Research article

Nitrate and nitrite bacterial reduction at alkaline pH and high nitrate concentrations, comparison of acetate versus dihydrogen as electron donors

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ABSTRACT

This study assesses bacterial denitrification at alkaline pH, up to 12, and high nitrate concentration, up to 400 mM. Two types of electron donors organic (acetate) and inorganic (dihydrogen) were compared. With both types of electron donors, nitrite reduction was the key step, likely to increase the pH and lead to nitrite accumulation. Firstly, an acclimation process was used: nitrate was progressively increased in three cultures set at pH 9, 10, or 11. This method allowed to observe for the first time nitrate reduction up to pH 10 and 100 mM nitrate with dihydrogen, or up to pH 10 and 400 mM nitrate with acetate. Nitrate reduction kinetics were faster in the presence of acetate. To investigate further the impact of the type of electron donor, a transition from acetate to dihydrogen was tested, and the pH evolution was modelled. Denitrification with dihydrogen strongly increases the pH while with acetate the pH evolution depends on the initial pH. The main difference is the production of acidifying CO_2 during the acetate oxidation. Finally, the use of long duration cultures with a highly alkaline pH allowed a nitrate reduction up to pH 11.5 with acetate. However, no reduction was possible in hydrogenotrophy as it would have increased the pH further. Instead, bacteria used organic matter from inoculum to reduce nitrate at pH 11.5. Therefore, considering bacterial denitrification in a context of alkaline pH and high nitrate concentration an organic electron donor such as acetate is advantageous.

1. Introduction

Nitrate pollution is a global environmental issue in which industrial effluents represent a major source of pollution. Bacterial denitrification may be the most economical and efficient way to deal with these effluents compared with other physicochemical nitrate removal techniques (Kapoor and Viraraghavan, 1997; Mohsenipour et al., 2014). Bacterial denitrification is usually studied for nitrate removal in wastewater treatment context, where authors seek to optimize the performance of denitrification working at neutral pH values and relatively moderate nitrate concentrations (0.1–10 mM). However, some industrial wastes are characterized by higher pH and nitrate concentration ranges. For example, in a radioactive waste repository, nitrate concentrations are expected to reach 1000 mM (Francis and Hatcher, 1980; Glass and Silverstein, 1999). Other effluents, such as those produced during stainless steel production or the explosives industry, may contain several tens (Fernández-Nava et al., 2008) to hundreds of mM of nitrate (Marecik et al., 2013). Nitrate pollution may also occur in alkaline environments. Effluents from a stainless steel factory may have a pH of 9.6 (Fernández-Nava et al., 2008). In the context of radioactive waste repositories, alkaline pH between 9 and 13, generated by the presence of cementitious materials, are expected (Albrecht et al., 2013; Alquier et al., 2014; Bertron et al., 2014; Durban et al., 2018). In addition, the question of the electron donor type (organic or inorganic) reducing nitrate is relevant when considering industrial contexts. Usually, in domestic wastewater treatment processes, the presence of organic matter provides electrons for nitrate reduction. However, in some industrial context, the organic matter could be missing, leaving inorganic compounds as the only alternative for electron source. For instance, in a radioactive waste repository, dihydrogen generated in situ and/or organic acids are expected depending on the waste type (Gales et al., 2004; Grebliunas and Perry, 2016; Libert et al., 2011; Pedersen, 1997).

Bacterial denitrification is a respiration process in which nitrate is reduced to nitrite, nitric oxide, nitrous oxide and finally dinitrogen. The

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use of organic versus inorganic matter to provide electron for nitrate reduction results in fundamental bacterial activity differences. For instance, the reactions with organic acetate and inorganic dihydrogen are presented in reactions (1) and (2) respectively. Acetate is a potential source of electrons and inorganic carbon. Furthermore, during denitrification with acetate (heterotrophic metabolism) there is a generation of CO_2 which has a strong impact on pH.

$$5\ CH_3COO^- + 8\ NO_3^- \rightarrow 10\ CO_2 + 4\ N_2 + 5\ H_2O + 9\ OH^- \quad (1)$$

$$2\ NO_3^- + 5\ H_2 \rightarrow 4\ H_2O + N_2 + 2\ OH^- \quad (2)$$

These two particular electron donors were compared because they are widely used in bacterial denitrification contexts (Dhamole et al., 2007; Fernández-Nava et al., 2008; Karanasios et al., 2010), and are likely to be present in the industrial contexts mentioned above. Usually, denitrification is studied with organic electron donors to optimize nitrate removal yields. Indeed, during denitrification with dihydrogen (hydrogenotrophic metabolism), slower growth and nitrate reduction kinetics are generally observed (Albina et al., 2019; Ergas and Reuss, 2001; Mateju et al., 1992). Authors explain it with: (i) the low solubility of dihydrogen which makes it less accessible, (ii) the need for hydrogenotrophic (autotrophic) bacteria to assimilate inorganic carbon (Chang et al., 1999; Ghafari et al., 2009; Liu et al., 2014). In addition, alkaline pH and high nitrate concentration also strongly affect bacterial denitrification. High nitrate concentration inhibits bacterial activity (Banihani et al., 2009; Dhamole et al., 2007; Glass and Silverstein, 1999). As a result, only a few studies have evaluated bacterial denitrification in environments with nitrate concentrations greater than 100 mM (Denariaz et al., 1989; Dhamole et al., 2007; Miao et al., 2015; Napier and Bustamante, 1988). High nitrate concentrations tend to cause considerable nitrite accumulations (Albina et al., 2019). Nitrite has been described as an inhibitor of bacterial activity with known toxicity (Baumann et al., 1997; Cua and Stein, 2011; Yarbrough et al., 1980). Nitrite could, for instance, form free radical compounds with oxygen within cells (Richardson and van Spanning, 2007; Titov and Petrenko, 2003; Van Der Vliet et al., 1997). The pH generally affects the activity of enzymes; the pH limits tolerated by bacteria are between 11.5 and 12 (Albina et al., 2020; Janto et al., 2012; Shapovalova et al., 2008; Sorokin, 2005). In alkaline medium, denitrifying activity slows down and nitrite accumulation is generally observed (Albina et al., 2019; Tang et al., 2011; Vasiliadou et al., 2006).

Accordingly, the management of wastes and effluents in an environment that combines high pH and high nitrate concentration is problematic, the bacterial activity will likely be severely slowed down. The issue is even more challenging when there is only an inorganic source of electron donors. As a result, the review of the literature revealed a lack of information regarding bacterial denitrification at alkaline pH or with high nitrate concentrations, and this lack is even greater when both conditions are considered simultaneously. To date, denitrification has not yet been tested for a combination of pH above 9 and nitrate concentration above 120 mM (Albina et al., 2019). However, the nitrate depollution issue needs to be addressed even in a difficult environment. For instance, high nitrate concentrations could impact the speciation and mobility of radionuclides in radioactive waste (Albrecht et al., 2013; Francis and Hatcher, 1980).

