Comparison of aerobic denitrifying activity among three cultural species with various carbon sources

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Abstract Abilities of three aerobic denitrifiers such as *Alcaligenes faecalis*, *Microvirgula aerodenitrificans* and *Paracoccus pantotrophus* were compared from the viewpoints of nitrate removal efficiency and organic matter utilization. First, the effect of carbon source was investigated. Although nitrate reduction was observed in all strains under aerobic conditions, a change of carbon source considerably affected the denitrification ability. In the case of *P. pantotrophus*, nitrate and nitrite were completely removed in three days under sodium acetate or leucine as a carbon source. In the case of *A. faecalis*, sufficient nitrate removal was observed only when sodium acetate or ethanol was added. *P. pantotrophus* and *A. faecalis* showed a higher ability of nitrate removal than that of *M. aerodenitrificans*. Therefore, *P. pantotrophus* was selected in order to investigate the effects of concentration and repetitive addition of carbon. Sodium acetate was used as a sole carbon source. Nitrate was not reduced when the carbon concentration was below 500 mgC/L. However, when carbon source was added repeatedly, nitrate was reduced under 100 mgC/L after the optical density of the bacterium reached above 1.0. This result indicated that a high enough level of bacterial density was necessary to express aerobic denitrification activity.

Keywords Aerobic denitrification; *Alcaligenes faecalis*; nitrate reduction; *Microvirgula aerodenitrificans*; *Paracoccus pantotrophus*

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Introduction

Denitrifying bacteria are usually characterized by the ability to use nitrogen oxides (nitrate and nitrite) as electron acceptors when producing gaseous nitrogen. Most denitrifiers are aerobic bacteria; therefore oxygen has priority to be used as an electron acceptor comparing to nitrate. Thus, it is known that oxygen inhibits the denitrification activity.

However, it has been reported that some cultural species can denitrify even under aerobic conditions. *Paracoccus pantotrophus*, first isolated by Robertson and Kuenen (1983) and renamed from *Thiosphaera pantotropha*, is the most well investigated aerobic denitrifier. Aerobic denitrification ability has been also reported in other bacteria, such as *Microvirgula aerodenitrificans* (Patureau *et al*., 1998, 2001), *Alcaligenes faecalis* (Vanniel *et al*., 1992; Robertson *et al*., 1995), *Thauera mechernichensis* (Sholten *et al*., 1999), and *Nitrosomonas eutropha* (Zart *et al*., 1998). These bacteria have nitrogen reductases tolerant to oxygen (Bell *et al*., 1991; Gupta, 1997).

Aerobic denitrification can be applied to wastewater treatment. A conventional activated sludge system is adopted in many municipal wastewater treatment plants. Generally, nitrogen cannot be removed during the system because the conventional activated sludge process has only aerobic tanks. However, if aerobic denitrifiers are introduced, nitrogen removal can be achieved without modification of the system.

Aerobic denitrification can also be applied to remediation of groundwater. In agricultural areas, there is serious contamination with nitrate in groundwater caused by high nitrogen loading, such as fertilizer and animal wastewater. In order to remove nitrate in the natural environment, creation of anoxic conditions is needed. It is usually aerobic around the nitrogen sources. Therefore, actual remediation sites, such as watersheds, artificial submerged areas and paddy fields, are placed far from the pollution source, and nitrate contamination

remains wide even if a denitrification method is applied. If it becomes possible to remove nitrate around its sources using aerobic denitrifiers, it can be a more effective way to prevent nitrate contamination in groundwater.

In order to understand the more effective places to apply aerobic denitrifiers, the nitrate reduction ability of three denitrifiers was compared. Influences of several factors, such as carbon source, carbon and nitrate concentrations, and bacterial density, were investigated.

