Environ. Eng. Res. Vol. 10, No. 6, pp. 283~293, 2005
Korean Society of Environmental Engineers

KINETICS OF AUTOTROPHIC DENITRIFICATION FOR THE BIOFILM FORMED ON SULFUR PARTICLES : Evaluation of Molecular Technique on Monitoring Biomass Growth

Sungyoun Kim[1], Am Jang[2], I-Tae Kim[1], Kwang-Soo Kim[1], and In S. Kim[3*]

[1]Construction Environment Research Department, Korea Institute of Construction Technology, 2311 Daewha-Dong, Ilsan-Gu, Goyang-Si, Gyeonggi-Do 411-712, Republic of Korea

[2]Dept. of Civil & Environmental Engineering, The University of Cincinnati, Cincinnati, Ohio 45221-0071, USA

[3]Dept. of Environmental Science and Engineering, Gwangju Institute of Science and Technology 1 Oryong-Dong, Buk-Gu, Gwangju 500-712, Republic of Korea

(received June 2005, accepted December 2005)

Abstract : Characteristics of sulfur-based autotrophic denitrification in a semi-continuous type reactor and the kinetic parameters were studied. Enriched autotrophic denitrifying culture was used for the reactor operation. Biomass growth on sulfur particles and in the liquid medium was monitored using the DAPI staining method. From the result of ion concentration changes and the biomass growth, maximum specific growth rate, μ_{max}, and the half velocity constant, K_M, were estimated as 0.61 d^{-1} and 3.66 mg/L, respectively. Growth yield coefficient, Y values for electron acceptor and donor were found as 0.49 gVSS/g N and 0.16 gVSS/g S. The biomass showed specific denitrification rate, ranging 0.86-1.13 gN/g VSS-d. A half-order equation was found to best simulate the denitrification process in the packed bed reactor operated in the semi-continuous mode.

Key Words : Autotrophic denitrification, kinetics, packed-bed, sulfur, biofilm, half-order

INTRODUCTION

Practical application of sulfur-based autotrophic denitrification process requires basic knowledge of the characteristics of autotrophic denitrifiers and its kinetics. In sulfur-based autotrophic denitrification process, bacterial communities responsible for nitrate removal are growing either suspended in the liquid phase or attached to the sulfur medium. Among them, predominant denitrifiers grow in the attached phase in steady state. The relatively constant biomass concentration in the sulfur-based autotrophic denitrification reactor therefore improves the system stability.

Development of a model equation for the sulfur-based autotrophic denitrification should be based on the consideration of possible factors that control the reaction. The presence of nitrate and nitrite as electron acceptors, elemental sulfur as an electron and energy source, the biomass concentration of autotrophic denitrifiers, anoxic condition, alkalinity, time passage and tempe-

† Corresponding author
E-mail: iskim@gist.ac.kr
Tel: +82-62-970-2436, Fax: +82-62-970-2434

rature are some of the important factors in the simulation. Factors influencing the performance of denitrification were investigated by Koenig and Liu[1], that the smaller the sulfur particle size, the faster the nitrate reduction along the height of packed column. The optimal pH and temperature conditions for the growth of *Thiobacillus denitrificans* were found[2] as between pH 7.5 and 8.0, and 30°C. Oh, *et al.*[3,4] reported that the inhibition to autotrophic denitrification occurred at nitrate concentration higher than 660 mgNO_3^--N/L, and the simultaneous reactions of autotrophic and heterotrophic denitrification were observed at chemical oxygen demand (COD) concentrations as high as 2,000 mg/L. The inhibiting effects of sodium chloride (NaCl) and sulfate (SO_4^{2-}) ion on the autotrophic denitrification were reported by Jung, et al.[5], at the levels of 5 g/L and 10 g/L and higher, respectively. The presence of oxygen is the most well known inhibitory factor both in autotrophic and heterotrophic denitrification process, as the approach was applied on the *Activated Sludge Model No. 2* (ASM2).[6] Oxygen tolerance of autotrophic denitrifying bacteria is known to be higher than heterotrophic denitrifiers,[7-9] thus the column operation with higher influent flow rate is allowed for autotrophic denitrification column, compared to heterotrophic denitrification. The study by Wang[10] indicated that there were two kinetic patterns governing the autotrophic denitrification process, suggesting that nitrate removal kinetics followed a half-order reaction when the biofilm thickness on the sulfur particle controls the reaction rate by nitrate diffusion. The reaction kinetic follows zero-order when the biofilm is thin so that the reaction rate is controlled by the nitrate degradation rate. The stoichiometric equation for the sulfur-based autotrophic denitrification is expressed by Koenig and Liu[1] as equation 1.

$$NO_3^- + 1.05S + 0.74H_2O + 0.28CO_2 \rightarrow 0.47N_2 + 1.05SO_4^{2-} + 1.09H^+ + 0.06C_5H_7O_2N \quad (1)$$

During the denitrification process, nitrate removal takes place by sequential reduction of nitrogen oxides. There are three intermediates in the denitrification process, nitrite, nitric oxide and nitrous oxide. Among the three intermediate species, only nitrite was considered in this study. Previous research for the gas composition in the sulfur-based autotrophic denitrification process reported that the production of intermediate gases was hardly observed.[11] The result can be verified with the study with agricultural water system,[12] which significantly suppressed the emission of N_2O gas by elemental sulfur addition to the system, by inducing sulfur-based autotrophic denitrification. In this study, denitrification is therefore considered as two separate steps, nitrate reduction and nitrite reduction.

In the denitrification process, production and reduction of nitrite must be given more attention than nitrate reduction, as it is more toxic than nitrate itself in the environment. Factors governing the nitrite accumulation have been reported as pH,[13,14] source of electron donor,[15,16] and the presence of oxygen,[17] although in most cases more than one factor are involved.

The reduction of specific nitrogen oxides is considered to depend solely on their substrate concentrations according to the *Michaelis-Menten* equation (Eq. 2). In the autotrophic denitrification process using sulfur particles, electron acceptors such as nitrate and nitrite concentration are only considered as substrate, but the elemental sulfur as an electron donor can be ignored as it is in the solid phase. As an excessive amount of sulfur is always available in the reactor, the change of the sulfur concentration during the process is negligible. As a result, nitrate or nitrite accumulation would occur when maximum reduction rate (v_{max}) of either substrate is lower than the other.

$$v = (v_{max}\ S) / (K_M + S) \quad (2)$$

Where v is reaction rate, v_{max} is maximum reaction rate, S is either nitrate or nitrite concentration, and K_M is the *Michaelis-Menten* coefficient. The values of v_{max} and K_M can be

determined using the Lineweaver-Burk plot, by conversion of the equation 2 to 3.

$$1 / v = (K_M / v_{max}) (1/S) + (1 / v_{max}) \quad (3)$$

The relationship between 1/v and 1/S is plotted linearly with the slope (K_M/v_{max}) and the y intercept ($1/v_{max}$).

