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Influence of Reactive Media Composition and Chemical Oxygen Demand as Methanol on Autotrophic Sulfur Denitrification

Qambrani, Naveed Ahmed and Sang-Eun Oh*

Department of Biological Environment, Kangwon National University, Chuncheon, Kangwon-do, Republic of Korea

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Sulfur-utilizing autotrophic denitrification relies on an inorganic carbon source to reduce the nitrate by producing sulfuric acid as an end product and can be used for the treatment of wastewaters containing high levels of nitrates. In this study, sulfur-denitrifying bacteria were used in anoxic batch tests with sulfur as the electron donor and nitrate as the electron acceptor. Various medium components were tested under different conditions. Sulfur denitrification can drop the medium pH by producing acid, thus stopping the process half way. To control this mechanism, a 2:1 ratio of sulfur to oyster shell powder was used. Oyster shell powder addition to a sulfur-denitrifying reactor completely removed the nitrate. Using 50, 100, and 200 g of sulfur particles, reaction rate constants of 5.33, 6.29, and 7.96 $mg^{1/2}/l^{1/2}\cdot h$ were obtained, respectively; and using 200 g of sulfur particles showed the highest nitrate removal rates. For different sulfur particle sizes ranging from small (0.85–2.0 mm), medium (2.0–4.0 mm), and large (4.0–4.75 mm), reaction rate constants of 31.56, 10.88, and 6.23 $mg^{1/2}/l^{1/2}\cdot h$ were calculated. The fastest nitrate removal rate was observed for the smallest particle size. Addition of chemical oxygen demand (COD), methanol as the external carbon source, with the autotrophic denitrification in sufficiently alkaline conditions, created a balance between heterotrophic denitrification (which raises the pH) and sulfur-utilizing autotrophic denitrification, which lowers the pH.

Keywords: Autotrophic denitrification, sulfur particle size, oyster shell, sulfur-denitrifying bacteria, heterotrophic denitrification

High nitrate concentrations in water bodies have caught the attention of the authorities, as <10 and <100 mg NO_3^--N/L are the recommended limits for humans and animals, respectively [11]. Anthropogenic sources like wastewaters, fertilizers, and septic tanks are the main causes of nitrate contamination in water bodies. They may lead to the acidification of soils and water bodies [25], eutrophication [8], and increased N_2O [2], CO_2, and CH_4 emission [16]. Therefore, NO_3^--N in the environment contributes toward global warming and climate change [18]; and the ingestion of nitrate-contaminated water may lead to methemoglobinemia in infants and stomach and gut cancers [3, 15].

Several methods have been employed to remove nitrate contamination, including electrodialysis [26], ion exchange [10], and reverse osmosis [21]; however, most of these methods are either expensive or require high operational costs. Elemental sulfur-based autotrophic denitrification is easy to handle and inexpensive, since it is a by-product of oil processing; in addition, sulfur particles act as the filter media so that no secondary tank is required [12]. Autotrophic colorless sulfur bacteria, such as *Thiobacillus* spp., are facultative denitrifying bacteria that can use sulfur as an electron donor to reduce nitrate into free nitrogen gas. The following stoichiometric reaction shows the sulfur denitrification process [Eq. (1)] [7].

$$NO_3^- + 1.114S^0 + 0.699H_2O + 0.337CO_2 + 0.0842HCO_3^- + 0.0842NH_4^+ \rightarrow 0.5N_2 + 1.114SO_4^{2-} + 1.228H^+ + 0.0842C_5H_7O_2N \quad (1)$$

Despite its advantages, sulfate production and alkalinity destruction are the drawbacks of this method, which cause a decrease in pH. The optimum pH for *Thiobacillus denitrificans* growth is known to be between pH 6 and 8 [1]. Oyster is a dominant product of shellfish and this industry produces large amount of oyster shell waste. As oyster shells are 96% $CaCO_3$, this waste product can be used as a source of alkalinity to control the pH [27]. Different authors have reported the use of oyster shell for alkalinity source and pH control in sulfur-based denitrification [17, 24, 28].

