Korean J. Chem. Eng., **25**(3), 542-547 (2008)

Application of spent sulfidic caustics for autotrophic denitrification in a MLE process and their microbial characteristics by fluorescence *in situ* hybridization

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(*Received 27 July 2007 • accepted 29 September 2007*)

Abstract−Spent sulfidic caustics (SSCs) produced from petrochemical plants contain a high concentration of hydrogen sulfide and alkalinity, and some organic matter. Most of the SSCs are incinerated with the auxiliary fuel causing secondary pollution problems. The reuse of this waste is becoming increasingly important in terms of economical and environmental viewpoints. To denitrify wastewater with a low COD/N ratio, additional carbon sources are required. Therefore, autotrophic denitrification has received increasing attention. In this research, SSCs were injected as electron donors for sulfur-based autotrophic denitrification in a modified Ludzack-Ettinger (MLE) process. According to the variations in the SSCs dosage, the efficiencies of COD, nitrification and TN removal were evaluated. Heterotrophic denitrification by organic matter and autotrophic denitrification by SSCs were also investigated. As a result, adequate injection of SSCs showed stable autotrophic denitrification. To investigate some of the harmful effects of SSCs, fluorescence *in situ* hybridization (FISH) for nitrifying bacteria and *Thiobacillus denitrificans* was performed. Ammonia-oxidizing bacteria (AOB) and *Nitrospira* genus showed a similar pattern. Excessive injection of SSCs made nitrifying bacteria decrease and nitrification failure occur because of the high pH caused by the SSCs. The distribution of *T. denitrificans* was relatively uniform as SSCs were injected. This result means that *T. denitrificans* are available at high pH.

Key words: Autotrophic Denitrification, Spent Sulfidic Caustics, Fluorescence *In Situ* Hybridization, *Thiobacilus denitrificans*, Nitrifying Bacteria

INTRODUCTION

The total nitrogen (TN) can be removed by nitrification and denitrification in a biological wastewater treatment plant. Nitrification is divided into two mechanisms: nitritation (from NH_4^+-N to NO_2^--N) by ammonia-oxidizing bacteria (AOB), and nitratation (from NO_2^--N to NO_3^--N) by nitrite-oxidizing bacteria (NOB) [1,2]. The TN removal efficiency depends on the COD/N ratio because conventional heterotrophic denitrification is restricted to pathways using organic carbon as an energy source. It is generally reported that, for most readily available organic carbon sources, a COD/N ratio from 3.0 to 6.0 enables stable denitrification [3-7]. For this reason, addition of an external carbon source is required for wastewater with a low COD/N ratio. However, this increases the operating cost. As alternatives, biological autotrophic denitrification, which considers insufficient carbon sources compared with the nitrogen content, has attracted increasing attention recently because of its lower chemical cost and sludge production when compared to heterotrophic denitrification [8,12]. Autotrophic denitrification using reduced sulfur compounds (H_2S, S, $S_2O_3^{2-}$, $S_4O_6^{2-}$, SO_3^{2-}) consumes alkalinity depending on the reduced sulfur species that are oxidized. Eq. (1) is a stoichiometric equation showing an example of sulfur-based autotrophic denitrification.

$$NO_3^- + 1.1S + 0.4CO_2 + 0.76H_2O + 0.08HCO_3^- + 0.08NH_4^+ \rightarrow 0.08C_5H_7O_2N + 0.5N_2 + 1.1SO_4^{2-} + 1.28H^+ \quad (1)$$

With regard to TN removal, ammonia nitrification and autotrophic denitrification consume more alkalinity than heterotrophic denitrification. In an effort to supply adequate alkalinity, sulfur and limestone autotrophic denitrification (SLAD) systems have been studied [11-16].