The reduction rate of nitrite is only dependent on the nitrite concentration, in other words, production of nitrite from nitrate reduction. Therefore if nitrate reduction rate is higher than nitrite reduction rate, it would result in nitrite accumulation. Reaction rates of nitrate and nitrite can be expressed differently as Equation 4 and 5.

$$v_A = - (1/X)(dS_A/dt) \quad (4)$$

$$v_B = -v_A - (1/X)(dS_B/dt) \quad (5)$$

Where v_A is nitrate reduction rate, v_B is nitrite reduction rate, S_A is the nitrate concentration, S_B is the nitrite concentration, X is the biomass concentration, and t is time.

In the sulfur-based autotrophic denitrification, the measurement of biomass growth within the packed media is not easily conducted, because the biomass grows mostly in the form of biofilm on sulfur particles, which evaporate during the conventional MLVSS (Mixed Liquor Volatile Suspended Solid) measurement process. This has hampered the development of accurate kinetic models. Recently the DAPI (4'6'-diamidino-2-phenylindole hydrochloride) staining method was applied to several studies for the monitoring of biomass growth in sulfur-based autotrophic denitrification process.[18,19] In this study, this approach for the measurement of biomass growth was evaluated by the simulation of the activity of autotrophic denitrifying bacteria, both in the attached and suspended forms. Using the results of active biomass growth and ion concentration changes including nitrate and nitrite, successful development of kinetic equation was attempted to express nitrate removal reaction in sulfur packed autotrophic denitrification reactor.

The objectives of this study are; 1) to find out kinetic parameters of sulfur-based autotrophic denitrification in the sulfur-packed reactor, 2) to simulate nitrate reduction in a semi-continuous type reactor, 3) to evaluate the cell counting method in kinetic studies.

MATERIALS AND METHODS

Enrichment of Seeding Culture

Sulfur-based autotrophic denitrification culture was enriched in 200 L master culture reactor. Initial culture was originally from the return sludge in a local municipal wastewater treatment plant, and the culture was operated under autotrophic condition, for around two years in a semi-continuous type operation. Feeding solution contained 722 mg/L KNO_3, 1.0 g/L $Na_2S_2O_3$ $5H_2O$, 1.5 g/L $NaHCO_3$, 5.6 mg/L K_2HPO_4, 1.0 mg/L $MnSO_4$ $4H_2O$, 5.74 mg/L NH_4Cl, 1.0 mg/L $CaCl_2$ $2H_2O$, 1.0 mg/L $FeCl_2$ $4H_2O$, and 1.0 mg/L $MgCl_2$ $6H_2O$.

Formation of Biofilm on the Sulfur Particle

Sulfur particles with a diameter of 2-4 mm were used in this study. Average mass of a particle was 67.7 mg/particle with ± 8.3 mg of standard deviation. A one-liter flask filled with 1 liter of sulfur particles was used as a denitrification reactor. Enriched culture was inoculated into the reactor with fresh nitrate medium in 3:7 volume ratios, resulting in a total 500 ml of liquid solution. Initial pH of the medium was maintained 8.0 (±0.1) by addition of 0.1 N HCl or NaOH if needed. The liquid medium was flushed with nitrogen gas for 15 minutes before it was introduced into the reactor to minimize dissolved oxygen level. It was then treated in the sulfur-packed reactor by a semi-continuous type operation. When nitrate and nitrite levels in the liquid medium were reduced below 5 mg N/L, it was replaced by a fresh medium, making the final NO_3^--N concentration to around 50 mg N/L. Synthetic medium consisted of 361 mg/L

KNO_3, 1.0 g/L $NaHCO_3$, 0.3 g/L KH_2PO_4, 0.1 g/L $MgSO_4$ $7H_2O$, 0.2 g/L NH_4Cl and 1ml/L of trace metal solution.[20] The flask was set on a digital shaker at 100 rpm. Nitrate monitoring and the replacement of fresh medium were repeated several times until the maximum denitrification rate did not change within 5%. The experiment was carried out at 35°C constant temperature room. Table 1 summarizes the experimental conditions.

Table 1. Conditions of experimental setup

Reactor volume	1 L
Packed sulfur particle volume	1 L
Liquid medium volume	500 ml
Volume % of culture in liquid medium	30 %
Cell numbers in master culture	7.40×10^8 cells / L
Initial cell numbers in liquid medium	2.22×10^8 cells /L
Operational mode	Semi-continuous (fill and draw)
Influent Nitrate-N concentration	50 mg N/L
Influent pH	8.0 (±0.1)
Temperature	35°C
Rotating speed	100 rpm

Sample Analysis

Liquid samples were taken from the reactor every 2-4 hours for the analysis of ion concentrations such as nitrate, nitrite and sulfate. To monitor biomass growth of autotrophic denitrifiers, liquid and sulfur particle samples were regularly taken for the measurement of volatile suspended solid (VSS) for liquid samples, and DAPI staining of the sulfur particle. Liquid samples were also taken for the total cell count. Specific anion levels such as chloride, nitrite, nitrate, phosphate and sulfate were determined by ion chromatography (DX-120 Ion Chromatography, Dionex, U.S.A.). Standard methods[21] were used for the measurement of VSS levels in the liquid samples. Total viable cell numbers for sulfur particle and bulk liquid samples were counted by DAPI staining method. The relationship between the cell numbers measured with DAPI method and the MLVSS levels was developed using number of liquid samples from the enriched culture of autotrophic denitrifier.