Numerous studies have shown the success and applicability of sulfur denitrification in drinking water [15], ground

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*Corresponding author
Phone: +82 33 250 6449; Fax: +82 33 241 6640;
E-mail: ohsangeun@kangwon.ac.kr

water [17], wastewater [13], and simultaneous autotrophic and heterotrophic denitrification [19]. Heterotrophic denitrification has been efficiently used for nitrate reduction in the presence of adequate amount of organic carbon. Different carbon sources have been used such as methanol, ethanol, acetate, *etc*. Because of the external carbon source, the process becomes expensive and produces a large amount of sludge [12, 19].

The objectives of this batch mode study were as follows: (i) to analyze the effects of reactive media components (*e.g.*, oyster shell powder addition, sulfur granule size and amount), and (ii) to determine the effect of different chemical oxygen demand (COD) levels (0, 95.4, 150, 276, and 644 mg/l) on sulfur-utilizing autotrophic denitrification.

MATERIALS AND METHODS

Culture Preparation

Sulfur-denitrifying bacterial (SDB) culture was prepared in an anoxic master culture reactor (MCR) with a working liquid volume of 1 L to provide a consistent supply of sulfur-denitrifying microorganism culture. Granular sulfur particles (500 g) of 4.0–4.75 mm in diameter were placed in the reactor. Anaerobic sludge from the municipal wastewater treatment plant in Chuncheon, South Korea was used to initially inoculate the MCR. The medium in the reactor was nutrient/mineral/buffer (N/M/B) solution as previously described [19], enriched with 100 mg NO_3^--N/L. Before sealing, the reactor was purged with N_2 gas for 10 min to remove oxygen. The reactor was operated at 30 ± 1°C by wasting and feeding 40% of the total liquid volume (1 L), every fourth day. The fill and draw mode operation lasted for two months. Before transferring the acclimated culture from the MCR to the serum bottle reactors, the sulfur particles in the MCR were agitated with N_2 gas to detach the biomass from the sulfur particles, and then it was used for nitrate removal by SDB in batch mode operation.

Batch Mode Reactor Operation

The reactor consisted of a 550 ml serum bottle equipped with a plastic cap and butyl rubber stopper. To each reactor was added 500 ml of N/M/B solution. Acclimated culture (10–20 ml) from the MCR was also added to each reactor and incubated under different conditions (*e.g.*, oyster shell powder addition, sulfur granule size, and sulfur amount). Two types of reactors with and without oyster shell powder addition (50 g) were run; each reactor received insufficient initial alkalinity (126 mg/l $CaCO_3$), 100 g sulfur particles (4.0–4.75 mm in diameter), and 50 mg NO_3^--N/L. The reactors with 50, 100, and 200 g of sulfur particle amounts (4.0–4.75 mm in diameter), received sufficient alkalinity (612 mg/l $CaCO_3$) and were each enriched with 80 mg NO_3^--N/L. Sulfur particle sizes were selected according to diameter: small (0.85–2.0 mm), medium (2.0–4.0 mm), and large (4.0–4.75 mm), and each reactor contained 100 mg NO_3^--N/L and 100 g of sulfur particles. In order to test the effects of organic carbon on denitrification, different methanol concentrations (95.4, 150, 276, and 644 mg/l COD) were added into the reactors. Before sealing, each reactor was purged with N_2 gas to remove oxygen; all tests were performed at 30 ± 1°C. The aqueous samples were collected by syringe, filtered through a 0.2 μm membrane filter (Fisher Scientific), and stored at 4 ± 1°C until analysis.

Analysis

The samples were analyzed for nitrite, nitrate, and sulfate ion concentrations by ion chromatography (IC) with conductivity detection (ICS-900, equipped with an AS14 anion column; Dionex Corp., Sunnyvale, CA, USA). The pH, COD concentration, and alkalinity were measured as described in the Standard Methods [5].