Spent sulfidic caustics (SSCs) produced from the olefin process of petrochemical plants, such as naphtha cracking and ethylene production, contain a high concentration of hydrogen sulfide, alkalinity resulting from caustic soda, and some non-biodegradable organics such as phenols, benzene and toluene [17,18, see Table 1]. Under the Waste Management Act in Korea, SSC is classified as a hazardous waste which requires high attention. Most SSCs are incinerated with auxiliary fuel resulting in air pollution problems, and thus operating cost is relatively high. Therefore, the reuse of SSCs is becoming increasingly important both economically and environmentally.

Table 1. Characteristics of SSCs used in this study

Item	Value
pH	13.1-13.5 (13.3)[a]
TOC (mg/L)	1,104-1,638 (1,314)
S^{2-} (mg/L)	15,200-17,600 (16,400)
Alkalinity (mg$CaCO_3$/L)	50,000-64,000 (57,300)
Phenols (mg/L)	1.8-33.8 (17.8)
Benzene (mg/L)	7.8-63.1 (28.6)
Toluene (mg/L)	0.2-7.8 (2.9)
Ethylbenzene (mg/L)	N.D.[b]
Xylene (mg/L)	N.D.

[a]( ) is mean value
[b]N.D.: not detected

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Byun et al. [19] implemented batch tests for identifying autotrophic denitrification using SSCs.

In this research, SSCs were injected into the anoxic tank in the modified Ludzack-Ettinger (MLE) process because they have a high hydrogen sulfide concentration and are quite alkaline. Through variation of the SSCs dosage, the optimum dosage of SSCs for the highest denitrification efficiency and the effects of SSCs on simultaneous heterotrophic and autotrophic denitrification were evaluated.

Recently, the microbial community has been analyzed by using advanced molecular techniques, overcoming traditional precision errors. Thus, for a better monitoring of bacteria content in the microbial community, fluorescence in situ hybridization (FISH) with rRNA-targeted oligonucleotide probes has been utilized and proven as an effective method for the analysis of the microbial community without the need for labor-intensive procedures [20-22].

The efficiency of nitrification is limited by pH, temperature, and inhibitory chemicals. However, denitrification is performed easily in a biological treatment plant, and denitrifying bacteria are widely distributed in nature [23,24]. Thus, the distribution ratio of nitrifying bacteria using the FISH method was investigated due to some toxic effects, as SSCs were applied for autotrophic denitrification. It is generally known that *T. denitrificans* perform sulfur-based autotrophic denitrification [8]. The distribution of *T. denitrificans* in the anoxic tank depending on SSCs dosage has also been investigated.

MATERIALS AND METHODS

1. Reactor Operation

The MLE process applied to the SSCs for autotrophic denitrification consists of the anoxic, aerobic (1), aerobic (2) tank and clarifier, as shown in Fig. 1. The effective volumes of the anoxic, aerobic (1) and aerobic (2) tanks were 12 L, 6 L and 6 L, respectively. This process has external and internal recycle for the sludge and nitrate recirculation, respectively. Generally, the MLE process provides for control over the function of nitrate removed through variation of the internal recycle ratio. To determine the optimum SSCs dosage, the internal recycle ratio was fixed in 1Q. SSCs were injected from the SSCs storage tank. The reactor was inoculated with activated sludge obtained from a municipal sewage treatment plant and were acclimated with the synthetic wastewater, whose concentrations are shown in Table 2.

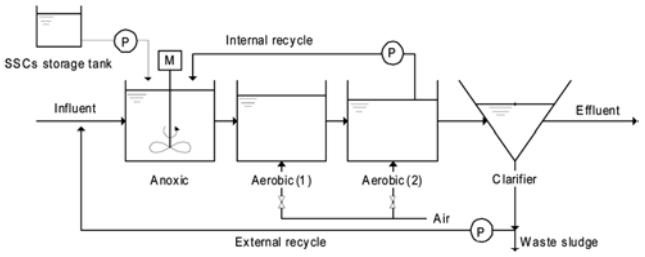

Fig. 1. Schematic diagram of the MLE process applied to SSCs for autotrophic denitrification.