DAPI(4',6'-diamidino-2-phenylindole hydrochloride) Staining

Five grams of wet sulfur particles were taken into the 15-ml capped tube with 10-ml washing buffer (PBS, pH 7.2), and treated three times in vortex for five minutes each. Detached cells in liquid buffer were then washed twice with 1.5 ml PBS buffer in 14,000 rpm centrifuge for 4 minutes. After washing, 1 ml of 4% paraformaldehyde was added and the samples were stored at 4°C for more than 1 hour. The cells were then washed again with PBS buffer. In each sample in pellet-form, 1-ml of fixing solution (PBS: EtOH (96%) = 1:1) was added and stored at -20°C. Fixed samples were then diluted as wished with PBS buffer. Regarding the microbial concentrations of individual sample, dilution factors can be varied from 10^{-1} to 10^{-12}. Slides with 12 wells of 5-mm diameter each were used for DAPI staining. In each well, 6.5 µl of diluted sample was loaded and dried in air. The slide was then immersed in the serial 50, 80 and 98% ethanol solutions for 3 minutes each and dried. In a dark chamber, the slides were stained with DAPI staining solution for five minutes, rinsed with deionized water, and dried. Mounting solution was then applied to each well of slide, followed by a cover glass. Stained cells were observed using an epifluorescent microscope (Axiolab, Zeiss, Germany) under x1000 magnification. The cell counting was conducted over ten random microscopic views, at excitation wavelength of 365 nm.

RESULTS AND DISCUSSIONS

Kinetic Parameters

It took a total of nine days for the biomass growth in the semi-continuous reactor to become

stabilized and the maximum denitrification rate remained at 5% ranges. Figure 1 illustrates the increase of viable cell numbers in the sulfur particles and in the liquid medium. The numbers were converted from the numbers in unit mass of sulfur and a unit volume of the liquid medium, to the total cell numbers present in the whole reactor. Total cell numbers increased rapidly for the first five days of operation and then suspended in narrow ranges. It was observed that the majority of the viable cells presented as attached to the sulfur particles and less than 5% were in the liquid suspension. The result also indicates the rapid biofilm formation and the stabilization of the process within five days of operation, so that the constant microbial concentration in the sulfur particles ensures the process stability and the high performance of nitrogen removal.

The number of total viable cells in the reactor was then converted to the values of active biomass concentration, by developing the relationship between total cell numbers and the conventional MLVSS levels. Enriched liquid culture samples in different concentrations were used for the DAPI staining methods and the MLVSS measurement. Figure 2 shows the linear relationship between the viable cell numbers and the MLVSS values. The conversion factor found in this relationship was used in later experiment in the determination of kinetic parameters.

From the relationship found in the Figure 2, the total biomass growth in the reactor can be

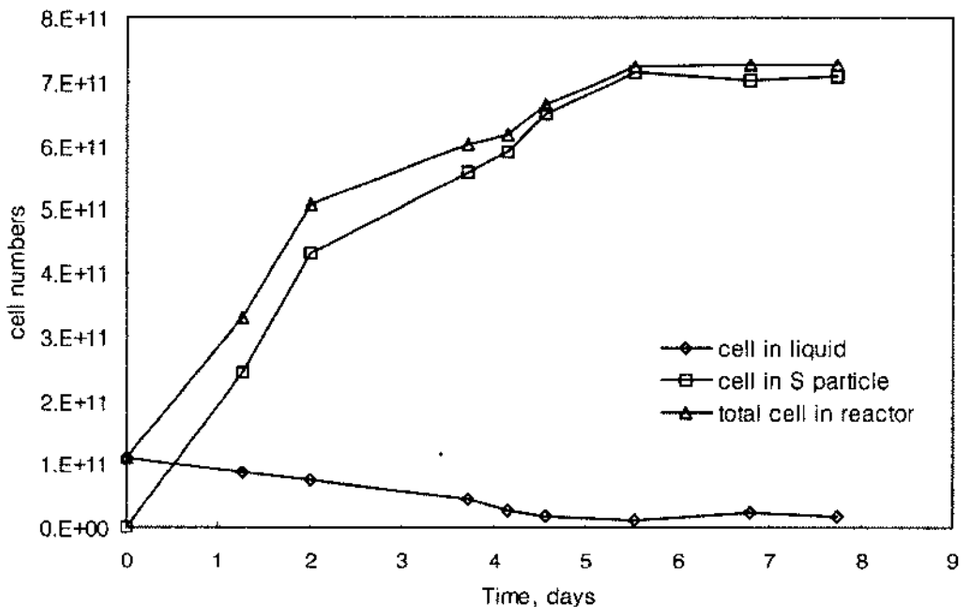

Figure 1. Biomass growth monitored using the DAPI staining method during the semi-continuous experiment.

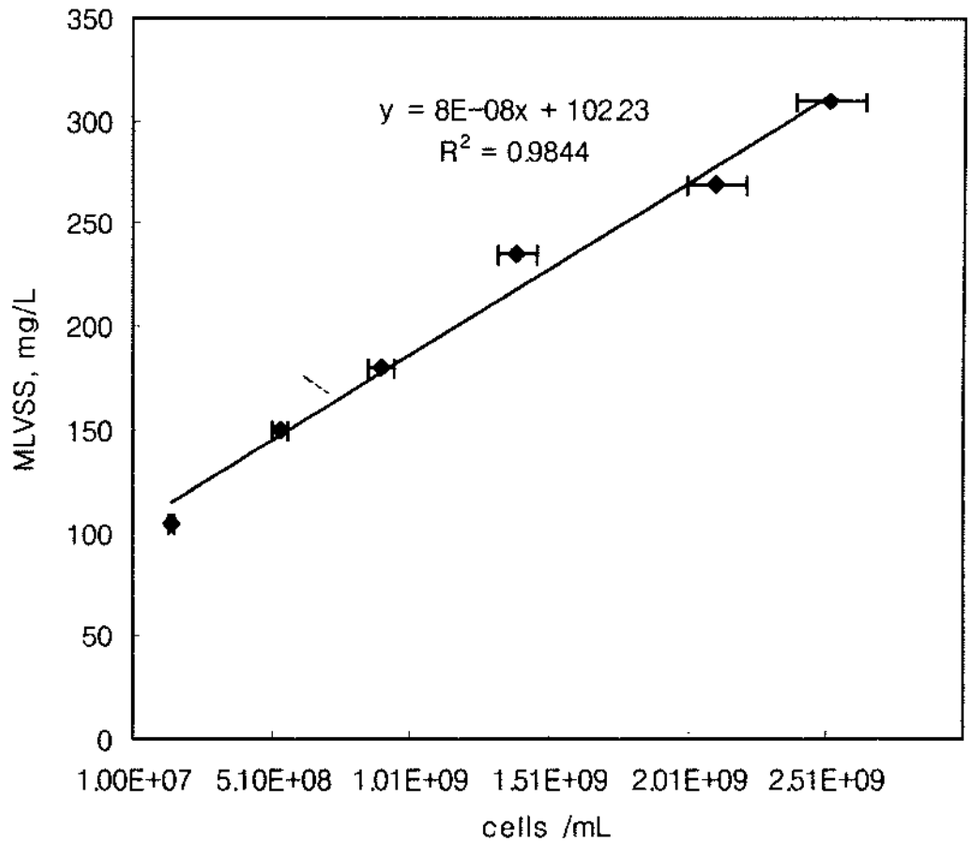

Figure 2. Linear relation between the cell numbers measured by DAPI staining and MLVSS values.

