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Adaptation of neutrophilic *Paracoccus denitrificans* to denitrification at highly alkaline pH

Pierre Albina[1,2] · Nadège Durban[1,2] · Alexandra Bertron[2] · Maud Schiettekatte[2] · Achim Albrecht[3] · Jean-Charles Robinet[3] · Benjamin Erable[1]

Abstract
Bacterial denitrification is widely documented at neutral pH in order to improve the removal of nitrate in wastewater treatment processes. However, certain industrial contexts generate alkaline waste and effluent containing nitrate that must be denitrified. To obtain more information on denitrification at alkaline pH, this study evaluated the possibility of adapting a neutrophilic denitrifying strain, *Paracoccus denitrificans*, to alkaline pH. Firstly, *P. denitrificans*' denitrifying activity was evaluated without acclimation in batch bioreactors at pH 7.0, 8.0, 9.0 and 10.0. Then, two acclimation methods using successive batch bioreactors and a continuous bioreactor allowed *P. denitrificans* to be gradually exposed to alkaline pH: from 8.5 to 11.2 in 26 and 72 days respectively. Results showed that *P. denitrificans* could grow and catalyse nitrate reduction (i) at pH 9.0 without acclimation, (ii) at pH 10.5 in successive batch cultures with progressively increasing pH and (iii) at pH 10.8 in continuously fed culture with a hydraulic retention time (HRT) of 8 days. It was shown that denitrification affected the pH despite the presence of carbonate buffering of the *P. denitrificans* growth medium. With acetate as an electron donor, the pH of a carbonate buffered medium tends towards pH 10 during the process of denitrification.

Keywords Nitrate reduction · High pH · Denitrifying bacteria · Acclimation · Batch and continuous bioreactors

Highlights
- First demonstration of denitrification with *P. denitrificans* at alkaline pH (> 9.5)
- Comparison of culture-dependent methods for bacterial acclimation
- *P. denitrificans* survived temporarily to pH 11.2 in a continuous bioreactor
- Hydraulic retention time of 8 days allowed *P. denitrificans* to adapt to pH 10.8

Responsible Editor: Gerald Thouand

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Introduction

Nitrate pollution is a persistent health and environmental problem on a worldwide scale. Among the sectors giving rise to large fluxes of nitrate, several industries generate effluent and waste also associated with alkaline pH levels. The disposal of radioactive waste deep underground faces a nitrate leaching issue with an expected pH between 9 and 13 (Francis and Hatcher 1980; Stroes-Gascoyne et al. 2011; Albrecht et al. 2013; Durban et al. 2018), and effluent from a stainless steel plant can reach pH 9.6 (Fernández-Nava et al. 2008). Denitrification has also been tested in alkaline soils (pH ≈ 10) polluted by agriculture, sampled in the former Lake Texcoco in Mexico (Ruiz-Romero et al. 2009).

Bacterial denitrification is an efficient and economical solution for nitrate removal in a polluted environment (Kapoor and Viraraghavan 1997; Mohsenipour et al. 2014). It is a reduction of nitrate to dinitrogen via four successive reduction steps ($NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$). The bacterial denitrification process is particularly

well-documented in the literature for a pH range from neutral to sub-alkaline (pH 7 to 9) (Karanasios et al. 2010; Wang et al. 2017; Xu et al. 2019). Several studies have evaluated denitrification in alkaline contexts to meet the industrial needs mentioned above. Some studies used alkaliphilic inoculate already adapted to high pH levels (Yoshida 2011; Rizoulis et al. 2012; Rafrafi et al. 2017), whilst others tested the acclimation of neutrophilic consortia to alkaline pH. Dhamole et al. succeed in acclimating activated sludge from pH 7.5 to 11.5 (Dhamole et al. 2008). However, with consortium inocula, it is rather unclear whether there is an enrichment of the population by a selection of alkaliphilic bacteria or whether bacteria evolve and adapt to alkaline pH. Therefore, the fundamental question that is addressed here is whether it is possible for a neutrophilic bacterium to adapt to alkaline pH.

