ELSEVIER

FEMS Microbiology Ecology 48 (2004) 379–386

FEMS MICROBIOLOGY Ecology

www.fems-microbiology.org

Use of chlorate as a selective inhibitor to distinguish membrane-bound nitrate reductase (Nar) and periplasmic nitrate reductase (Nap) of dissimilative nitrate reducing bacteria in sediment

Iman Rusmana [1], David B. Nedwell *

Department of Biological Sciences, University of Essex, Wivenhoe Park, Colchester CO4 3SQ, UK

Received 28 July 2003; received in revised form 20 February 2004; accepted 20 February 2004

First published online 19 March 2004

Abstract

The use of chlorate as a selective inhibitor of dissimilative nitrate reduction was studied using pure cultures of *Comamonas testosteroni* (a denitrifier) and *Klebsiella pneumoniae* (a nitrate-ammonifier) isolated from estuarine sediment, and in sediment slurry. Pure culture experiments demonstrated that chlorate selectively inhibited membrane-bound nitrate reductase (Nar) activity, probably by blocking nitrate transporters (NarK). Sediment slurry experiments showed that chlorate inhibited nitrate reduction and N_2O formation, but did not inhibit nitrite reduction and its N_2O formation, indicating that chlorate selectively inhibited only the first step of nitrate reduction. Chlorite chemically oxidized nitrite to nitrate and could not be used as a selective inhibitor of nitrite metabolism, although chlorite apparently selectively inhibited formation of N_2O from nitrite. Chlorate can be used as a specific inhibitor to distinguish between nitrate reduction by Nap or Nar in natural communities of microorganisms.

Keywords: Chlorate; Chlorite; Membrane-bound nitrate reductase; Periplasmic nitrate reductase

1. Introduction

Dissimilatory nitrate reduction plays a key role in the biological nitrogen cycle. There are two distinct processes of dissimilatory nitrate reduction: denitrification, reduction of nitrate to gaseous nitrogen (N_2O or N_2), and nitrate ammonification, reduction of nitrate to ammonium. These processes are widely distributed among bacteria. Denitrification is performed by organisms with respiratory type metabolism, such as *Pseudomonas* species, and nitrate ammonification by bacteria with a fermentative type of metabolism such as *Aeromonas*, *Klebsiella*, *Wolinella* and *Vibrio* spp. [1]. Both processes occur under oxygen-limited or anaerobic condition. Although the complete pathway for microbial denitrification has been established as: $NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$, and the pathway of nitrate ammonification has been published as $NO_3^- \rightarrow NO_2^- \rightarrow NH_4^+$, with N_2O as byproduct [1], distinguishing the processes in mixed cultures or complex natural microbial communities is very difficult, even using a tracer isotope ^{15}N technique, due to the common substrate (NO_3^-) and gaseous intermediates or byproduct (N_2O) [1].

Due to the similarities between chemical structures of chlorate and nitrate, and of chlorite and nitrite, chlorate has been used as a selective inhibitor to study nitrification in activated sludge [2], flooded soil [3], acid forest soil [4,5], grassland soil [4] and in the Rhone River plume [6]. Chlorate has been also used in molecular studies of dissimilative nitrate reduction to differentiate between the activities of two different nitrate reductases, Nar and Nap [7], in *Pseudomonas* sp. G-179 [8], *Paracoccus denitrificans* [9], the archaeon *Haloferax volcanii* [10] and *Anabaena* sp. PCC-7120 [11].

* Corresponding author. Tel.: +44-1206-87211; fax: +44-1206-873416.

E-mail address: nedwd@essex.ac.uk (D.B. Nedwell).

[1] Present address: Department of Biology, Bogor Agricultural University, Jl. Pajajaran, Bogor 16144, Indonesia.

0168-6496/$22.00 © 2004 Federation of European Microbiological Societies. Published by Elsevier B.V. All rights reserved.
doi:10.1016/j.femsec.2004.02.010

Chlorate and chlorite may enter the environment as by-products of bleaching processes in the textile, pulp and paper industries [12], as disinfectant in several applications, or as a by-product of ozonation of drinking water treated with chlorine [13]. Chlorate is chemically stable in water, and conventional water and physical treatments are not effective in removing this oxyanion of chlorine [14]. Many studies investigating the toxicity of chlorate/chlorite have been made in algae, bacteria and fungi [15,16], including *Aspergillus nidulans* [17] and *Salmonella typhimurium* [18]. Previously, there was a suggestion that chlorate toxicity was caused by simple competition with nitrate reduction [19]. Other researchers [20,21] suggested that chlorite, a reduction product of chlorate, was responsible for chlorate toxicity to *Proteus mirabilis* [20], *Rhodobacter capsulatus* and *Rhodobacter sphaeroides* [21]. However, few data are available on the selective inhibition mechanism of chlorate on dissimilative nitrate reduction, and most are for model organisms such as *Escherichia coli* which are not representative of estuarine nitrate utilizing populations. Therefore, this study was undertaken to investigate the specificity of chlorate as a selective inhibitor of dissimilative nitrate reduction both in pure cultures of *Comamonas testosteroni* (a denitrifier) and *Klebsiella pneumoniae* (a nitrate ammonifier), isolated from estuarine sediment, and in sediment slurry, as a potential tool for investigating nitrogen cycling in estuarine environments.