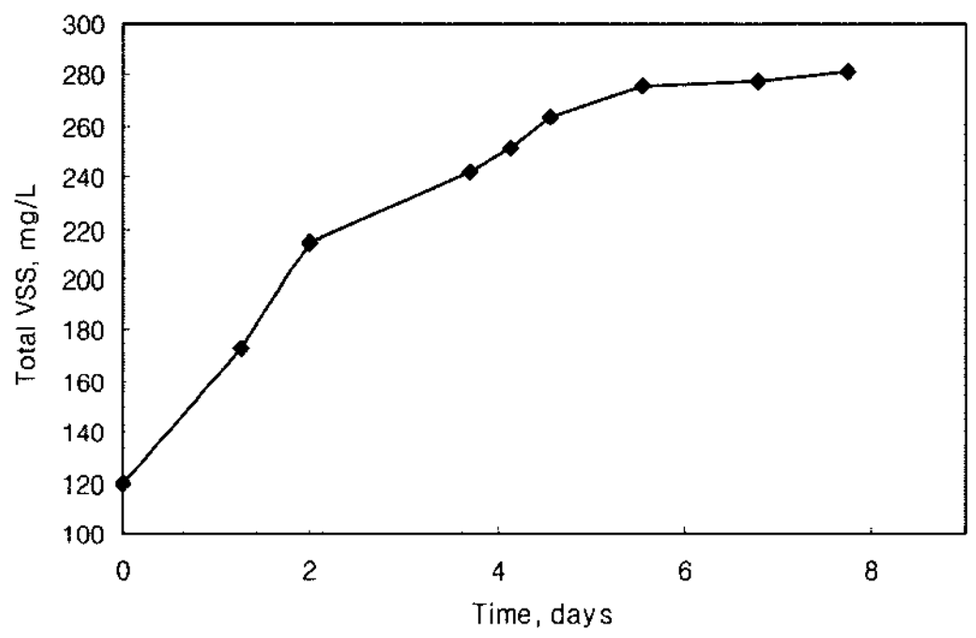

Figure 3. Total biomass growth in the reactor.

expressed in MLVSS values (Figure 3).

From the biomass growth data, specific growth rate $\mu(d^{-1})$ during the growth phase and the doubling time (d) of the culture can be determined. In a closed system where growth is the only process affecting cell concentration, Equation 6 expresses the biomass growth as a function of time.

$$X = X_0 \, e^{\mu t} \tag{6}$$

X_0 is the viable cell concentrations at time zero. Equation 6 represents exponential growth. Taking natural logarithms:

$$\ln X = \ln X_0 + \mu t \tag{7}$$

According to Equation 7, a plot of lnX versus

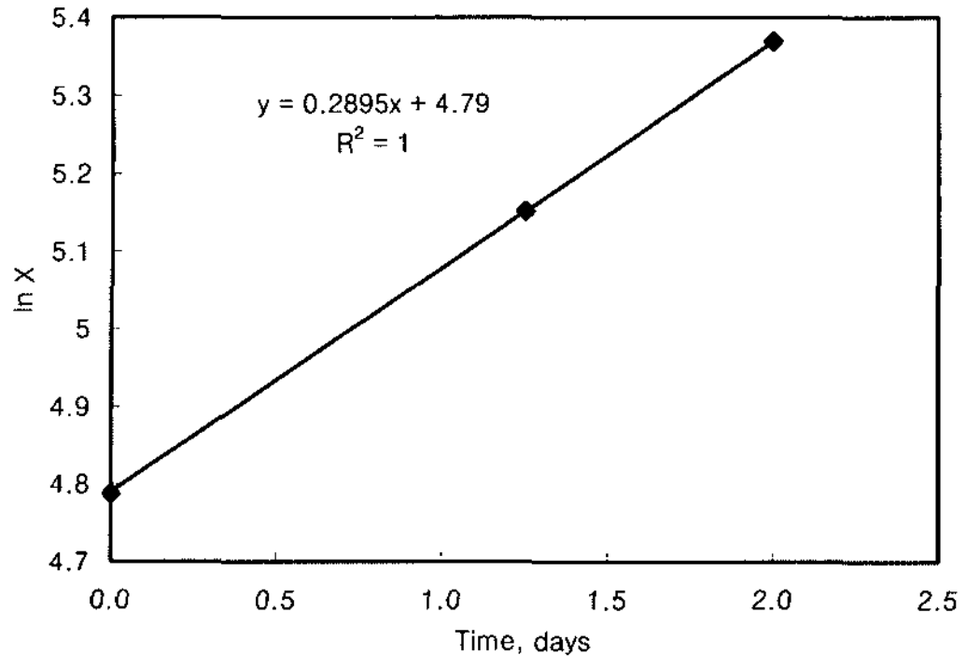

Figure 4. Calculation of specific growth rate.

time gives a straight line with slope μ. In Figure 3, no lag phase is evident, and the growth reaches stationary phase at around day five. Since the first three data points show the exponential growth clearly, those data were used to determine the specific growth rate (Figure 4).

Fitting a straight line to the data gives a slope of 0.29. Therefore, specific growth rate (μ) equals to 0.29 d^{-1}. This is also regarded as maximum specific growth rate (μ_{max}).

Doubling time refers at the time when the biomass concentration X equals to $2X_0$. By substituting X to $2X_0$ in the equation 7, doubling time is expressed as Equation 8.

$$t_d\ (d) = \ln 2 / \mu \qquad (8)$$

In this experiment, doubling time of the culture is 2.4 d. Using the Equations 2 and 3, substrate concentration K_M was determined as 2.4 mg NO_3^--N/L.