Materials and methods

Microorganisms

Paracoccus pantotrophus (ATCC 35512), *Alcaligenes faecalis* (IFO 14479) and *Microvirgula aerodenitrificans* (LMG 18919) were obtained from American Type Culture Collection (ATCC), Institute of Fermentation Osaka (IFO) and Belgian Co-ordinated Collections of Micro-organisms (BCCM), respectively. The strain was delivered in freeze-dried form. Each strain was cultivated in 500 mL liquid medium using an Erlenmeyer flask with a silicone stopper (Aram, Japan). It prevents microorganisms and contaminants from entering the culture while allowing free air exchange. It was shaken at 90 rpm at 28°C in a bioshaker (Bio-Shaker BR-300LF, Taitec, Japan) to keep aerobic conditions. After exponential growth, a sample was taken from the flask and kept at –80°C in a 1.5 ml cryogenic vial (Nalgene, USA). The components of the medium are shown in Table 1.

Precultivation

When each strain was used, it was thawed at room temperature, and then precultivated. The precultivating stage was divided into two steps. After cultivation without nitrate, it was cultivated in a medium including 100 or 200 mgN/L KNO_3. The other components in each medium were the same as described above, when carbon source effects were investigated. Sodium acetate (4 gC/L) was used as a carbon source instead of polypeptone and yeast extract when concentration and repetitive addition effects were investigated. At the second step of

Table 1 Components of media

	Mineral		Carbon	
P. pantotrophus	Na_2HPO_4	4.2 g/L	Polypeptone	10 g/L
	KH_2PO_4	1.5 g/L	Yeast extract	2 g/L
	NH_4Cl	0.3 g/L		
	$MgSO_4 \cdot 7H_2O$	0.1 g/L		
	Trace element solution			
	(See right column)	2.0 mL/L		
A. faecalis	$MgSO_4 \cdot 7H_2O$	1 g/L	Polypeptone	10 g/L
			Yeast extract	2 g/L
M. aerodenitrificans	$(NH_4)_2SO_4$	1 g/L	Succinic acid	1 g/L
	$MgSO_4 \cdot 7H_2O$	1 g/L	Peptone	10 g/L
	$FeCl_3 \cdot 7H_2O$	2 mg/L		
	$MnSO_4 \cdot H_2O$	2 mg/L		

Trace element solution	
EDTA	50.0 g/L
$ZnSO_4 \cdot 7H_2O$	22.0 g/L
$CaCl_2$	5.54 g/L
$MnCl_2 \cdot 4H_2O$	5.06 g/L
$FeSO_4 \cdot 7H_2O$	4.99 g/L
$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	1.10 g/L
$CuSO_4 \cdot 5H_2O$	1.57 g/L
$CoCl_2 \cdot 6H_2O$	1.61 g/L

precultivation, 1.5 mL of the medium including bacteria was taken and added to a new medium with nitrate. After exponential growth, the medium was centrifuged for 5 minutes at 10,000 rpm and dispersed in phosphate buffer. The phosphate buffer contained (g/L distilled water): 1.16 Na_2HPO_4, 0.2 KH_2PO_4, 0.2 KCl, and 8.0 NaCl. It was centrifuged for 5 minutes at 10,000 rpm before washing and dispersed in phosphate buffer again for the main experiment.

Condition setting

Conditions of the three experiments were summarized in Table 2.

Carbon source effects. Denitrification ability of each strain was investigated under five carbon sources: sodium acetate, ethanol, D(+)-glucose, L-leucine, and peptone. Initial concentrations of carbon source and nitrate were set at 1 gC/L and 200 mgN/L, respectively. The mineral composition for *P. pantotrophus* (Table 1) was adopted for *P. pantotrophus* and *A. faecalis*. For *M. aerodenitrificans*, its own mineral medium was used.

Concentration effects. *P. pantotrophus* was used in order to investigate the effects of carbon and nitrate concentrations. Sodium acetate was used for a sole carbon source. Initial carbon concentration was set from 100 mgC/L to 1,000 mgC/L at intervals of 100 mgC/L, whereas nitrate concentration was set at 200 mgN/L or 20 mgN/L. Twenty conditions were set in total.