Half-Order Kinetic Model

Generally, it is considered that substrate consumption inside biofilms follows Monod kinetics, which is represented either as a first-order or zero-order reaction depending on the Ks value. Since, the Monod model has given the previously reported low values of saturation constant Ks for autotrophic denitrification, researchers tried to use a half-order kinetic model for sulfur utilizing autotrophic denitrification [1, 4, 14]. Sulfur-utilizing denitrifying bacteria form a biofilm on the surface of sulfur particles. Denitrification occurs inside the biofilm as sulfur is dissolved by the action of an enzyme from inside into the biofilm and nitrate diffuses from the bulk liquid into the biofilm. The reaction products (SO_4^{2-}, N_2, *etc*) are released into the bulk liquid. Substrate transport in the biofilm pores is not fully effective, so a zero-order reaction in the biofilm changes into a half-order reaction at the biofilm surface [14]. Therefore, a half-order reaction kinetic model was selected to obtain the denitrification rate constants. The half-order kinetic model is expressed as follows:

$$-\frac{dC}{dt} = -kC^{1/2} \tag{2}$$

$$C^{1/2} = C_o^{1/2} - \left(\frac{k_{1/2v}}{2}\right)t \tag{3}$$

$$C^{1/2} = C_o^{1/2} - \frac{1}{2}k_{1/2v}t \tag{4}$$

where C and C_o are the remaining and initial concentrations, respectively, and *t* is the incubation time. The plot of square root $C^{1/2}$ vs. t should be a straight line. The half-order reaction rate can be calculated from the slope of the line. The specific surface area of the reactive media (ω) is obtained by multiplying the specific surface area of the particles by the factor (1 – ε), where ε is the porosity of the reactor media [6].

$$k_{1/2v} = \omega k_{1/2a} \tag{5}$$

where the half-order reaction rate constant per unit volume, $k_{(1/2)v}$, is directly proportional to the half-order reaction rate constant per unit area of biofilm, $k_{(1/2)a}$, by the factor "ω", the specific surface area of the reactor media.

RESULTS AND DISCUSSION

Effect of Oyster Shell Powder on Nitrate Removal

In order to evaluate the effects of pH and buffer on sulfur denitrification, two experiments were run using 50 mg NO_3^--N/L; the first experiment was carried out under insufficiently alkaline conditions (126 mg $CaCO_3$/L) without

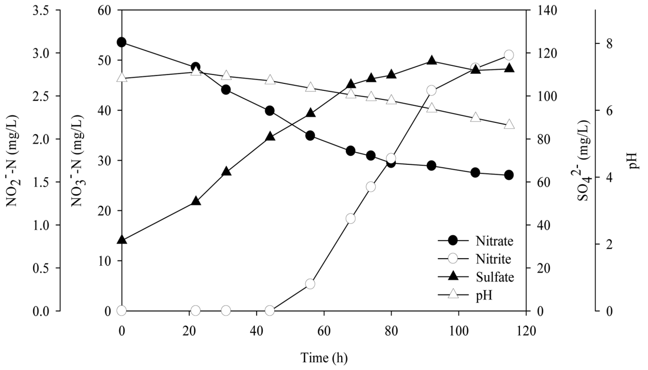

Fig. 1. Profiles of nitrate, nitrite, and sulfate concentrations and pH versus time (without buffer addition).

buffer and the second experiment used 50 g of oyster shell powder containing 96% $CaCO_3$ [27]. According to Eq. (1), the required stoichiometric amount of alkalinity for complete removal of 50 mg/l nitrate-nitrogen is 219.0 mg/l $CaCO_3$. Fig. 1 illustrates the denitrification profile under insufficiently alkaline conditions. Here, about half of the NO_3^--N (26.5 mg/l) was removed and 2.97 mg NO_2^--N/L accumulated in the system as the pH dropped significantly from 7.0 to 5.5 owing to the decrease in alkalinity from 126 to 9 mg $CaCO_3$/L in 5 days. According to Eq. (1), the alkalinity requirement for denitrification is 4.38 mg $CaCO_3$/L; so, for 26.5 mg NO_3^--N/L, 116.07 mg $CaCO_3$/L was required. The alkalinity consumption in the experiment was 117 mg $CaCO_3$/L, which is approximately the stoichiometric alkalinity requirement. The SO_4^{2-}/N ratio was 3.01, which was very low compared with the SO_4^{2-}/N ratio; that is, 7.64 as per the stoichiometry of Eq. (1). If the accumulated nitrite was considered, then the ratio might be 3.39, which is in agreement with previously published ratios of 3.37–7.64 [9].