Based on the biomass growth and nitrate removal data, specific growth rate, μ, and the observed growth yield of the autotrophic denitrifiers, Y_{obs}, based on the electron acceptor and donor were estimated as 0.29 d^{-1}, and 0.49 g VSS/g N and 0.16 g VSS/g S, respectively. The biomass in the reactor showed specific denitrification rate, *q*, ranging 0.86-1.13 g N/g VSS-d. Table 2 summarizes the kinetic parameters for sulfur-based autotrophic denitrification as estimated in this study and other data available in the literatures.

The changes of pH levels before and after complete denitrification reflected that the synthetic medium provided high enough alkalinity for the activity of autotrophic denitrifiers. The pH levels of fresh medium also kept the reactor environment in optimum condition for autotrophic denitrification. As Koenig and Liu[22] stated, limestone addition was not necessary in this experiment in which alkalinity was provided as a form of bicarbonate at an alkalinity/N ratio of higher than 2, and the pH of medium in the reactor was maintained within 7.2-7.5 ranges.

Denitrification Rate

The fill-and-draw mode of operation was conducted a total nine times and the average result of the three nitrate reduction cycles is illustrated in Figure 5. It took 8.7 days to complete the nine-repetition of denitrification test. From the 5th cycle, the patterns of nitrate and nitrite reduction and the sulfate production

Table 2. Kinetic parameters for sulfur-based autotrophic denitrifiers

Electron donor	Y (mgVSS/ mgN)	Y (mgVSS/ mgS^0)	Specific growth rate, μ(d^{-1})	K_M (mg/L)	Max. sp. denitrification rate (gN/gVSS d)	References
$S_2O_3^{2-}$	0.57	-	2.64	0.2	4.23	[2]
$S_2O_3^{2-}$	0.4 -0.5	-	2.9-4.8	3 - 10	8.40	[3]
$S_2O_3^{2-}$	0.37	-	-	-	-	[24]
S^0	-	-	-	-	0.1-0.2	[25]
S^0	-	-	-	-	0.15	[1]
S^0	-	0.10	1	-	-	[26]
S^0	0.33	-	-	-	0.19-0.24 (gN/gTOC d)	[27]
S^0	0.49	0.16	0.29	2.4	1.13	This study

became stabilized and did not change in 5% ranges. Figure 5 is the average of three cycles (6^{th} to 8^{th} cycles) of nitrate removal test with standard error bars.

Figure 5 suggests that while nitrate reduction took place rapidly with the addition of fresh medium, the nitrite concentration increased until the nitrate concentration decreased and reached around 6.5 mg/L. As Vidal, *et al.*[9] reported, rapid initial decrease of nitrate induced the accumulation of nitrite, however this is not the case in a packed-bed denitrification process where nitrite production is relatively lower. The production of nitrite could be reduced by improving anaerobic condition, either by the reduction of flow rate in column process, or by enlarging the cross sectional area to reduce advective flux of oxygen. Nitrite reduction was then followed to be relatively slower than nitrate reduction and was completed after around 1.2 days.

Similar results were reported by Kimura, *et al.*[23], from the combined elemental sulfur-based denitrification and membrane filtration process. In their research, the batch test produced very high nitrite concentration, comprising about 80% of influent nitrate concentration and the nitrite reduction followed when nitrate removal was completed. However in the long-term operation with a continuous feeding, a substantial reduction in nitrate concentration occurred immediately without any nitrite accumulation. Likewise, the relatively high nitrite concentrations in this experiment have never been observed in

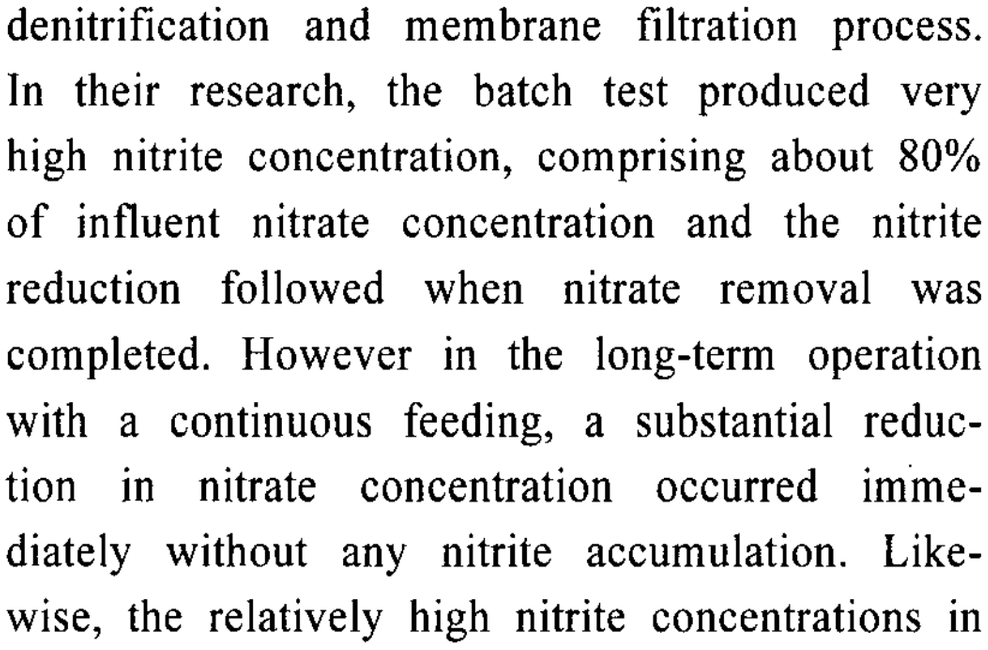

Figure 5. Average nitrate-N, nitrite-N and sulfate profiles for S-based denitrification.