Repetitive addition effects. Effect of repetitive addition of carbon source was investigated using *P. pantotrophus*. Sodium acetate was used as a sole carbon source. Initial carbon source concentration was set at 500 mgC/L, 300 mgC/L or 100 mgC/L, whereas nitrate concentration was set at 200 mgN/L.

Table 2 Overview of the three experiments

	Precultivation		Main
	Without nitrate	With nitrate	
			
(1) Carbon source effects			
P. pantotrophus	Polypeptone 10 g/L Yeast extract 2 g/L Without nitrate	Polypeptone 10 g/L Yeast extract 2 g/L Nitrate 200 mgN/L	Carbon source 1 gC/L Sodium acetate, ethanol, glucose, leucine, or peptone
A. faecalis	Polypeptone 10 g/L Yeast extract 2 g/L Without nitrate	Polypeptone 10 g/L Yeast extract 2 g/L Nitrate 200 mgN/L	
M. aerodenitrificans	Peptone 10 g/L Succinic acid 1 g/L Without nitrate	Peptone 10 g/L Succinic acid 1 g/L Nitrate 100 mgN/L	Nitrate 200 mgN/L
(2) Concentration effects			
P. pantotrophus	Sodium acetate 4 gC/L Without nitrate	Sodium acetate 4 gC/L Nitrate 200 mgN/L	Sodium acetate (10 levels) Nitrate (2 levels)
(3) Repetitive addition of carbon			
P. pantotrophus	Sodium acetate 4 gC/L Without nitrate	Sodium acetate 4 gC/L Nitrate 200 mgN/L	Sodium acetate (3 levels) Nitrate 200 mgN/L

Sampling and analysis

Ten millilitres of sample was taken from the medium using a sterilized Pasteur pipette. Optical density at 580nm (OD_{580}) was measured by a spectrophotometer (U-2010, Hitachi, Japan). After measuring OD_{580}, the sample was filtrated using a cellulose-acetate filter (pore size: 0.45 μm, Advantec, Japan) for removing bacteria. Total organic carbon (TOC) in the filtrate was analyzed by TOC-500 (Shimadzu, Japan). Nitrate (NO_3^-) and nitrite (NO_2^-) were determined using an ion chromatograph (IC-7000, Yokogawa, Japan). Ammonium (NH_4^+) was analyzed by the phenate method.

Results and discussion

Carbon source effects

Figures 1, 2, and 3 show the temporal changes of OD_{580}, nitrate, and nitrite in the cases of *P. pantotrophus*, *A. faecalis* and *M. aerodenitrificans*, respectively. *P. pantotrophus* and *A. faecalis* showed similar behaviors. Rapid increase in bacterial density accompanied with

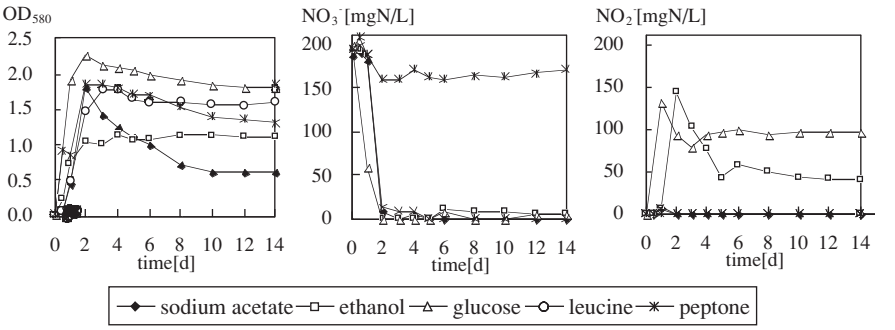

Figure 1 Temporal changes of OD, nitrate, and nitrite in the case of *P. pantotrophus*

