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Autotrophic denitrification for nitrate and nitrite removal using sulfur-limestone

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Abstract

Sulfur-limestone was used in the autotrophic denitrification process to remove the nitrate and nitrite in a lab scale upflow biofilter. Synthetic water with four levels of nitrate and nitrite concentrations of 10, 40, 70 and 100 mg N/L was tested. When treating the low concentration of nitrate- or nitrite-contaminated water (10, 40 mg N/L), a high removal rate of about 90% was achieved at the hydraulic retention time (HRT) of 3 hr and temperature of 20–25°C. At the same HRT, 50% of the nitrate or nitrite could be removed even at the low temperature of 5–10°C. For the higher concentration nitrate and nitrite (70, 100 mg N/L), longer HRT was required. The batch test indicated that influent concentration, HRT and temperature are important factors affecting the denitrification efficiency. Molecular analysis implied that nitrate and nitrite were denitrified into nitrogen by the same microorganisms. The sequential two-step-reactions from nitrate to nitrite and from nitrite to the next-step product might have taken place in the same cell during the autotrophic denitrification process.

Key words: nitrate; nitrite, autotrophic denitrification; sulfur-limestone; denitrifying bacteria

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Introduction

Inorganic nitrogen compounds, i.e., ammonium, nitrate and nitrite, are common wastewater contaminants. Nitrogen removal is important in preventing a wide range of public-health and environmental impacts. Inorganic nitrogen can contribute to eutrophication in natural waterbodies, like rivers and lakes. Ammonium is toxic to aquatic organisms. Nitrate can be easily transformed into nitrite and nitrite is a dangerous cancer inducer and may cause the disease of methemoglobinemia in infants. High concentrations of nitrate and nitrite severely limit the utilization of groundwater for drinking purposes. Therefore, there is a great need to remove N-compounds from various types of water.

Biological processes combining sequential nitrification and denitrification are commonly used for N-compounds removal. The process of denitrification involves the reduction of nitrate to nitrite by anaerobic facultative bacteria that utilize nitrate as electron acceptor. Denitrifying bacteria are generally heterotrophic and need organic matters as electron donor. Nowadays the most common approach for the removal of nitrogen is the heterotrophic denitrification, such as the anoxic/oxic process in most wastewater treatment plants as tertiary treatment. However, there are some unsolvable problems in this process, including the residual organic matters which cause secondary contamination, residual nitrate and nitrite due to the incomplete heterotrophic denitrification.

For water and wastewater with a low BOD/N ratio, autotrophic denitrification is an interesting alternative to the heterotrophic one, due to its two major advantages (Soares, 2002; Rocca et al., 2007; Sierra-Alvarez et al., 2007): (1) no need for an external organic carbon source (methanol, ethanol or acetate) which lowers the cost and risk of secondary contamination; and (2) lower cell yield of autotrophic bacteria and therefore less sludge production, which minimizes the handling of sludge. Autotrophic denitrification is accomplished by denitrifying bacteria which utilizes the inorganic materials other than organic carbon as electron donors while reducing nitrate to elemental nitrogen gas. Studies on autotrophic denitrification processes have currently been divided into three major directions: hydrogen-based process (Lee and Rittmann, 2003), in which hydrogen gas is used as electron donor;

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sulfur-based autotrophic denitrification, in which sulfur compounds, such as elemental sulfur or thiosulfate, are utilized (Moon et al., 2004, 2006); and ANAMMOX process, which applies anaerobic ammonia oxidization bacteria to convert ammonium to nitrogen gas with nitrite as the electron acceptor (Tsushima et al., 2007; Khin and Annachhatre, 2004). Because the anaerobic ammonia oxidization bacteria are hard to cultivate, which greatly hinders the application of ANAMMOX process, and hydrogen gas is difficult to handle and generating hydrogen is usually expensive, recently much more attention has been on sulfur-based process. A few species of sulfur-utilizing autotrophic denitrifiers, such as *Thiobacillus denitrificans* (Moon et al., 2008; Koenig et al., 2005) and *Thiomicrospira denitrificans* (Brettar et al., 2006), have been found to reduce nitrate to nitrogen gas. The following is a stoichiometric equation, which shows an example of elemental-sulfur-utilizing autotrophic denitrification (Koenig and Liu, 2001).

$$1.06NO_3^- + 1.11S + 0.3CO_2 + 0.785H_2O \Longrightarrow$$
$$0.5N_2 + 1.11SO_4^{2-} + 1.16H^+ + 0.06C_5H_7O_2N$$

Energy for autotrophic denitrifying microorganisms is derived from the oxidation reactions of inorganic elements such as hydrogen or various sulfur compounds (H_2S, S, S_2O_3). Autotrophic denitrifiers utilize inorganic carbon compounds (e.g., CO_2, HCO_3^-) as their carbon source. In this process hydrogen ions are produced, indicating that alkalinity is consumed by the reaction. Therefore alkaline, like limestone, is usually added in the sulfur-based autotrophic denitrification reactors.

