Removal of high NO_3^- concentrations in saline water through autotrophic denitrification by the bacterium *Thiobacillus denitrificans* strain MP

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Abstract Autotrophic denitrification by *Thiobacillus denitrificans* MP isolated from mangrove was investigated in both a sulphur-limestone column reactor and a fermenter. More than 97.5% of the nitrate (NO_3^-) in the 250 mg NO_3^--N/L strong influent was removed after 14.3 hours in the column reactor. Influent NO_3^- was completely depleted in the lower part of the column as the hydraulic retention time increased and a slight pH drop was also observed along the reactor column due to the exhaustion of the buffering ability of the limestone. Trace amounts of oxygen present in the lower part of the reactor column resulted in the accumulation of nitrite and subsequent inhibition of further denitrification. The species composition of the bacterial community in the higher parts of the reactor column was morphologically more diverse than in the lower part. Denitrification by *T. denitrificans* MP reached an optimal level when the dissolved oxygen was maintained between 1.5–2% of saturation level in the automated fermenter. The stoichiometric ratios of ΔSO_4^{2-} produced/ΔNO_3^--N removed were 6.81 and 9.32 in the reactor column and fermenter, respectively. This study suggests that efficient removal of high NO_3^- concentrations in water or wastewater can be achieved using autotrophic bacteria immobilized on surfaces of sulphur granules in the column system.

Keywords Autotrophic denitrification; dissolved oxygen; inhibition; retention time; stoichiometry; *Thiobacillus denitrificans* MP

Introduction

High concentrations of nitrate (NO_3^-) are often found in drinking water and post-treatment wastewater. In recent years, nitrate concentrations of groundwater have increased in many parts of the world due to increasing usage of nitrogenous fertilizers and discharge of domestic wastewater. Nitrate in drinking water is a health hazard to both humans and animals (Kross *et al*., 1993). Current recommendations are <10 and <100 mg/L nitrate-N for water used for humans and animals, respectively (Hunter, 1999). Because of the adverse effects on health and the concern over deteriorating water quality, nitrate removal technologies have drawn increasing attention in recent years worldwide (Halling-Sorensen and Jorgensen, 1993).

Autotrophic denitrification using *Thiobacillus denitrificans* has been investigated before (Sikora and Keeney, 1976; Batchelor and Lawrence, 1978a; Koenig and Liu, 1996). *T. denitrificans* is a small, Gram-negative, rod-shaped bacterium capable of fixing carbon dioxide and using nitrate, nitrite or nitrogenous gas (N_2O) as a terminal electron acceptor under anoxic conditions. The two-step process of autotrophic denitrification using elemental sulphur as an electron donor can be shown as: first step (reduction of nitrate, NO_3^-): $6NO_3^- + 2S^\circ + 2H_2O = 6NO_2^- + 2SO_4^{2-} + 4H^+$; second step (reduction of nitrite, NO_2^-): $6NO_2^- + 3S^\circ = 3N_2 + 3SO_4^{2-}$

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The stoichiometric reaction using *T. denitrificans* has been proposed as follows (Batchelor and Lawrence, 1978b):

$$NO_3^- + 1.10\,S^\circ + 0.40\,CO_2 + 0.76\,H_2O + 0.08\,NH_4^+ \xrightarrow{\textit{Thiobacillus denitrificans}\ \text{MP}} 0.5\,N_2 + 1.10\,SO_4^{2-} + 1.28\,H^+ + 0.08\,C_5H_7O_2N \quad [1]$$