2. Materials and methods

2.1. Chemical analyses

Nitrate, nitrite and ammonium were analysed colorimetrically [22]. Nitrate was analysed as nitrite after samples had been passed through a copper–cadmium reduction column [22]. Ammonium was measured using a modified indophenol blue method with dichloroisocyanurate as chlorine donor and developed in the dark [23–25]. N_2O was measured with a gas chromatograph (GC) fitted with an electron capture detector [26,27].

Chlorate was analysed by ion exchange chromatography (series 2000 I, Dionex Corp) with a 1.7-mM $NaHCO_3$ and 1.8-mM Na_2CO_3 eluent, at a flow rate of 3 ml min^{-1} and regenerant 0.07% solution of concentrated sulphuric acid at a flow rate of 8 ml min^{-1}. Peak areas of samples were measured with an integrator (Dionex Corp) and compared to those of known standards.

2.2. Bacterial isolates

Comamonas testosteroni [28] and *K. pneumoniae* [29], isolated from sediment of Colne estuary, East Coast of England, United Kingdom, were used as model examples of a denitrifier and a dissimilative nitrate ammonifier, respectively. *C. testosteroni* has both membrane-bound nitrate reductase (Nar) and periplasmic nitrate reductase (Nap), while *K. pneumoniae* has only Nar [30]. The estuary and the sample sites used have been described in [27].

2.3. Inhibition by chlorate of nitrate reduction by C. testosteroni and K. pneumoniae

Denitrifying bacteria were grown on the medium of Barford et al. [31] with 1.87 mM ammonium and 15 g l^{-1} NaCl. Ammonium was added as a source of nitrogen for assimilation so that nitrate was only used dissimilatively. Sodium acetate (2.72 g l^{-1}) was the carbon source for growth of *C. testosteroni* and glycerol for *K. pneumoniae*. The medium was made anoxic by gassing with oxygen-free N_2 (OFN) to remove dissolved oxygen and oxygen in the headspace.

An initial experiment with five different concentrations of chlorate showed that 10 mM chlorate was the minimum concentration required to inhibit nitrate reduction by both bacteria (data not shown). This concentration of chlorate was used to investigate more detail of its effect on both *C. testosteroni* and *K. pneumoniae*. The experiments were conducted using anoxic medium in triplicate flasks sealed with suba-seals. Anaerobiosis was established by gassing the medium with OFN for 15 min. Flasks were incubated on a shaker (150 rev min^{-1}) at 20 °C, and chlorate or chlorite (10 mM final concentration) was added after 24 h. Subsamples of headspace gas and culture were taken daily up to 5 days. Nitrous oxide concentration was measured in the subsamples of headspace gas, while nitrate, nitrite and ammonium in the cultures were analysed using the methods described previously.

2.4. Inhibitory concentration of chlorate and chlorite on N_2O formation in sediment

Sediment was taken from 0 to 2 cm layer of sediment at the Hythe, Colne estuary. The slurry was made by mixing sediment 50% (v/v) with anaerobic artificial seawater (2% salinity, made with Tropic Marine salts, Chorley Wood, UK) and placed in an Erlenmeyer flask fitted with a subaseal. Anaerobic conditions were maintained by gassing with OFN for 15 min. An initial experiment to determine the optimum inhibitory concentration of chlorate and chlorite was conducted with 20 ml volumes of slurry in 50-ml bottles. A series of flasks of slurry with initial concentrations of either 1 mM nitrate or nitrite were dispensed, each with initial concentrations of 0, 1, 10, 20, 50 and 100 mM chlorate or chlorite (triplicate

flasks at each concentration). After incubation for 3 h at 20 °C, N_2O concentration in the headspace was analysed using the GC. The optimum concentration of chlorate/chlorite that inhibited N_2O formation in this experiment was used for the next experiment.

2.5. Inhibition by chlorate/chlorite of nitrate/nitrite reduction in sediment slurry

Aliquots of 120 ml slurry were transferred into 200-ml flasks and sealed with subaseals and gassed again with OFN for 15 min. The minimum concentration of the inhibitors (20 mM) determined from the previous experiment was used to examine the effects of chlorate and chlorite on nitrate and nitrite reduction. Flasks of slurry were set up as described with initial concentrations of 1 mM of either nitrate or nitrite and incubated on a shaker (150 rev min^{-1}; at 20 °C) for up to 6 h. Sub-samples of headspace gas and slurry were taken hourly. N_2O concentration was measured in sub-samples of headspace gas, while nitrate, nitrite and ammonium in slurry sub-samples were analysed using the methods described previously. To determine the effect of chlorate on the gaseous end products of denitrification, similar experiments were conducted with and without acetylene block. Acetylene was used to inhibit further reduction of N_2O to N_2, and total N_2 production in the slurry was calculated as

(N_2O formation in presence of acetylene)

– (N_2O formation in absence of acetylene).

3. Results

3.1. Inhibition by chlorate of nitrate reduction in pure cultures of C. testosteroni

Nitrate, nitrite and N_2O concentrations in the cultures of *C. testosteroni* are shown in Fig. 1. Addition of chlorate after 24 h inhibited nitrate removal over the following two days by *C. testosteroni* by 56%. Nitrite concentration increased on the first day in both treatments, prior to chlorate addition, and decreased after chlorate addition until the end of incubation. N_2O production appeared to be inhibited by chlorate. The concentration of chlorate during the incubation period was stable, indicating that there was no removal of chlorate from the cultures.