The reactor was operated with different operating conditions shown in Table 3. To estimate the amount of heterotrophic denitrification only, no SSCs were injected in the A condition initially. Based on Eq. (1), 2.5 mg sulfur is required for autotrophic denitrification as 1 mg nitrate-nitrogen is removed. Therefore, the operating conditions varied according to the different S/N ratios, such as in the B, C and D conditions.

2. Oligonucleotide Probes

The following rRNA-targeted oligonucleotides were used: EUB 338(II), Nso190, Ntspa662, Nit3 and Td626. Oligonucleotides were synthesized and fluorescently labelled with fluorescein isothiocyanate (FITC) or a hydrophilic sulfoindocyanine dye (CY3) at the 5' end by CoreBioSystem (Seoul, Korea). All probe sequences, hybridization conditions and references are given in Table 4.

3. Fluorescence *In Situ* Hybridization

All *in situ* hybridization was performed by using the procedure described by Manz et al. [25]. Samples were fixed by immersing in a freshly prepared paraformaldehyde solution (4% in phosphate buffered saline, PBS) overnight at 4 °C. Thereafter, the samples were rinsed with a PBS solution. Each sample was immobilized on a gelatin-coated glass slide. The sample was finally dehydrated by successive passage through an ethanol solution, and air dried. The fixed samples were hybridized by first spiking sequentially 8 μL of hybridization buffer (0.9 M NaCl, 20 mM Tris-HCl (pH 7.2), 0.01% sodium dodecyl sulphate (SDS)), formamide at the concentrations shown in Table 4 and 2 μL of fluorescent probes. Then they were quickly transferred to a pre-warmed moisture chamber under a temperature condition of 46 °C. Finally, the slide was dipped into a washing solution at 48 °C. After the hybridization, digital images of the

Table 2. Characteristics of the synthetic wastewater used in this study

Item	pH	COD_{Cr} (mg/L)	NH_4^+-N (mg/L)	Alkalinity (mg $CaCO_3$/L)
Concentration	7.0-7.4 (7.3)[a]	63-74 (70)	29-36 (33)	254-307 (295)

[a]( ) is mean value during the operating days

Table 3. Operating conditions

Condition	A	B	C	D
Injected SSCs amount (mL/L)	0	2	4	6
Influent sulfur concentration (mg S/L)	0	30.4-35.2	60.8-70.4	91.0-105.4
Influent NH_4^+-N concentration (mg NH_4^+-N/L)	31-35	31-36	30-36	29-36
Influent S/N ratio	0	0.85-1.14	1.70-2.38	2.51-3.65
HRT (hr)	8	8	8	8

Table 4. Oligonucleotide probes applied

Probe	Specificity	Sequence (5'-3')	Target site[a]	% FA[b]	[NaCl] (mM)[c]	Reference
EUB338(II)	Eubacteria	GCAGCCACCCGTAGGTGT	338-355	20	215	[29]
Nso190	Ammonia-oxidizing *β-Proteobactria*	CGATCCCCTGCTTTTCTCC	190-208	20	215	[30]
Nit3	*Nitrobacter* spp.	CCTGTGCTCCATGCTCCG	1035-1048	40	46	[31]
Ntspa662	*Nitrospira* genus	GGAATTCCGCGCTCCTCT	662-679	20	215	[32]
Td626	*Thiobacillus denitrificans*	GTTCAAAACGCCATTCCC	605-622	30	102	[33]

[a]16S rRNA position according to *Escherichia coli* numbering
[b]Formamide concentration in the hybridization buffer
[c]Sodium chloride concentration in the washing buffer

aggregates were taken by a fluorescence microscope (Zeiss Axioskop 2plus, Germany) and visualized by using Zeiss Axiovision digital imaging software. Analyses were performed with the standard software package with the Carl Zeiss Imaging Solution system (Zeiss, Germany).