To evaluate the adaptability of a bacterium to alkaline pH, a neutrophilic single strain *Paracoccus denitrificans* was confronted with alkaline pH. The success of its adaptation was evaluated through its activity (acetate and nitrate reduction). *P. denitrificans* was chosen as the model strain because it is able to produce the four reductases leading to the complete reduction of nitrate to N_2 (Qu et al. 2016), and it has a versatile metabolism adaptable to various environments (Blaszczyk 1993; Chih-Cheng and Szu-kung 1998). *P. denitrificans* has already been thoroughly investigated as a denitrifying model; it is a Gram-negative, non-mobile, facultative anaerobic bacterium. The optimal pH for *P. denitrificans* activity is around pH 8 (Thomsen et al. 1994; Chih-Cheng and Szu-kung 1998).

To successfully adapt bacteria to a stressful environment, acclimation procedures that apply progressive environmental modifications have been tested. The key to the acclimation procedure to alkaline pH is certainly related to the balance between the rate of the pH elevation and the time of bacteria generation, as adaptation occurs through mutations and selection (Brooks et al. 2011). It has already been shown that bacterial strains are capable of adapting, on time scales from an hour to a day, to different environmental changes, such as glucose (Rosenzweig et al. 1994) or oxygen (Rainey and Travisano 1998) limitation. In the present work, two acclimation procedures, successive inoculation in batch reactors and a continuous reactor, inspired by Kim et al. (2011), were compared with cultivation without an acclimation method:

- Direct batch exposure in bioreactors where the pH was initially set at 7.0, 8.0, 9.0 or 10.0.
- A gradual increase from pH 8.5 to 11.2 by successive inoculations in batch bioreactors (ΔpH ≈ 1 every 10 days)
- A gradual increase from pH 8.5 to 11.2 in a continuous-feed bioreactor (pH increase over 72 days)

Materials and methods

Bacterial strain *P. denitrificans* and growth medium

P. denitrificans was purchased from the DMSZ-German Collection of Microorganisms and Cell Cultures GmbH (strain n°413). The growth medium was based on DSMZ growth medium 81 recommended for *P. denitrificans*' optimal activity. Distilled water was supplemented with 1 g NH_4Cl, 0.5 g $MgSO_4$, 0.01 g $CaCl_2$, 0.05 g $Fe(NH_4)$citrate, 4.2 g (50 mM) $NaHCO_3$ and 10 mL of trace solution (ATCC) per litre. A total of 20 mM acetate and 10 mM nitrate were added, this ratio being used to prevent acetate from becoming limiting. The final medium COD was 1.2 g/L. Carbonate ($NaHCO_3$) was added as a buffer solution to stabilise the pH. The pH of the growth medium was adjusted by NaOH and HCl 1 M. Bioreactors were stirred at 200 rpm, and the temperature was set at 30 °C, the optimal temperature for *P. denitrificans* (Blaszczyk 1993; Thomsen et al. 1994; Chih-Cheng and Szu-kung 1998).

Batch bioreactors

Batch bioreactors were 150-mL hermetic glass flasks, containing 100 mL of growth medium flushed with N_2 after each opening to maintain anaerobic conditions.

Independent batch cultures

Four batch bioreactors at pH 7.0, 8.0, 9.0 and 10.0 were inoculated simultaneously with 1 mL of a preculture at an optical density of 0.2 (preculture = 1 week of culture at pH 8.0, with 20 mM acetate and 10 mM nitrate).

Acclimation by successive cultures

One millilitre of the same preculture as in independent batch cultures was used to inoculate the first culture at pH 8.5. When the culture reached maximal optical density (OD), 5 mL of this culture was used to inoculate the second culture at pH 9.5. This re-inoculation process was used successively at pH 10.5 and 11.2. Finally, the pH gradually increased from 8.5 to 11.2 in 26 days (Δ pH ≈ 1 every 10 days).