3.2. Inhibition by chlorate of nitrate reduction in pure cultures of K. pneumoniae

Nitrate, nitrite, ammonium and N_2O concentrations in the cultures of *K. pneumoniae* are shown in Fig. 2. The data show that both nitrate reduction and ammonium formation were completely inhibited after chlorate addition, indicating that chlorate inhibited nitrate ammonification by *K. pneumoniae*. In the control, nitrite concentration increased during the first day and then decreased as nitrite was reduced to ammonium. In the chlorate treatment, nitrite concentrations did not decrease after chlorate addition, indicating that nitrite reduction to ammonium was also inhibited by chlorate. N_2O production was completely inhibited by chlorate.

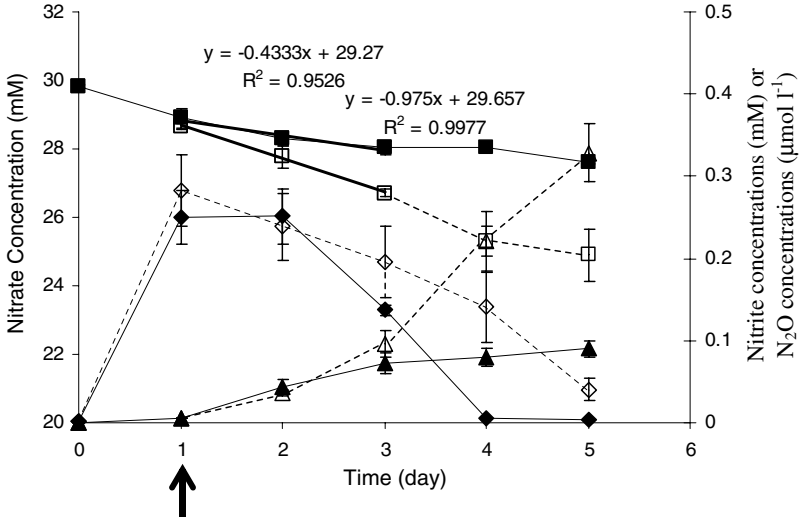

Fig. 1. Nitrate, nitrite, N_2O concentrations in the cultures of *C. testosteroni* either with or without addition of 10 mM chlorate: →, chlorate addition; □, nitrate in control (without chlorate addition); ■, nitrate with 20 mM chlorate addition; ◇, nitrite in control; ◆, nitrite with 20 mM chlorate addition; △, N_2O in control and ▲, N_2O with 20 mM chlorate addition. Bars indicate SEs ($n = 3$). Bold lines indicate linear regression of nitrate concentration versus time for 2 days after chlorate addition.

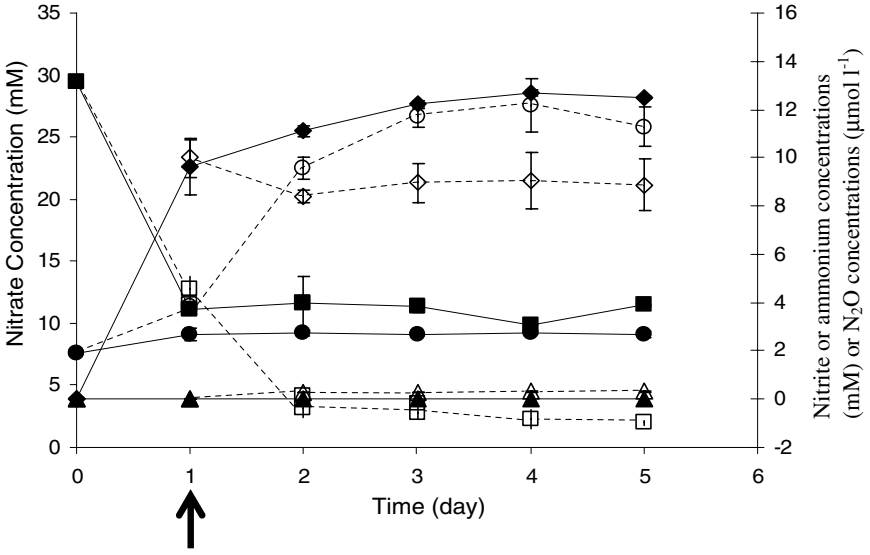

Fig. 2. Nitrate, nitrite, N_2O concentrations in the cultures of *K. pneumoniae* either with or without addition of 10 mM chlorate: →, chlorate addition; □, nitrate in control (without chlorate addition); ■, nitrate with 20 mM chlorate addition; ◇, nitrite in control; ◆, nitrite with 20 mM chlorate addition; △, N_2O in control; ▲, N_2O with 20 mM chlorate addition; ○, ammonium in control and ●, ammonium with 20 mM chlorate. Bars indicate SEs ($n = 3$).

In the control, N_2O production mostly occurred towards the end of the experiment. Chlorate concentration was stable throughout the experiment, and there was no reduction of chlorate in the cultures.

3.3. Optimum concentrations of chlorate and chlorite that inhibit nitrate/nitrite reduction in slurry

The initial experiment to examine the inhibitory effect of chlorate and chlorite showed that N_2O production from nitrate was inhibited by 10 mM of either chlorate or chlorite. In contrast, N_2O production from nitrite was only inhibited by chlorite but not by chlorate. The minimum concentration of chlorate and chlorite required to completely inhibit N_2O production was 20 mM, and this concentration was used for further experiments.