4. Analytical Methods

All samples for each condition were tested within seven days of sampling. For each sample, the NO_3^--N, NO_2^--N and SO_4^{2-} concentrations were determined by ion chromatography (DX-300, DIONEX, USA). The soluble chemical oxygen demand (SCOD) and NH_4^+-N concentrations were measured with an auto analyzer (AA3, Bran+ Luebbe, Germany) after filtration of the sample through a 0.45 μm membrane filter. Phenols and BTEX concentrations were measured by gas chromatography mass spectrometry (HP 5973N, USA). The pH and dissolved oxygen concentrations were measured with an Orion Research pH meter (Model230A, USA) and a YSI DO meter (Model58, USA), respectively. The sulfur content was measured by using an inductively coupled plasma atomic emission spectrophotometer (Thermo Jarrell Ash, USA), and the alkalinity and suspended solids were measured by Standard Methods [26]. The stored samples were kept refrigerated at 4 ºC until tested.

RESULTS AND DISCUSSION

1. Removal Efficiencies of COD, NH_4^+-N and TN

In many cases, the organic matter available in the wastewater may be a limiting factor for successful nitrogen removal in many treatment plants. If the wastewater has a low C/N ratio, most readily biodegradable organic matters will be removed in the anoxic tank. In this research, the mean removal ratio of COD in the anoxic, aerobic (1) and aerobic (2) tank was 97.3, 2.0 and 0.7%, respectively. As shown in Fig. 2(a), injection of SSCs increased COD concentration in the effluent. The mean COD removal ratio of the A, B, C and D conditions was 88.7, 85.4, 77.9 and 67.5%, respectively. Compared with the A condition, the increased COD concentration of the B, C and D conditions was 2.4, 5.4 and 8.1 mg/L, respectively. This result explains why approximately 1.4 mg COD/L of the effluent increased according to the injection of 1 mL SSCs/L influent. Thus, it is estimated that the SSCs have 1,400 mg/L of hardly biodegradable organic matter caused by the petroleum refinery process.

Fig. 2(b) shows the NH_4^+-N concentrations of each condition. Nitrification efficiencies of the A, B and C conditions were above 95%, whereas the D condition was about 30%. Considering the variation of the pH (Fig. 2(d)), this sharp decrease of nitrification efficiency in the D condition is presumed to be due to the pH effect. Villaverde et al. [27] reported that the optimal pH of *Nitrosomonas* spp. and *Nitrobacter* spp. was in the range of 7.9-8.2 and 7.2-7.6, respectively. High pH ranged from 8.4 to 9.9 in the aerobic (1) tank of the D condition. This was caused the nitrification failure, which did not consume alkalinity; thus the denitrification failed.

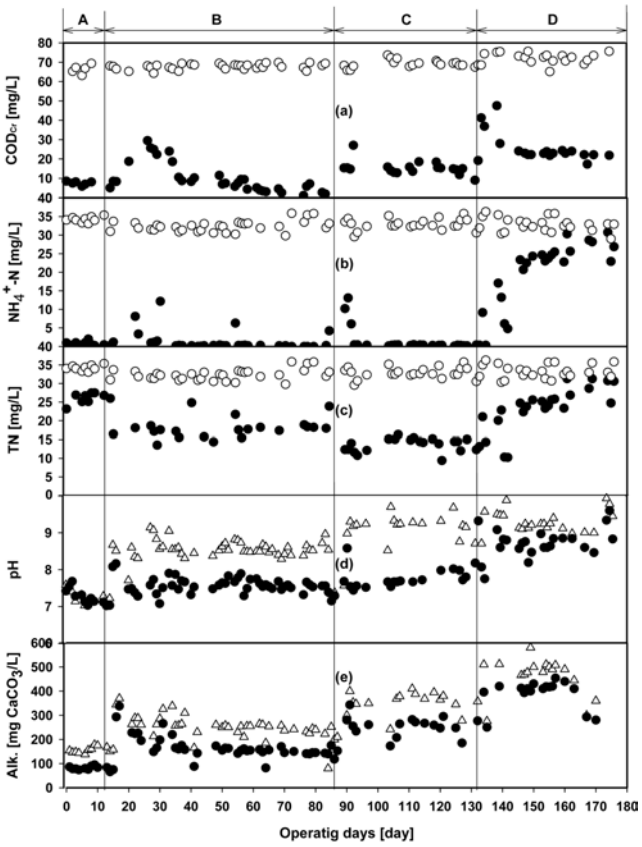

Fig. 2. Results of the reactor operation in each condition: (a) COD; (b) NH_4^+-N; (c) TN; (d) pH; and (e) alkalinity; influent (○), anoxic tank (△), effluent (●).