Continuous bioreactor

A glass bioreactor of 2.3 L was inoculated with 23 mL of preculture at 0.2 OD (preculture = 1 week culture at pH 8.0, with 20 mM acetate and 10 mM nitrate). The reactor was fed with the fresh medium by a peristaltic pump. In order to slowly increase the pH in the reactor and minimise cell washout, the feeding flow was adjusted to 0.2 ml min^{-1}, the lowest that was possible without

clogging the pipes. Therefore, the HRT was set to 8 days, (HRT = volume of reactor/influent flow rate (Najafpour 2015)). Anaerobic conditions were maintained by a constant N_2 gas flow of 80 mL min^{-1}. The pH was continuously increased from 8.5 to 11.2 over 72 days by increasing the feeding medium pH. The overall pH increase rate was 0.04 units per day. From day 72 to day 87 and from day 92 to day 125, the feeding was stopped and the reactor was monitored in batch mode. Two successive batch periods were set at pH 10.8 to obtain a better reading of *P. denitrificans* activity. Between these two batch periods (from day 87 to 92), the pH was increased to 10.8 by temporary continuous feeding.

Growth medium analysis

Optical density

Bacterial cell density was evaluated by measuring OD at 600 nm with a spectrophotometer (JENWAY 7315).

Chemical analysis

After sample filtration at 0.22 μm (Minisart PES, Fisher Scientific), soluble nitrate, nitrite and acetate concentrations were quantified by high-performance ion chromatography (Dionex ICS-3000) coupled to a conductometric detector with a chemical suppressor (Thermo Scientific ADRS 600, 4 mm).

A Thermo Scientific IonPac AS11-HC analytical column (4 × 250 mm) was used with an AG11-HC guard column (4 × 50 mm) and a KOH elution gradient. The details of the method are described in (Alquier et al. 2013).

pH monitoring

Fisherbrand pH probes with gel electrolyte connected to a multi-channel Consort data logger (model C3060) were used to monitor the pH.

Results

Acclimation of batch cultures

Independent batch cultures

According to Fig. 1a, the maximum OD decreased as the pH increased. At pH 10.0, a pronounced drop in OD marked a decline in the growth of *P. denitrificans*. The nitrate reduction yield was 100% from pH 7.0 to pH 9.0 (Fig. 1b). At the same time, the acetate reduction yield was around 65%. Therefore, the molar ratio of acetate/nitrate consumed was 0.65, which is close to the stoichiometric ratio of the complete bacterial denitrification into dinitrogen occurring with acetate as the electron donor. According to reaction (1), the acetate/nitrate molar ratio is 5/8 = 0.625.

$$5\ CH_3COO^- + 8\ NO_3^- \rightarrow 10\ CO_2 + 4\ N_2 + H_2O + 13\ OH^- \quad (1)$$

At pH 10.0, no reduction was detectable. Without any particular acclimation, *P. denitrificans* was able to maintain an observable denitrifying activity only up to pH 9.0. The activity of *P. denitrificans* stopped at a pH between 9.0 and 10.0.

Acclimation by successive inoculations

In this experiment, the pH values investigated were shifted to obtain information on the activity of *P. denitrificans* at different pH, especially between pH 9.0 and 10.0. According to Fig. 1c), the growth of *P. denitrificans* fell by more than 60% between pH 8.5 and pH 9.5. At pH 10.5 and 11.2, a negligible cellular proliferation of *P. denitrificans* cells was observed. The nitrate consumption yield was 100% at pH 8.5 and pH 9.5, Fig. 1d). Despite lower OD values compared with the independent batch experiments, *P. denitrificans* was able to remove all the nitrate, so *P. denitrificans* was successfully adapted to pH 8.5 and 9.5. The consumption of acetate decreased with increasing pH. At pH 9.5 the acetate/nitrate ratio was 0.45—below the stoichiometric ratio of 5/8. It can be considered that the denitrification was partial; a part of nitrate was not reduced to the dinitrogen stage. At pH 10.5, the nitrate reduction yield was 10%. Thus, acclimation by successive inoculations enabled *P. denitrificans* to maintain a low nitrate reduction at pH up to 10.5 with negligible cell growth.