3.4. Inhibition by chlorate and chlorite of nitrate/nitrite reduction in slurry

Nitrate and nitrite concentrations during 6 h of incubation in the slurry with chlorate, chlorite and control treatments are shown in Fig. 3. Chlorate and chlorite both apparently inhibited nitrate removal from the slurry compared to the control; chlorite apparently inhibiting nitrate removal more than chlorate. However, chlorate did not inhibit nitrite removal which was identical to that in the control (see Fig. 3(b)). In contrast, chlorite apparently inhibited both nitrate (Fig. 3(a)) and nitrite reduction but from nitrite being immediately removed in the presence of chlorite (Fig. 3(b)), and from the presence of high nitrate immediately after chlorite addition to slurry containing nitrite (data not shown), it was evident that the effect of chlorite was due to an immediate chemical oxidation of nitrite to nitrate.

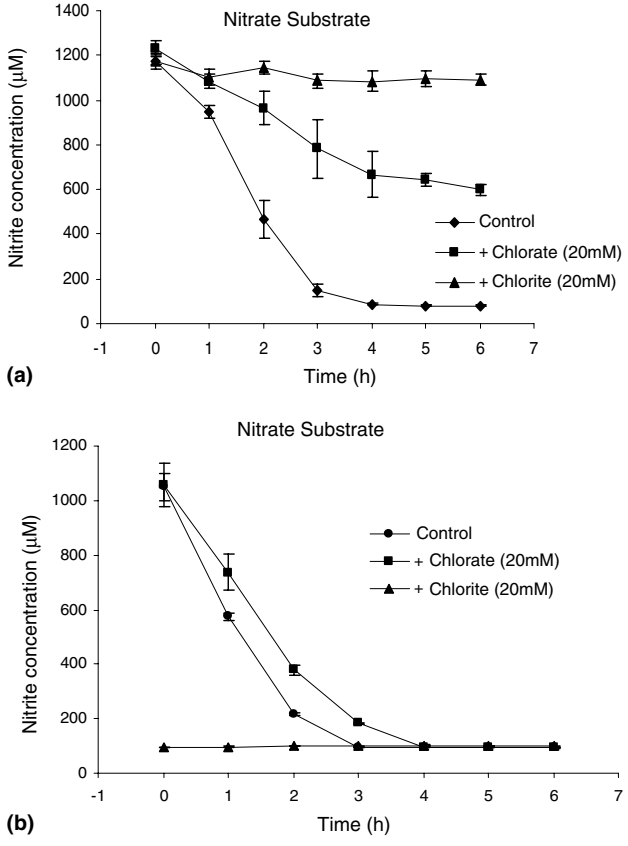

Fig. 3. Nitrate and nitrite concentration during 6 h of incubation with chlorate, chlorite and control treatments in sediment slurry. (a) Nitrate substrate and (b) nitrite substrate. Bars indicate SEs ($n = 3$).

Nitrite concentrations in the slurry with nitrate added are shown in Fig. 4. There was a peak of nitrite concentration from nitrate reduction in the control after 2 h incubation, which then decreased due to further reduction of nitrite. Addition of both chlorate and chlorite apparently inhibited reduction of nitrate to nitrite. However, addition of chlorite produced a high concentration of nitrate immediately after addition of nitrite, and the apparent inhibitory effect of chlorite was an artefact of chemical oxidation of nitrite to nitrate by chlorite.

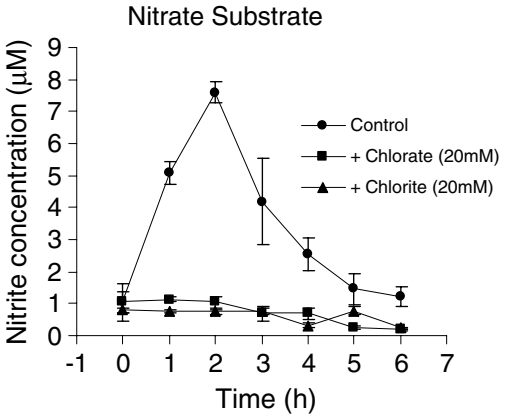

Fig. 4. Changes in nitrite concentration in slurry with added nitrate, with chlorate, chlorite and control treatments. Bars indicate SEs ($n = 3$).

N_2O concentration up to 6 h of incubation in the control, chlorate added, and chlorite added is shown in Fig. 5. The N_2O concentrations increased until incubation for 3 h in the controls with either nitrate or nitrite added. N_2O production from nitrate was inhibited by both chlorate and chlorite, while N_2O production from nitrite was inhibited by chlorite but not by chlorate. This implied that chlorite actually did inhibit nitrite reduction to N_2O, even though most nitrite was oxidized back to nitrate.

3.5. Effect of chlorate on nitrate reduction in sediment slurry and its gaseous end-product with acetylene block technique

Nitrate, nitrite and N_2O concentrations in sediment slurry with and without acetylene block are shown in Fig. 6. The data show that the acetylene block treatment did not affect the rate of nitrate reduction (the linear regressions for nitrate removal ± acetylene were not