Therefore, the objective of this work is to evaluate the bacterial denitrification possibilities in environments characterized simultaneously by alkaline pH and high nitrate concentrations. Denitrification was assessed by monitoring nitrate and nitrite reductions. The experiments were carried out with either organic acetate or inorganic dihydrogen. A new approach, based on predictive pH modelling allowed to assess the strong impact of the electron donor type on the pH evolution. To maximize the probability of observing bacterial activity in this context, long culture times and acclimation procedures (gradual increases in pH or nitrate concentration) were used (Albina et al., 2020). Activated sludge from urban wastewater treatment plant was used to match with industrial contexts and to maximize the inoculum survivability. Indeed, activated sludge communities can adapt and reduce nitrate at high pH (Durban et al., 2020), high nitrate concentrations (Miao et al., 2015) and in hydrogenotrophy (Rezania et al., 2005).

2. Materials and methods

2.1. Reactor medium

The reactor medium was cement leachate, a mineral medium representative of an aqueous system in a concrete dominated environment as in a radioactive waste repository. The cement leachate was manufactured from crushed, hydrated CEM V/A cement pastes. One gram per litre of crushed cement was poured into water and mixed at 900 rpm for 24 h. The cement leachate was filtered and then stored in hermetic bottles saturated with dinitrogen. The average composition of the cement leachate is shown in Table 1.

In the heterotrophic reactors, sodium acetate was added about three times a week to maintain its concentration at 20 mM. In the hydrogenotrophic reactors, dihydrogen (1 bar) was bubbled at 80 mL min^{-1}. The leachate was supplemented with sodium nitrate at various concentrations (10–400 mM) and 50 mM sodium bicarbonate as a pH buffer (and as the inorganic carbon source for hydrogenotrophic bacteria). The pH was adjusted by additions of NaOH and HCl (10 M) to the reactors.

2.2. Inoculum

Activated sludge was collected in the aeration basin of the wastewater treatment plant at Castanet near Toulouse, France. The collected sample was characterized by a nitrate concentration of 0.7 ± 0.4 mM, a pH of 7.6 ± 0.01, and 6.5 ± 1.5 g/L of suspended solids. The sample was centrifuged (4610 g, 10 min, 25 °C), then 1 g/L of the activated sludge was added to the fresh medium to initiate precultures. The precultures were carried out for about a week to adapt the bacteria to cement leachate at pH adjusted to 9–10 and nitrate utilization (10–50 mM) with acetate or dihydrogen depending on the experiment that was to follow. The reactors were then inoculated with a 50/50 (v/v) mixture of preculture and fresh cement leachate.

2.3. Reactor set-up

The reactors were glass bottles (volume 1 L) agitated at 300 rpm (magnetic stirrer) and maintained at a temperature of 30 °C. They were kept anaerobic by a constant flow of dinitrogen (1 bar, 80 mL min^{-1}) in heterotrophy and of dihydrogen (1 bar, 80 mL min^{-1}) in hydrogenotrophy. The sequential batch reactors were operated in successive stages: a first batch period, during which nitrate reduction was monitored, and a second period of partial renewal by fresh medium 50/50% (v/v) containing a higher concentration of nitrate. These two periods were repeated in cycles. During each stage of medium renewal, the agitation was stopped for a few hours before changing the medium. This operation allowed to keep the majority of the biomass decanted in the bottom volume of the reactor. The majority of experiments were conducted in sequential batch reactors and were therefore subdivided into several batch periods; others were conducted with only one batch period. All the experimental set-ups are summarized in Table 2. The experiments involving dihydrogen had shorter duration for operational

Table 1
Average chemical composition of the cement leachate.

Concentration (mM)							pH
Na	K	Ca	Mg	Al	Fe	Si	
0.53 ±0.11	0.14 ±0.01	0.97 ±0.26	0.01 ±0.00	0.04 ±0.01	<0.01	0.23 ±0.07	11.4 ±0.2

Table 2
Summary of the experimental set-ups reviewed in this study.

Experimental set-up	Reactor set-up	Electron donor	Nitrate (mM)	pH	Duration (days)
High nitrate concentration with acetate	SBR[a]	Acetate (20 mM)	50–400	9, 10, 11	108
Highly alkaline pH with acetate	Batch	Acetate (20 mM)	50	11.5, 12	234
High nitrate concentration with dihydrogen	SBR[a]	H_2 (1 bar, 30 °C)	10–100	9, 10, 11	83
Highly alkaline pH with dihydrogen	Batch	H_2 (1 bar, 30 °C)	50	11.5, 12	186
Transition from acetate to dihydrogen only	SBR[a]	Acetate (20 mM) to H_2 (1 bar, 30 °C)	50–100	9–11	48

[a] SBR stands for sequential batch reactors.

and safety reasons.

2.4. Analytical techniques

2.4.1. pH monitoring

The pH was monitored using FisherBrand gelled electrolyte probes connected to a Consort data acquisition system (model C3060). CO_3^{2-} and HCO_3^- concentrations were determined together by titration with hydrochloric acid (HCl).

2.4.2. Biomass analysis

Bacterial population diversity was analysed by sequencing 16S RNA. At the end of the experiment, the biomass was centrifuged (4610 g, 10 min, 4 °C), the total DNA present in the solid was extracted using QIAGEN DNeasy PowerBiofilm kits. The samples were sent to the Research and Testing Laboratory (RTL, Texas, USA), where the DNA was sequenced by PCR with the bacterial primers (515F GTGCCAGCMGCCGCGGTAA - 806R GGACTACHVGGGTWTCTAAT) according to RTL protocols. Subsequent data analyses of the DNA quality, DNA sequence alignment, clustering in operational taxonomic units, and assignment were also performed by RTL according to their protocols.

2.4.3. Chemical analysis

After filtration at 0.2 µm by cellulose acetate filters (Minsisart PES, Fisher Scientific) acetate, nitrate, and nitrite concentrations were determined by high-precision ion chromatography (Dionex ICS-2000 and ICS-3000); the method is described in Alquier et al. (2014).

3. Results and discussions

3.1. Denitrification at high nitrate concentration and alkaline pH

3.1.1. Denitrification in the presence of acetate

This experiment was carried out in three sequential batch reactors set at pH 9, 10 and 11. Three successive batch periods (vertical lines in Fig. 1) allowed to increase the initial 50 mM nitrate concentration to 200 and 400 mM respectively on day 14 and 49. At the start of each batch period, the pH was readjusted to 9, 10 or 11. In Fig. 1 (A), the coloured numbers indicate the average nitrate reduction kinetics in $mM.d^{-1}$, in Fig. 1 (B), they indicate the nitrite concentration that was reduced during each batch period as calculated from equation (3).

$$[NO_2^-]_{reduced} \quad [NO_3^-]_{reduced} + [NO_2^-]_{initiale} - [NO_2^-]_{final} \tag{3}$$

During the batch period at 50 mM nitrate, the reactor initially set at pH 9 had higher nitrate reduction rate. However, its nitrite reduction was low: only 15 mM of nitrite was reduced building a 40 mM nitrite accumulation, Fig. 1 (B). ***NB: The non-zero nitrite concentrations observed at the beginning of each batch period were caused by the possible presence of nitrite in the inoculum.*** In reactors at pH 10 and 11, the nitrate was not fully consumed, therefore the nitrite accumulation was lower. Regarding the pH, it decreased in both reactors at initial pH 10 and 11, while it decreased then increased in the reactor at initial pH 9. Thus, the pH evolution was dependant of the initial pH. During the batch period at 200 mM nitrate, nitrate was reduced at the same rate regardless of the initial pH. The higher nitrite accumulation in the reactor at pH 9 during the former batch period probably led to a subsequent slowdown in bacterial activity. While the reactor at initial pH 11.0 acidified to 9.9, which could explain why the nitrate reduction kinetics in the reactor at pH 11 was not very different from those of reactors at initial pH 9 and 10. In the batch period at 400 mM nitrate, nitrate was reduced at the