Fig. 2(c) shows the TN concentrations in the influent and the effluent of each condition. As the internal recycle ratio of all conditions was 1Q in this research, TN removal efficiency in the A, B, C and D conditions was 23.7, 43.5, 58.7 and 29.9%, respectively. Compared with the A condition without SSCs injection, 35% of the additional TN removal occurred in the C condition.

2. Autotrophic Denitrification Caused by SSCs

In the A condition, on which was conducted only heterotrophic denitrification, 7.2 mg COD was removed as 1 mg nitrate-nitrogen was denitrified. The available organic matter in SSCs for heterotrophic denitrification was about 2,500 mg COD/L, because the mean concentration of organic matter in the SSCs was 1,300 mg TOC/L or 3,900 mg COD/L, and it was estimated that the concentration of non-biodegradable organic matter was 1,400 mg COD/L. The efficiency of the autotrophic denitrification in the B, C and D conditions was calculated as follows:

$$aD = tD - hD \quad (2)$$

where aD=the efficiency of autotrophic denitrification, tD=the efficiency of total denitrification, and hD=the efficiency of heterotrophic denitrification, which was considered the addition of available organic matter by SSCs.

Fig. 3 shows the efficiency of total, heterotrophic and autotrophic denitrification calculated by Eq. (2). It was supposed that the TN was removed by denitrification pathways because most of the COD

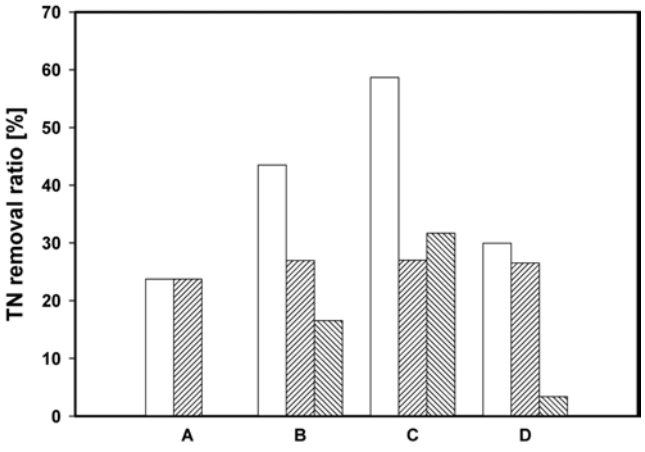

Fig. 3. The efficiencies of total (□), heterotrophic (▨) and autotrophic (▧) denitrification in each condition.

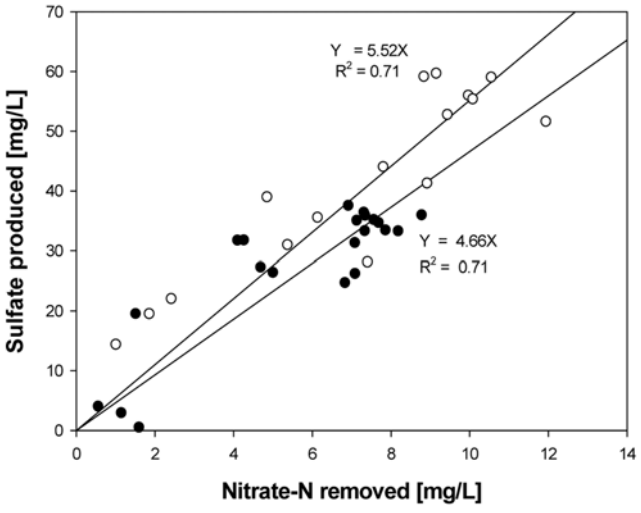

Fig. 4. Relationship between nitrate-nitrogen removed and sulfate produced in the B (●) and C (○) condition.