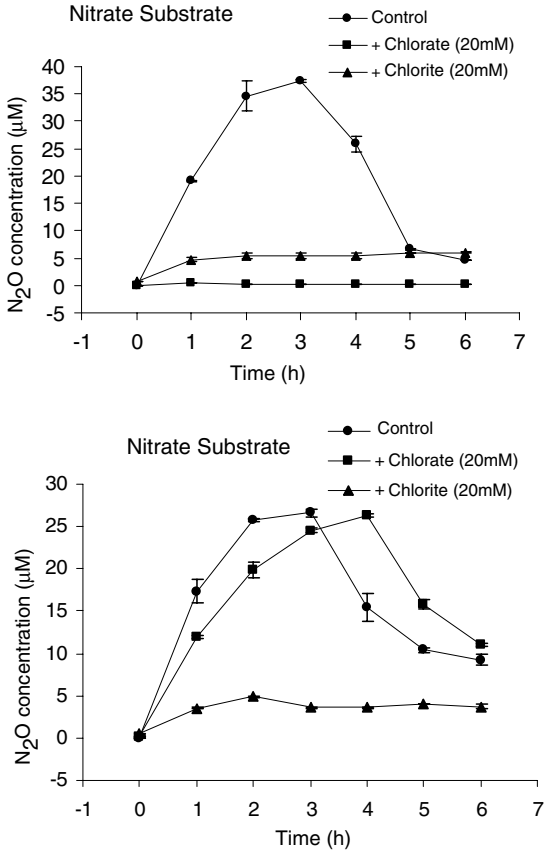

Fig. 5. N_2O concentration in the headspace of slurry with nitrate or nitrite added as substrates, with chlorate, chlorite and control treatments. Bars indicate SEs ($n = 3$).

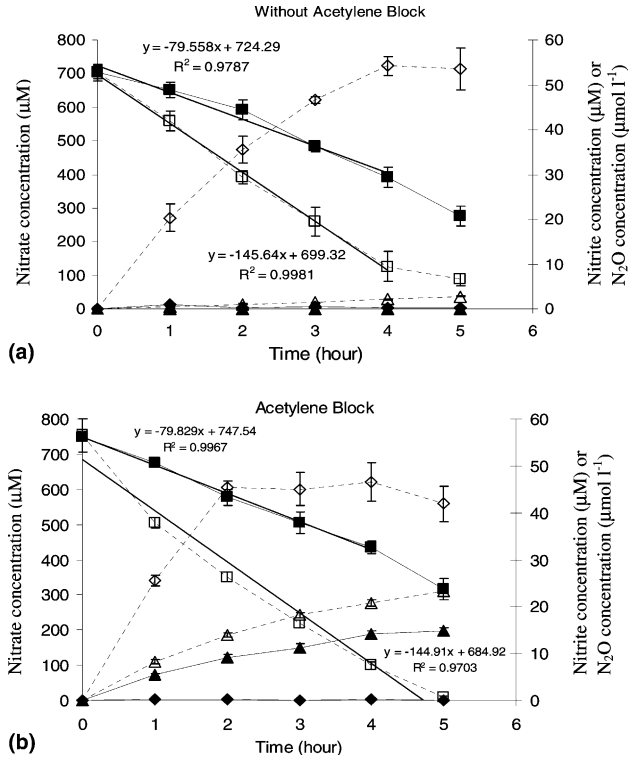

Fig. 6. Nitrate, nitrite and N_2O concentrations in sediment slurry: (a) without acetylene block, (b) with acetylene block; □, nitrate in control (without chlorate addition); ■, nitrate with 20 mM chlorate addition; ◇, nitrite in control; ◆, nitrite with 20 mM chlorate addition; △, N_2O in control and ▲, N_2O with 20 mM chlorate addition. Bars indicate SEs ($n = 3$). Bold lines indicate linear regression of nitrate concentration with time.

significantly different). However, chlorate inhibited the rate of nitrate reduction by 45% of that in the control in the series of flasks either with or without acetylene (45.3% without acetylene and 44.9% with acetylene), and completely inhibited nitrite production. N_2O concentration in the headspace increased with time in controls both with and without acetylene, and also in the chlorate treatment with the acetylene block. However N_2O accumulation in chlorate treatment without the acetylene block was completely inhibited. N_2O concentration in the control with acetylene was approximately 100 times greater than without acetylene block, confirming that the majority of the nitrate was denitrified to N_2. In addition, N_2 formation was slightly inhibited by chlorate. Chlorate concentration in sediment slurry was stable in the slurry both with and without acetylene inhibition, and there was no removal of chlorate during the incubation period.

4. Discussion

4.1. Effect of chlorate on nitrate reduction in C. testosteroni and K. pneumoniae

Much of the research done on denitrification and nitrate ammonification has been carried out with model organisms such as *E. coli* that have probably little relevance to estuarine sediments. We used *C. testosteroni* that has Nar and Nap as a model denitrifier from sediment and *K. pneumoniae* that has only Nar as a model nitrate ammonifier. Chlorate concentration in both *K. pneumoniae* and *C. testosteroni* cultures did not change with time, confirming that no chlorate was being reduced during the experiments, although it has been reported that Nar can reduce chlorate [8,32–34]. It can be concluded that chlorate inhibited Nar activity in both the denitrifier and nitrate ammonifier. Our results showed that there was complete inhibition by chlorate of both nitrate removal and ammonium accumulation in *K. pneumoniae* which has only the *nar* gene. In contrast, in *C. testosteroni* which has both *nar* and *nap*, only some 50% of nitrate removal and N_2 formation (as determined by acetylene inhibition) was inhibited by chlorate. Others [8,32–34] have demonstrated that chlorate does not inhibit nitrate reduction by Nap, and the residual nitrate reduction activity by *C. testosteroni* was presumably because of continued activity by this enzyme even in the presence of chlorate.