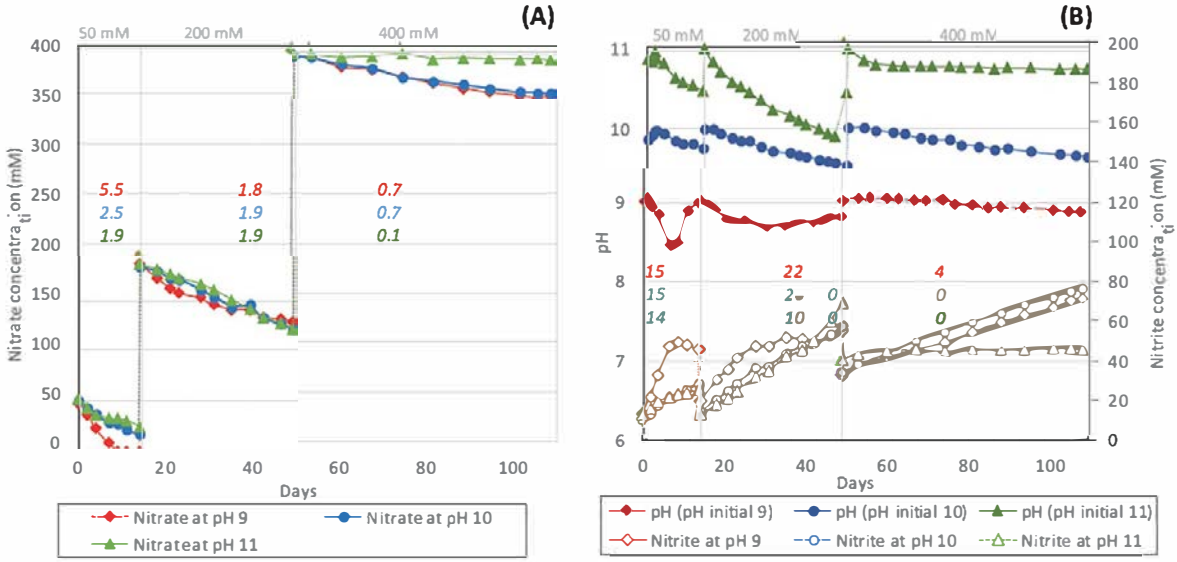

Fig. 1. Influence of a nitrate increase from 50 to 400 mM on the dynamics of nitrate reductions (A), and the evolution of pH and nitrite concentrations (B) in heterotrophic reactors maintained at 20 mM acetate and supplemented with 50 mM bicarbonate. The coloured numbers above the curves in graph (A) indicate the average nitrate reduction kinetics in $mM.d^{-1}$. Those above the curves in graph (B) indicate the nitrite concentration reduced (mM) during each batch period.

same rate at initial pH 9 or 10. Nitrite was only reduced in the reactor at initial pH 9. It is difficult to draw a definitive conclusion at pH 11 given the very low concentration of nitrate reduced compared to the initial concentration. Nitrate reduction is therefore possible at least up to pH 10 and 400 mM nitrate.

High nitrate concentrations slow down bacterial activity (Bollag and Henninger, 1978; Dhamole et al., 2008; Glass et al., 1997; Glass and Silverstein, 1999). Among other things, high nitrate concentration seems to generate nitrite accumulation. Nitrite can inhibit bacterial activity at relatively low concentrations, of the order of 10 mM (Albina et al., 2019; Bollag and Henninger, 1978; Yarbrough et al., 1980). For example, nitrite can form cytotoxic free-radical species or block various metabolic activities (Van Spanning and Richardson, 2007; Yarbrough et al., 1980). In these experiment during the batch period at 400 mM nitrate, the nitrite accumulation increased from 40 to 80 mM which could explain the low nitrate reductions. In the reactor at pH 9, the nitrite accumulation in the first batch period slowed the subsequent nitrate reduction in the second batch period. This can explained by a denitrification regulation in order to limit internal accumulations of nitrite (Chen and Strous, 2013; Van Spanning and Richardson, 2007). A genetic regulation by transcription factors of the fumarate and nitrate reductase regulatory family has been observed in bacteria (Arai et al., 1997; Crack et al., 2016; Kuroki et al., 2014; Silvestrini et al., 1994). Various forms of metabolic regulation also have been observed. For example, competition for electrons between denitrification reductases was shown (Kornaros et al., 1996; Kucera et al., 1986; Thomsen et al., 1994). The nitrate reduction could also be controlled by the bacterial population evolution. In bacterial communities, rather than considering an uniform reaction, the denitrification is segmented by partially denitrifying bacteria that share the reduction steps between them (Lycus et al., 2017; Roco et al., 2017). Thus, in denitrifying communities, rather than performing all four stages of denitrification, most bacteria will express only one or a few reduction steps. Since nitrite accumulation is a key parameter in denitrification, several authors have simply categorized the bacterial communities into two types: "nitrate respiring" bacteria that only reduce nitrate to nitrite and cause nitrite accumulation, and "true denitrifier" bacteria that are capable of reducing nitrate and nitrite to dinitrogen (Dhamole et al., 2007; Glass and Silverstein, 1998; Liessens et al., 1992; Szekeres et al., 2002; Wilderer et al., 1987). Growth kinetics observed for "nitrate respiring" bacteria can be three times higher than for "true denitrifier" bacteria (Turk and Mavinic, 1987). Consequently, it is generally reported that, as long as nitrate is present, nitrate respiring bacteria dominate the community and nitrite accumulation occurs. When nitrate becomes limiting, true denitrifier bacteria dominate the population and nitrite accumulation disappears (Glass and Silverstein, 1998). Therefore, in the reactor at pH 9 during batch phase at 50 mM nitrate, it was assumed that at day 7 the nitrite accumulation and nitrate limitation caused an evolution of the denitrifying community towards "true denitrifier" bacteria. In the following batch period at 200 mM, the lower proportion of "nitrate respiring" bacteria in the reactor at initial pH 9 finally slowed the reduction of nitrate.

3.1.2. *Denitrification in the presence of dihydrogen*

In this dihydrogen-fed experiment, the initial 10 mM nitrate concentration was increased to 50 mM on day 5 and then to 100 mM on day 19, in three sequential batch reactors at pH 9, 10 or 11, Fig. 2. In the reactor set at pH 11, acetate was added on day 75 in an attempt to restart bacterial activity.