In the second experiment, a 2:1 sulfur to oyster shell powder ratio was used (Fig. 2) under insufficient initial alkaline conditions. In this case, NO_3^--N was completely removed in 115 h and the pH was 7.45 at the end of the experiment. The SO_4^{2-}/N ratio was 11.13, which is comparable with the previously reported value of 11.1 [22]. Oyster shell powder dissolves slowly in the medium and 50 g of oyster shell powder provides 1,000 mg $CaCO_3$/L in 10 days [17]. Since oyster shell powder was added in this experiment, it dissolved slowly and maintained the pH in the desired range. This result suggests that controlling the pH and alkalinity of wastewater is important for the efficient removal of nitrate ions by SDB under optimum pH conditions.

Effect of Sulfur Amount and Particle Size

Elemental sulfur is an apolar and insoluble mineral, which renders it to be an important rate-limiting factor during the denitrification process. Therefore, the amount of sulfur and its particle size can be important for increasing or decreasing the rate of denitrification. In a previous study, it was observed that a stoichiometrically lower amount of sulfur (compared with NO_3^--N) stopped the sulfur denitrification process [23]. Thus, providing comparatively more than the stoichiometric amount of sulfur should increase the reaction rate. To prove this, three excess amounts of sulfur particles were tested. Three reactors with 50, 100, and 200 g of sulfur particles (4.0–4.75 mm in diameter), respectively, were each enriched with 80 mg NO_3^--N/L. As shown in Fig. 3, nitrate was completely removed at 80 h for the 200 g batch, compared with 130 h for the 50 g batch. Half-order reaction rate constants were calculated for the various sulfur amounts. For 50, 100, and 200 g of sulfur particles, reaction rate constants of 5.33, 6.29, and 7.96 mg$^{1/2}$/l$^{1/2}$·h were obtained, respectively (Table 1). This

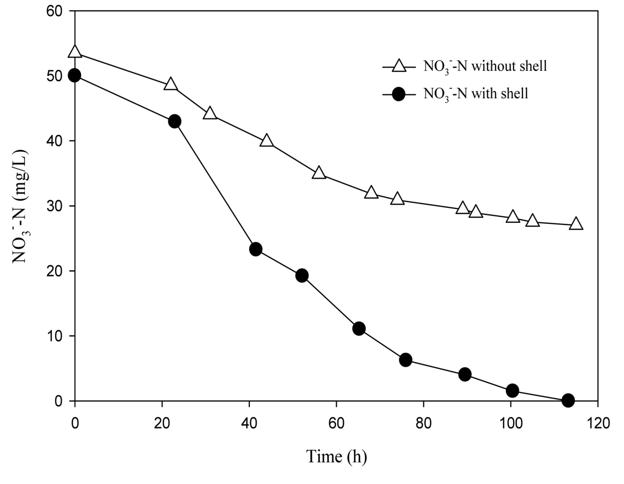

Fig. 2. Nitrate concentration versus time under conditions with or without oyster shell powder addition.