Previous studies demonstrated that the system is highly efficient and comparable to heterotrophic denitrification systems in terms of the effectiveness for treating nitrate-contaminated water. The nitrate removal efficiency of the reactor system was observed to be affected by various operational variables, such as temperature, influent nitrate concentration, hydraulic retention time and salinity (Kruithof *et al.*, 1988; Koenig and Liu, 1996; Flere and Zhang, 1998, 1999; Zhang, 2002). Oxygen and pH could also affect the denitrification activities of *T. denitrificans*. The highest rates of denitrification by *T. denitrificans* were observed for the pH between 7 and 8 (Koenig and Liu, 1996). Most investigators consider oxygen an inhibitor to the denitrification process (Halling-Sorensen and Jorgensen, 1993). Oxygen either represses the nitrate reductase enzyme or acts as a competing electron acceptor, therefore preventing the reduction of nitrate (Payne, 1973). However, beneficial effects of oxygen in the denitrification process were also observed (Wood, 1986). The level of oxygen was observed to affect the concentration profiles in a range of reactor systems (Flere and Zhang, 1998; Zhang, 2002). In particular, a decrease of pH and an increase in sulphate concentration could result in the reactor system under aerobic conditions (Zhang, 2002).

The major problems associated with the application of autotrophic denitrification are the high concentrations of sulphate, low pH and DO (dissolved oxygen) in the effluent. Therefore, the objectives of this study were to: i) investigate the microbial denitrification in selected reactor systems; ii) investigate the effect of DO on the removal of nitrate; and iii) investigate the stoichiometric ratio between nitrate reduced and sulphate produced under different operational conditions.

Materials and methods

Enrichment of microorganisms

A bacterium was isolated from sediment samples from Mai Po Nature Reserve of Hong Kong and identified as *Thiobacillus denitrificans* MP. *T. denitrificans* MP was cultured and fed in a synthetic medium to the column reactor system incubated at 22 °C. The medium consisted of tapwater containing 5.0 g/L $Na_2S_2O_3{\cdot}5H_2O$, 2.0 g/L K_2HPO_4, 2.0 g/L KNO_3, 1.0 g/L $NaHCO_3$, 0.5 g/L NH_4Cl, 0.5 g/L $MgCl_2{\cdot}6H_2O$ and 0.01 g/L $FeSO_4{\cdot}7H_2O$ (Koenig and Liu, 1996).

Experimental apparatus

The experiments were divided into two parts. The first phase was carried out in a pilot-scale column reactor packed with elemental sulphur as described before (Koenig and Liu, 1996). Limestone granules (approximately 0.6–1.1 cm) were mixed into the packed sulphur bed to prevent the pH from dropping below 6.5. The reactor column had an inside diameter of 8.4 cm and was packed with elemental sulphur particles (approximately 0.56–1.12cm) to a height of 165 cm. Sampling ports were attached on the side at the height of 20, 40, 60, 80, 120, 140, 160 cm, respectively. The reactor was fed continuously in the upflow mode by means of adjustable peristaltic pumps (Cole-Parmer, Chicago, USA). The temperature was maintained at 22 ± 2 °C.

The second phase was carried out in a fermenter (Electrolab 300, Tewkesbury, UK). The temperature was maintained at 30±1 °C. Concentrations of DO and pH were recorded continuously at a 10-minute interval during the execution of the experiments.

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Experimental program

The cultures of *T. denitrificans* MP were introduced into the packed bed column reactors at the beginning of the experiment. After a period of incubation, the bacteria attached to the sulphur particles and formed a biofilm. Synthetic wastewater with 250 mg/L NO_3^--N was fed into the reactors at an upflow rate of 0.6–0.7 L/hr. After reaching steady-state conditions, the upflow rates were step-wise reduced to 0.5 L/hr.

In view of the influence of DO on autotrophic denitrification, it was decided to conduct another series of experiments in a fermenter system. Sulphur particles with the attached biofilm from the column reactor were transferred into the fermenter fed with the same synthetic wastewater as in the reactor column. DO levels in the fermenter were increased by releasing one of the ports on the cover to allow atmospheric O_2 to penetrate.

Wastewater characteristics

The synthetic saline wastewater contained 1.773 g/L KNO_3, 0.1 g/L NH_4Cl, 1.67 g/L $NaHCO_3$, 0.2 g/L K_2HPO_4, and 32.96 g/L NaCl and was used for both of the systems described above.

Sampling and analysis

Samples from the packed bed column reactor were collected from the influent, at the five different heights of the reactor, and from the exiting effluent. The pH value of all samples was measured using a pH meter at the time of sampling. The analytical methods used to determine nitrate, nitrite and sulphate are those as described in APHA (2000). External standards were used in calibration and both blank controls and standards were implemented at every 10 samples' intervals during analysis.