Although chlorate is structurally similar to nitrate, the nitrate transporter discriminates against chlorate and does not allow it to pass through the membrane [35]. This is probably caused by the different charge in which chlorate is more electronegative than nitrate [36]. However, the discrimination of nitrate transporters can be removed by adding low concentrations of detergents such as Triton X-100, which then allow intact cells to reduce chlorate [37]. This suggests that inhibition of Nar activity by chlorate was probably due to inhibition, presumably by blocking the NarK nitrate transporter, of nitrate transport into the cytoplasm where the active site of Nar is located, rather than inhibition of the Nar activity itself. NarK was first identified in *E. coli* and is located directly upstream from the structural genes encoding Nar [38]. The function of NarK as a nitrate/nitrite antiporter was previously proposed, and Rowe et al. [39] demonstrated evidence that NarK in *E. coli* was a nitrite efflux protein. There are two types of NarK [35]; NarK1 was a nitrate/proton symporter and NarK2 was a nitrate/nitrite antiporter [40]. A hypothetical mechanism of the chlorate inhibition is as shown in Fig. 7.

There was no chlorate reduction in either the pure cultures of *C. testosteroni* and *K. pneumonia* or in sediment slurry. Recently, some bacteria have been reported that respire using chlorate as a terminal electron acceptor [14,20,41–44]. These bacteria have a single enzyme that can dismute chlorite to chloride [20]. The chlorate reduction pathway is suggested as:

$$ClO_3^- \rightarrow ClO_2^- \rightarrow Cl^-$$

Moreover, Kengen et al. [43] reported that this enzyme is periplasmic, but Wallace et al. [42] reported that the activity of the enzyme was found in both the membrane and soluble fractions of the cell lysate. Bruce et al. [44] suggested that there was no correlation between this enzyme and the denitrification enzyme. This supports the proposal that chlorate cannot pass through the cell membrane, and therefore that chlorate inhibits transport by NarK.

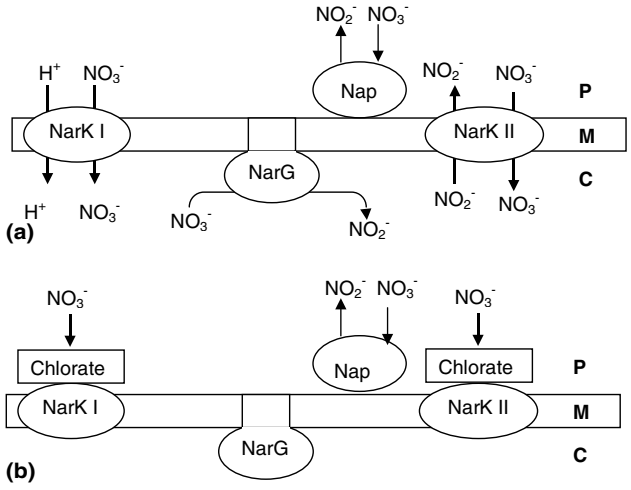

Fig. 7. Hypothetical mechanisms of chlorate inhibition on Nar activity: (a) no chlorate inhibition and (b) chlorate inhibition. P, periplasm; M, membrane and C, cytoplasm.

4.2. Effects of chlorate and chlorite on nitrate and nitrite reduction in slurry

Chlorate clearly inhibited nitrate reduction, but not nitrite reduction. The slurry experiment results showed inhibition by chlorate of nitrate reduction and N_2O formation in the slurry with nitrate added, but lack of inhibition of nitrite reduction or N_2O formation in the slurry with nitrite added, indicating that chlorate only inhibited the first step of nitrate reduction pathways. This conclusion was confirmed by lack of inhibition of nitrite removal by chlorate addition. Nitrate removal in the slurry was not inhibited completely by chlorate, but only by about 45% of that of the control. This was presumably because chlorate has different effects on the two different dissimilative nitrate reductases-membrane bound nitrate reductase (Nar) and periplasmic nitrate reductase (Nap) that are known to have different physiology, biochemistry and genetics [8,32,33,35]. Previous laboratory studies have shown that Nar but not Nap can reduce chlorate [8,32–34] but our data showed that the chlorate concentration in the slurry was stable, and that even Nar activity in these sediments was not removing chlorate and that any inhibitory effect of chlorate was consistent throughout the period of the experiments.

The differential mode of action of chlorate on Nar and Nap was also shown on N_2O production with and without C_2H_2 inhibition. These data showed that N_2 production during denitrification was not inhibited by chlorate. N_2 gas is the end product of denitrification [8,32] with complete reaction as follows:

$$NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$$

Almost all true denitrifiers such as *P. denitrificans*, *Paracoccus pantotropha*, *Pseudomonas aeruginosa* and *P. denitrificans* either have both Nar and Nap or only have Nap [45]. However, nitrate ammonifiers such as *Klebsiella oxytoca*, *K. pneumoniae*, *Bacillus subtilis* and *Bacillus stearothermophilus* have only Nar [45]. This suggests that in the microbial community in the sediment slurry chlorate inhibited dissimilative nitrate reduction to ammonium, but did not completely inhibit denitrification as that by Nap continued. Inhibition of N_2O production by chlorate in this slurry experiment presumably indicated that N_2O was only produced by dissimilative nitrate reduction by Nar. In contrast, in freshwater sediment Kelso et al. [1] reported that N_2O was produced by dissimilative nitrate reduction to ammonium. Chlorite apparently inhibited nitrate reduction because of its ability to oxidize nitrite to nitrate chemically. This was shown by formation of nitrate in slurry with nitrite treatment soon after chlorite added. The possible reaction of nitrite oxidation by chlorite is as follows:

$$2NO_2^- + ClO_2^- \rightarrow 2NO_3^- + Cl^-$$

Our data indicate that chlorate is likely to be a useful tool for field ecological studies on anaerobic nitrogen cycling in sediment, to differentiate between the contribution of Nar and Nap to nitrate removal and its gaseous end-product accumulation.