Sulfur-based autotrophic denitrification has been studied in the treatment of drinking water (Sierra-Alvarez et al., 2007; Wang and Qu, 2003), for the simultaneous removal of S and N from petrochemical industries (Cai et al., 2008; Kleerebezem and Mendezà, 2002), for the removal of N from metal plating wastewaters (Flores et al., 2006), municipal wastewater (Jang et al., 2005; Lau et al., 2006), highly-concentrated wastewaters such as baker's yeast effluent (Kalyuzhnyi et al., 2006) and nitrified leachate (Koenig and Liu, 2002). There are also some studies concentrating on fundamental research areas like the kinetic parameters (Zeng and Zhang, 2005; Liu and Koenig, 2002; Oh et al., 2001) or media composition (Moon et al., 2006). However, very few real applications of this process were found, especially in the developing countries, probably because the lab studies usually demanded pure bacterial inoculants, relatively high running temperature and sufficient nutrient addition. There is no detailed information on the utilization of this process for the treatment of slightly contaminated water at a relatively short retention time, under the natural temperature conditions, and with no external nutrient addition. Moreover, almost no study has been carried out on the autotrophic reduction of nitrite although nitrite makes an even greater risk to human health and aqueous living beings. So far no detailed analysis on the microbial community of autotrophic nitrite denitrification has been reported. The objectives of this study were: (1) to investigate the feasibility of autotrophic denitrification in the long-term treatment of nitrate- and/or nitrite-contaminated water under the stringent temperature and nutrient conditions; (2) to find out the effect of temperature, pH and hydraulic retention time (HRT) on the autotrophic nitrate and nitrite removal efficiency, (3) to compare the microbial community of the microorganisms in nitrite and nitrate autotrophic denitrification processes.

1 Experimental

1.1 Experimental set-up

Two anaerobic up-flow biofilters, as shown in Fig. 1, were used for the continuous treatment of the nitrate- and nitrite-contaminated wastewater. The lab scale reactors consisted of a cylindrical glass tube with the effective volume of about 3.2 L. Sulfur-limestone 2.7 L, with the sulfur/limestone ratio of about 1:1.5 (m/m) and the average diameter of 3–15 mm, was used as media. Due to the cold weather, inoculation was carried out by seeding some digested sludge from municipal wastewater treatment plant into the sulfur-limestone, soaking them in 100 mg N/L nitrate solution and then cultivating at 30°C for 6–8 days in an incubator. After cultivation, the light yellow sulfur-limestone turned blackish. Then 0.6 L of this cultivated sulfur-limestone was mixed with 2.4 L of uncultivated one, and installed into the reactor. The nitrite-fed reactor was started up 9 months later, using 1.0 L of the mature media from the nitrate-fed reactor as seed and mixing with 2.0 L uncultured sulfur-limestone. No temperature controller was installed over the reactors in order to simulate the natural temperature variation. Synthetic nitrate or nitrite solution, without any other chemical added, was used as the substrate for each reactor. The influent nitrate concentration was controlled at 10 mg N/L to represent the eutrophicated surface water, 40 mg N/L as the ground water or effluent from municipal wastewater treatment plant, 70 and 100 mg N/L as the industrial wastewater.

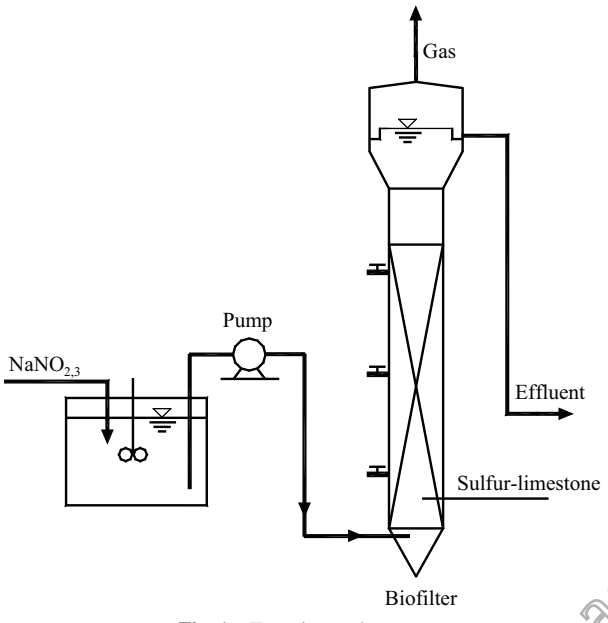

Fig. 1 Experimental set-up.

Synthetic nitrite-contaminated water was prepared with the same N concentration for comparison. Tap water was acidic (pH 6.0–6.2) and the water temperature during the 13-months running time was 5–32°C. The effluent was collected and measured every day for nitrate and nitrite. The effluent sulfate and gas component was measured once every four or five days. And sludge samples were taken once every 1–2 months from the reactor for the analysis of microbial community.

Batch tests were also carried out for studying the effects of each running parameter on nitrate and nitrite removal in the autotrophic denitrification process. A certain amount of cultivated media was taken from the continuously running reactor, sealed in the 125 mL serum bottles and incubated at 28, 18, and 8°C, while shaking at the speed of 100 strokes/min. Nitrate and nitrite with the target concentration of 10, 40, 70 and 100 mg N/L were injected into the serum bottle as the sole substrate. Samples were taken from the serum bottle after the scheduled time interval to measure the concentrations of nitrate and nitrite to investigate their variation under various conditions.

1.2 Analytical method

Nitrate and nitrite determination were performed according to the standard method (APHA, 1998). The ultraviolet spectrophotometric method was applied for nitrate determination and the 1,2-ethanediamine, N-1-naphthalenyl-, dihydrochloride spectrophotometric method was used for the nitrite measurement. Gas chromatography apparatuses (GC-14B/TCD, Shimadzu, Japan) were used for the N_2 and O_2 analysis. Sulfate measurement was carried out following the barium chromate spectrophotometric method.