Scanning electron microscopy (SEM)

Sulphur particles with attached biofilms were taken from the reactor column at three different heights (20, 100, 160 cm). Preparation of samples for SEM followed the procedures described elsewhere (Gu *et al.*, 1996). The prepared samples were then observed on a Leica Cambridge S440 SEM.

Results and discussion

Concentration profiles in the reactor

Concentration of NO_3^--N decreased from the initial 250 mg/L in the influent to 3.3 mg/L in the effluent at 120 cm height (Figure 1). At the same time, the concentrations of SO_4^{2-} increased from 209 mg/L to 1,860 mg/L in the effluent for the same column distance. The pH values showed relatively small changes from 8.20 to 6.75 along the entire reactor column. According to equation [1], autotrophic denitrification reaction produces significant amounts of H^+ and results in decrease of pH in the column (Suzuki *et al.*, 1992). The limestone in the reactor neutralizes the H^+ produced (Flere and Zhang, 1999) and therefore the pH in all trials never dropped below 6.5. Although low levels of NO_2^- were detected in the samples from all heights of column (Figure 1), they never exceeded 2% of the influent NO_3^--N.

The rates of both nitrate removal and sulphate production were relatively slow in the lower part of the reactor column (below 40 cm) followed by a more rapid phase of denitrification and sulphate production before the concentrations finally leveled off as the upflow reached the height of 160 cm (Figure 1). The initial "slow phase" was correlated with the high concentration of NO_2^- in the lower part of the reactor column between 20 and 40 cm (Figure 1). Nitrite concentration reached as high as 8 mg/L. Clearly, the peak of NO_2^- inhibited reduction of NO_3^-, specifically nitrite reductase. The low rate of nitrate removal

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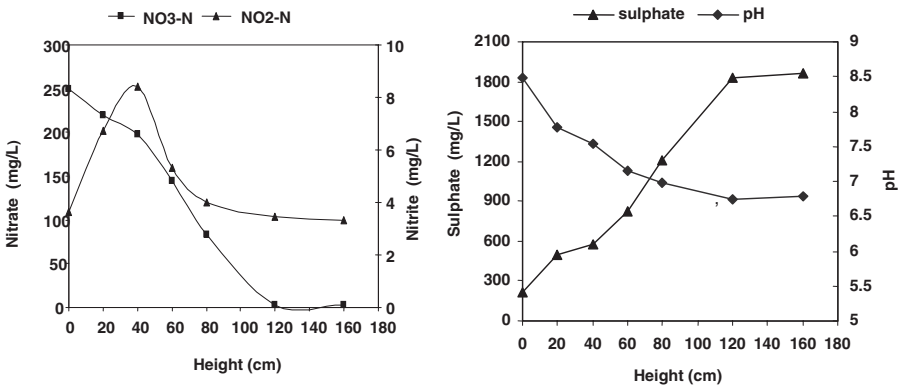

Figure 1 Sulphate (SO_4^{2-}), nitrate nitrogen (NO_3^--N), nitrite nitrogen (NO_2^--N) and pH profiles within the column reactor packed with sulphur particles and limestone granules and fed with synthetic wastewater at an upflow rate of 0.5 L/hr

and the accumulation of nitrite are largely due to the relatively high concentration of dissolved oxygen in the lower part of the reactor column, which inhibits the activity of both nitrate reductase and nitrite reductase in *T. denitrificans* (Justin and Kelly, 1978). The rate of nitrate removal increased when the dissolved oxygen was consumed by bacteria, and when the upflow reached the middle part of the column and higher.