Acclimation in a continuous bioreactor

From day 0 to day 72, the pH of the medium in the continuous flow bioreactor was progressively raised from 8.5 to 11.2 by modulating the pH of the fresh medium supply. Starting from day 72, no further denitrifying activity was detectable from *P. denitrificans*, and therefore, the continuous supply of fresh medium was stopped. A first batch period was operated simultaneously to a manual pH decrease to 10.8 (batch period means the interruption of fresh medium supply). During this first batch period of 15 days, the pH decreased to 10.6. Then for 5 days, the fresh medium was supplied again to increase the pH from 10.6 to 10.8 for the start of the second batch period on day 92. During the second batch period, the pH decreased again from 10.8 to 10.4 in 33 days. In order to facilitate the analysis of the results, the timeline of the experiment was segmented into four distinct periods defined by the pH, the OD and the nitrate consumption yield evolutions, as reported in Fig. 2a:

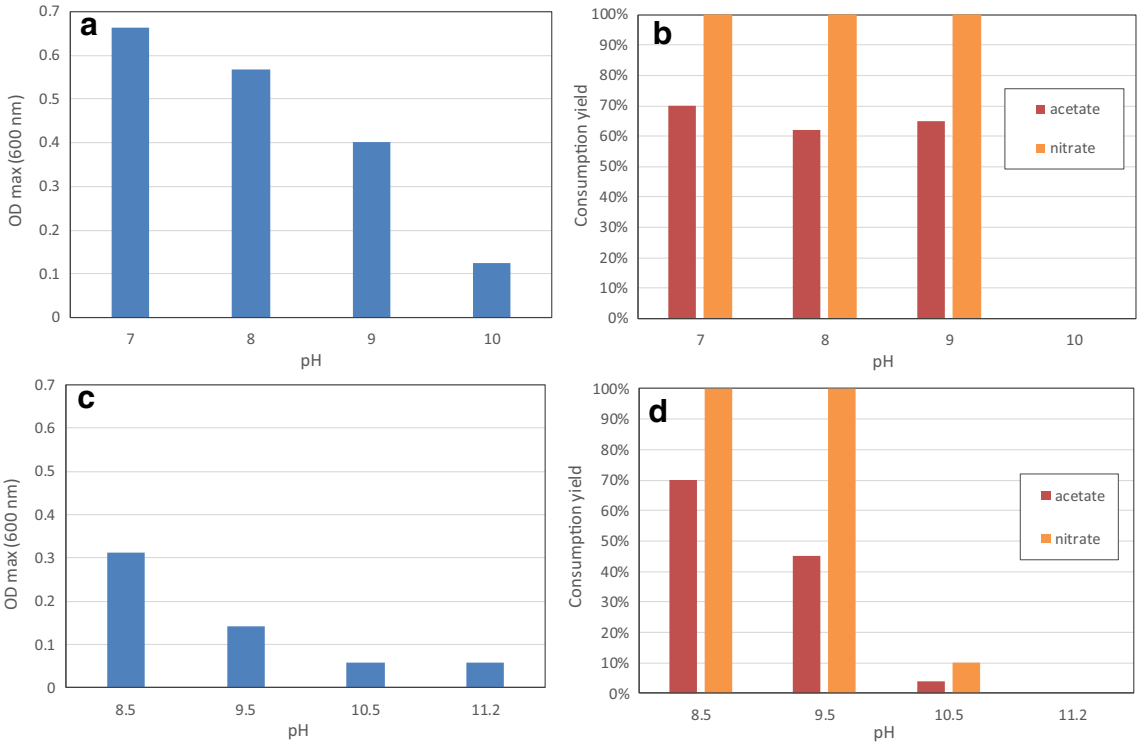

Fig. 1 Effect of the pH increase on OD evolution and on acetate and nitrate consumption yields for *P. denitrificans* batch cultures without acclimation (**a** and **b**) and for *P. denitrificans* batch cultures with successive inoculations (**c** and **d**)