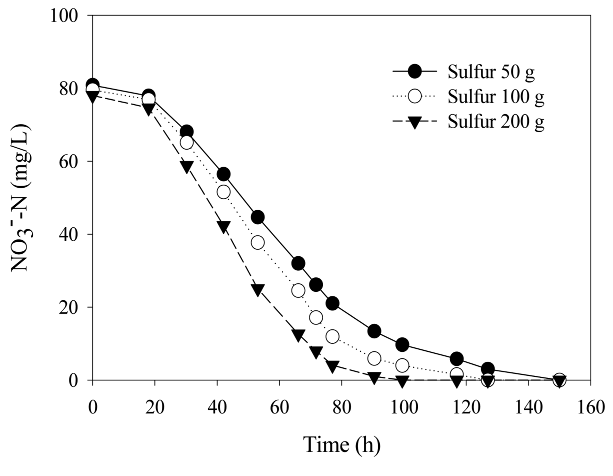

Fig. 3. Nitrate concentration versus time for different amounts of sulfur particles.

Table 1. Half-order reaction rate constants at different NO_3^--N concentrations, depending on sulfur particle amounts and sizes in sulfur-utilizing autotrophic denitrification.

Media composition	Specific surface area of sulfur particles "ω" (dm^2/dm^3)	$k_{1/2a}$	Half-order reaction rate constant $k_{1/2v}$ ($mg^{1/2}/l^{1/2}\cdot h$)
Sulfur particle amount[a] (4.0–4.75 mm in diameter)			
50 g		0.0648	5.33
100 g	82.29	0.0765	6.29
200 g		0.0967	7.96
Mean sulfur particle size[b] (mm in diameter)			
1.23 (0.45–2.0)	293.88	0.1074	31.56
3.00 (2.0–4.0)	120.00	0.0907	10.88
4.38 (4.0–4.75)	82.29	0.0757	6.23

[a] Initial NO_3^--N concentration = 80 mg/l.

[b] Initial NO_3^--N concentration = 100 mg/l.

indicates that an increasing amount of sulfur particles caused an increased rate of denitrification. Increasing the amount of sulfur particles in the reactor provides more surface area for bacteria to make biofilm; therefore, more bacteria will take part in the process and the rate of denitrification will be accelerated accordingly.

In order to determine the effect of sulfur particle size, three sulfur particle sizes were selected according to diameter: small (0.85–2.0 mm), medium (2.0–4.0 mm), and large (4.0–4.75 mm). Each reactor contained 100 mg NO_3^--N/L and 100 g of sulfur particles. Fig. 4 indicates the changes in nitrate concentrations for different sulfur particle diameters. For the small (0.85–2.0 mm), medium (2.0–4.0 mm), and large (4.0–4.75 mm) sulfur particle diameters, reaction rate constants of 31.56, 10.88, and 6.23 $mg^{1/2}/l^{1/2}\cdot h$ were calculated (Table 1). This clearly shows that the smaller the sulfur particle size, the faster the rate of denitrification; and the larger the sulfur particle size, the slower the rate of denitrification. These results are in accordance with previous studies. Moon *et al.* [17] found half-order reaction rate constants of 2.883, 2.494, and 0.677 $mg^{1/2}/l^{1/2}\cdot h$ for sulfur particles smaller than 2.0 mm, between 2.0 and 4.0 mm, and larger than 5.0 mm, respectively. Koenig and Liu [14] also obtained half-order reaction rate constants of 2.94–3.60, 1.47–2.04, and 1.12–1.29 $mg^{1/2}/l^{1/2}\cdot h$ for sulfur particle sizes of 2.8–5.6, 5.6–11.2, and 11.2–16.0, respectively.

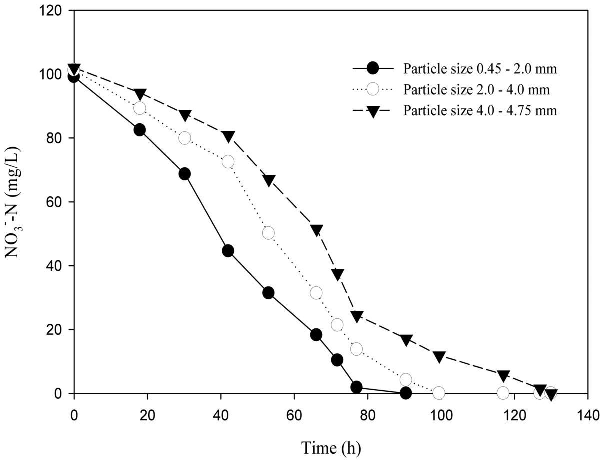

Fig. 4. Nitrate removal versus time for different sulfur particle sizes.

