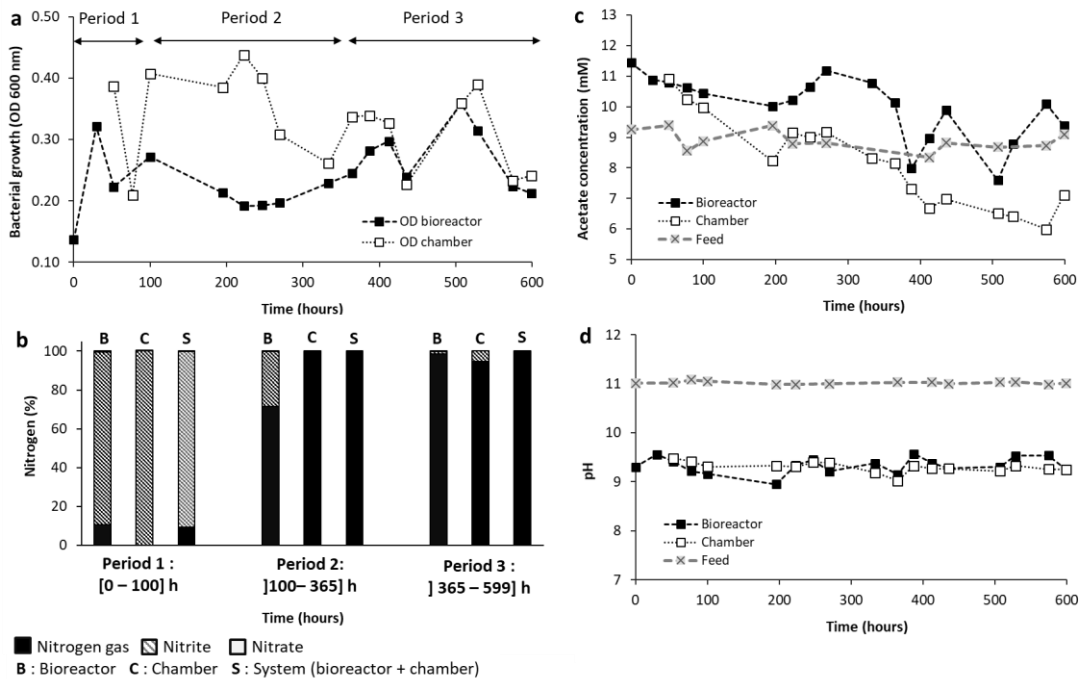
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**Figure 2:** Bacterial growth and changes in pH (a), nitrate and nitrite concentrations (b) with (reactor R.A\_YE: white) and without (reactor R.A: black) yeast extract during batch tests in closed bioreactors.



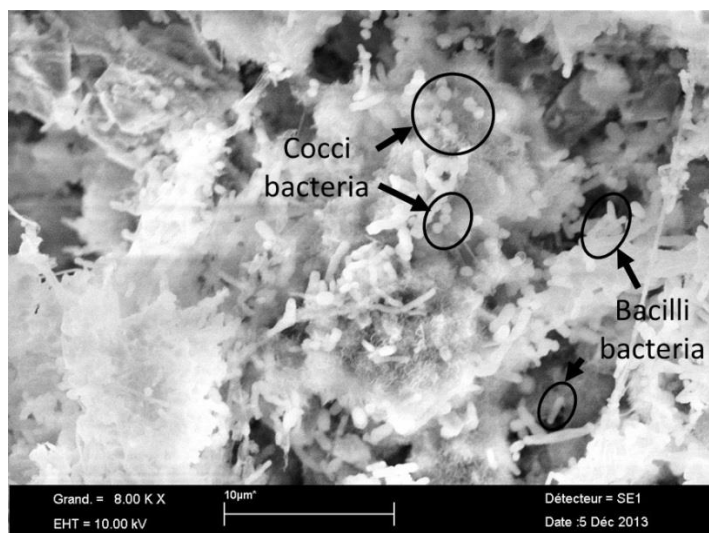
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**Figure 3:** Microbial growth (OD 600nm) (a), pH (b), nitrate concentration (c), and nitrite concentration (d), in fed-batch bioreactors initially inoculated with a sedimentary microbial consortium and containing 2 or 4 solid specimens of CEM V cement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