- [8.5–10.5]: The OD reached a maximum value of 0.36. Both nitrate and nitrite reductions were almost total (Fig. 2b and d).
- [10.5–11.2]: The OD decreased whilst the concentration of nitrate increased in the bioreactor. Nitrate reduction and bacterial growth were negligible whilst fresh medium supplied 10 mM nitrate continuously at 0.2 mL min^{-1}.
- [10.8–10.6]: During the first batch phase, the OD remained constant; a slight reduction of 1.2 mM of nitrate (10%) was observed.
- [10.8–10.4]: During the second batch phase, the OD increased and 45% of the nitrate was reduced. Nitrate consumption and cell growth clearly accelerated during the second batch period. During the first batch period, *P. denitrificans* was still slowed down by the former alkaline pH at 11.2; then, during the second batch period, *P. denitrificans* successfully adapted to pH 10.8.

Discussion

Without acclimation, *P. denitrificans* could reduce nitrate at a pH of up to 9.0 but not at pH 10.0. In other words, *P. denitrificans* could not adapt to a pH rise greater than one pH unit. With the method using successive cultures in batch bioreactors, the pH was increased from 8.5 to 11.2 in 26 days by pH shifts of one pH unit (0.7 for the last iteration). *P. denitrificans* was able to reduce nitrate up to pH 10.5 with this method. The exposure time at each increasing pH was about 1 week (Table 1). Considering the generation time of *P. denitrificans*, which was estimated at 20 h at pH 9 in a control culture (results not presented), 7 days offered the possibility for several generations to multiply during the time of the successive batch experiments. However, increasing the pH by one pH unit likely induced a sharp change that caused cell growth inhibition.

Using the continuous pH acclimation procedure, the pH was progressively elevated from 8.5 to 11.2 in 72 days (Table 1). As a result, *P. denitrificans* was able to reduce nitrate at pH 10.8 and survive at pH 11.2 for 24 h. However, the growth of *P. denitrificans* became particularly slow at pH above 10.5. The bacterial cells were leached out of the bioreactor by the flow of the fresh medium. The interruption of fresh medium flow, i.e. the transition from continuous to batch operation, made it possible to restore the growth of *P. denitrificans* in the bioreactor and consequently to observe a further quantifiable reduction of nitrate. In conclusion, at pH > 10.5, batch periods were necessary to detect the denitrifying activity of *P. denitrificans*. These results highlight the importance of time and of progressive changes in the acclimation method.