Effect of hydraulic retention time (HRT)

When the hydraulic retention time was 30.5 hrs, more than 98% of total nitrate was removed from the water before the upflow reached a height of 60 cm (Figure 2). In comparison, similar removal rates were observed at 80 and 120 cm for HRTs of 26.9 and 24.7, 18.3 and 14.3 hrs, respectively (Figure 2). The appearance of observable black patches in the lower part of the column and emission of H_2S gas suggested the presence of sulphate-reducing bacteria and the development of anaerobic conditions in the lower part of the column at HRT 30.5 hrs. As the HRTs were reduced to 26.9, 24.7, 18.3 and 14.3 hrs, the denitrification process was divided into three phases: an initial slow, then an accelerated, and finally a decelerated phase (Figure 2). In all cases, more than 97.5% of the total nitrate was effectively removed.

The change of HRT also affects the pH profile in the reactor column. The lower the HRT, the greater the pH drop along the total length of the reactor column (Figure 2). The

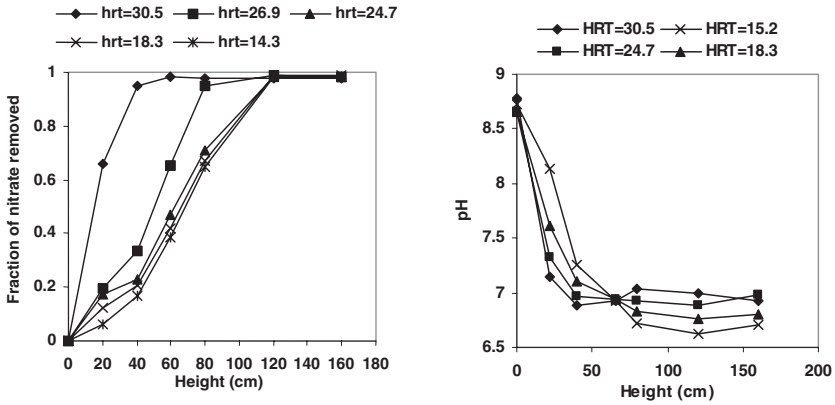

Figure 2 Effect of hydraulic retention times (HRTs) on NO_3^- removal and pH profiles in the column reactor inoculated with *T. denitrificans* MP

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reduction of HRT reduces the contact between the solution and the limestone, thus the buffering effect of the limestone is also reduced. This information is very important to the design of efficient reactors since a balance between the HRT and a near neutral pH is critical for high removal of NO_3^- by *T. denitrificans* (Koenig and Liu, 1996). Other more effective buffering materials should be tested because lower HRT makes treatment more efficient and economical.

The microbial community of the reactor

At the lower part of the column, *T. denitrificans* MP developed a dense, morphologically uniform biofilm (Figure 3). The extracellular metabolites form a network attaching the bacteria firmly onto the sulphur particle surfaces. At the upper part of the column, the species composition of the biofilm was more diverse (Figure 3). A more diverse bacterial community was observed on sulphur granules at the top part of the column as well as in the upflow water. It is possible that, at the top part of the column, the upflow carries more organic materials in the form of metabolites of *T. denitrificans* MP and dead bacterial cells, and microorganisms of different nutritional requirements may grow. From our results, these bacteria may or may not actively participate in the nitrate removal and further investigation is needed to substantiate this hypothesis. Further investigations are needed of this aspect to understand the microbial ecology and activity.

Effects of DO (dissolved oxygen) levels on denitrification in the fermenter system

Higher concentration of oxygen (89% of saturation) inhibited the denitrification in the early phase (Figure 4). As the oxygen level gradually decreased to around 2% of saturation because autotrophic bacteria such as *T. denitrificans* MP can utilize oxygen as an electron acceptor under aerobic conditions (Holt *et al.*, 1994), the rate of denitrification increased when the DO level was maintained at 1.5–2% of saturation. At 97.8 hrs, the level of DO was artificially increased to around 40% of saturation, which again slowed down the denitrification in the fermenter. Similar changes in sulphate production rate were also observed, which was slow in the initial phase when the oxygen level was high, and increased when the oxygen level decreased, but slowed down again when the oxygen level was increased to around 1.3% of saturation. The pH continued to decrease throughout the whole process, but the rate of decrease was more pronounced when nitrate removal was accelerated.