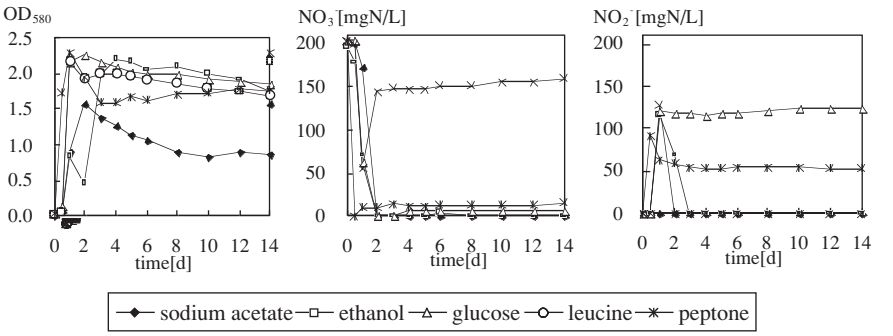

Figure 2 Temporal changes of OD, nitrate, and nitrite in the case of *A. faecalis*

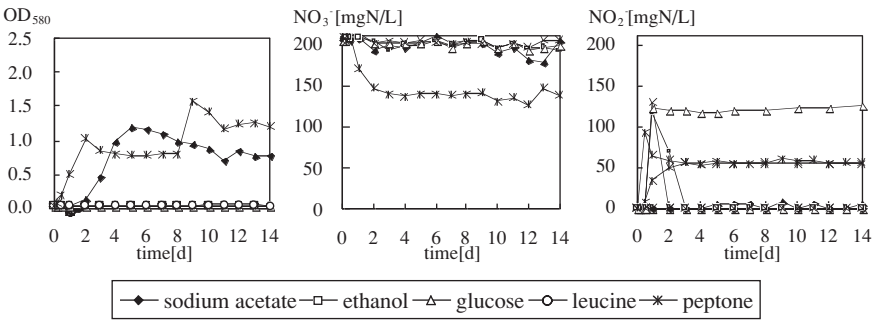

Figure 3 Temporal changes of OD, nitrate, and nitrite in the case of *M. aerodenitrificans*

nitrate reduction and nitrite production was observed at the beginning of the experiment. When bacterial density was settled followed by a slight decrease, no more reduction and nitrite production occurred. In the case of *M. aerodenitrificans*, on the other hand, increase of OD_{580} was slower than those of *P. pantotrophus* and *A. faecalis*. The nitrate reduction and nitrite production were observed only when peptone was added. OD_{580} under sodium acetate was also increased, but no reduction of nitrate was observed.

Figure 4 shows the composition of the remaining nitrogen at the end of the experiment. Nitrate removal during the precultivation is also shown. In the case of *P. pantotrophus*, added nitrate was almost reduced when sodium acetate, ethanol, glucose or leucine was used as a carbon source. When sodium acetate or leucine was used, added nitrate and produced nitrite were completely reduced as shown in Figure 1. In the case of *A. faecalis*, complete reduction of nitrate and nitrite was observed when sodium acetate or ethanol was added as a carbon source. When glucose or peptone was used, a larger amount of nitrite remained without reduction. As shown in Figure 3, *M. aerodenitrificans* showed a lower ability of nitrate reduction.

Concentration effects

Figure 5 shows the values of OD_{580} of *P. pantotrophus* under various concentrations of carbon and nitrate on day 5. Initial OD_{580} values ranged from 0.03 to 0.05 and increased in all