In the batch period at 10 mM nitrate, in the reactors at initial pH of 9 and 10, the nitrate was completely reduced, Fig. 2 (A). In the reactor at initial pH of 11, only 5 mM nitrate was reduced. Nitrate and nitrite reduction kinetics were correlated to the initial pH of the reactor. In the batch period at 50 mM nitrate, nitrate was completely reduced at pH 9 with reduction kinetics comparable to those for heterotrophy under the same conditions. Nitrite was only reduced in the reactor at pH 9. At pH 10, the reduction of nitrate slowed down. At pH 11, nitrate was not reduced at all. During the batch period at 100 mM, the nitrate reduction was still going on at pH 9 and 10. Nitrate reduction was therefore possible up to pH 10 and 100 mM nitrate in hydrogenotrophy. No bacterial reduction was going on at pH 11 and higher nitrate concentration than 10 mM. However, the addition of acetate into the reactor at pH 11 on day 75 initiated bacterial activity: 40 mM nitrate was suddenly reduced and nitrite accumulated. Analyses of the bacterial communities were carried out on the final biomass of the three reactors. In reactors at pH 9 and 10, the genus *Thauera* sp., including several hydrogenotrophic denitrifiers species, was overwhelmingly represented (Fida et al., 2017; Macy et al., 1993). However, in the reactor at initial pH 11, the final addition of acetate resulted in the dominance of a heterotrophic

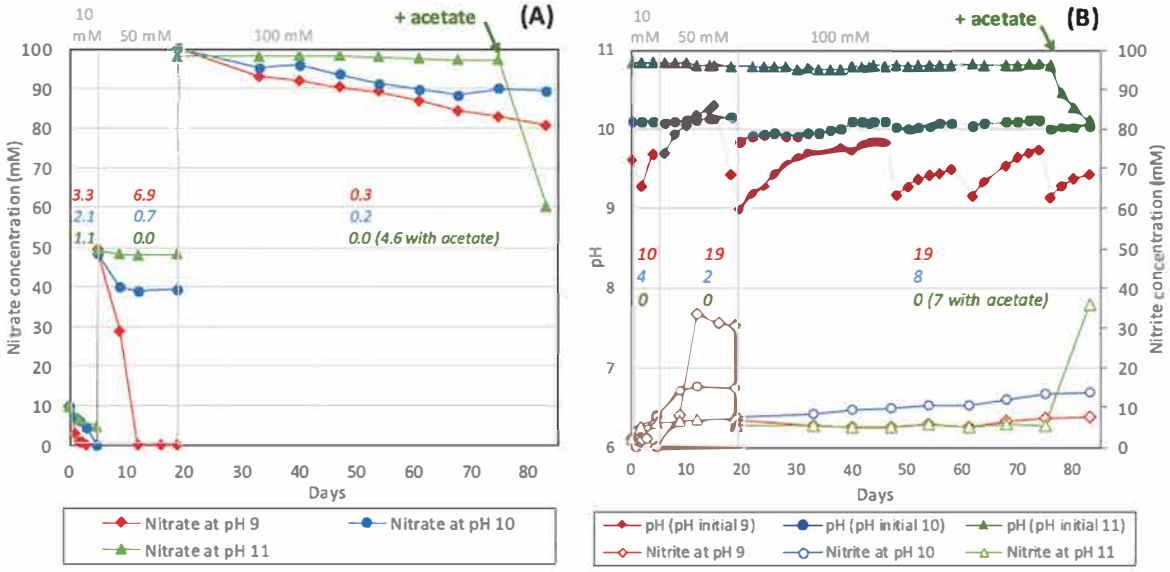

Fig. 2. Influence of a nitrate increase from 10 to 100 mM on the dynamics of nitrate reductions (A), and the evolution of pH and nitrite concentrations (B) in hydrogenotrophic reactors fed with a constant flow of dihydrogen (1 bar, 80 mL min $^{-1}$) and 50 mM bicarbonate. The coloured numbers above the curves in graph (A) indicate the average nitrate reduction kinetics in mM.d $^{-1}$. Those above the curves in graph (B) indicate the nitrite concentration reduced (mM) during each batch period.

denitrifying bacteria: *Halomonas* sp., (Alquier et al., 2014; Rafrafi et al., 2017). Therefore, heterotrophic denitrification was advantageous at pH 11. This conclusion can be explained by the pH evolution. Indeed in reactors at pH 9 or 10, regular manual addition of HCl was necessary to readjust the pH which was constantly increasing. These additions are indicated by the pH steps (dotted lines) in Fig. 2 (B). In the reactor at initial pH 11, the pH remained constant as bacterial activity was low. After the addition of acetate, the medium quickly acidified to pH 10.2 during nitrate reduction. There was an opposite pH evolution as soon as the denitrification used acetate as the electron donor. It is quite clear that in these alkaline pH ranges, the more the pH is acidified, the more the bacterial activity is facilitated.

According to these results, hydrogenotrophic denitrification seems to tolerate lower pH and nitrate concentration compared to heterotrophic denitrification. Studies on heterotrophic denitrification tested pH from 7.5 to 9.5 and nitrate concentrations from 10 to 100 mM and observed high nitrate reduction rate around 60-600 mM d^{-1} (Fernández-Nava et al., 2008; Glass and Silverstein, 1998; Thomsen et al., 1994). Some specific operating conditions, such as biomass concentration, even allowed nitrate reduction kinetics to reach 5000 mM d^{-1} (Kucera et al., 1986). In this present study, the nitrate reduction rates were globally lower. Experimental conditions, designed to approach the context of radioactive waste repositories, such as the low density of the inoculum, or the poor cementitious medium, slowed down the nitrate reduction rates. Under hydrogenotrophy, studies report nitrate reduction kinetics between 1 and 50 mM d^{-1}. The pH and nitrate concentration ranges generally tested are from pH 6.5 to 9.5 and nitrate concentrations from 1 to 10 mM (Chang et al., 1999; Lee and Rittmann, 2003; Rezania et al., 2005; Vasiliadou et al., 2006). These huge differences between the two types of electrons donors were explained by the low dihydrogen solubility (Karanasios et al., 2010), and the need for (autotrophic) hydrogenotrophic bacteria to assimilate inorganic carbon (Chang et al., 1999; Ghafari et al., 2009; Liu et al., 2014). Despite these differences, some authors have achieved kinetics of the order of 300 mM d^{-1} under hydrogenotrophic conditions by increasing dihydrogen partial pressure which increased its solubility and its availability to bacteria (Epsztein et al., 2016). It is also possible to increase dihydrogen solubility using specifically designed reactors i.e. porous membrane, hollow fibre, and

