Characteristics of nitrogen removal and microbial distribution by application of spent sulfidic caustic in pilot scale wastewater treatment plant

S. Park, J. Lee, J. Park, I. Byun, T. Park and T. Lee

ABSTRACT

Since spent sulfidic caustic (SSC) produced from petrochemical industry contains a high concentration of alkalinity and sulfide, it was expected that SSC could be used as an electron donor for autotrophic denitrification. To investigate the nitrogen removal performance, a pilot scale Bardenpho process was operated. The total nitrogen removal efficiency increased as SSC dosage increased, and the highest efficiency was observed as 77.5% when SSC was injected into both anoxic tank (1) and (2). FISH analysis was also performed to shed light on the effect of SSC dosage on the distribution ratio of nitrifying bacteria and *Thiobacillus denitrificans*. FISH results indicated that the relative distribution ratio of ammonia-oxidizing bacteria, *Nitrobacter* spp., *Nitrospira* genus and *Thiobacillus denitrificans* to eubacteria varied little with the pH of the tanks, and SSC injection did not give harmful effect on nitrification efficiency. These results show that SSC can be applied as an electron donor of autotrophic denitrification to biological nitrogen removal process effectively, without any inhibitory effects to nitrifying bacteria and sulfur-utilizing denitrifying bacteria.

Key words | fluorescence in situ hybridization (FISH), nitrifying bacteria, nitrogen removal, spent sulfidic caustic, *Thiobacillus denitrificans*

INTRODUCTION

Biological nitrogen removal (BNR) mechanism consists of nitrification and denitrification. In treating sewage wastewater, heterotrophic denitrification which is effective and reliable in removing nitrate is usually used (Kim et al. 2002). When the COD/N ratio of wastewater is low, organic matters (ex. methanol) should be supplemented for heterotrophic denitrification, and it can cause high operating costs. Autotrophic denitrification has attracted a great attention on account of its lower maintenance costs and sludge production compared to the heterotrophic denitrification. Therefore, many studies about the nitrogen removal based on the autotrophic denitrification have been implemented so far (Claus & Kutzner 1985; Koenig & Liu 1996; Zhang & Lampe 1999; Oh et al. 2001).

Autotrophic denitrification could be implemented using reduced sulfur compounds (H\textsubscript{2}S, S\textsuperscript{0}, S\textsubscript{2}O\textsubscript{3}\textsuperscript{2-}, S\textsubscript{4}O\textsubscript{6}\textsuperscript{2-}, SO\textsubscript{4}\textsuperscript{2-}), and the stoichiometric equations of autotrophic denitrification using sulfur compounds are as follows (McCarty 1972);

1. \[\begin{align*}
1.1S + NO_3^- + 0.4CO_2 + 0.76H_2O + 0.08HCO_3^- \\
+ 0.08NH_4^+ \rightarrow 0.08C_3H_7O_2N + 0.5N_2 + 1.1SO_4^{2-} \\
+ 1.28H^+ 
\end{align*}\]  
(1)

2. \[\begin{align*}
0.844S_2O_3^{2-} + NO_3^- + 0.347CO_2 + 0.434H_2O \\
+ 0.086HCO_3^- + 0.086NH_4^+ \rightarrow 0.086C_3H_7O_2N \\
+ 0.5N_2 + 1.689SO_4^{2-} + 0.697H^+ 
\end{align*}\]  
(2)

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0.421H₂S + 0.421HS⁻ + NO₃⁻ + 0.346CO₂
+ 0.086HCO₃⁻ + 0.086NH₄⁺ → 0.086C₃H₇O₂N
+ 0.5N₂ + 0.842SO₄²⁻ + 0.434H₂O + 0.262H⁺   \( \text{(3)} \)

Autotrophic denitrification consumes alkalinity as shown in Equation (1) to Equation (3), therefore, it has to be supplied alkalinity and sulphur source for the sulfur-based autotrophic denitrification.