The DNA analysis was carried out for the sludge samples. DNA was extracted from 0.3 g sludge samples following the protocol described by Kageyama et al. (2003) and Zhou et al. (2007). With a MJ Mini^{TM} thermal cycler (Biorad, US), the extracted DNA was amplified with two pairs of specific primers to detect two different nitrite reductase genes (Forward primer, nirS1F: CCT AYT GGC CGC CRC ART; Reverse primer, nirS6R: CGT TGA ACT TRC CGG T; Forward primer, nirK1F: GCM ATG GTK CCS TGG CA; Reverse primer, nirK5R: GCC TCG ATC AGR TTR TGG) (Braker et al., 1998). For PCR amplification, the total volume of 25 μL of reaction mixtures contained 2.5 μL template, 400 nmol/L of each primer, 1.25 units of rTaq DNA polymerase, 200 μmol/L dNTP mixture and 1×PCR buffer (Takara, Japan). Amplification conditions for the reaction included an initial denaturation at 95°C for 5 min, 30 cycles of denaturation at 94°C for 30 sec, annealing at 60°C (for *nirK* primers) and 65°C (for *nirS* primers) for 40 sec, and extension at 72°C for 30 sec; with the last cycle followed by a 5-min extension at 72°C. The amplicons were examined by electrophoresis for 30 min at 100 V in 1.5% agarose LO3 (Takara Shuzo, Japan) gel. Then the gel was stained with ethidium bromide and photographed under ultraviolet (UV) light.

The amplicons were checked further by restriction fragment length polymorphism (RFLP) analysis. Five types of restriction enzymes, *Alu*I, *Hinf*I, *Mbo*I, *Msp*I and *Rsa*I, were used for digestion. The 15 μL digestion mixture included 0.5 μL of restriction enzymes, 1.5 μL buffer, 5 μL of PCR amplicons and 8 μL of sterilized distilled water. Digestion was completed over night at 37°C and the resulting restriction fragments were checked by electrophoresis in 3% agarose gel.

To compare the microbial community, the PCR amplicons were analyzed with denatured gradient gel electrophoresis (DGGE) using the Dcode universal mutation detection system (Biorad, USA). Electrophoresis was performed on 0.8-mm-thick polyacrylamide gels (8% acrylamide/bisacrylamide, 37.5:1 (*V*/*V*)), with a denaturing gradient ranging from 20% to 50% (100% corresponding to 7 mol/L urea and 40% formamide), at a constant temperature of 60°C in 1XTAE buffer for 270 min at 200 V. After electrophoresis, the gels were stained in 10 μg/mL ethidium bromide solution for 20 min and visualized under UV light.

The specific DGGE bands were excised from the gels with sterile scalpels. The DNA fragments were extracted from the PAGE gel using an EZ spin column PAGE gel DNA extraction kit (Bio Basic Inc., Canada). DNA (2.5 μL) eluted from each DGGE band was used for reamplification. The amplicons were further purified with EZ spin column PCR product purification kit (Bio Basic Inc., Canada), then sent to Shanghai Sangon Biotech Co., Ltd. (China) for sequencing. The sequences obtained were compared with those deposited in the GenBank DNA database (http://www.ncbi.nlm.nih.gov/ and http://blast.ddbj.nig.ac.jp/top-e.html).

2 Results and discussion

2.1 Continuous test on nitrate and nitrite removal

The nitrate removal efficiency of the continuous reactors is shown in Fig. 2. To clearly describe the running performance of nitrate-fed reactor and to avoid the complexity caused by the great amount of data under various running conditions, the data obtained in the 13 months were sorted into four groups according to the influent concentrations although the different concentrations were fed into the same reactor in turn. The performance at the concentrations of 70 and 100 mg N/L is not shown due to the similarity.

Figure 2a shows the NO_3-N removal from 10 mg N/L raw water under different running conditions (Fig. 2b). Because the tap water in this city contains nitrate of about 3 mg N/L, the real influent nitrate was 13 mg N/L. The experiment started in the coldest days of winter, and the low removal efficiency in the beginning proved that the cold weather was unfavorable to the start up of the denitrification reactor no matter at what concentration. From March (day 15 in Fig. 2a and b) when the temperature ascended up to 15°C, restarting the reactor came to be successful and fast. Nitrogen removal increased to a much higher level in the following days (day 15–32 in Fig. 2a and b). The highest removal rate of about 99% was obtained at HRT of 3 hr. However, when HRT was