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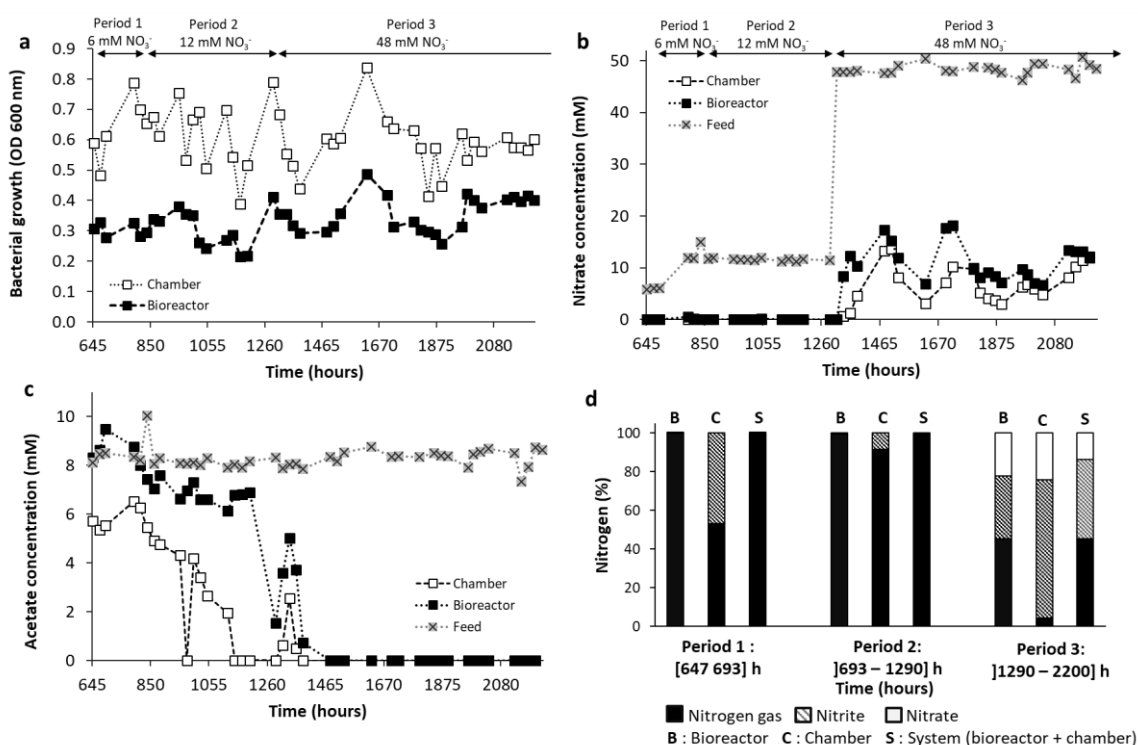
**Figure 4:** Monitoring of sedimentary microcosm culture under dynamic conditions – (a) bacterial growth in the bioreactor and in the exposure chamber, (b) nitrogen mass balance in the bioreactor, the chamber and on the global system (i.e. bioreactor + chamber), (c) acetate concentrations and (d) pH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



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

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

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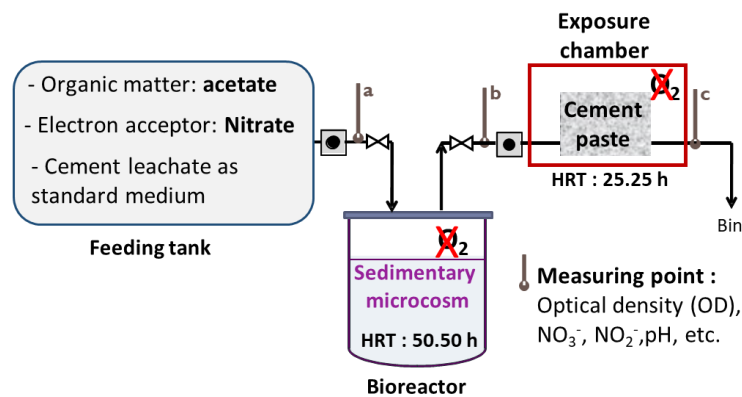
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Supplementary data