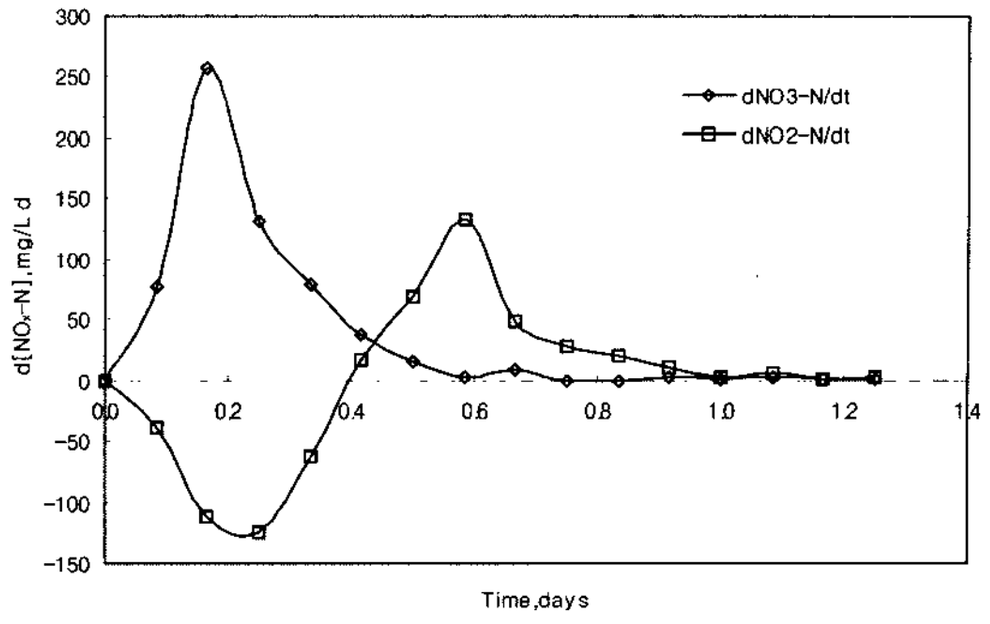

Figure 6. Rates of nitrate and nitrite reduction.

previous continuous column tests in this laboratory, where nitrite concentration was always less than 5 mgNO_2^--N/L.

Figure 6 illustrates time course of reduction rates for nitrate and nitrite. During the first four hours of reaction, a rapid increase in the nitrate reduction occurred, and then the reduction rate decreased gradually until the entire nitrate was removed. On the other hand, nitrite accumulation was observed at the beginning of the reaction, and then the reduction rate increased in opposite direction as the nitrate reduction rate decreased. The maximum nitrate and nitrite reduction rates were 0.26 and 0.13 kg/m^3d, respectively.

Simulation of S-based Autotrophic Denitrification

Based on the time course of nitrate removal, it can be determined which order kinetics the nitrate reduction reaction follows. Depending on the reaction order, each reaction can be described with following equations (Eq. 9 - 11).

$$\text{Zero order: } C = C_0 - k_0 t \quad (9)$$

$$\text{Half order: } C^{1/2} = C_0^{1/2} - 1/2\, k_{1/2} t \quad (10)$$

$$\text{First order: } \ln C = \ln C_0 - k_1 t \quad (11)$$

Where C_0 and C are the substrate concentrations at time zero and t, and k_0, $k_{1/2}$ and k_1 are the reaction coefficients of zero, half and first order, respectively. The t is time. It was found that the denitrification process in the sulfur-packed reactor followed half-order reac-

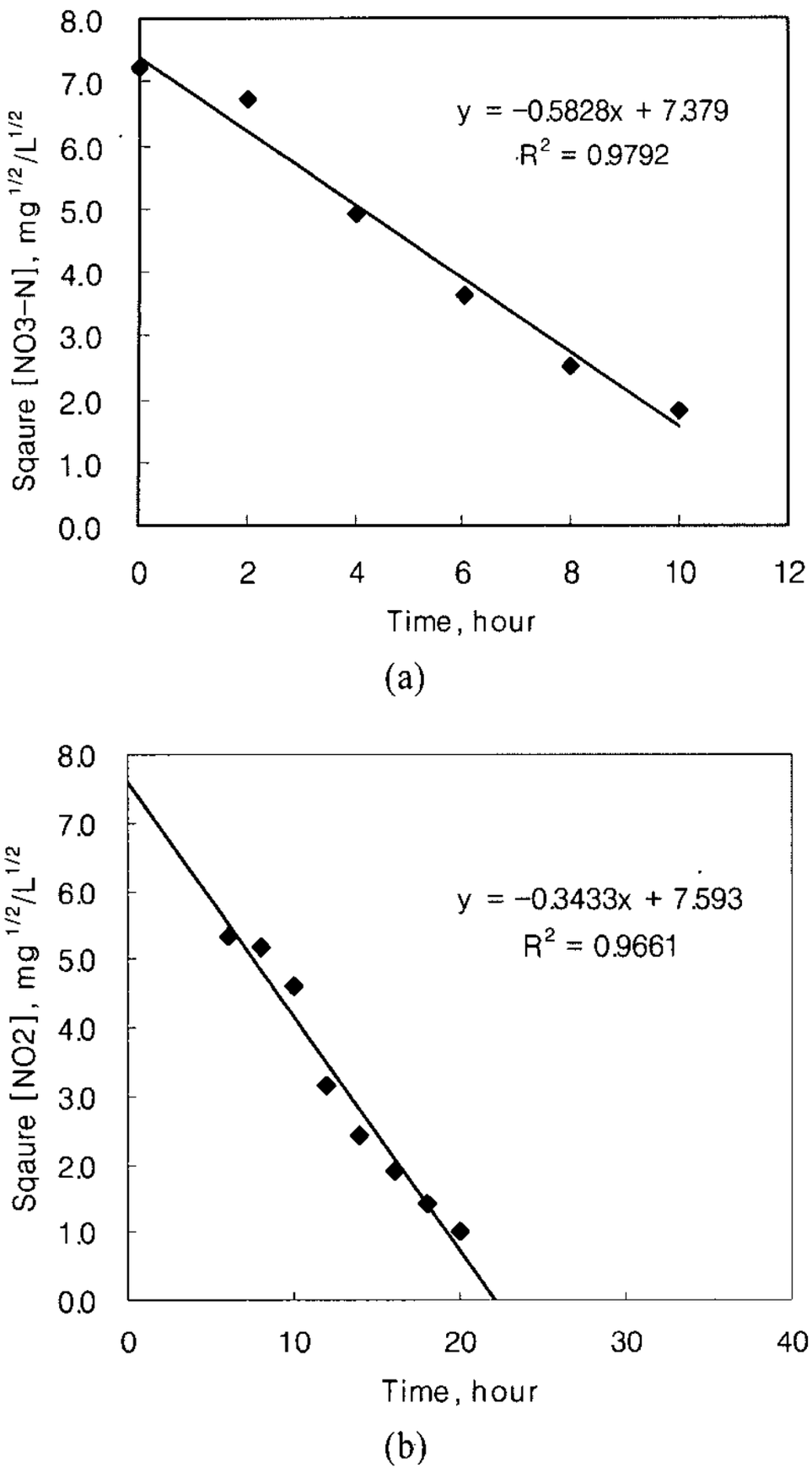

Figure 7. Linear relationship between time and square root of the nitrate and nitrite concentrations: (a) Time vs. square nitrate-N, (b) Time vs. square nitrite-N.

tion, and the half-order reaction coefficients for nitrate and nitrite were determined as 1.17 and 0.69 $mg^{1/2}/L^{1/2}$ h, respectively (Figure 7(a), (b)).