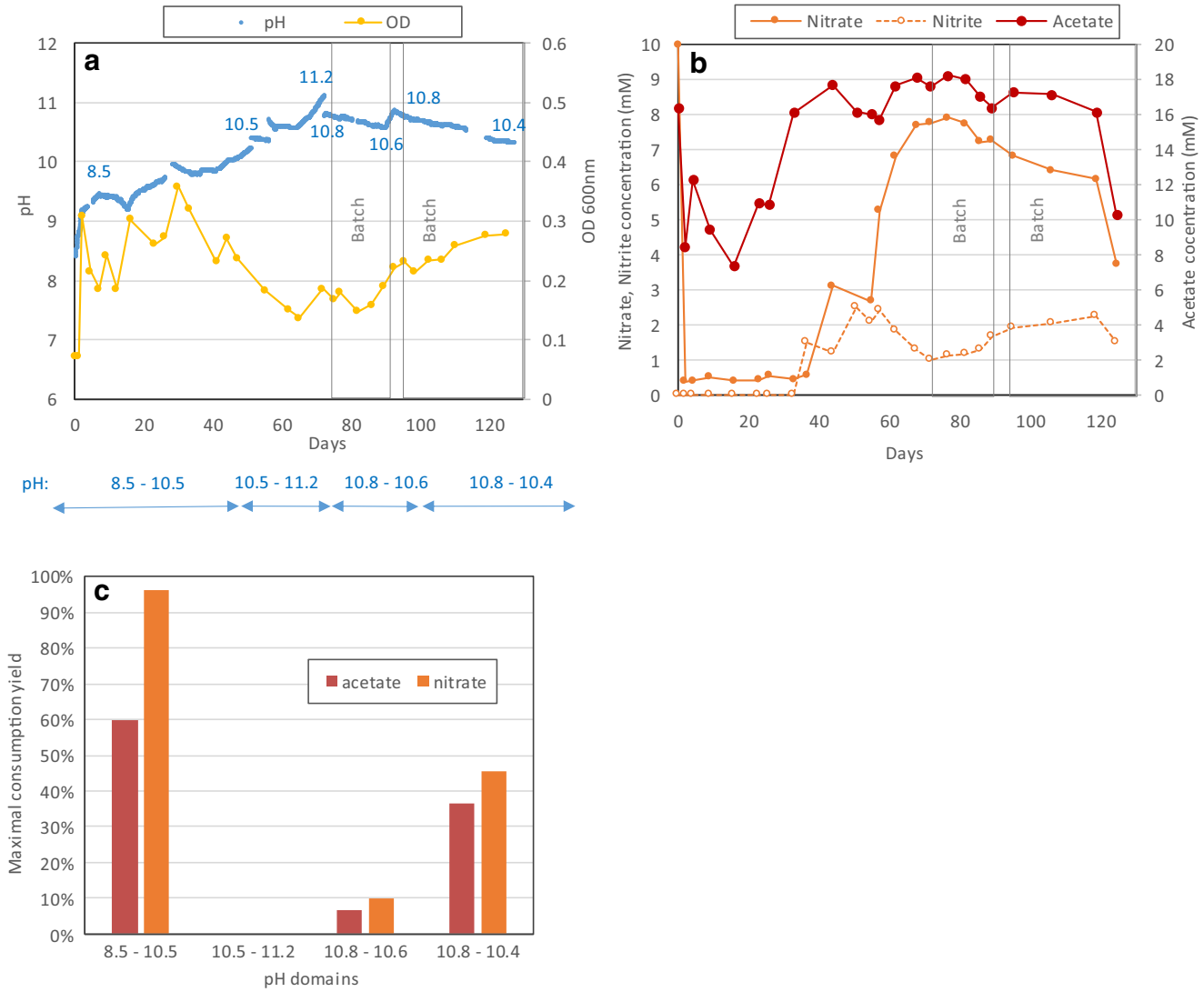

Fig. 2 Effect of the pH increase on the evolutions of OD and pH (**a**), nitrate, nitrite, and acetate concentrations (**b**), acetate and nitrate consumption yield (**c**) for *P. denitrificans* cultivation in a continuous flow bioreactor

Table 1 Overview of the initial pH, nitrite reduction, duration of each batch culture or continuous culture period and the pH evolution for each experimental test condition

	pH	Nitrite reduction	Time (days)	pH evolution
Cultures without acclimation	7	Total	14	↗ to 8
	8	Total	14	↗ to 9
	9	Total	14	↗ to 9.4
	10	Null	14	=
Cultures acclimated by successive inoculations	8.5	Total	4	↗ to 9.2
	9.5	Total	10	↗ to 9.6
	10.5	Total	12	=
	11.2	Null	12	=
Culture acclimated in a continuous flow bioreactor	8.5–10.5	Total	52	↗
	10.5–11.2	Null	20	↗
	10.8–10.6	Null	15	↘
	10.8–10.4	Total	33	↘