Effect of Methanol Addition on Nitrate Removal

The autotrophic denitrification process does not require an external source of organic carbon; in contrast, heterotrophic denitrification requires organic carbon as the electron donor. Usually, sulfur-denitrifying autotrophic culture is composed mainly of autotrophs; however, heterotrophic denitrifying bacteria were present with autotrophic denitrifying bacteria in a ratio of 5:2 in an inorganic medium-packed bed reactor growing on dead bacterial biomass [4]. Heterotrophs can be very useful if an external source of carbon is provided; in fact, they can start growing heterotrophically in the presence of organics by producing H^+ into the medium to balance the proton loss, and sulfate production will be decreased owing to the heterotrophic part in nitrate removal. Equation (6) shows methanol as the electron donor in a heterotrophic denitrification reaction as follows [12].

$$NO_3^- + 0.833CH_3OH + 0.167H_2CO_3 \rightarrow 0.5N_2 + 0.75H_2O + HCO_3^- \quad (6)$$

In this study, COD (as methanol) was used at 0, 95.4, 150, 276, and 644 mg/l. Media contained sufficient alkalinity and were enriched with 80 mg NO_3^--N/L. Fig. 5 shows the effects of various levels of COD (as methanol) on denitrification. With increasing amounts of COD in the reactor, the denitrification process occurred quickly and sulfate production in the end was also decreased, which

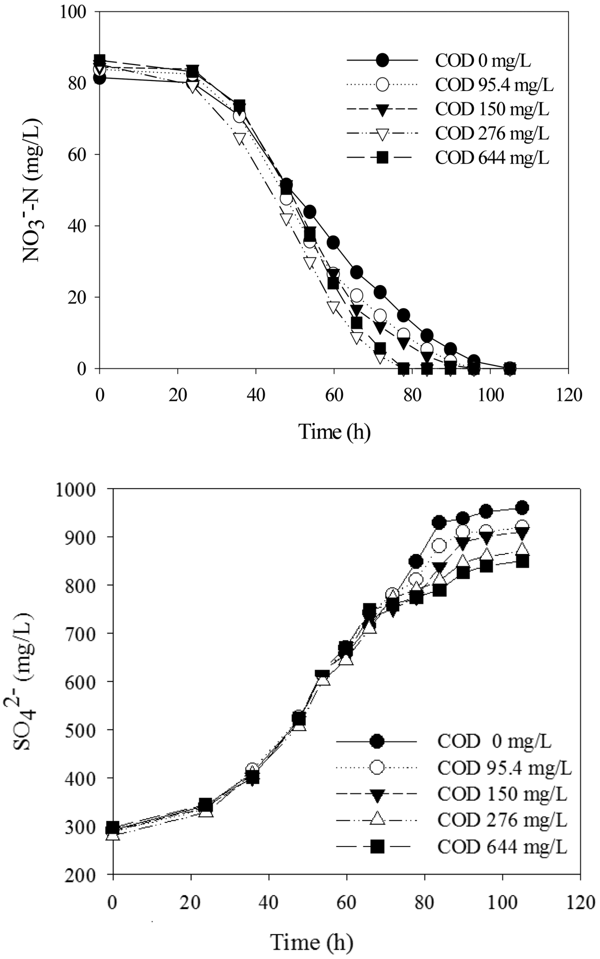

Fig. 5. Nitrate and sulfate concentration versus time in the presence of different concentrations of COD (as methanol).

suggests that some heterotrophic denitrification also occurred. Since heterotrophic denitrification can produce H^+ and autotrophic denitrification can consume H^+, the balance of protons occurred and less amount of sulfate was produced in the presence of COD. Even with stoichiometrically high concentrations of COD (644 mg/l), there was no hindrance in the denitrification process and it occurred smoothly, as previously described [20]. The addition of COD increased the possibility of the presence of some heterotrophs in the mixed culture, suggesting that further experiments are required to exploit its benefits.