Taking half-order equations for the expression of denitrification process, nitrate and nitrite reduction rates can be simulated as;

$$\text{Nitrate reduction rates: } r_1 = -k_1 C_1^{1/2} \quad (12)$$

$$\text{Nitrite reduction rates: } r_2 = -r_1 - k_2 C_2^{1/2} = k_1 C_1^{1/2} - k_2 C_2^{1/2} \quad (13)$$

Where r_1 and r_2 are the reduction rates for nitrate and nitrite, C_1 and C_2 are nitrate and nitrite concentrations, k_1 and k_2 are the half-order reaction coefficients for nitrate and nitrite, respectively. Equations 12 and 13 can be further integrated to express nitrate and nitrite concentrations.

Figure 8 shows the comparison between empirical nitrogen removal data and the simulation using the equations 12 and 13. The model equation fits very well with the nitrate reduction, but nitrite reduction showed large discrepancy with the simulation. Compared to the estimated values, higher amounts of nitrite were produced during the denitrification process and lasted longer than estimated. Furumai, *et al.*[13] found similar result from their experiment and reported that there were strong pH dependences on nitrite accumulation below pH 7.4. They also found out that the high sodium biocarbonate contents in feeding medium significantly decreased nitrite accumulation. Wilderer, *et al.*[14] also pointed out that nitrite reduction was inhibited by the changes of pH and temperature, while Korner and Zumft[17] suggested that nitrite utilization in heterotrophic denitrification was inhibited by the high nitrate concentration.

It was also reported by Iwamoto, *et al.*[15] that denitrification rate, especially nitrite reduction rate was dependent on the electron donor used in the system. As the availability of electrons from the insoluble sulfur particles is lower than that from the liquid thiosulfate medium, it results in slower nitrite reduction in elemental sulfur-packed reactor compared to thiosulfate-based denitrification. In this experiment, several

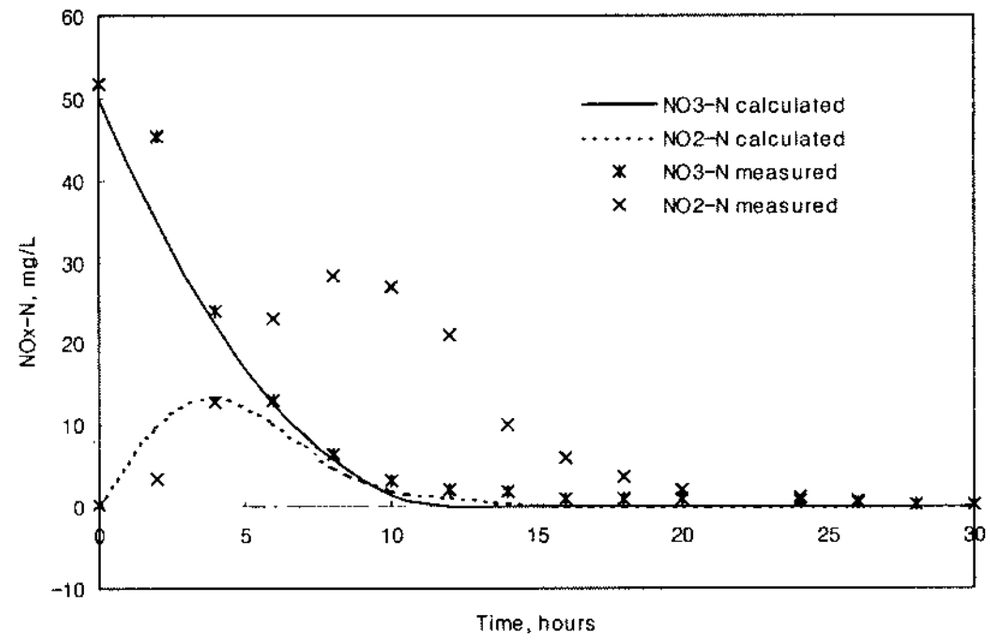

Figure 8. Half-order model fit for denitrification rate at 50 mg NO_3^--N/L with comparison to experimental data.

factors could account for the nitrite accumulation: availability of electron from solid sulfur particles, competition with nitrate as an electron acceptor, different nitrate and nitrite utilization rates, and the differences between nitrite production and utilization rates. The dependence of nitrite reduction rate on the pH and temperature changes cannot be explained with the experimental results.

It appeared from the semi-continuous test that the sulfur-based denitrification process, nitrate and nitrite reduction took place in order, and the total denitrification rate was controlled by the rate-limiting nitrite reduction step. The nitrate removal followed half-order reaction kinetics; however the simulation of nitrite removal was poor. In this experiment the deficiency of alkalinity could be the reason for the nitrite accumulation as the reactor was filled with sulfur particles only. In a continuous column reactor, generally limestone granules are provided together with sulfur particles to support additional alkalinity. If the reactor was operated in continuous mode with sufficient alkalinity source, the accumulation of nitrite could be reduced significantly.

CONCLUSIONS

Sulfur-based autotrophic denitrification in semi-continuous reactor was studied and its kinetic parameters and the reaction characteristics were found. The reduction rates of nitrate and nitrite were determined as 0.26 and 0.13 kgN/m^3 d, respectively. Specific growth rate (μ) of the autotrophic denitrifiers was found to be 0.24-0.32 d^{-1}. The observed growth yield (Y_{obs}) based on the electron acceptor and donor, were estimated as 0.49 g VSS/g N and 0.16 g VSS/g S, respectively. The biomass in the reactor showed specific denitrification rate, q, ranging 0.86 -1.13 g N/g VSS-d.

Model equation for the autotrophic denitrification process successfully simulated nitrate reduction but nitrite simulation was poor. The reasons for the difference in experimental nitrite reduction and the calculated values may include the slow availability of electrons from solid sulfur particles and the high nitrate reduction rate. Compared to continuous system, operation in semi-continuous mode may also cause the nitrite accumulation. The lower rate of nitrite utilization compared to nitrate, as a result, affected the overall denitrification rate. Regarding the high toxicity of nitrite, further studies are required for extensive reaction kinetics of nitrite production and the reduction.