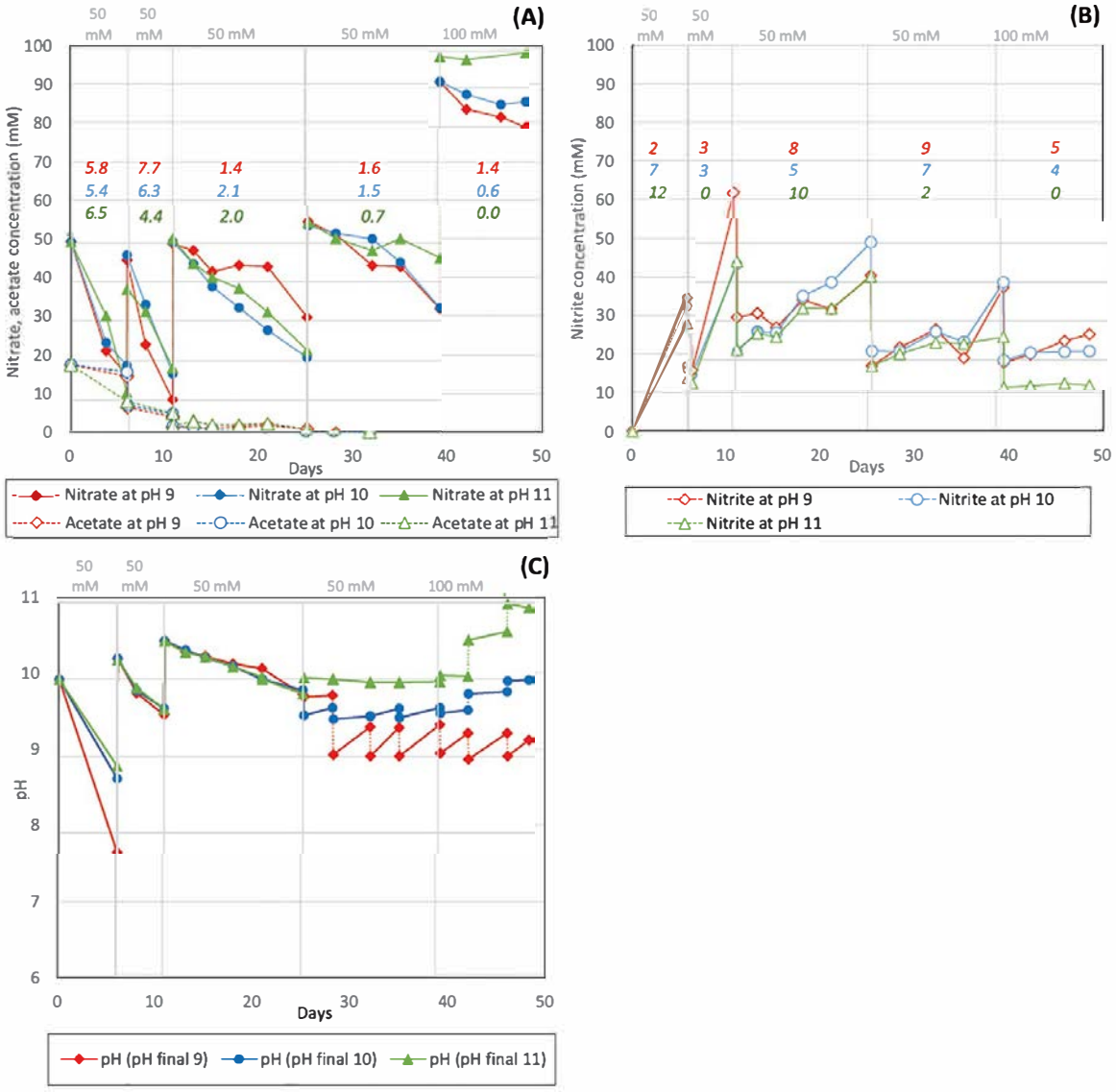

Fig. 3. Influence of a transition from acetate to dihydrogen on the dynamics of nitrate reductions (A), on the evolution of nitrite concentration (B), and pH (C). Reactors fed with a constant flow of dihydrogen (1 bar, 80 mL min^{-1}), 50 mM bicarbonate and 20 mM acetate only at the initial day. The coloured numbers above the curves in graph (A) indicate the average nitrate reduction kinetics in mM.d^{-1}. Those above the curves in graph (B) indicate the nitrite concentration reduced (mM) during each batch period.

silicone tube reactors (Karanasios et al., 2010). In this present study, the limited solubility of dihydrogen allowed only 0.74 mM of dihydrogen to be dissolved at 30 °C and 1 bar. Even though dihydrogen was supplied continuously, adding 20 mM soluble acetate provided an electrons donor 27 times more concentrated, thus dihydrogen was a limiting kinetic factor. In addition, dihydrogen provides 2 electrons when oxidized to protons while acetate provides 8 electrons when oxidized to CO_2, so there are potentially 108 times more electrons available in the presence of 20 mM acetate. This limitation of electron availability surely slowed down bacterial activity.

3.2. Transition from acetate and dihydrogen to dihydrogen only

In this experiment, the reactors were continuously fed with dihydrogen gas, and 20 mM acetate was added only on the first day of the experiment. Four batch periods were successively carried out with an initial nitrate concentration about 50 mM, the fifth batch period was initiated at 100 mM nitrate, Fig. 3. During the fourth batch period the reactors were adjusted at pH 9, 9.5, and 10. During the last batch period pH was adjusted at pH 9, 10 and 11, the reactors were named after this last batch period. The dotted pH jumps represent manual pH adjustments.

The rapidly consumed acetate reached negligible concentrations on day 25, from that day the remaining bacteria were considered using mainly hydrogenotrophic metabolism (Fig. 3). During the two initial batch periods, when acetate was still present, high nitrate reduction rates were observed despite the initial pH at 10.3. That can be explained by the pronounced acidification of 2.5 pH units, Fig. 3 (C). As the durations of the batch periods were short, denitrification was incomplete and nitrite accumulated up to 60 mM. Between day 10 and day 25, the acetate concentration became limiting and, as a result, the nitrate reduction kinetics were slowed down. From day 25, during hydrogenotrophic metabolism, the pH was adjusted manually to finally reach pH 9, 10 and 11 in the last batch period, at the same time nitrate concentration was increased from 50 mM to 100 mM in the last batch period. Therefore, the nitrate reduction was possible up to pH 10 and 100 mM nitrate, which confirms the previous findings in hydrogenotrophy, Fig. 2. It appears that nitrate reduction was strongly affected by pH rather than the nitrate concentration. Indeed, when the reactor at pH 9 had an increase from 50 to 100 mM its nitrate reduction rate did not change. On the opposite increasing pH above 9.5 in the reactors strongly decreased the reduction rates. The relatively low pH sensitivity in heterotrophy and high pH sensitivity in hydrogenotrophy can again be explained by the pH evolution during denitrification. When acetate was still present the denitrification caused acidification, which allowed high nitrate reduction rate, as in Fig. 1. On the opposite, as in Fig. 2, in the presence of dihydrogen the denitrification caused alkalinization.