In the pH evolution column, the symbol "↗" means pH increase, "↘" means pH decrease, "=" means that the pH evolution was negligible

To adapt to alkaline pH, *P. denitrificans* is thought to use pH sensors to trigger pH regulation systems. Bacterial pH sensors have been described in the literature, usually as transcriptional factors, sigma factors or other DNA binding proteins (Krulwich et al. 2011). In *Escherichia coli*, the Na/H transporter also plays a role in detecting pH changes (Krulwich et al. 2011). Then, at alkaline pH, several pH regulation systems, such as the membrane transporters Na/H or K/H or drugs/H, would be stimulated (Padan et al. 2005; Ling et al. 2018). To adapt to long-term environmental change, new adaptive mechanisms can emerge by means of spontaneous mutations acquired over generations and selected in response to the environmental pH pressure. According to the evolutionary convergence theory, two distinct bacterial species facing the same environmental pressure may develop a similar adaptive characteristic. Therefore, the adaptive mechanisms acquired by *P. denitrificans*, confronted to a high pH, could be similar to, or at least serve the same purpose as, those described for other alkaliphilic bacteria:

- Modifications of the cell wall to shift the permeability to protons (Horikoshi 1999; Preiss et al. 2015; Ling et al. 2018).
- Creation of microenvironments protected from alkaline pH by cells structuring into biofilm, or cell membrane structuration into micro-domains (Preiss et al. 2015; Sanhueza et al. 2015).
- Stimulation of the synthesis of membrane transporters maintaining proton flows towards the cytosol: Na/H anti-transporters, K/H anti-transporters and the Mrp(Na/H) anti-transporter (Horikoshi 1999; Preiss et al. 2015).
- Modifications in the amino acid sequence of proteins causing structural changes (multiplication of hydrogen, hydrophobic bonds etc.) to adapt their pH tolerance (Shirai et al. 1997, 2007; Dubnovitsky et al. 2005).
- Synthesis of chaperone proteins that restore the functional structure of proteins denatured by high pH (Sanhueza et al. 2015; Ling et al. 2018).

Results also showed that, during bacterial denitrification, the pH of the medium could increase or decrease despite the presence of carbonate buffering the solution. In batch cultures with an initial pH below pH 9.5, the pH systematically increased. In the continuous bioreactor, during the two batch periods at pH 10.8, the pH decreased. Therefore, according to the initial pH, two opposite pH evolutions were observed: acidification starting from pH 10.8 and alkalinisation at pH < 9.5. Similarly, in the literature, 'self-acidification' at high pH of 10.0 or 11.0 (Mateju et al. 1992; Glass and Silverstein 1998; Durban et al. 2018) and 'self-alkalinisation' at pH 7.0 (Li et al. 2014, 2015) have been reported in denitrifying cultures.

Considering a theoretical reaction in which all nitrate is reduced to dinitrogen in the presence of acetate and bacteria, it is possible to model the pH evolution from denitrification reaction (1) using the same calculation as Albina et al. (2019). During the reaction, the CO_2 production can acidify the solution whilst the OH^- can alkalinise the solution. Considering that, in the 8–12 pH domain, OH^- reacts with the CO_2, producing HCO_3^- and CO_3^{2-} according to reaction (2), the reaction (1) can be transformed into reaction (3).

$$10\ CO_2 + 13\ OH^- \rightarrow 7\ HCO_3^- + 3\ CO_3^{2-} + 3\ H_2O \quad (2)$$

$$5\ CH_3COO^- + 8\ NO_3^- \rightarrow 7\ HCO_3^- + 3\ CO_3^{2-} + 4\ N_2 + 4\ H_2O \quad (3)$$