Caustics (sodium hydroxide) are utilized in petroleum refining process to remove hydrogen sulphide from various hydrocarbon streams. The caustics which absorb hydrogen sulphide in the facility are generally termed as spent sulfidic caustic (SSC). Accordingly, SSC contains a high concentration of hydrogen sulphide and alkalinity; therefore, it has been adapted for nitrogen removal of sewage by autotrophic denitrification in our previous studies (Byun et al. 2008; Park et al. 2008; Park et al. 2009).

However, SSC is classified as hazardous wastes under the U.S. Resource Conservation Recovery Act (RCRA), and it is suspected that SSC has some toxic effects on microorganisms mainly due to high pH. Since it contains a high concentration of alkalinity, SSC application to biological process may have some impacts on microbial diversity and distribution as well as their performance.

Nitrifying bacteria (i.e. ammonia-oxidizing bacteria [AOB] and nitrite-oxidizing bacteria [NOB]) shows a low substrate affinity and maximum growth rates (Focht & Verstraete 1977; Koops et al. 2003). Nitrifying bacteria is also highly sensitive to toxic substances and sudden changes of pH and temperature (Prosser 1989; Byun et al. 2008).

In biological nitrogen removal process, nitrification is very important because it could be a limiting step for denitrification, and complete nitrification is required to meet the treatment standards for wastewater.

In this study, pilot scale Bardenpho process was operated for evaluating the applicability of SSC, and we investigated the effect of SSC injection on the change of the relative distribution ratio of nitrifying bacteria and *Thiobacillus denitrificans*, which play an important role in BNR process, using FISH analysis.

**MATERIALS AND METHODS**

**Reactor operation**

In our previous study, the lab. scale reactor of modified Ludzack-Ettinger (MLE) process was operated (Park et al. 2008). For better performance and extended application, the pilot scale reactor of Bardenpho process consisted of anoxic tank (1), aerobic tank (1), aerobic tank (2), anoxic tank (2) and aerobic tank (3) was put in operation in this study, as presented in **Figure 1**. The effective volume of each tank was 0.59 m³ and the all tanks were inoculated with the activated sludge obtained from a municipal sewage treatment plant (Park et al. 2009). The characteristics of the domestic wastewater used in this study are shown in **Table 1**. 2 rectangular ciliary media (0.5 × 1 m) were packed in each aerobic tank at 2.4 v/v %. The mixed liquor suspended solids (MLSS) were recycled from the aerobic tank (2) to the anoxic tank (1) at 200% and the sludge recycle ratio was 100%. SSC was injected into the anoxic tank after adjusting pH, from 13.3 to 11.5 with the addition of sulphuric acid (H₂SO₄). The characteristics of SSC are shown in **Table 2**. The pilot scale reactor of Bardenpho

**Figure 1** | Schematic diagram of the pilot scale reactor of Bardenpho process used in this study.
process was operated under the different 5 phases, as shown in Table 3. In phase A, no SSC was injected in order to evaluate heterotrophic denitrification using only organic matters in the domestic wastewater. Equation (1) represents that 2.5 mg of sulphur is required for denitrifying 1 mg of NO$_2^-$-N, and the SSC dosage in each phase was determined based on this stoichiometric equation. The durations of each operation mode were 34, 28, 30, 35, 25 days in phase A, B, C, D and E, respectively.

Oligonucleotide probes

The following 16S rRNA-targeted oligonucleotide probes were used: EUB338, Nso190, Ntspa662, Nit3 and Td626. The oligonucleotide probes were synthesized and labeled fluorescently with a fluorescein isothiocyanate (FITC) or with a hydrophilic sulfoindocyanine dye (CY3) at the 5' end by CoreBioSystem (Seoul, Korea). All oligonucleotide probe sequences, hybridization conditions, and references are provided in Table 4.