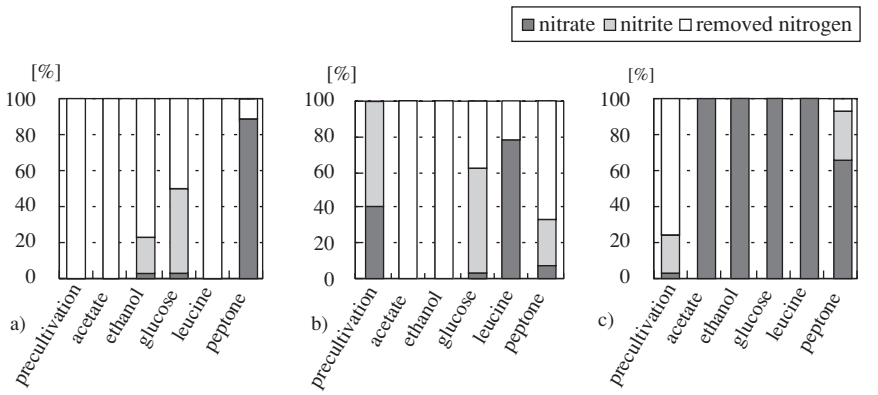

Figure 4 Remaining nitrogen composition at the end of the experiment. The cases of *P. pantotrophus* (a), *A. faecalis* (b), and *M. aerodenitrificans* (c) are shown

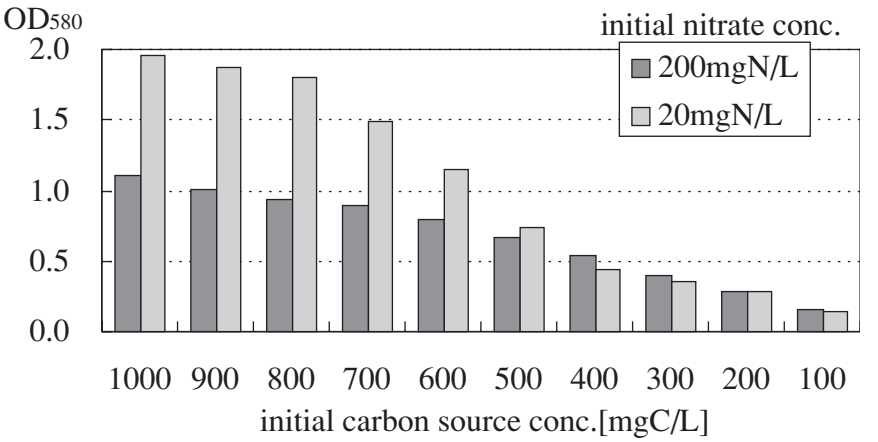

Figure 5 Optical density of *P. pantotrophus* on day 5

cases. Positive correlation was observed between the initial concentration of carbon and the increase in OD_{580}. Added sodium acetate was almost used up on day 5 in all cases.

Figure 6 shows the concentrations of remaining nitrate and nitrite on day 5. Whether the initial concentration of nitrate was high or low, added nitrate was not removed when the carbon concentration was below 500 mgC/L. When the initial nitrate concentration was 200 mgN/L, nitrate was not removed completely and nitrite was accumulated even under the highest carbon concentration. On the other hand, when the initial concentration was 20 mgN/L, all added nitrate was removed when the carbon concentration was above 700 mgC/L.

Effect of repetitive addition of carbon source

Previous results indicated that the concentration of carbon might affect nitrate removal. However, as shown in Figures 7 and 8, when carbon source was added repetitively, added nitrate was removed even under 100 mgC/L. When carbon concentration was 500 mgC/L or 300 mgC/L, initially added nitrate was completely removed in 3 days. Nitrite production was also observed, but produced nitrite was rapidly reduced. After initial nitrate was reduced, 200 mgN/L nitrate was added again. It was reduced more rapidly compared to the initial nitrate. When carbon concentration was 100 mgC/L, OD_{580} increased more slowly compared to the other two conditions. Initially added nitrate began to be reduced on day 8, when OD_{580} reached around 1.0. When carbon concentration was set at 500 mgC/L or 300 mgC/L, OD_{580} reached 1.0 in 2 days. Those results indicated that sufficient density of bacteria is needed for *P. pantotrophus* to express the aerobic denitrification ability. Even after bacterial density increases, *P. pantotrophus* does not form flocs. The concentration of