In conclusion, elemental sulfur as an electron donor was successfully used for nitrate removal. The use of oyster shell powder showed better results than conventional methods for buffering the medium and maintaining pH, as autotrophic denitrification is a pH decreasing process. Oyster shell powder slowly released $CaCO_3$ in the medium, so it is suitable for maintaining the pH and provides sufficient alkalinity to carry out sulfur-based autotrophic denitrification. In addition, the size and amount of sulfur particles have an effect on the sulfur denitrification process. Smaller sized and increasing amounts of sulfur particles accelerated the rate of denitrification. Heterotrophic denitrification occurred along with autotrophic sulfur denitrification in the reactor supplemented with an external carbon source (COD as methanol), even at high concentrations (644 mg/l). Collectively, the results of this study indicate that autotrophic sulfur denitrification is affected by experimental parameters that can be optimized for the process.

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References

1. Batchelor, B. and A. W. Lawrence. 1978. Autotrophic denitrification using elemental sulfur. *J. Water Pollut. Control Fed.* **50:** 1986–2001.
2. Beaulieu, J. J., J. L. Tank, S. K. Hamilton, W. M. Wollheim, R. O. Hall Jr., P. J. Mulhollande, *et al.* 2011. Nitrous oxide emission from denitrification in stream and river networks. *Proc. Natl. Acad. Sci. USA* **108:** 214–219.
3. Bouchard, D. C., M. K. Williams, and R. Y. Surampalli. 1992. Nitrate contamination of groundwater: Sources and potential health effects. *J. Am. Water Works Assoc.* **84:** 85–90.
4. Claus, G. and H. J. Kutzner. 1985. Physiology and kinetics of autotrophic denitrification by *Thiobacillus denitrificans*. *Appl. Microbiol. Biotechnol.* **22:** 283–288.
5. Clesceri, L. S., A. D. Eaton, A. E. Greenberg, and M. A. H. Franson (eds.). 1996. *Standard Methods for the Examination of Water and Wastewater*, 19th Edition Supplement. American Public Health Association, Washington, DC.
6. Darbi, A. and T. Viraraghavan. 2003. A kinetic model for autotrophic denitrification using sulphur: Limestone reactors. *Water Qual. Res. J. Can.* **38:** 183–192.
7. Driscoll, C. T. and J. J. Bisogni. 1978. The use of sulfur and sulfide in packed bed reactors for autotrophic denitrification. *J. Water Pollut. Cont. Fed.* **50:** 569–577.
8. EPA. 2008. *Integrated Science Assessment for Oxides of Nitrogen and Sulfur – Environmental Criteria*. USEP Agency, Washington, DC.
9. Flere, J. M. and T. C. Zhang. 1999. Nitrate removal with sulfur-limestone autotrophic denitrification processes. *J. Environ. Eng.* **125:** 721–729.
10. Fonseca, A. D., J. G. Crespo, J. S. Almeida, and M. A. Reis. 2000. Drinking water denitrification using a novel ion-exchange membrane bioreactor. *Environ. Sci. Technol.* **34:** 1557–1562.
11. Gu, J. D., W. Qiu, A. Koenig, and Y. Fan. 2004. Removal of high NO_3-concentrations in saline water through autotrophic denitrification by the bacterium *Thiobacillus denitrificans* strain MP. *Water Sci. Technol.* **49:** 105–112.