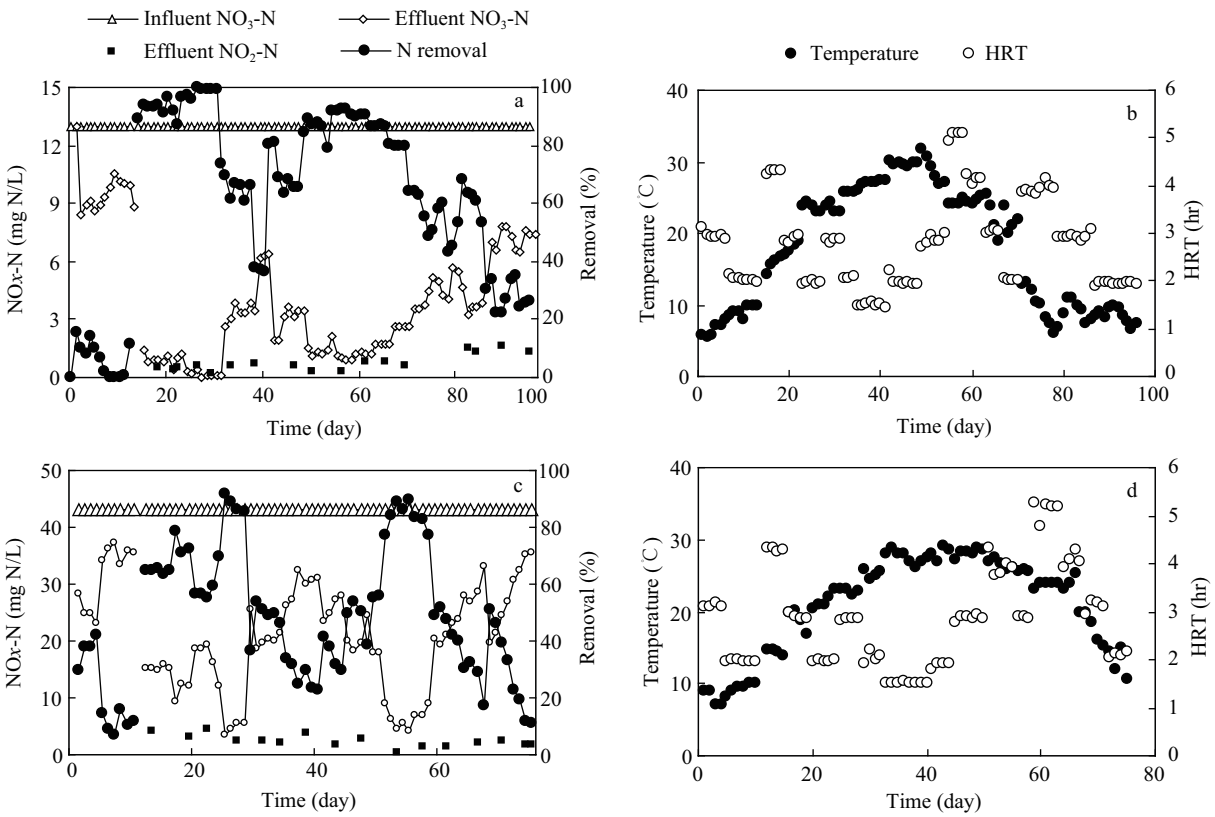

Fig. 2 Running performance of the continuous reactor treating nitrate-contaminated water of 10 mg N/L (a, b) and 40 mg N/L (c, d).

shortened to 2 hr and further to 1.5 hr (day 33–47 in Fig. 2a and b), the removal efficiency dropped greatly. After a few months of good treatment, the treatment efficiency of the reactor showed a slowdown (day 48–70 in Fig. 2a and b) even under the optimal conditions. The reason was later found to be that the continuously multiplying sludge had covered the media and hindered the mass transfer between nitrate and sulfur. This problem was solved later by discharging part of the sludge and mixing the media again. In autumn and winter (day 71–98 in Fig. 2a and b) when the temperature continuously dropped, the treatment performance turned worse. However, after a year of running, even when the water temperature was as low as 5–10°C (day 75–87), about 50% of the influent nitrate could be removed at the HRT of 3 hr. Since 'water bloom', the most serious problem of eutrophicated natural water bodies, does not take place in the cold winter, bacterial survival, rather than nitrogen removal, turned out to be the biggest concern in the biological treatment system. This study verified that the autotrophic bacteria were able to survive the cold temperature and also remain the removal efficiency of about 50%.

The same trend was found at the influent nitrate concentration of 40 mg N/L, as shown in Fig. 2c and d, yet the removal rate was relatively lower than that at 10 mg N/L. Because the tap water in this city contains nitrate of about 3 mg N/L, the real influent nitrate was 43 mg N/L. At the optimum HRT of 4 hr, 80%–90% of the nitrate could be removed. Higher nitrate concentration of 70 and 100 mg/L required longer HRT of 5 and 6 hr to achieve the removal rate of 70%–80%.

Comparing the different influent nitrate concentrations, it was clear that autotrophic denitrification process at short HRT was suitable for low concentration nitrate water, like eutrophicated surface water. Nitrogen in ground water and effluent from municipal wastewater treatment plant (mostly 30–40 mg NO_3-N/L) may also get a relatively high removal rate of 80%–90% at the HRT of 4 hr. But for the contaminated water with nitrate higher than 70 mg/L, longer HRT is required to get the satisfactory removal rate.

Throughout the whole period of continuous experiment, the effluent nitrite remained low regardless of the influent nitrate concentration or HRT, which indicated that the health risk caused by intermediate nitrite in the heterotrophic denitrification could be avoided in autrotrophic denitrification process. This could be an advantage of this process. Nitrogen gas produced by the denitrifiers was detected by gas-chromatography and accounted for 92%–94% of the produced gas, which verified that the reduction reaction was complete, and the final product of both nitrate and nitrite reduction was nitrogen gas.

A nitrite-fed reactor was started in summer, seeded with the sludge from the nitrate-fed reactor. The start-up turned out to be fast and successful. The running performance of the nitrite-fed reactor (Fig. 3) showed great similarity to the nitrate treatment. Good removal efficiency was obtained at a long HRT or low concentration influent. e.g., at the HRT of 3 hr, more than 95% of the 10 mg N/L influent nitrite could be removed by the autotrophic denitrification process, while the 100 mg N/L nitrite could be removed by 60%–70% only when the HRT was longer than 5 hr. Treatment efficiency remained 50%–60% at the most unfavorable temperature of 5–10°C when treating the water containing 10 mg N/L nitrite at the HRT of 3 hr.