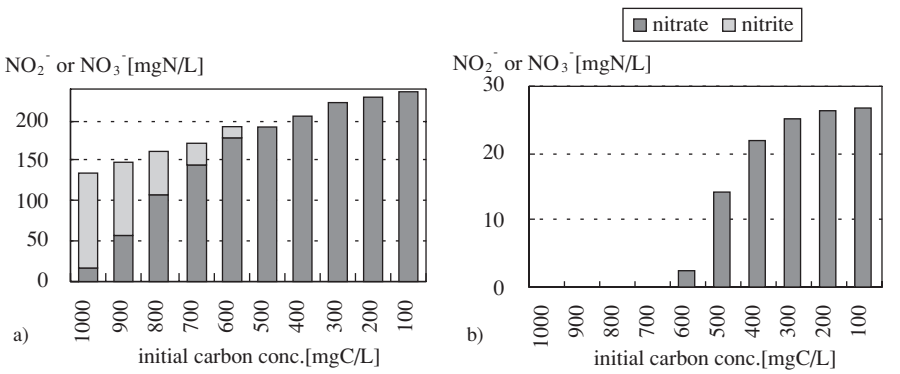

Figure 6 Nitrate and nitrite concentrations on day 5. Initial concentration of nitrate was 200 mgN/L (a) or 20 mgN/L (b)

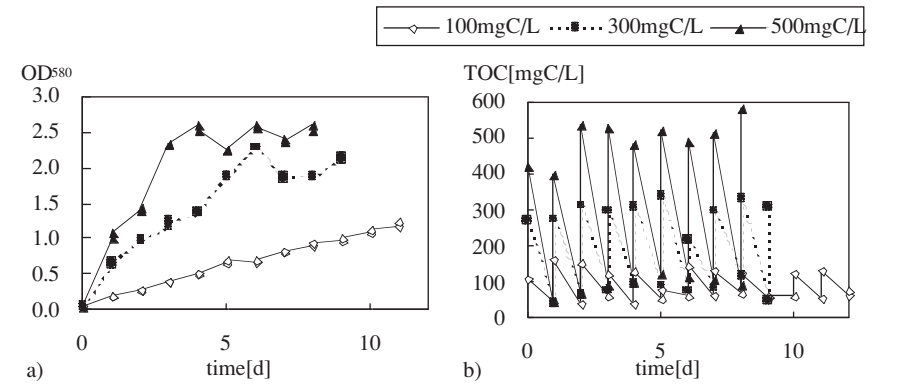

Figure 7 Temporal changes of OD_{580}(a) and TOC(b) when carbon source was added repetitively

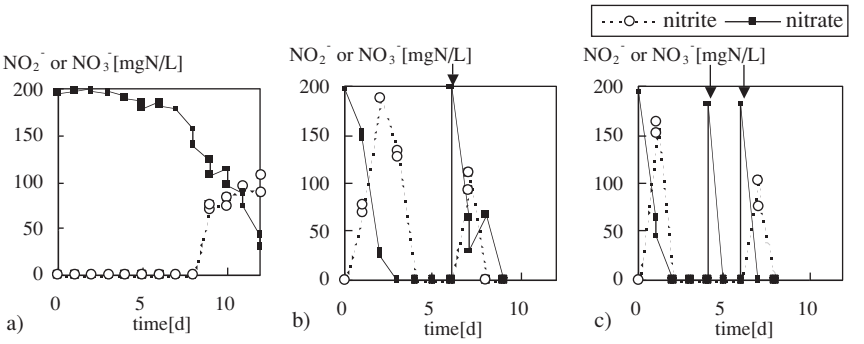

Figure 8 Temporal changes of nitrate and nitrite when carbon was added repetitively. The initial concentration of carbon was set at 100 mgC/L (a), 300 mgC/L (b), or 500 mgC/L (c). The arrows (↓) show the addition of nitrate.

dissolved oxygen (DO) was 4.8 mgO/L even under 500 mgC/L on day 8. Therefore, the influence of bacterial density on nitrate removal cannot be caused by the formation of anoxic sites. Patureau *et al*. (2001) also reported that aerobic denitrification activity of *M. aerodenitrificans* appeared in the presence of high numbers of bacteria. From those results, it is suggested that *P. pantotrophus* can remove nitrate even in low carbon concentration, if the bacterial density is kept high.