Therefore, the final pH can be calculated by the Henderson-Hasselbalch equation according to the initial pH (determined by the initial HCO_3^- and CO_3^{2-} concentrations) and the concentration of nitrate reduced to dinitrogen. Equation (4) expresses the pH evolution during complete denitrification with acetate.

$$\begin{aligned} \mathrm{pH} &= 10.32 + \mathrm{Log}\left(\frac{\left[CO_3^{2-}\right]_{\mathrm{initial}} + \frac{3}{8}\left[NO_3^-\right]_{\mathrm{reduced}}}{\left[HCO_3^-\right]_{\mathrm{initial}} + \frac{7}{8}\left[NO_3^-\right]_{\mathrm{reduced}}}\right) \\ \mathrm{pKa}\left(HCO_3^-/CO_3^{2-}\right) &= 10.32 \\ \mathrm{pH} &= \mathrm{pKa} + \mathrm{Log}\left(\frac{\left[CO_3^{2-}\right]_{\mathrm{final}}}{\left[HCO_3^-\right]_{\mathrm{final}}}\right) \\ \text{with :} \quad &= \mathrm{pKa} + \mathrm{Log}\left(\frac{\left[CO_3^{2-}\right]_{\mathrm{initial}} + \left[CO_3^{2-}\right]_{\mathrm{produced}}}{\left[HCO_3^-\right]_{\mathrm{initial}} + \left[HCO_3^-\right]_{\mathrm{produced}}}\right) \\ &= \mathrm{pKa} + \mathrm{Log}\left(\frac{\left[CO_3^{2-}\right]_{\mathrm{initial}} + \frac{3}{8}\left[NO_3^-\right]_{\mathrm{reduced}}}{\left[HCO_3^-\right]_{\mathrm{initial}} + \frac{7}{8}\left[NO_3^-\right]_{\mathrm{reduced}}}\right) \end{aligned} \quad (4)$$

According to the equation given above, during the heterotrophic denitrification with acetate, if the initial carbonate concentration is negligible compared with the nitrate reduced, the pH tends to 10.0 (10.32 + log (3/7) = 10.0) regardless of the initial pH. This explains why, in the cultures at pH 10.8, the medium acidified, while, in the culture below pH 9.5, the medium alkalinised. In our study with 50 mM carbonates for batch cultures at pH 7, 8, 8.5, 9 and 9.5, where both nitrate and nitrite were totally reduced (see Table 1), the theoretical final pH calculated would be respectively 9.1, 9.1, 9.2, 9.4 and 9.6. The data from the theoretical evaluation are relatively close to the experimental data presented in Table 1 except for the culture at pH 7. In the culture at pH 7, the very rapid depletion of nutrients could ultimately lead to lysis of bacterial cells (apoptosis phenomena) leading to a significant release of protons and, consequently, decreasing the pH. In this simple synthetic medium, it is therefore possible to predict the pH evolution during denitrification. In more complex and heterogeneous

media such as wastewater, several compounds can interfere with the pH evolution (phosphate, carbonate, organic acids, etc.). However, whatever the type of alkaline environment, the production of CO_2 by the oxidation of acetate or other organic compounds would be a mean of acidification. On the other hand, the chemical nature of the medium may influence the rapidity of the pH evolution. Natural buffers such as carbonates (water hardness) or phosphates would slow down the pH evolution.

Conclusion

The acclimation method, especially the rate of the pH rise, impacted the activity of *P. denitrificans*. Without pH acclimation, neutrophilic *P. denitrificans* was active at pH 9.0. When a progressive pH elevation was applied for a period of 72 days with a continuous supply of fresh medium in a bioreactor, *P. denitrificans* was able to adapt and reduce nitrate at pH up to 10.8. Denitrification also affected the pH despite the presence of a buffer. It has been highlighted that, in synthetic media in which carbonates are in excess, the pH tends to 10.0 during the denitrification with acetate.

Funding information This research was financially supported by the French National Radioactive Waste Management Agency (Andra) and by the University of Toulouse.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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