Fluorescence in situ hybridization

The suspended sludge from each tank was taken 3 days before alternating the operating mode for FISH analysis. The samples were fixed via immersion overnight in a freshly prepared 4% paraformaldehyde solution at 4°C. Thereafter, the samples were rinsed in a phosphate buffered saline (1XPBS) solution. Each sample was immobilized on a glass slide coated with gelatine. The sample was finally dehydrated via successive passage through an ethanol solution, and then air-dried. The fixed samples were hybridized first by sequentially spiking with 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 as shown in Table 4, and 2 µL of fluorescent probes (25 ng/µL). The samples were then quickly transferred to a pre-warmed moisture chamber at a temperature of 48°C. After the hybridization, digital images of the aggregates were obtained with a fluorescence microscope (Zeiss Axioskop 2plus, Germany) and visualized with Zeiss Axiovision digital imaging software. Analyses were conducted with the standard software package using the Carl Zeiss Imaging Solution system (Zeiss, Germany).

Analytical method

All samples for each condition were taken from the sampling port which is located in the middle part of reactor. They were stored at a temperature of 4°C and they were tested within 7 days of sampling. The NO$_2^-\text{-N}$, NO$_3^-\text{-N}$ and SO$_4^{2-}$ concentrations were determined via ion chromatography (DX-300, DIONEX, USA). The soluble chemical oxygen demand (SCOD$_{Cr}$) and NH$_4^+$-N concentrations were measured with an auto analyzer (AA3, Bran + Luebbe, Germany) after the filtration of the sample through a 0.45 µm membrane filter. Phenols and BTEX concentrations were measured via gas chromatography mass spectrometry (HP 5973N, USA). The sulfur content was determined using an inductively coupled plasma atomic emission spectrophotometer (Thermo Jarrell Ash, USA), and the alkalinity, mixed liquor suspended solids, total nitrogen (TN) and total chemical organic demand

<table>
<thead>
<tr>
<th>Item</th>
<th>Value</th>
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<tbody>
<tr>
<td>pH</td>
<td>13.1 ~ 13.5 (13.3)$^\dagger$</td>
</tr>
<tr>
<td>TOC (mg/L)</td>
<td>1.104 ~ 1.638 (1.314)</td>
</tr>
<tr>
<td>S$^{2-}$ (mg/L)</td>
<td>15,200 ~ 17,600 (16,400)</td>
</tr>
<tr>
<td>Alkalinity (mg CaCO$_3$/L)</td>
<td>50,000 ~ 64,000 (57,300)</td>
</tr>
<tr>
<td>Phenols (mg/L)</td>
<td>1.8 ~ 33.8 (17.8)</td>
</tr>
<tr>
<td>Benzene (mg/L)</td>
<td>7.8 ~ 63.1 (28.6)</td>
</tr>
<tr>
<td>Toluene (mg/L)</td>
<td>0.2 ~ 7.8 (2.9)</td>
</tr>
<tr>
<td>Ethylbenzene (mg/L)</td>
<td>N.D.$^\dagger$</td>
</tr>
<tr>
<td>Xylene (mg/L)</td>
<td>N.D.</td>
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</table>

$^\dagger$ | Mean value. N.D.; not detected
(TCOD$_{Cr}$) were measured via Standard Methods (APHA 1998). The dissolved oxygen (DO) concentrations and pH were measured with a DO meter (YSI, Model 58, USA) and pH meter (Orion, Model 520A, USA), respectively.

### RESULTS AND DISCUSSION

#### Organic matter removal

Organic matter concentration was monitored with TCOD$_{Cr}$ and SCOD$_{Cr}$. About 98.6% of eliminated COD$_{Cr}$ was removed in anoxic tank (1) through heterotrophic denitrification in phase A. The COD$_{Cr}$ removal efficiency and COD$_{Cr}$ concentration in effluent are shown in Table 5.

As it was summer season and rained frequently in Korea, influent COD$_{Cr}$ concentration was low in phase A and B compared to the other conditions. Therefore, it was considered that SCOD$_{Cr}$ removal efficiencies were relatively low in phase A and B. Despite of the fluctuations of influent SCOD$_{Cr}$ concentration (34.4 – 74.0 mg/L), the effluent SCOD$_{Cr}$ concentrations were stable, ranging from 19.0 to 20.4 mg/L. SSC contains a non-readily biodegradable organic matter of 1,400 mg/L caused by petroleum refinery process, therefore, SSC injection could cause the increase of COD$_{Cr}$ concentration in effluent (Park et al. 2008). Biofilm process has many advantages due to the biofilm formed on a media (Atkinson 1975; Adler 1987), and suspended and attached growth processes facilitate the improvement of COD$_{Cr}$ removal, nitrification and denitrification (Tchbanoglous et al. 2003). In this pilot scale study, COD$_{Cr}$ concentrations in effluent did not increase because the ciliary media was equipped in all aerobic tanks for improving process performance.