3.3. pH evolution during denitrification

This opposite pH evolution can be explained through calculation. In a medium buffered by carbonate, the Henderson-Hasselbalch equation allows determining the pH from carbonate concentration (Bhagavan and Ha, 2015; Davies and Moores, 2010) according to equation (4). The final concentration of CO_3^{2-} and HCO_3^- species can be expressed by their initial concentration together with the produced or consumed concentration of CO_3^{2-} and HCO_3^-, which can be expressed in nitrate equivalents according to equations (1) or (2). The calculation, further detailed in Albina et al. (2019) results in equations (5) and (6). These equations express the final pH according to the nitrate concentration reduced to dinitrogen. The equations are applicable in the pH range between 8 and 12.

$$\mathrm{pH} \quad \mathrm{pKa} + \mathrm{Log}\left(\frac{[CO_3^{2}]_{\mathrm{final}}}{[HCO_3]_{\mathrm{final}}}\right) \quad 10.32 + \mathrm{Log}\left(\frac{[CO_3^{2}]_{\mathrm{initial}} \pm [CO_3^{2}]}{[HCO_3]_{\mathrm{initial}} \pm [HCO_3]}\right) \tag{4}$$

$$\mathrm{pH} \quad 10.32 + \mathrm{Log}\left(\frac{[CO_3^{2}]_{\mathrm{initial}} + \frac{3}{8}[NO_3]}{[HCO_3]_{\mathrm{initial}} + \frac{7}{8}[NO_3]}\right) \tag{5}$$

$$\mathrm{pH} \quad 10.32 + \mathrm{Log}\left(\frac{[CO_3^{2}]_{\mathrm{initial}} + [NO_3]}{[HCO_3]_{\mathrm{initial}} \quad [NO_3]}\right) \tag{6}$$

Equation (6) applies only when the initial bicarbonate concentration is higher than the denitrified nitrate concentration ($[HCO_3^-]$initial > $[NO_3^-]$). Otherwise, HCO_3^- is depleted and carbonate species cannot act as a buffer. According to equations (5) and (6), in a reactor with 50 mM carbonate, different pH evolution curves are plotted as a function of the initial pH and the reduced nitrate concentration, Fig. 4.

If the electron donor is acetate, the pH tends towards 10 during denitrification regardless of the initial pH. With dihydrogen, the pH increases regardless of the initial pH. Therefore, there is a fundamental difference in the pH evolution during denitrification between an organic or inorganic electron donor. The major reason is the production of CO_2 by the acetate oxidation, which as the potential to acidify the solution. Thus the hydrogenotrophic denitrification causes rapid alkalinization. In addition, in hydrogenotrophy, another phenomenon must be taken into account. Indeed, in the absence of organic carbon for growth, bacteria have to reduce and assimilate inorganic carbon. The consumption of inorganic carbon, in the CO_2 form (Blombach and Takors, 2015) or HCO_3^- form (Chollet et al., 1996; Tong, 2013) unbalances the carbonate buffer in favour of the CO_3^{2-} form, which increases the pH of the medium. Therefore, an additional explanation of the differences between heterotrophic and hydrogenotrophic denitrification is presented in this study. As the pH evolution is opposite between the two metabolisms, it strongly impacts the denitrification, especially in an alkaline environment.

The evolution of the pH has been further investigated by considering the key step of nitrite reduction. It can be stated from reactions (7) and (8) that the nitrite influence on pH could be a cause of its adverse effect. Indeed by comparing the OH^- generation during nitrite reduction to nitric oxide, reactions (7) and (8), with the OH^- generation during the whole denitrification, reactions (1) and (2), it appears the nitrite reduction is the key step which produces OH^- and generate alkalinity.

$$H_2 + 2\ NO_2 \rightarrow 2\ NO + 2\ OH \tag{7}$$

$$CH_3COO + 8\ NO_2 + 3\ H_2O \rightarrow 2\ CO_2 + 16\ NO + \mathbf{9\ OH} \tag{8}$$

Consequently, nitrite generally accumulates, so that denitrification takes place in two stages: reduction from nitrate to nitrite then reduction from nitrite to dinitrogen. Therefore, in presence of acetate, using the method of Albina et al. (2019), the pH evolution during the reduction of nitrate to nitrite and then of nitrite to dinitrogen can be respectively expressed with equations (9) and (10); in the pH range between 8 and 12. The pH evolution curves in Fig. 5 were plotted from these equations. In the presence of dihydrogen, the pH evolution during the denitrification is only caused by the nitrite reduction. Thus, the pH evolution during nitrite reduction is similar to Fig. 4 (B).

$$NO_3 \rightarrow NO_2 \quad \mathrm{pH} \quad 10.32 + \mathrm{Log}\left(\frac{[CO_3^{2}]_{\mathrm{initial}} \quad \frac{2}{8}[NO_3]}{[HCO_3]_{\mathrm{initial}} + \frac{6}{8}[NO_3]}\right) \tag{9}$$

$$NO_2 \rightarrow N_2 \quad \mathrm{pH} \quad 10.32 + \mathrm{Log}\left(\frac{[CO_3^{2}]_i + \frac{5}{8}[NO_2]}{[HCO_3]_i \quad + \frac{1}{8}[NO_2]}\right) \tag{10}$$

NB: The final pH 10 during denitrification in Fig. 4 (A), is different from the final pH 11 during nitrite reduction in Fig. 5 (B). Indeed in Fig. 5 (B), the acidifying effect of nitrate reduction is not considered. To compare both figures, the final pH of Fig. 5 (A) and the carbonate generated during nitrate reduction must be considered.

Therefore, with acetate during the reduction of nitrate to nitrite, the pH decreases regardless of its initial value, Fig. 5 (A). Then, during the reduction of nitrite to dinitrogen, the pH equilibrates towards 11, Fig. 5

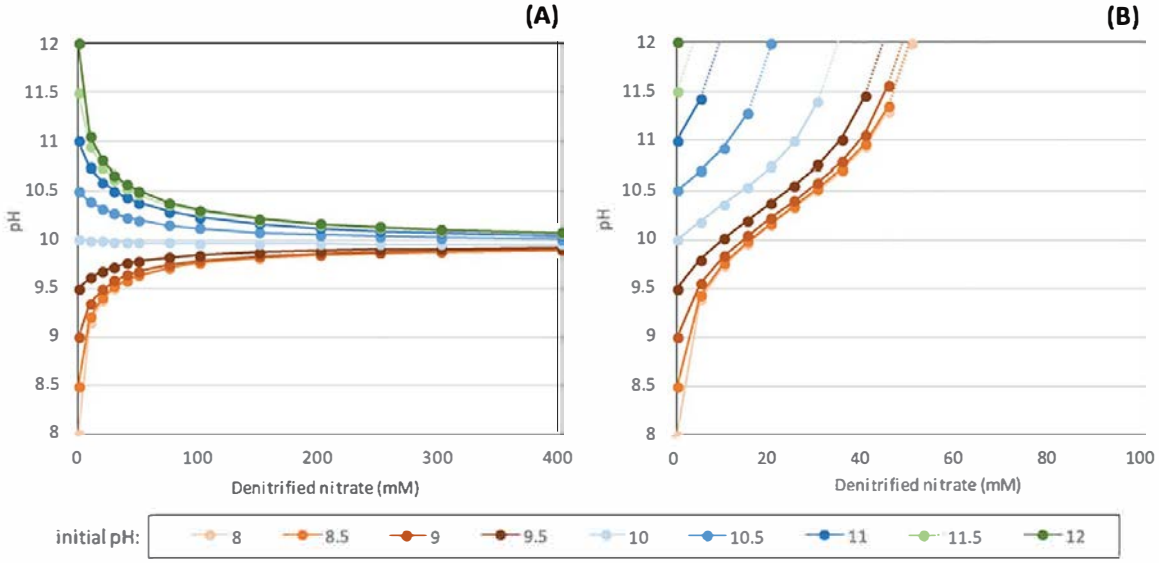

Fig. 4. pH evolution during the microbial denitrification process as a function of the initial pH and type of electron donor (acetate (A) or dihydrogen (B)), according to equations (5) and (6).