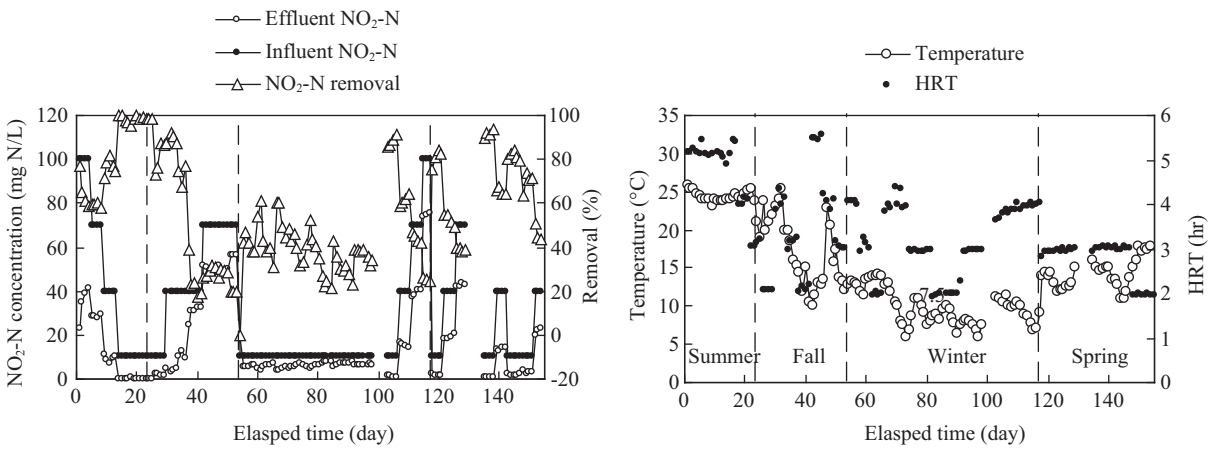

Fig. 3 Running performance of the continuous reactor treating nitrite-contaminated water.

The excess sludge in this process was low. About 300 mL of sludge were discharged from the nitrate-fed reactor, after 415 days operation. No excess sludge was collected from the nitrite-fed reactor. Moreover, the sludge increase in the reactor could be obviously detected only during the summer when the temperature was over 20°C. Almost no excess sludge could be discharged in winter. However, discharging the excess sludge and remixing the media were found necessary in summer because the heavy excess sludge would cover the media, hamper the mass transfer and further affect the reduction efficiency. On the other hand, the excess sludge was of excellent settling ability and easy to be disposed. From the process management, the sulfur-based autotrophic denitrification system required only cheap materials including sulfur and limestone. As for the running cost, since no chemical other than nitrate/nitrite was added in the experiment, the chemical consumption was not considered. Electricity was consumed only on water pumping. Additionally, according to the analysis, nitrogen and oxygen gas accounted for 82%–94% and 17%–5% in the biogas, respectively, which indicated that the treatment of biogas might be unnecessary or at least easy. Therefore, the autotrophic denitrification using elemental sulfur as electron donor proved to be a cheap and easy-to-manage process. Requiring of no carbon source, nutrients or oxygen supply, low and medium concentration nitrate and nitrite, i.e., less than 40 mg N/L, were removed efficiently at a short HRT of 3–4 hr in a mature anaerobic biofilter system.

One of the disadvantages of this process is the high concentration of effluent sulfate. In this study, the effluent sulfate varied with the influent concentrations, removal efficiency and the operational parameters such as HRT and temperature. To find the regularity of sulfate production, a comprehensive factor was necessary. Figure 4 shows the correlation between reduced nitrate/nitrite and the produced sulfate. Although the treatment performance under different running conditions throughout the entire experiment was quite different, good linear correlation was found for both nitrate and nitrite reduction. In order to reduce 1 mol of nitrate, 0.93 mol of sulfur had to be consumed and 89 g sulfate was produced, while for the reduction of 1 mol nitrite, about 0.54 mol of sulfur was oxidized into 53 g sulfate. Sulfate in the local tap water was about 1 mol/L, and the dissolved oxygen (DO) in the raw water might have consumed part of the sulfur. These factors explained the intercept of the trend lines. It might indicate that the raw water with low DO may save the sulfur consumption and therefore lower the running cost. Furthermore, when the influent nitrate or nitrite concentration was lower than 40 mg N/L, the effluent sulfate remained lower than 250 mg/L, which is the upper limit of the sulfate in drinking water criteria in many countries including China.

2.2 Effect of influent concentration, temperature and pH on the nitrate and nitrite removal

Since the temperature was always changing during the continuous test, the effect of each parameter on the nitrate

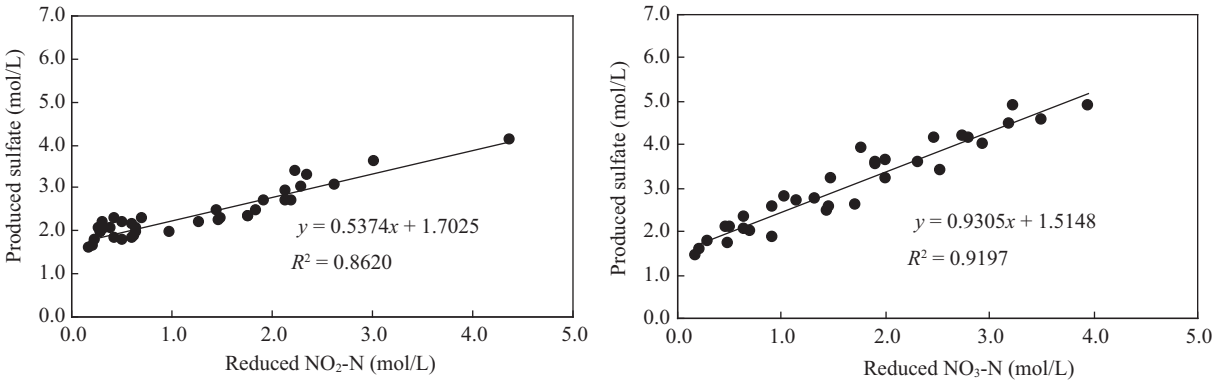

Fig. 4 Correlation between the reduced N and produced sulfate.

and nitrite reduction efficiency remained unclear. Batch test was performed to clarify this question. Results of batch test (Fig. 5) revealed that the surrounding factors and the running parameters have great impact on the autotrophic denitrification process.