Acknowledgements

This work was supported by a studentship from the Quality for Undergraduate Education (QUE) Project, Department of Biology, Faculty of Mathematics and Sciences, Bogor Agricultural University, Indonesia to I.R. Thanks are also due to J. Takeuchi (Department of Biological Sciences, University of Essex, UK) for information about the molecular analysis of *nar*G and *nap*A genes in the bacterial isolates.

References

[1] Kelso, B.H.L., Smith, R.V., Laughlin, R.J. and Lennox, D. (1997) Dissimilatory nitrate reduction in anaerobic sediments leading to river nitrite accumulation. Appl. Environ. Microbiol. 63, 4679–4685.

[2] SurmaczGorska, J., Gernaey, K., Demuynck, C., Vanrolleghem, P. and Verstraete, W. (1996) Nitrification monitoring in activated sludge by oxygen uptake rate (OUR) measurements. Water Res. 30, 1228–1236.

[3] Chen, D.L., Chalk, P.M., Freney, J.R., Smith, C.J. and Luo, Q.X. (1995) Estimation of nitrification rates in flooded soils. Microbiol. Ecol. 30, 269–284.

[4] Pennington, P.I. and Ellis, R.C. (1993) Autotrophic and heterotrophic nitrification in acidic forest and native grassland soils. Soil Biol. Biochem. 25, 1399–1408.

[5] Bauhus, J., Meyer, A.C. and Brumme, R. (1996) Effect of the inhibitors nitrapyrin and sodium chlorate on nitrification and N_2O formation in an acid forest soil. Biol. Fertil. Soil. 22, 318–325.

[6] Feliatra, F. and Bianchi, M. (1993) Rates of nitrification and carbon uptake in the Rhone river Plume (Northwestern Mediterranean sea). Microbiol. Ecol. 26, 21–28.

[7] Hochstein, L.I. and Tomlison, G.A. (1988) The enzymes associated with denitrification. Ann. Rev. Microbiol. 42, 231–261.

[8] Bedzyk, L., Wang, T. and Ye, R.W. (1999) The periplasmic nitrate reductase in *Pseudomonas* sp. strains G-179 catalyzes the first step of denitrification. J. Bacteriol. 181, 2802–2806.

[9] Sears, H.J., Ferguson, S.J., Richardson, D.J. and Spiro, S. (1993) The identification of periplasmic nitrate reductase in *Paracoccus denitrificans*. FEMS Microbiol. Lett. 113, 107–111.

[10] Bickelsandkotter, S. and Ufer, M. (1995) Properties of a dissimilatory nitrate reductase from halophilic Archaeon *Haloferax volvanii*. J. Biosci. 50, 365–372.

[11] Rai, A.K. and Tiwari, S.P. (1999) Mutants of the Cyanobacterium *Anabaena* sp. PCC 7120 altered in nitrate transport and reduction. Curr. Microbiol. 39, 237–243.

[12] Rosemarin, A., Lehtinen, K. and Notini, M. (1990) Effect of treated and untreated softwoodpulp mill effluents on Baltic sea algae and invertebrates in model ecosystems. Nord. Pulp. Pap. Res. J. 2, 83–87.

[13] Siddiqui, R.A., Warnecke-Eberz, U., Hengsberger, A., Schneider, B., Kostka, S. and Friedrich, B. (1993) Structure and function of a periplasmic nitrate reductase in *Alcaligenes eutrophus* H16. J. Bacteriol. 175, 5867–5876.