ACKNOWLEDGEMENT

This work was supported in part by the Korea Science and Engineering Foundation (KOSEF) through the Advanced Environmental Monitoring Research Center (ADEMRC) and, in part by the sustainable Water Resource Research Center (SWRRC) through the Water Reuse Technology Center (WRTC) at the Gwangju Institute of Science and Technology.

REFERENCES

1. Koenig, A. and Liu, L.H., Kinetic model of autotrophic denitrification in sulfur packed-bed reactors, *Wat. Res.*, **35**(8), 1969-1978 (2001).
2. Claus, G. and Kutzner, H.J., Physiology and kinetics of autotrophic denitrification by *Thiobacillus denitrificans*. *Appl. Microbiolo. Biotechnol.*, **22**, 283-288 (1985).
3. Oh, S. E., Kim, K. S., Choi, H. C., Cho, J., and Kim, I. S., Kinetics and physiological characteristics of autotrophic denitrification by denitrifying sulfur bacteria, *Wat. Sci. Tech.*, **42**(3-4), 59-68 (2000).
4. Oh, S. E., Chae, K. J., and Kim, I. S., The characteristics of autotrophic denitrification by denitrifying colorless sulfur bacteria using microbial respirometer, *J. of KSEE*, **22**(9), 1651-1659 (2000).
5. Jung, H. J., Kim, S. Y., Kim, S. M., and Kim, I. S., Determination of maximum inhibition concentration and inhibition coeffi-

cient of sodium chloride and sulfate ion on sulfur based autotrophic denitrification, *J. of KSEE,* **26**(9), 960-965 (2004).
6. Activated Sludge Model No. 2. Task group on municipal modeling for design and operation of biological wastewater treatment processes. IAWQ Scientific and Technical Report No. 3. IAWQ, London (1995).
7. Seitzinger, S. P., Denitrification in freshwater and coastal marine ecosystems: ecological and geochemical significance, *Limnol. Oceanogr.*, **33**, 702-724 (1988).
8. Le Cloirec, P. and Martin, G., Total dissolved inorganic nitrogen removal with two coupled autotrophic biological nitrification and denitrification processes-laboratory studies. *Aquaculture*, **39**, 16-23 (1990).
9. Vidal, S., Rocha, C., and Galvao, H., A comparison of organic and inorganic carbon controls over biological denitrification in aquaria, *Chemosphere*, **48**, 445-451 (2002).
10. Wang, Z., Application of biofilm kinetics to the sulfur/lime packed bed reactor for autotrophic denitrification of groundwater, *Wat. Sci. Tech.*, **37**(9), 97-104 (1998).
11. Yoo, Y.-B., The effect of organics on the sulfur utilizing denitrification., Thesis for Master's Degree in the Department of Environmental Science and Engineering, Kwangju Institute of Science and Technology, Gwangju, Korea (2001).
12. Hasegawa, K., Hanaki, K., Matsuo, T., and Hidaka, S., Nitrous oxide from the agricultural water system contaminated with high nitrogen, *Chemosphere-Global Change Science*, **2**, 335-345 (2000).
13. Furumai, H., Tagui, H., and Fujita, K., Effect of pH and alkalinity on sulfur-denitrification in a biological granular filter, *Wat. Sci. Tech.*, **34**(1-2), 355-362 (1996).
14. Wilderer, P. A., Jones, W. L., and Dau, U., Competition in denitrification systems affecting reduction rate and accumulation on nitrite, *Wat. Res.*, **21**, 239-245 (1987).
15. Iwamoto, M., Asanuma, N., and Hino, T., Effect of pH and electron donors on nitrate and nitrite reduction in ruminal microbiota, *Animal Science Journal*, **72**(2), 117-125 (1987).
16. Sung, D. W., Song, S. H., Kim, J. H., and Yoo, Y. J., Effects of electron donors in nitrate removal by nitrate and nitrite reductases, *Biotechnol. Bioprocess Eng.*, **7**, 112-116 (2002).
17. Korner, H. and Zumft, W. G., Expression of denitrification enzymes in response to the dissolved oxygen level and respiratory substrate in continuous culture of *Pseudomonas stutzeri*, *Appl. Emviron. Microbiol.*, **55**, 1670-1676 (1989).
18. Ahn, Y. H., Jang, A., Bum, M. S., Tartavaty, R., Jung K. J., and Kim, In S., Quantification of denitrifiers in sulfur-based denitrification process, *Proc. of Korean Society of Water Quality Conference*, Choongju, 439-443 (2002).
19. Tartavaty, R., Ahn, Y. H., Kim, S. Y., and Kim, In. S., Analysis of microbial community in sulfur-based denitrification process as revealed by fluorescence *in situ* hybridization, *Proc. of Korean Society of Environmental Eng.*, Fall Conference. 241-242 (2002).
20. Atlas, R. M., *Handbook of Microbiological Media*, 2nd edn., CRC Press (1997).
21. APHA-AWWA-WEF., American Public Health Association (APHA), *Standard Methods for the Examination of Water and Wastewater* 19th edition. Washington DC, USA (1995).
22. Koenig, A. and Liu, L.H., Use of limestone for pH control in autotrophic denitrification: continuous flow experiments in pilot-scale packed bed reactors, *Journal of Biotechnology*, **99**, 161-171 (2002).
23. Kimura, K., Nakamura, M., and Watanabe, Y., Nitrate removal by a combination of elemental sulfur-based denitrification and membrane filtration, *Wat. Res.*, **36**, 1758-1766 (2002).
24. Yamamoto-Ikemoto, R., Komori, T., Nomura, M., Ide, Y., and Matsukami, T., Nitrogen removal form hydroponic culture

wastewater by autotrophic denitrificaiton using thiosulfate, *Wat. Sci. Tech.*, **42**(3-4), 369-376 (2000).

25. Batchelor, B. and Lawrence, A. W., Autotrophic denitrification using elemental sulfur, *J. Water Pollut. Control Fed.*, **50**, 1986-2001 (1978).
26. Rittmann, B. E. and McCarty, P. L., *Environmental Biotechnology: Principles and application.*, McGraw-Hill Companies, N.Y. (2001).
27. Hashimoto, S., Furukawa, K., and Shioyama, M., Autotrophic denitrification using elemental sulfur, *J. Fermentation Technology*, **65**(6), 683-692 (1987).