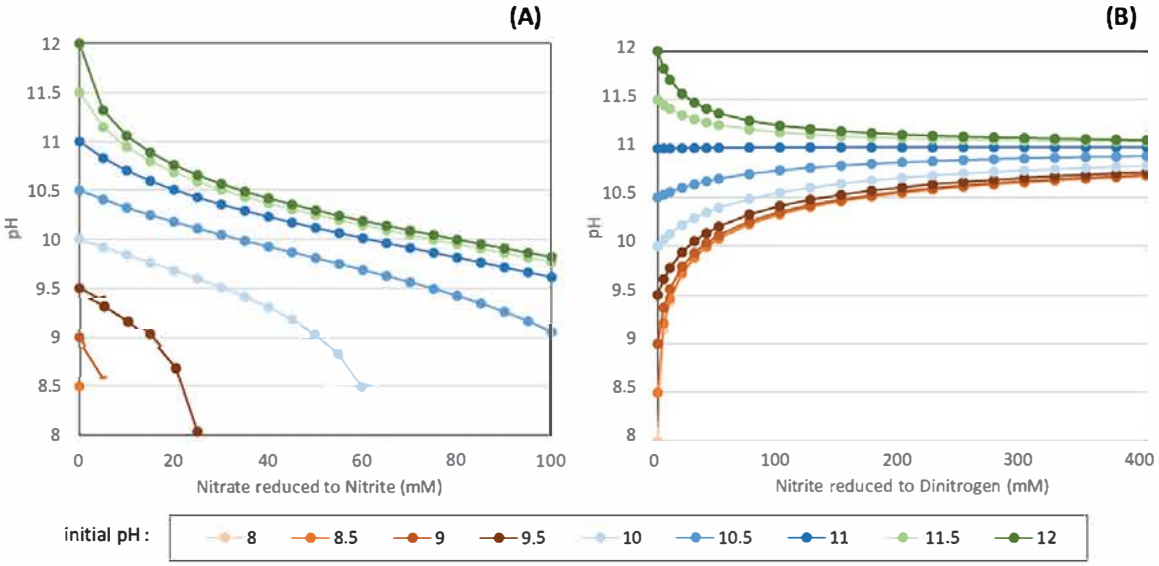

Fig. 5. pH evolution during nitrate or nitrite reduction with acetate as a function of the initial pH: nitrate reduction to nitrite (A), nitrite reduction to dinitrogen (B).

(B). Experimentally, when nitrate is reduced to nitrite and nitrite accumulates it causes a pH decrease. When nitrate becomes limiting, the reduction of nitrite causes an alkalinization toward pH 11. This shift in pH evolution due to nitrite reduction is visible in Fig. 1 (B) for the reactor at initial pH 9 on days 10 and 30. When considering the whole denitrification with acetate, the pH would always tend to 10, regardless of the initial pH. On the contrary under hydrogenotrophy, the pH increases during nitrite reduction regardless of the initial pH and bacteria may be less inclined to resorb a nitrite accumulation. This could explain why the nitrate concentrations tolerated by hydrogenotrophic bacteria are lower especially at alkaline pH. Bacteria have to choose between reducing nitrite and raising the pH or letting the nitrite accumulate at levels that may be become toxic, both solutions making their environment more inhibiting.

3.4. *Denitrification at highly alkaline pH*

3.4.1. *Denitrification in the presence of acetate*

Two reactors, of initial pH 11.5 and 12, containing 20 mM acetate and 50 mM nitrate, were monitored in batch mode, Fig. 6. Due to the high pH, the reactors were monitored over longer times periods (about 200 days). In the reactor at initial pH 12, manual acidification was carried out down to pH 11 on day 195 in an attempt to activate nitrate reduction.

In the reactor at initial pH 11.5, nitrate reduction was initiated on day 90 and accelerated on day 180 when the medium acidified to pH 11, Fig. 6 (A). In the reactor at pH 12, the nitrate concentration remained stable until day 195. By day 195, manual acidification to pH 11 allowed the start of nitrate and nitrite reduction. The medium was then simultaneously acidified, without operational intervention, from pH 11 to pH 10.3. In Fig. 6 (B), the evolution of pH and nitrite concentration are presented together to illustrate their interdependence. Indeed, in both

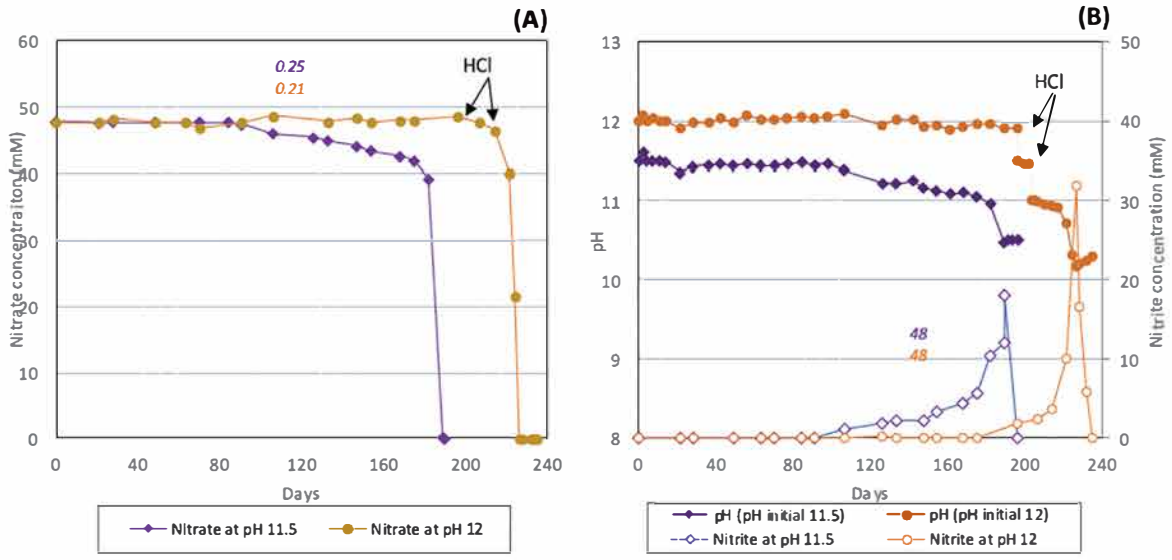

Fig. 6. Influence of highly alkaline pH with 50 mM nitrate on the dynamics of nitrate reduction (A), and on the evolutions of pH and nitrite concentration (B) in heterotrophic reactors maintained at 20 mM acetate and supplemented with 50 mM bicarbonate. The coloured numbers above the curves in graph (A) indicate the average nitrate reduction kinetics in mM.d ^{1}. Those above the curves in graph (B) indicate the final nitrite concentration reduced in mM.

reactors, the acidifications were correlated to the nitrate reduction. Then, when the nitrite accumulated started to be reduced there was pH evolution shift, as explained in section 3.2. During nitrite reduction acidification stopped, and in the reactor at initial pH 12 the medium even started to alkalinize. In Fig. 6, assuming that all the nitrate and the nitrite were reduced to dinitrogen it is possible to confront the results with the model section 3.2. Starting with 50 mM nitrate at pH 11.5 or pH 11.0 (the reactor at initial pH 12 was manually acidified to pH 11) the experimental final pH were respectively 10.5 and 10.3. According to Fig. 4 (A), the theoretical pH are respectively 10.5 and 10.4. Theoretical and experimental pH values were, therefore, really close.