[14] Herman, D.C. and Frankenberger Jr., W.T. (1998) Microbial-mediated reduction of perchlorate in groundwater. J. Environ. Qual. 27, 750–754.
[15] Van Wijk, D.J. and Hutchinson, T.H. (1995) The ecotoxicity of chlorate to aquatic organisms. A critical review. Ecotoxicol. Environ. Saf. 32, 244–253.
[16] Van Wijk, D.J., Kroon, S.G. and Garttener-Arends, I.C. (1998) Toxicity of chlorate and chlorite to selected species of algae, bacteria, and fungi. Ecotoxicol. Environ. Saf. 40, 206–211.
[17] Cove, D.J. (1976) Chlorate toxicity in *Aspergillus nidulans*: the selection and characterization of chlorate resistant mutants. Heredity 36, 191–203.
[18] Anderson, R.C., Buckley, S.A., Callaway, T.R., Genovese, K.J., Kubena, L.F., Harvey, R.B. and Nisbet, D.J. (2001) Effect of sodium chlorate on *Salmonella typhimurium* concentrations in the weaned pig gut. J. Food Prot. 64, 255–258.
[19] Coates, J.D., Michaelidou, U., Bruce, R.A., O'Connor, S.M., Crespi, J.N. and Achenbach, L.A. (1999) Ubiquity and diversity of dissimilatory (per)chlorate-reducing bacteria. Appl. Environ. Microbiol. 65, 5234–5241.
[20] de Groot, G.N. and Stouthamer, A.H. (1969) Regulation of reductase formation in *Proteus mirabillis*. I. Formation of reductases and enzymes of the formic hydrogenlyase complex in the wild type and in chlorate resistant mutants. Arch. Microbiol. 66, 220–233.
[21] Roldan, M.D., Reyes, F., Moreno-Vivian, C. and Castillo, F. (1994) Chlorate and nitrate reduction in the phototrophic bacteria *Rhodobacter capsulatus* and *Rhodobacter sphaeroides*. Curr. Microbiol. 29, 241–245.
[22] Parsons, T.R., Maita, Y. and Lalli, C.M. (1984) A Manual of Chemical and Biological Methods for Seawater Analysis. Pergamon Press, Oxford.
[23] Harwood, J.E. and Kuhn, A.L. (1970) A colorimetric method for ammonia in natural waters. Water Res. 4, 805–811.
[24] Gravitz, N. and Gleye, L. (1975) A photochemical side reaction that interferes with phenol-hypochlorite assay for ammonia. Limnol. Oceanogr. 20, 1015–1017.
[25] Krom, M.J.D. (1980) Spectrophotometric method determination of ammonia: a study of modified Berthelot reaction using salicylate and dichloroisocyanurate. Analyst 105, 305–317.
[26] Ogilvie, B.G., Rutter, M. and Nedwell, D.B. (1997) Selection by temperature of nitrate-reducing bacteria from estuarine sediments: species composition and competition for nitrate. FEMS Microbiol. Ecol. 23, 11–22.
[27] Robinson, A.D., Nedwell, D.B., Harrison, R.M. and Ogilvie, B.G. (1998) Hypernutrified estuaries as sources of N_2O emission to the atmosphere: the estuary of the River Colne, Essex, UK. Mar. Ecol. Prog. Ser. 164, 59–71.
[28] Rusmana, I. (2003) Physiology of nitrous oxide production in estuarine dissimilative nitrate reducing bacteria. PhD. Thesis, University of Essex, Colchester, UK.
[29] Lloyd, D.L. (1999) Temperature effects on competition, selection and physiology of estuarine nitrate-respiring bacteria. PhD. Thesis, University of Essex, Colchester, UK.
[30] Takeuchi, J. (personal communication).
[31] Barford, C.C., Montoya, J.P., Altabet, M.A. and Mitchell, R. (1999) Steady-state nitrogen isotope effects of N_2 and N_2O production in *Paracoccus denitrificans*. Appl. Environ. Microbiol. 65 (3), 989–994.
[32] Zumft, W. (1997) Cell biology and molecular basis of denitrification. Microbiol. Mol. Biol. Rev. 61, 533–616.
[33] Moreno-Vivian, C., Cabello, P., Luque, M.M., Blasco, R. and Castillo, F. (1999) Prokaryote nitrate reduction: molecular properties and functional distinction among bacterial nitrate reductases. J. Bacteriol. 181, 6573–6584.
[34] Philippot, L. and Hojberg, O. (1999) Dissimilatory nitrate reductases in bacteria. Biochem. Biophys. Acta 1446, 1–23.
[35] Moir, J.B. and Wood, N.J. (2001) Nitrate and nitrite transport in bacteria. Cell. Mol. Life Sci. 58, 215–224.
[36] Madigan, M.T., Martinko, J.M. and Parker, J. (2000) Brock Biology of Microorganisms. Prentice Hall International, Inc, London.
[37] Alefounder, P.R. and Ferguson, S.J. (1980) The location of dissimilatory nitrite reductase and the control of dissimilatory nitrate reductase by oxygen in *Paracoccus denitrificans*. Biochem. J. 192, 231–240.
[38] Noji, S., Nohno, T., Saito, T. and Taniguchi, S. (1989) The *nark* gene product participates in nitrate transport induced in *Escherichia coli* nitrate-repiring cells. FEBS Lett. 252, 139–143.
[39] Rowe, J.J., Ubbink-Kok, T., Molenaar, D., Konings, W.N. and Driessen, A.J.M. (1994) NarK is a nitrite-extrusion system involved in anaerobic nitrate respiration by *Escherichia coli*. Mol. Microbiol. 12, 579–586.
[40] Wood, N.J., Alizadeh, T., Richardson, D.J., Ferguson, S.J. and Moir, J.W.B. (2002) Two domains of a dual-function NarK protein are required for nitrate uptake, the first step of denitrification in *Paracoccus pantotrophus*. Mol. Microbiol. 44, 157–170.
[41] Logan, B.E., Bliven, A.R., Olsen, S.R. and Patnaik, R. (1998) Growth kinetics of mixed cultures under chlorate-reducing conditions. J. Environ. Eng. ASCE 124, 1008–1011.
[42] Wallace, W., Beshear, S., Williams, D., Hospadar, S. and Owens, M. (1998) Perchlorate reduction by a mixed culture in an up-flow anaerobic fixed bed reactor. J. Ind. Microbiol. Biotechnol. 20, 126–131.
[43] Kengen, S.W.M., Rikken, G.B., Hagen, W.R., van Ginkel, C.G. and Stams, A.J.M. (1999) Purification and characterization of (per)chlorate reductase from the chlorate –respiring starin GR-1. J. Bacteriol. 181, 6706–6711.
[44] Bruce, R.A., Achenbach, L.A. and Lindgren, O. (1999) *Dechlorimonas agitatus* strain CKB gen. Nov., sp. Nov., a novel dissimilatory chlorate reducer from a paper mill. Environ. Microbiol. 1, 319–331.
[45] Richardson, D.J., Berks, B.C., Russel, D.A., Spiro, S. and Taylor, C.J. (2001) Functional, biochemical and genetic diversity of prokaryotic nitrate reductases. Cell. Mol. Life Sci. 58, 165–178.