As the reaction proceeded, the oxygen level was lowered to near zero after 83 hrs by purging the system with pure N_2 (Figure 5). When the oxygen level was artificially adjusted between 1.7–2.1% of saturation after 84 hrs of incubation, the reaction was accelerated. The rates of nitrate removal, sulphate production and pH change increased as the oxygen level was kept within this range.

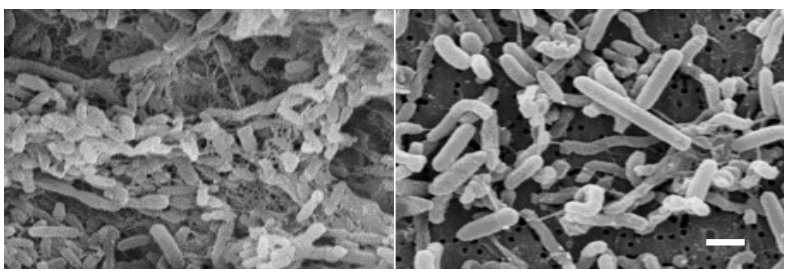

Figure 3. Scanning electron micrographs showing biofilm of *Thiobacillus denitrificans* MP on the surface of sulphur particles taken from the lower part (20 cm from the influent port) (*left*) and upper part (160 cm) (*right*) of the reactor column (scale bar, 2 μm)

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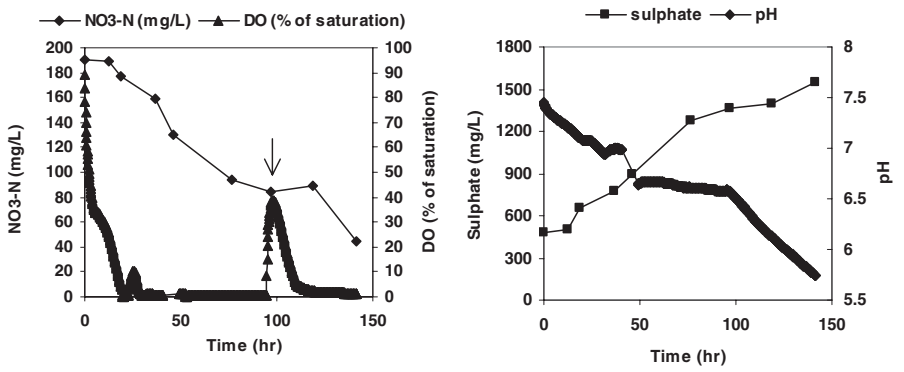

Figure 4. Concentration profiles of NO_3^- and sulphate, saturation of DO and pH in the fermenter system. Note the surge of DO at 97.8 hrs (indicated by an arrow) by manipulation and the slow down of denitrification

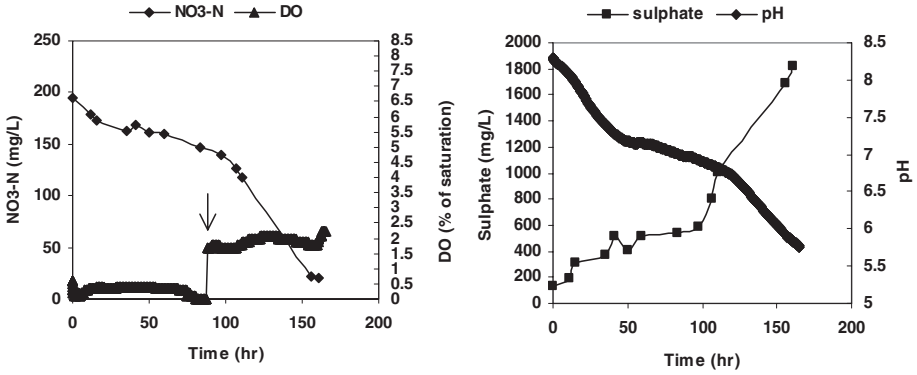

Figure 5. Concentration profiles of NO_3^-, saturation of DO, SO_4^{2-} and pH in the fermenter system when the initial DO was 1.3% of saturation and adjusted to between 1.7 and 2.1 indicated by an arrow