2.2.1 Influent concentration

Influent concentration, similar to the continuous test, was a decisive factor of the time required for N reduction. High concentrations of both nitrate and nitrite required long HRT to achieve a high removal rate (Fig. 5a). For instance, the 100 mg N/L nitrate was mostly removed after 8 hr at 28°C, while 10 mg N/L nitrate was removed by 90% in only 150 min. The reduction speed at high concentration turned out to be slightly faster than that of low concentration.

2.2.2 Temperature

High temperatures favored autotrophic nitrification as the batch test (Fig. 5b) clearly showed. Reduction of 10 mg N/L of nitrate was accomplished within 150 min at 28°C, but was only half done in 250 min at 8°C. The reduction speed at 18°C was slightly slower than at 28°C but not much difference was found. This study indicated that starting-up the reactor in a cold season would be difficult and inefficient, but the reactor, once started, could sustain the cold winter if it was running at a suitable HRT.

2.2.3 pH

Although hydrogen ions are produced and alkalinity is consumed in the sulfur-based autotrophic denitrification process according to the stoichiometric equation (Koenig and Liu, 2001), high influent pH did not show much superiority to lower pH either in nitrate or nitrite reduction processes, as batch test showed (Fig. 5c and d). In fact, the influent nitrite at the pH value of 6.0 was reduced more quickly than that at pH of 7.5 (Fig. 5d), probably due to the good buffering ability of the limestone in the media. This finding might indicate that there was no need to add extra alkaline when treating nitrate/nitrite-contaminated water

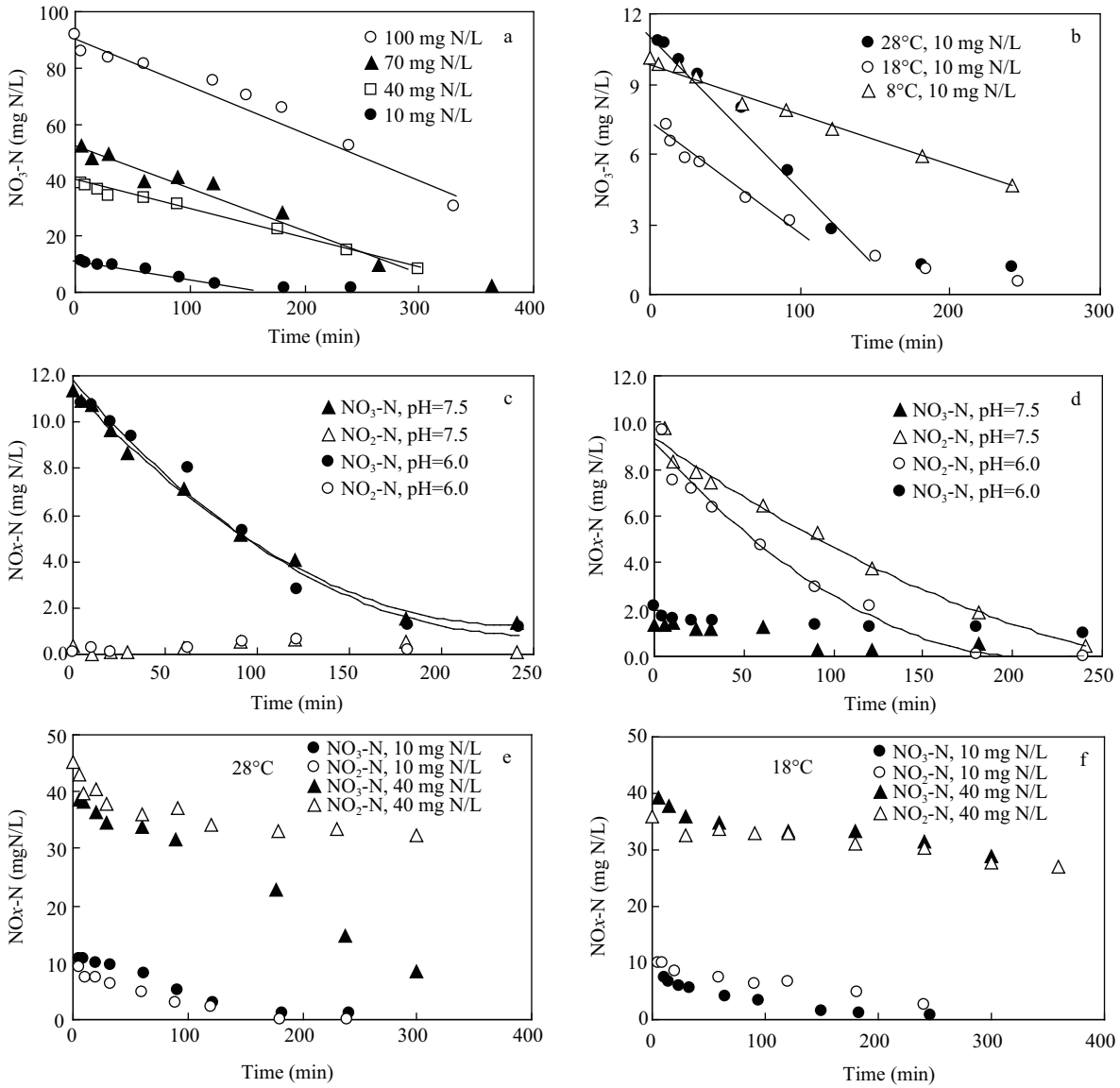

Fig. 5 Variation of NO_3-N and/or NO_2-N in batch test. (a) reduction of NO_3-N at different initial concentrations; (b) reduction of NO_3-N at different temperatures; (c) reduction of NO_3-N under different pH; (d) reduction of NO_2-N under different pH; (e, f) comparison of NO_3-N and NO_2-N reduction rate at 28 and 18°C, respectively.

with sulfur-limestone.