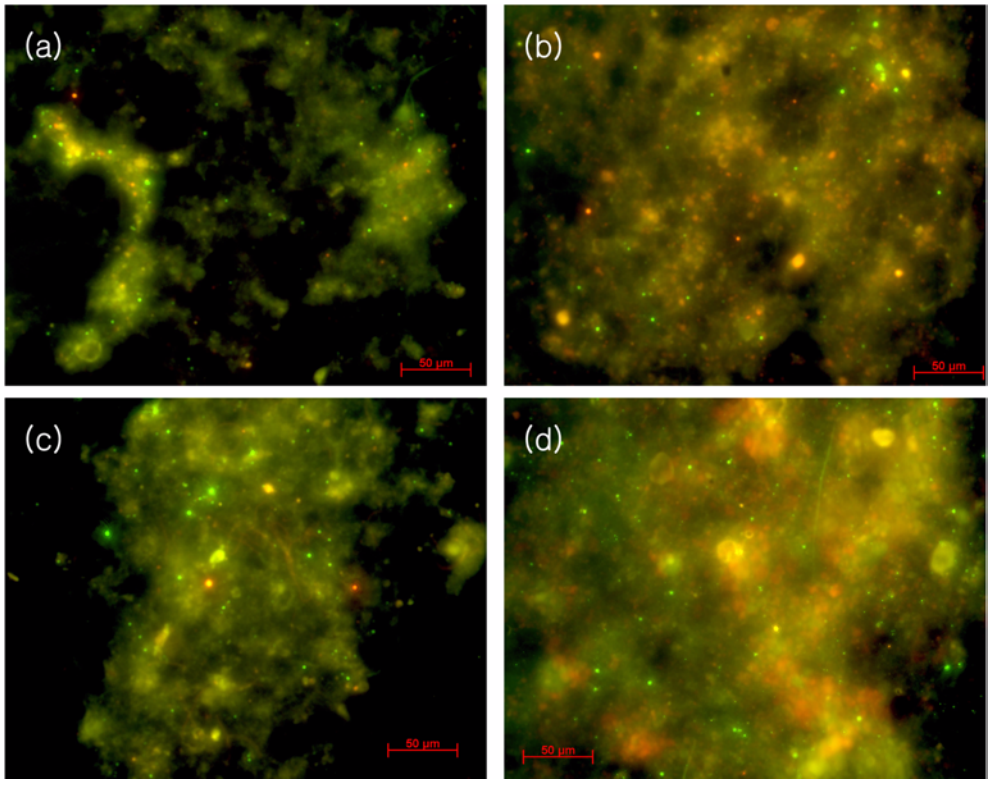

Fig. 5. FISH images; (a) EUB338(II) (green) and Td626 (red) (b) EUB338(II) (green) and Nso190 (red) (c) EUB338(II) (green) and Nit3 (red) (d) EUB338(II) (green) and Ntspa662 (red) probes. Overlapping labels are visualized in yellow.

removal took place in the anoxic reactor. Previously mentioned, TN removal efficiency in the A, B, C and D conditions was 23.7, 43.5, 58.7 and 29.9%, respectively. The autotrophic denitrification efficiency of the B, C and D conditions was 16.5, 31.7 and 3.4%, respectively. In this research, the C condition was the best for autotrophic denitrification using SSCs. In the D condition, autotrophic denitrification was only 3.4%, because the nitrification failure caused by the high pH in the aerobic tank affected the low alkalinity consumption. This low alkalinity consumption consequently produced the high pH in the anoxic and aerobic tank.