#### Nitrogen removal

Nitrification efficiency was averaged at 84.8, 58.8, 96.8, 97.3 and 83.0% in phase A, B, C, D and E, respectively. In early stage of phase B, nitrification failure was observed as shown in Figure 2. It is one of the phenomenon which might happen when the low loading rate lasts for a long time (Wobus & Röske 2000), and the growth of nitrifying bacteria could be inhibited or deactivate due to a lack of the substrate, when the low ammonia loading rate continues.
To solve this problem, internal recycle was changed from 200 to 400%, and hydraulic retention time (HRT) was decreased from 6 h to 5 h temporarily. Then, nitrification was performed successfully. From day 128 to 137, NH$_4^+$ concentration in effluent increased temporarily due to the sudden temperature drop (16.4°C → 13.8°C).

On the assumption that total nitrogen (TN) removal is implemented by autotrophic and heterotrophic denitrification, the autotrophic denitrification was calculated by deducting heterotrophic denitrification efficiency from total nitrogen removal efficiency as follows:

$$ TR_{auto} = TR_{total} - TR_{hetero} $$

where $TR_{auto} = $ the TN removal efficiency by autotrophic denitrification, $TR_{total} = $ the TN removal efficiency, and $TR_{hetero} = $ the TN removal efficiency by heterotrophic denitrification.

In phase A, 13.9 mg TCOD$_{Cr}$ was required for denitrification of nitrate of 1 mg. The TN removal efficiency by heterotrophic denitrification was calculated based on this value as follows: (Park et al. 2008).

$$ TR_{hetero} = (C_{TCOD_{in}} - C_{TCOD_{eff}}/13.9)/(C_{TN_{in}} - C_{TN_{eff}}) \times 100 $$

Table 4 | Oligonucleotide probes used for FISH analysis

| Probe | Specificity | Sequence(5'-3') | Target site | % FA | $|NaCl| (mM)$ | Reference |
|-------|-------------|-----------------|-------------|------|-------------|-----------|
| EUB338 | Most bacteria | GCAGCCACCCGTAGGTG | 338–355 | 0–50 | - | Amann et al. (1990) |
| Nso190 | Betaproteobacterial ammonia-oxidizing bacteria | CGATCCCCCTGCTTTT | 190–208 | 55 | 0.020 | Mobbary et al. (1996) |
| Nit3 | Nitrobacter spp. | CTGTGCTCCATGCTCG | 1035–1048 | 40 | 0.056 | Wagner et al. (1996) |
| Ntspa662 | Genus Nitrospira | GGAATTCCCGCTCTCTT | 662–679 | 35 | 0.079 | Daims et al. (2001) |
| Td626 | Thiothrix denitrficans | GCTAAAGCGCATTC | 605–622 | 30 | 0.112 | Uki (2006) |

Table 5 | Organic matter removal efficiency and concentration of effluent

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<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
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<tbody>
<tr>
<td>TCOD$_{Cr}$ removal efficiency (%)</td>
<td>85.4 ± 4.47</td>
<td>80.1 ± 4.58</td>
<td>82.7 ± 3.36</td>
<td>82.9 ± 3.45</td>
<td>85.2 ± 3.47</td>
</tr>
<tr>
<td>TCOD$_{Cr}$ concentration in effluent (mg/L)</td>
<td>20.4 ± 5.66</td>
<td>22.3 ± 2.22</td>
<td>25.2 ± 4.13</td>
<td>24.0 ± 4.35</td>
<td>23.1 ± 6.01</td>
</tr>
<tr>
<td>SCOD$_{Cr}$ removal efficiency (%)</td>
<td>55.2 ± 7.72</td>
<td>54.0 ± 7.93</td>
<td>61.2 ± 6.01</td>
<td>64.9 ± 4.39</td>
<td>63.1 ± 8.85</td>
</tr>
<tr>
<td>SCOD$_{Cr}$ concentration in effluent (mg/L)</td>
<td>19.9 ± 2.77</td>
<td>20.4 ± 3.42</td>
<td>19.7 ± 3.46</td>
<td>19.0 ± 3.10</td>
<td>19.1 ± 5.83</td>
</tr>
</tbody>
</table>