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

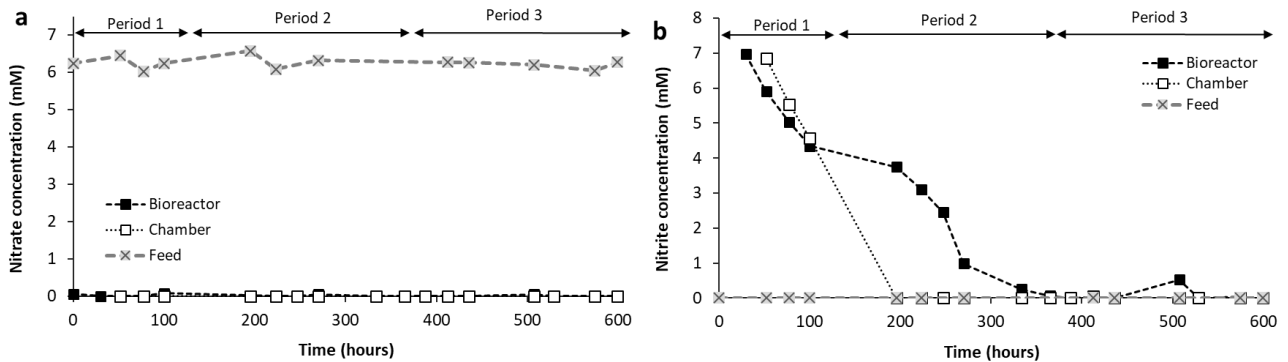
| Culture medium  | Starting time (hours) | Duration (hours) | [NO <sub>3</sub> ] (mM) | [C <sub>2</sub> H <sub>3</sub> O <sub>2</sub> ] (mM) | [acetate]/[Nitrate] Feed | Cement paste surface area (cm <sup>2</sup> ) |
|---|-----------------------|------------------|-------------------------|--|--------------------------|--|
| Cement leachate with adjusted pH (11) and 0.1% of yeast extract | 648                   | 144              | 5.99 ± 0.13             |  | 1.40 ± 0.03              |  |
|   | 792                   | 522              | 11.84 ± 0.90            | 8.27 ± 0.40  | 0.70 ± 0.01              | 339 cm <sup>2</sup>                          |
|   | 1314                  | 888              | 48.30 ± 1.06            |  | 0.17 ± 0.01              |  |

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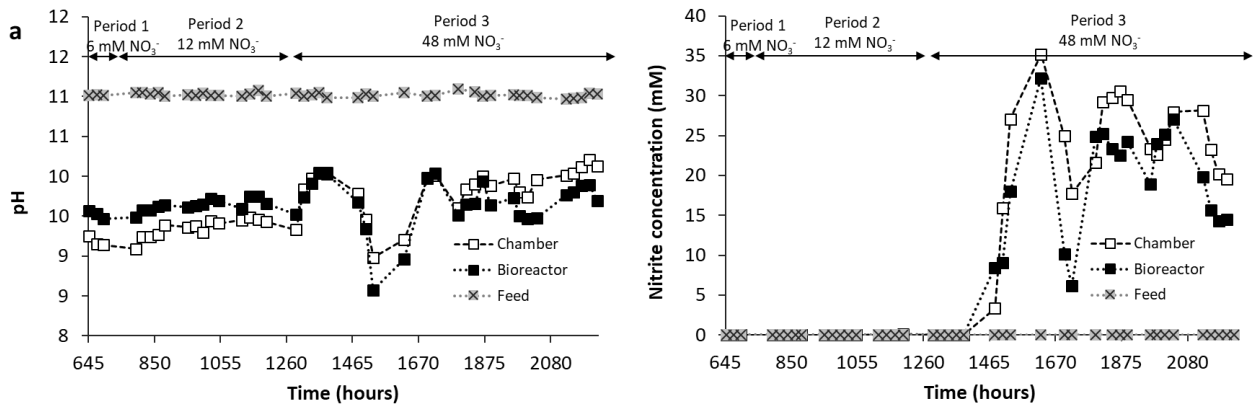
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**Figure A.1:** Scheme of the experimental set-up designed to study the impact of the cement leachate on the Buxton sediment’s microbial activity, with or without hardened cement paste specimen, under continuous supply (adapted from Rafrafi et al.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