Sulfate is the end product of sulfur-based autotrophic denitrification. As shown in Fig. 4, for 1 mg NO_3^--N being reduced, about 5.5 and 4.7 mg SO_4^{2-} was produced in the anoxic tank of the B and C conditions, respectively. Both values are lower than the theoretical value that 7.54 mg sulfate was produced per 1 mg nitrate-nitrogen removed, based on Eq. (1). For the lower sulfate production, Oh et al. [12] reported that some portion of the nitrate was removed heterotrophically and the remainder was denitrified by sulfur-based autotrophic denitrification. On the other hand, Zhang and Lampe [11] concluded that there exists some mechanism for sulfate being reduced after it is produced. Sulfate-reducing bacteria, known as obligate anaerobes, are one of the reasons for this. In this research, the conclusion by Oh et al. [12] was conducive because the obligate anaerobic condition was not achieved in the anoxic tank of the MLE process with external and internal recirculation.

3. Microbial Characteristics by FISH

To investigate the microbial characteristics, FISH was performed by the combination of two probes. Fig. 5 shows the digital images of the aggregates. Fig. 6 and Fig. 7 show the bacterial distribution ratios of *T. denitrificans* and the nitrifying bacteria, respectively.

As SSCs were applied for autotrophic denitrification, the distribution ratios of nitrifying bacteria were investigated because of some expected toxic effects. The distribution ratios of AOB (probe Nso190), *Nitrospira* genus (probe Ntspa662) and *Nitrobacter* spp. (probe Nit3) were displayed by the relative distribution of eubacteria. Fig. 7 shows the bacterial distribution ratios of the aerobic (1) tank for each condition.

AOB oxidize ammonia to nitrite and are important microorganisms which control nitrification [28]. The mean ratio of AOB was 13.1%, 16.1%, 15.6% and 8.2% in the A, B, C and D conditions, respectively. The ratio of AOB in the C condition compared with the A condition did not decrease, However, the ratio decreased in the D condition. This result indicates that high pH ranging from 8.4 to 9.9 led to the nitrification failure due to the decrease of AOB. The mean ratio of *Nitrospira* genus in the A, B, C and D condition was 10.9, 15.6%, 13.7 and 8.8%, respectively. This pattern was similar to that of AOB. However, the mean ratio of *Nitrobacter* spp. in the A, B, C and D condition was 13.1%, 6.6%, 4.2% and 4.4%, respectively. The injection of the SSCs caused the decrease of *Nitrobacter* spp. and was more harmful to *Nitrobacter* spp. than to *Nitrospira* genus. As a result, excessive injection of the SSCs made nitrifying bacteria decrease and nitrification failure occur.

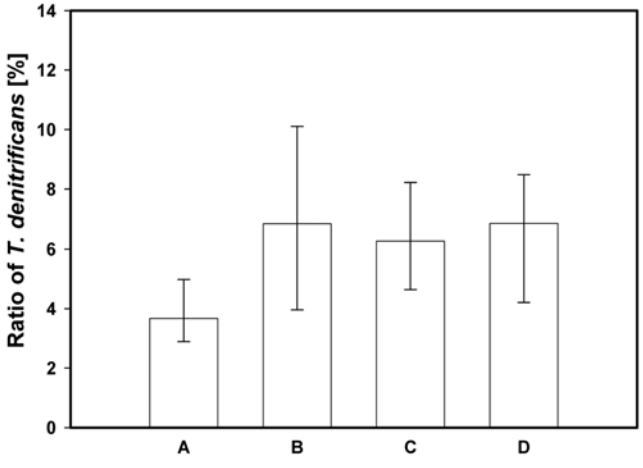

Fig. 6. Distribution ratio of *T. denitrificans* using probe Td626 in each condition.

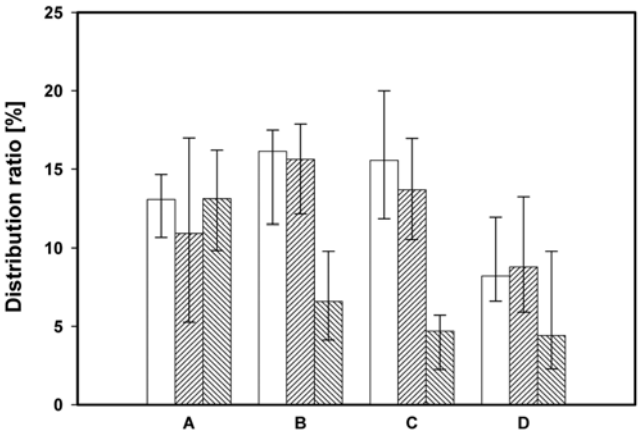

Fig. 7. Distribution ratio of AOB (□), *Nitrospira* genus (▨) and *Nitrobacter* spp. (▧) in each condition.