Conclusions and perspectives

The aerobic denitrification ability was compared for three cultural species and requisite conditions for *P. pantotrophus* to denitrify were investigated. Nitrate reduction under aerobic conditions was observed in all strains, but the ability of nitrate reduction was largely affected by the change of carbon source. Among three strains, *P. pantotrophus* and *A. faecalis* showed a higher ability of nitrate removal compared to *M. aerodenitrificans*. Even if optimum carbon (sodium acetate) was used, *P. pantotrophus* showed a low ability of aerobic denitrification when the concentration was below 500 mgC/L. However, if carbon was added repetitively, nitrate was reduced. When bacterial density reached 1.0, nitrate began to be reduced even under 100 mgC/L. Therefore, it is indicated that selection of an optimum carbon source and concentration of bacterial density are needed for effective use of aerobic denitrifiers.

References

Bell, L.C. and Ferguson, S.J. (1991). Nitric and nitrous oxide reductases are active under aerobic conditions in cells of *Thiosphaera pantotropha*. *Biochem. J*., **273**, 423–427.

Gupta, A.B. (1997). *Thiosphaera pantotropha*: a sulphur bacterium capable of simultaneous heterotrophic nitrification and aerobic denitrification. *Enzyme Microb. Tech*., **21**, 589–595.

Patureau, D., Godon, J., Dabert, P., Bouchez, T., Bernet, N., Delgenes, J.P. and Moletta, R. (1998). *Microvirgula aerodenitrificans* gen. nov., sp. nov., a new Gram-negative bacterium exhibiting co-respiration of oxygen and nitrogen oxides up to oxygen-saturated conditions. *Int. J. Syst. Bacteriol*., **48**, 775–782.

Patureau, D., Helloin, E., Rustrian, E., Bouchez, T., Delgenes, J.P. and Moletta, R. (2001). Combined phosphate and nitrogen removal in a sequencing batch reactor using aerobic denitrifier, *Microvirgula aerodenitrificans*. *Wat. Res*., **35**(1), 189–197.

Robertson, L.A. and Kuenen, J.G. (1983). *Thiosphaera pantotropha* gen nov. sp.nov., a facultatively anaerobic, facultatively autotrophic sulphur bacterium. *J. Gen. Microbiol*., **129**, 2847–2855.

Robertson, L.A., Dalsgaard, T., Revsbech, N.P. and Kuenen, J.G. (1995). Confirmation of aerobic denitrification in batch cultures, using gas-chromatography and N-15 mass-spectrometry. *FEMS Microbiol. Ecol*., **18**(2), 113–119.

Sholten, E., Lukow, T., Auling, G., Kroppenstedt, R.M., Rainey, F.A. and Diekmann, H. (1999). *Thauera mechernichensis* sp. nov., an aerobic denitrifier from a leachate treatment plant. *Int. J. Syst. Bacteriol.*, **49**, 1045–1051.

Vanniel, E.W.J., Braber, K.J., Robertson, L.A. and Kuenen, J.G. (1992). Heterotrophic nitrification and aerobic denitrification in *Alcaligenes faecalis* strain TUD. *Anton. Leeuw. Int. J. G.*, **62**(3), 231–237.

Zart, D. and Bock, E. (1998). High rate of aerobic denitrification by *Nitrosomonas eutropha* grown in a fermentor with complete biomass retention in the presence of gaseous NO_2 or NO. *Arch. Microbiol.*, **169**, 282–286.