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



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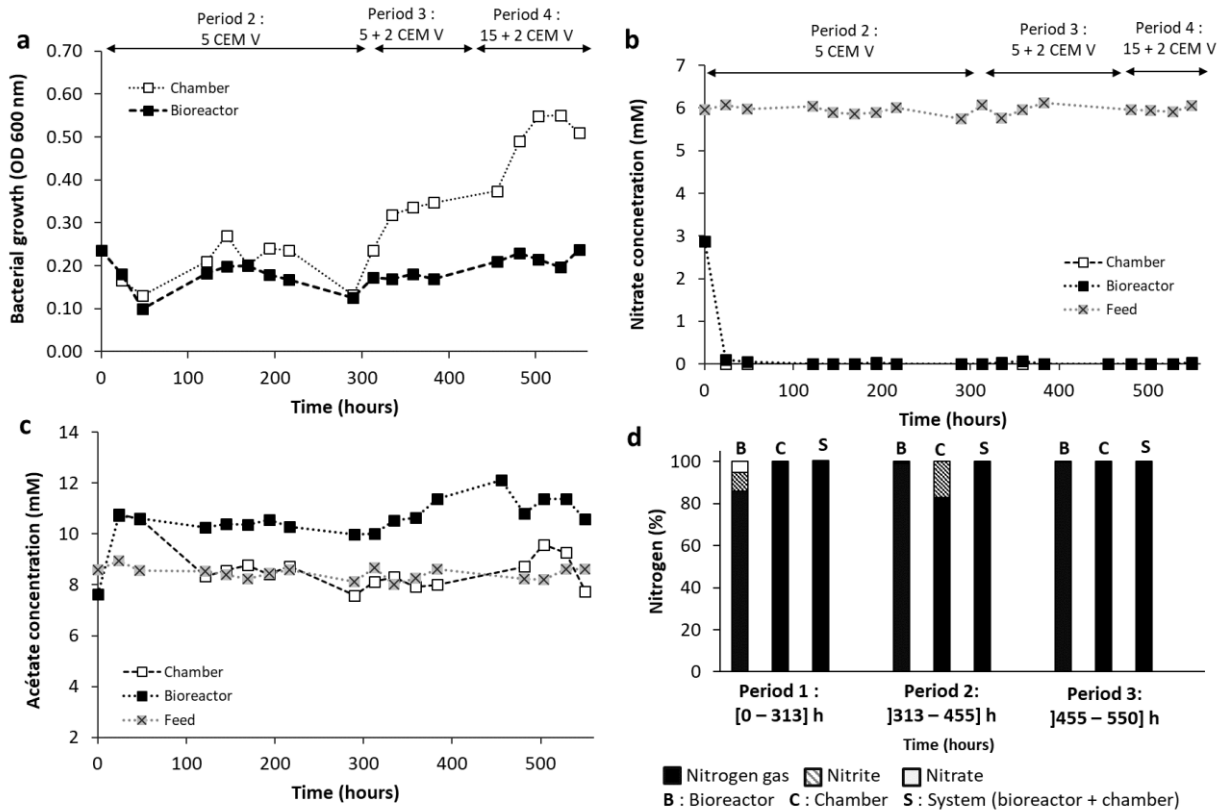
809 **Additional experiments**

810 After 3 days in batch operation, the bioreactor was continuously supplied with fresh standard  
 811 medium according to the same protocol as the one described in section 2.4.3. The bioreactor  
 812 was inoculated with the enrichment obtained from standard medium incubation (section  
 813 2.3.2). Five slices (h= 10 mm), 2 slices (h= 20 mm) and then 10 slides (h=10 mm) of cement  
 814 paste were added into the exposure chamber after approximately 1 day, 14 days, and 20 days  
 815 of culture, respectively. The surface available for microbial colonisation increased  
 816 progressively from 100 cm<sup>2</sup> with five cement pastes, to 156 cm<sup>2</sup> and to 356 cm<sup>2</sup> at the end of  
 817 the experiment (Table A.1).

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

|   | Time<br>(days) | [NO <sub>3</sub> <sup>-</sup> ]<br>(mM) | [C <sub>2</sub> H <sub>3</sub> O <sub>2</sub> H<br>] (mM) | [acetate]/N<br>Feed | Cement<br>paste<br>surface | Culture<br>medium  |
|---|----------------|---|---|---------------------|----------------------------|--|
| CEM V increase under<br>continuous supply | 1              |   |   |                     | 100 cm <sup>2</sup>        | Cement leachate<br>with adjusted pH<br>(11) and 0.1% of<br>yeast extract |
|   | 14             | 5.9 ± 0.1                               | 8.4 ± 0.25  | 1.4 ± 0.03          | 156 cm <sup>2</sup>        |  |
|   | 20             |   |   |                     | 339 cm <sup>2</sup>        |  |

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

827 **Table A.6:** The average nitrate reduction rate and the average values of OD for bacterial  
 828 growth assessment under dynamic conditions with increasing number of cement paste  
 829 specimens in the exposure chamber (acetate concentration kept constant at 8.5 mM)  
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| CEM V<br>paste<br>surface<br>area<br>(cm <sup>2</sup> ) | [NO <sub>3</sub> <sup>-</sup> ] <sub>inlet</sub><br>(mM) | Nitrate consumption rate<br>(mM/h) |                     |                  | Bacterial growth<br>(DO 600 nm) |                     |
|---|--|------------------------------------|---------------------|------------------|---------------------------------|---------------------|
|   |  | Bioreactor                         | Exposure<br>chamber | System           | Bioreactor                      | Exposure<br>chamber |
| <b>Experiment with cement paste addition</b>            |  |                                    |                     |                  |                                 |                     |
| 100   | 6.0  | 0.11 ± 0.018                       | 0.001 ±<br>0.001    | 0.079 ±<br>0.001 | 0.17 ± 0,04                     | 0.22 ± 0,05         |
| 156   |  | 0.12 ± 0.004                       | 0.001 ±<br>0.001    | 0.078 ±<br>0.002 | 0.20 ± 0,02                     | 0.39 ± 0,02         |
| 356   |  | 0.12 ± 0.001                       | 0.001 ±<br>0.001    | 0.079 ±<br>0.001 | 0.22 ± 0,02                     | 0.52 ± 0,03         |

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