T. denitrificans can reduce nitrate to nitrogen gas, while oxidize elemental sulfur or reduced sulfur compounds to sulfate. The efficiency of denitrification is very sensitive to pH and an optimum pH of most denitrifying bacteria is known to be around 7 and 8 [12]. Koenig and Liu [10] also reported that the highest autotrophic denitrification was observed at pH 7.0-8.0. Pure strains of *T. denitrificans* showed an optimum growth at pH 7.5-8.0 when thiosulfate was used as a sole energy source [9]. Whereas the mean pH of the A condition in the anoxic tank was 7.3, the mean pH of the B, C and D conditions was 8.5, 8.9 and 9.4, respectively.

To identify *T. denitrificans,* FISH was also performed with the combination of probe EUB338(II) and probe Td626 in the anoxic tank. Fig. 5(a) shows the digital image of aggregates, and Fig. 6 represents the distribution ratio of *T. denitrificans* in each condition. In the A condition, the mean ratio of *T. denitrificans* was 3.67%. Upon injecting the SSCs, the mean ratio of *T. denitrificans* increased to 6.83, 6.27 and 6.84% in the B, C and D conditions, respectively. In the B and C conditions, it is considered that the autotrophic denitrification was performed by *T. denitrificans*, and this can be also confirmed by the sulfate production. Even though the ratio of *T. denitrificans* reached 6.84% in the D condition, little autotrophic denitrification occurred due to the nitrification failure in the D condition. This explains that *T. denitrificans* can be available in pH 9.4. Further research is surely needed to determine the relationship between the activity and the distribution ratio of *T. denitrificans*.

CONCLUSIONS

From the viewpoint of industrial ecology, the reuse of waste is important. In this research, for the reuse of SSCs, the SSCs were applied to the biological nutrient removal process as electron donors for autotrophic denitrification. SSCs were chosen since they have a high hydrogen sulfide concentration and are quite alkaline. Through the variation of the SSCs dosage, the optimum dosage of SSCs was evaluated.

Compared with not injecting SSCs, 35% more TN was removed with an adequate injection of SSCs, when the S/N ratio ranged from 1.7 to 2.4. Autotrophic denitrification efficiency was calculated at 31.7%. When excessive SSCs were injected, autotrophic denitrification efficiency was only 3.4%. This means that nitrification failure caused by a high pH in the aerobic tank caused low alkalinity consumption. Further, this also affected the high pH in the anoxic and the aerobic tank. As NO_3^--N was reduced by 1 mg, about 5 mg SO_4^{2-} was produced in the anoxic tank. This value was lower than the theoretical value because some portion of the nitrate was removed heterotrophically and the remainder was denitrified by sulfur-based autotrophic denitrification.

To investigate some of the harmful effects of SSCs, fluorescence *in situ* hybridization (FISH) for nitrifying bacteria and *T. denitrificans* was performed. Both the AOB and *Nitrospira* genus showed similar patterns. Excessive injection of SSCs made nitrifying bacteria decrease and caused nitrification failure because of the high pH caused by the SSCs. The distribution ratio of *T. denitrificans* showed a relatively uniform value as SSCs were injected. This means that *T. denitrificans* are available at high pH.

In this research, we concluded that the reuse of SSCs in the biological nutrient removal process is possible. Also, some neutralization may be needed because excess injection of SSCs causes a high pH in the treatment process.

ACKNOWLEDGMENT

This study was supported by the Core Environmental Technology Development Project for Next Generation Program (Eco-Technophia-21), Korea Institute of Environmental Science & Technology, Republic of Korea.

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