Through a series of controlled experiments it was found that the optimal level of DO is around 2% of saturation for effective NO_3^- removal. This result is different from previous studies, in which trace amounts of oxygen were suggested to be a key reason for nitrite accumulation and inhibition of nitrate removal (Flere and Zhang, 1999). However, our results showed that the denitrifying rate in the fermenter system was increased to an optimum when the bacteria were exposed to a low level of DO maintained at around 2% of saturation. The activities of denitrifying microorganisms were observed to be enhanced after exposure to low levels of oxygen, possibly because some microorganisms need oxygen in order to synthesize haem in the electron transport system (Wood, 1986). Most of the microorganisms known to denitrify are not strict anaerobes, but rather facultative microorganisms, which use nitrate as a final electron acceptor under anoxic conditions (Halling-Sorensen and Jorgensen, 1993). It was shown that 1–2 mg/L dissolved oxygen (13.6–17.2% of DO saturation) in filtrate and 0.5 mg/L (6.8% of saturation) in suspended cultures did not influence the denitrification process (Halling-Sorensen and Jorgensen, 1993). However, a high level of oxygen is clearly inhibitory to the denitrification process. Therefore, it is essential to maintain the DO level to a narrow range so that the rate of nitrate removal can be maximized.

Stoichiometric ratio between nitrate reduced and sulphate produced

During autotrophic denitrification, *T. denitrificans* oxidizes elemental sulphur to sulphate under anoxic conditions while reducing NO_3^- to N_2. The mass of SO_4^{2-} produced per mg of

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NO_3^--N consumed, based on equation [1], is 7.54 mg SO_4^{2-}. In this study, a linear relationship with a slope of 6.81 mg SO_4^{2-}/mg NO_3^--N was achieved in the reactor system with a regression coefficient (r^2) of 0.94. Several different ratios (all in units of mg SO_4^{2-} produced/mg NO_3^--N consumed) were reported before, such as 11.1 (Schippers *et al.*, 1987), 6.4 (Sikora and Keeney, 1976), 9 or 9.9 (Hashimoto *et al.*, 1987), and 7.89 (Koenig and Liu, 1996). The reasons for the differences among them may be due to the different bacteria species involved in each of the reaction systems, column configurations, and operational conditions.

When the concentrations of SO_4^{2-} and NO_3^--N from the fermenter samples were plotted, a line with a slope of 9.32 mg SO_4^{2-}/mg NO_3^--N could be drawn with a regression coefficient (r^2) of 0.97. The stoichiometric relationship was more pronounced in the fermenter than in the reactor column. The ratio of ΔSO_4^{2-} produced/ΔNO_3^--N removed in the fermenter was significantly higher than in the reactor column (6.81 as discussed above).

Summary

The column reactor with an influent concentration of 250 mg/L NO_3^--N had a nitrate removal efficiency above 97.5% at HRTs between 14.3 and 30.5 hrs. At the same time, the sulphate concentrations increased from 190 mg/L in the influent to above 1,800 mg/L in the effluent. Low levels of nitrite were detected and pH dropped slightly along the height of the reactor column. The decrease of HRT reduced the nitrate removal rate along the height of the reactor column, but also increased the pH change along the column, as the time for the limestone to counterbalance the H^+ produced in the autotrophic denitrification decreased. The stoichiometric ratios between the nitrate removed and the sulphate produced were assessed to be 6.81 and 9.32 in the reactor column and fermenter, respectively. The SEM micrographs illustrated that *T. denitrificans* was the dominant bacteria in the reactor column. The species composition is more diverse at the top of the reactor than in the lower part. Utilization of sulphur by the bacteria resulted in the formation of microscopic pores on the sulphur surface. Bacteria were also observed to be attached on the surfaces of limestone. Both high and low levels of DO inhibit autotrophic denitrification by *T. denitrificans* MP by inhibiting the enzymes involved in the biochemical reactions. The maximum denitrification rate of *T. denitrificans* MP in this study occurred when the dissolved oxygen level was maintained between 1.5–2% of saturation.

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