12. Kim, E. W. and J. H. Bae. 2000. Alkalinity requirements and the possibility of simultaneous heterotrophic denitrification during sulfur-utilizing autotrophic denitrification. *Water Sci. Technol*. **42:** 233−238.
13. Kim, I., S. E. Oh, M. Bum, J. Lee, and S. Lee. 2002. Monitoring the denitrification of wastewater containing high concentrations of nitrate with methanol in a sulfur-packed reactor. *Appl. Microbiol. Biotechnol.* **59:** 91−96.
14. Koenig, A. and L. H. Liu. 2001. Kinetic model of autotrophic denitrification in sulphur packed-bed reactors. *Wat. Res.* **35:** 1969−1978.
15. Liu, H., W. Jiang, D. Wan, and J. Qu. 2009. Study of a combined heterotrophic and sulfur autotrophic denitrification technology for removal of nitrate in water. *J. Hazard. Mater.* **169:** 23−28.
16. Liu, L. and T. L. Greaver. 2009. A review of nitrogen enrichment effects on three biogenic GHGs: The CO_2 sink may be largely offset by stimulated N_2O and CH_4 emission. *Ecol. Lett.* **12:** 1103−1117.
17. Moon, H. S., K. Nam, and J. Y. Kim. 2006. Initial alkalinity requirement and effect of alkalinity sources in sulfur-based autotrophic denitrification barrier system. *J. Environ. Eng.* **132:** 971−975.
18. Mosier, A. R., A. D. Halvorson, C. A. Reule, and X. J. Liu. 2006. Net global warming potential and greenhouse gas intensity in irrigated cropping systems in Northeastern Colorado. *J. Environ. Qual.* **35:** 1584−1598.
19. Oh, S. E., M. S. Bum, Y. B. Yoo, A. Zubair, and I. S. Kim. 2003. Nitrate removal by simultaneous sulfur utilizing autotrophic and heterotrophic denitrification under different organics and alkalinity conditions: Batch experiments. *Water Sci. Technol.* **47:** 237−244.
20. Oh, S. E., K. S. Kim, H. C. Choi, J. Cho, and I. S. Kim. 2000. Kinetics and physiological characteristics of autotrophic denitrification by denitrifying sulfur bacteria. *Water Sci. Technol.* **42:** 59−68.
21. Ravindran, V., M. Pirbazari, and I. Ersever. 2007. Biological denitrification of reverse osmosis brine concentrates: II. Fluidized bed adsorber reactor studies. *J. Environ. Eng. Sci.* **6:** 519−532.
22. Schippers, J. C., J. C. Kruithof, F. G. Mulder, and J. W. van Lieshout. 1987. Removal of nitrate by slow sulphur/limestone filtration. *Aqua (Oxford)* **5:** 274−280.
23. Sierra-Alvarez, R., R. Beristain-Cardoso, M. Salazar, J. Gómez, E. Razo-Flores, and J. A. Field. 2007. Chemolithotrophic denitrification with elemental sulfur for groundwater treatment. *Water Res.* **41:** 1253−1262.
24. Sengupta, S., S. J. Ergas, and L. E. Lopez. 2007. Investigation of solid-phase buffers for sulfur-oxidizing autotrophic denitrification. *Water Environ. Res*. **79:** 2519−2526.
25. Vitousek, P. M., J. D. Aber, R. W. Howarth, G. E. Likens, P. A. Matson, D. W. Schindler, *et al*. 1997. Human alteration of the global nitrogen cycle: Sources and consequences. *Ecol. Appl.* **7:** 737−750.
26. Wan, D., H. Liu, J. Qu, and P. Lei. 2010. Bio-electrochemical denitrification by a novel proton-exchange membrane electrodialysis system − a batch mode study. *J. Chem. Technol. Biotechnol.* **85:** 1540−1546.
27. Yoon, G. L., B. T. Kim, B. O. Kim, and S. H. Han. 2003. Chemical-mechanical characteristics of crushed oyster-shell. *Waste Manage.* **23:** 825−834.
28. Zhang, T. C. and H. Zeng. 2006. Development of a response surface for prediction of nitrate removal in sulfur-limestone autotrophic denitrification fixed-bed reactors. *J. Environ. Eng.* **132:** 1068−1072.