2.2.4 HRT

HRT is a very important consideration, whether in the lab scale test or in real applications. Based on the data from this experiment, HRT of 3 hr was recommended for the eutrophicated surface water with the nitrate concentration of less than 10 mg N/L; 4 hr for ground water and wastewater treatment plant effluent which generally contains 20–40 mg N/L of nitrate; 6 hr or more was necessary to achieve good removal efficiency when treating the water containing more than 70 mg N/L nitrate or nitrite.

2.2.5 Substrate

Both nitrate and nitrite can be reduced effectively under the proper conditions in the autotrophic denitrification process, yet reduction rate of nitrate was always faster than that of nitrite at same initial concentration and under the same temperatures of 28 and 18°C, as shown in Fig. 5e and f. The autotrophic denitrifiers seemed to prefer nitrate to nitrite as substrate.

Some researchers (Chen et al., 2009) believed that the autotrophic denitrification pathway was like that of heterotrophic one, i.e., nitrate is first reduced into nitrite by one species of bacteria then into elemental nitrogen by other species. According to this pathway, in addition to the fact that reduction of nitrite was found to be slower than that of nitrate, there should be a temporary intermediate peak of NO_2-N, which should appear as NO_3-N is reduced and then decrease when N_2 is produced. Yet in our study, no such peak was found in the batch test. Along the whole process of nitrate reduction, nitrite remained almost unchanged (Fig. 5c and d). Therefore this study indicated that nitrite might not be the extracellular intermediate of nitrate reduction in the autotrophic denitrification process. Two possibilities may explain this phenomenon. First, the reduction of nitrate and nitrite were accomplished through different pathways by different microorganisms and neither of them affected the other. Second, nitrate and nitrite reduction is accomplished by the same microorganisms. Reduction of nitrate into nitrite and of nitrite into the next-step product may have taken place in the same cell so that the intermediate nitrite does not appear in the water phase. To answer this question, the microbial analysis was carried out.

Table 1 Sampling time and temperature

	Sampling time (day)	Room temperature (°C)	Season
NO_3-N fed reactor	59	15.7	Spring
	165	27.4	Summer
	224	28.3	Summer
	246	27.3	Summer
	268	24.0	Fall
	325	8.1	Winter
	352	7.5	Winter
	382	15.0	Spring
NO_2-N fed reactor	29	24.0	Fall
	47	18.0	Fall
	86	8.0	Winter
	113	7.5	Winter
	144	15.0	Spring

2.3 Comparison of the microbial community in nitrate and nitrite autotrophic denitrification

Sludge samples were taken from both reactors at different temperatures and seasons as shown in Table 1.

Bacterial DNA was extracted from these samples and PCR amplification was performed. For denitrifying bacteria, two pairs of specific primers, nirS1F/nirS6R and nirK1F/nirK5R, were applied to detect two types of nitrite reductase which contain cytochrome cd_1 and copper, respectively (Braker et al., 1998). Amplification using *nirS* primers were often found successful for the heterotrophic denitrification (such as the anoxic/oxic process), from the authors' research experience. Yet with the autotrophic denitrification bacteria in this experiment, both *nirK* and *nirS* primers functioned, with the amplicons of about 514 and 890 bp, respectively, which indicated that the autotrophic denitrification bacteria were quite different from heterotrophic ones, and that the bacteria in nitrate and nitrite autotrophic denitrification process produced both cytochrome cd_1 and copper containing nitrite reductase.

2.3.1 RFLP analysis of the *nirK* amplicons

To check the similarity of nitrate and nitrite denitrifiers, RFLP analysis was performed and five types of restriction enzymes, namely *Alu*I, *Hinf*I, *Mbo*I, *Msp*I and *Rsa*I, were applied to digest the *nirK* amplicons from both nitrate- and nitrite-fed bacteria. The restriction fragments of *nirK* amplicons are shown in Fig. 6. From the estimation of

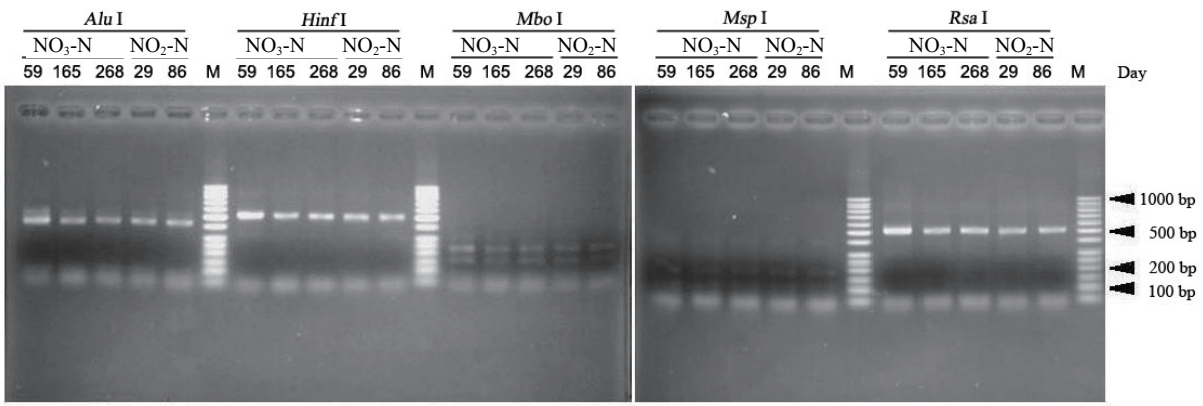

Fig. 6 Restriction fragments from *nirK* amplicons digested by different enzymes.