Therefore, bacterial denitrification at 50 mM nitrate is possible at pH 11.5 but impossible in these conditions at pH 12 and it seems that pH 11 is a threshold value for bacterial activity. However, the bacteria were able to survive for about 200 days at pH 12 and then initiate their denitrifying activity when the environmental conditions became more favourable. These results confirm conclusions from the bibliography in which the maximum physiologically tolerated pH limit for bacteria is reported to lie between pH 11 and 12 (Rizoulis et al., 2012; Sorokin, 2005; Watts et al., 2015). The pH has a strong impact on bacterial activity as the pH affects all enzyme activity, for instance, denitrification reductases have a functional optimum pH between 7.5 and 9.0 (Kucera et al., 1986; Thomsen et al., 1994). Therefore, using long time culture allowed to observe bacterial activity at a highly alkaline pH given the optimal pH for denitrification. Experimentation in highly alkaline environments requires rigorous medium pH control (Sorokin, 2005). Indeed, the medium pH can be quickly modified by bacterial activity, in particularly denitrification products as OH^- and CO_2 strongly impact

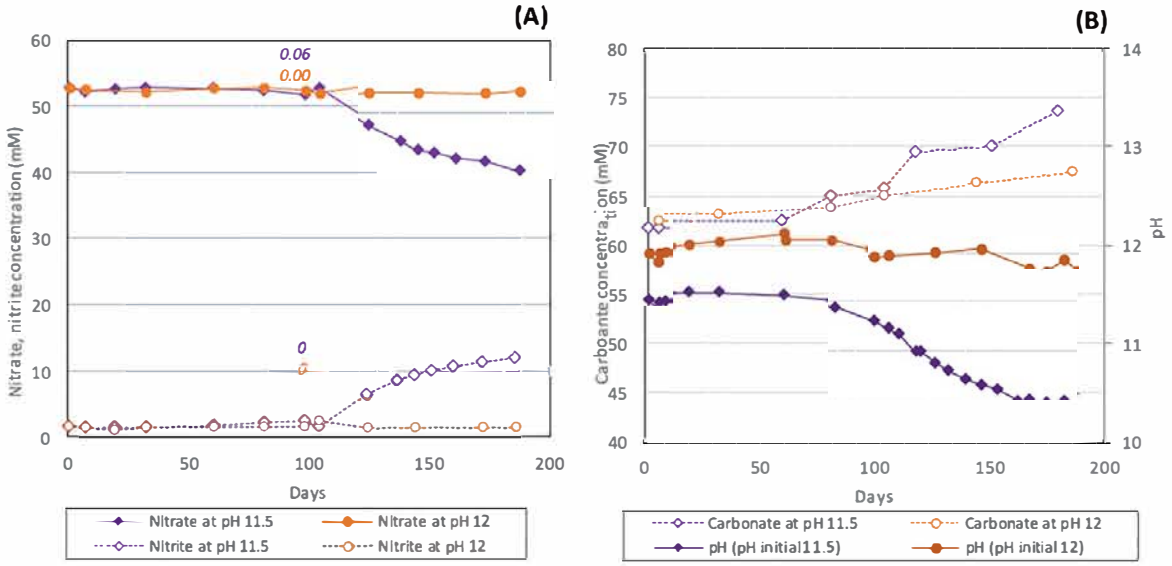

Fig. 7. Influence of highly alkaline pH with 50 mM nitrate on the evolution of nitrate and nitrite concentrations (A), and of pH and carbonate concentration (B) in hydrogenotrophic reactors fed with a constant flow of dihydrogen (1 bar, 80 mL min ^{1}), and 50 mM bicarbonate. The coloured numbers above the nitrate curves in graph (A) indicate the average nitrate reduction kinetics in mM.d ^{1}. Those above the nitrite curves in graph (A) indicate the final nitrite concentration reduced in mM.

the pH of the environment.

3.4.2. Denitrification in the presence dihydrogen

Two reactors adjusted to pH 11.5 and 12 were monitored in batch mode with 50 mM nitrate under a constant dihydrogen flow (1 bar, 80 mL min^{-1}), Fig. 7.

In the reactor initially at pH 11.5, only 10 mM nitrate was reduced while pH was acidified. The pH decreased despite the hydrogenotrophic conditions, which was contradictory with the above findings. The nitrate reduction started at day 105, after the initiation of the acidification when pH reached 11.2. Nitrite was not reduced and accumulated. The concentration of soluble carbonate ($HCO_3^- + CO_3^{2-}$) increased by 11 mM in the reactor at initial pH 11.5. In an airtight reactor with a medium initially devoid of organic matter, the increase in inorganic carbon can only have come from one source: the mineralization of the inoculum biomass. Therefore, it was hypothesised that a part of the biomass was oxidized into inorganic carbon by some of the bacteria, which were thereby able to reduce nitrate. Analyses of the bacterial communities were carried out on the final biomass of the reactor. The reactor popu-lation was composed of 93% of *Bacillus* sp. The genus *Bacillus* is described as denitrifying, heterotrophic, and haloalkaliphilic (Denariaz et al., 1989; Preiss et al., 2015). More importantly, some species, such as *Bacillus subtilis*, are described as saprophytes i.e. capable of utilizing organic matter from other bacteria by releasing digestive enzymes (Ochiai et al., 2007). The presence of a dominant alkalophilic hetero-trophic bacteria capable of oxidizing the biomass of other bacteria gives credence to the hypothesis that at pH 11.5 nitrate was reduced by heterotrophic bacteria using the biomass provided by the inoculum. Therefore, hydrogenotrophic denitrification was not possible at pH 11.5 in these experimental conditions. As for the reactor at pH 12, the nitrate was not reduced and the pH remained almost constant despite 185 days of experiment; the pH was too high for both heterotrophic and hydro-genotrophic metabolisms.

4. Conclusion

- Using acclimation protocols and long culture time, it was possible to observe bacterial denitrification at high pH and high nitrate concentration simultaneously.
- High nitrate concentration or high pH causes nitrite accumulation due to the difficulty for bacteria to reduce nitrite as it is toxic and increases the pH.
- Denitrification strongly affects the pH of the medium. During denitrification with acetate, the pH tends to 10 while during denitrification with dihydrogen the pH increases.
- Heterotrophic denitrification resulted in faster nitrate reduction kinetics and larger ranges of nitrate and pH tolerated by bacteria, 400 mM nitrate or pH 11.5.
- Hydrogenotrophic denitrifying bacteria tolerated concentrations of nitrate up to 100 mM or pH up to 11.
- Hydrogenotrophic metabolism is affected by (i) the low solubility of dihydrogen, (ii) the need for inorganic carbon assimilation, and (iii) the adverse pH evolution.
- Therefore, as soon as the nitrate concentration is greater than 50 mM or the pH is greater than 9, an organic electron donor such as acetate is advantageous for bacterial denitrification.

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Credit author statement

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jenvman.2020.111859.

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