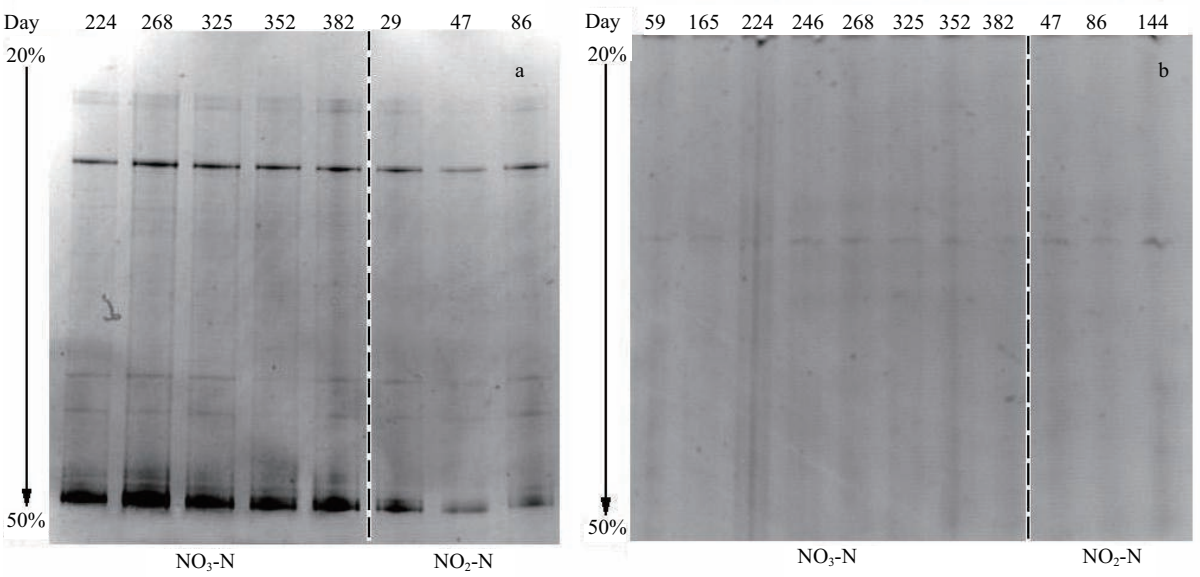

Fig. 7 DGGE band patterns of the *nirS* amplicons (a) and *nirK* amplicons (b). M: marker.

fragment length, it can be deduced that the denitrification bacteria in both reactors were actually more than one species. Moreover, all of *nirK* amplicons showed the same fragment pattern, which indicated that the microorganisms did not vary after 13 months of cultivation in spite of the wide variations of surrounding temperature and operational conditions, and that the denitrification bacteria in nitrate- and nitrie-fed bacteria might be of the same species, which implies that the system treating nitrate-contaminated water can be easily shifted to treat nitrite-contaminated water with no need of re-acclimation.

2.3.2 DGGE band patterns of the *nirS* genes

The PCR amplicons were analyzed with DGGE analysis to compare the microbial community in the samples taken from both reactors. Both *nirK* and *nirS* amplicons were loaded on the 8% polyacrylamide gels, with a denaturing gradient ranging from 20% to 50%. The same analysis was performed twice to verify the reoccurrence. Figure 7 shows the DGGE band patterns of the *nirK* and *nirS* genes. The same band patterns were observed for all the samples from both reactors. From the DGGE gel picture it could be clearly concluded that the same denitrifiers had involved in nitrate and nitrite reduction in the autotrophic denitrification process using elemental sulfur as the electron donor.

Sequences of the three clear bands observed in polyacrylamide gel were obtained, one from *nirK* amplicons and the other two from *nirS* amplicons. The *nirk* gene sequence was found to be similar to *Citromicrobium Bathyomarinum JL354* (Accession Number ADAE01000037) with the similarity of 98%. No similar gene sequence could be found in the gene bank for *nirS* fragments.

Since *nirK* and *nirS* genes were detected in both sludge samples from different substrates, in other words, nitrite reductase existed in both reactors. It would be reasonable to conclude that the nitrite had been the intermediate in autotrophic nitrate denitrification process. However, no nitrite variation was detected in the water phase when the nitrate was being reduced, as batch test showed (Fig. 5c). From the RFLP and DGGE analysis, it might be more clearly concluded that nitrate and nitrite were reduced by the same microorganisms in the autotrophic denitrification process. The two sequential steps from nitrate to nitrite and from nitrite to the next-step product may have taken place in the same cell so that the nitrite may not appear in the water phase as the extracellular intermediate in this process.

3 Conclusions

Autotrophic denitrification process with sulfur-limestone as the electron donor was feasible to remove the nitrate and nitrite, especially from the low concentration water such as eutrophicated surface water, underground water, or wastewater treatment plant effluent. For the higher concentration nitrate and nitrite removal, longer HRT was necessary. Requiring of no carbon source, nutrients or oxygen supply, autotrophic denitrification proved to be a cheap and easy-to-manage process. Influent concentration, HRT and temperature are important factors that affect the denitrification efficiency. pH did not affect the process obviously. The PCR-RFLP and PCR-DGGE analysis implied that nitrate and nitrite were denitrified into nitrogen by the same species of microorganisms. The nitrate reduction pathway in autotrophic denitrification process was discussed. The two sequential steps from nitrate to nitrite and from nitrite to the next product might have taken place in the same cells.

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