Table 4  | Oligonucleotide probes used for FISH analysis

Table 5  | Organic matter removal efficiency and concentration of effluent

To solve this problem, internal recycle was changed from 200 to 400%, and hydraulic retention time (HRT) was decreased from 6 h to 5 h temporarily. Then, nitrification was performed successfully. From day 128 to 137, NH$_4^+$ concentration in effluent increased temporarily due to the sudden temperature drop (16.4°C → 13.8°C).

On the assumption that total nitrogen (TN) removal is implemented by autotrophic and heterotrophic denitrification, the autotrophic denitrification was calculated by deducting heterotrophic denitrification efficiency from total nitrogen removal efficiency as follows:

$$ TR_{auto} = TR_{total} - TR_{hetero} $$

where $C_{TCOD_{in}} = $ TCOD$_{Cr}$ concentration of influent, $C_{TCOD_{eff}} = $ TCOD$_{Cr}$ concentration of effluent, $C_{TN_{in}} = $ TN concentration of influent, $C_{TN_{eff}} = $ TN concentration of effluent.

The heterotrophic and autotrophic denitrification efficiencies in each phase were shown in Table 6.

In phase A, TN removal efficiency was only 33.9%, and it increased in phase B, C, D and E. SSC injection made TN removal efficiency improve by performing autotrophic
denitrification using SSC. After injecting SSC, about 17.8, 32.6, 43.1 and 39.9% of TN was removed additionally in phase B, C, D and E, respectively. The highest TN removal and autotrophic denitrification efficiency was shown in phase D, where the SSC were injected into both anoxic tank (1) and anoxic tank (2).

**Nitrifying bacteria**

To investigate the effect of the SSC addition on the relative distribution of bacterial groups within the microbial community, FISH analysis was implemented with the combination of EUB338 and each probe. The results showed an insignificant change; however, the pattern of the distribution ratio in each tank under the different operating condition seemed to be meaningful.

The distribution ratio of AOB in aerobic tank (1) was 7.46, 9.59, 8.40, 8.35 and 9.23% in phase A, B, C, D and E (Figure 3). In phase B, AOB increased about 2.13% due to the fact that the pH was closer to the optimum pH. Nitrifying bacteria has a different optimum pH for growth depending on the species, and it is generally between 7.0 and 8.0 (Villaverde et al. 1997). The pH was maintained below 7.0 in phase A, and it increased with the SSC injection in other phases. It was also shown that AOB distribution ratio was relatively high in phase C, D and E, because the influent NH$_4^+$-N concentration increased. The NH$_4^+$-N loading rate increased 14.6, 18.4 and 20.9% in phase C, D and E compared to phase A. The distribution ratio of *Nitrobacter* spp. was 5.98, 7.37, 7.52, 7.55 and 8.18% in A, B, C, D and E phase, respectively, and *Nitrospira* genus was 4.82, 6.91, 6.41, 6.32 and 5.78%, respectively.

The distribution ratio of nitrifying bacteria (the sum of AOB and NOB) was highest in aerobic (1) tank, and it decreased in order of aerobic tank (2) and aerobic tank (3). It was due to that the ammonia of 78.4% was removed in aerobic tank (1), and the small portion of nitrification was done in aerobic tank (2) and aerobic tank (3). Consequently, it was concluded that SSC injection did not give significant influence to the distribution ratio of nitrifying bacteria and nitrification efficiency.

DO is usually considered as a key factor which affect the microbial community. There was no change in DO concentration depending on the operation mode, and the values of anoxic tank (1), aerobic tank (1), aerobic tank (2), anoxic tank (2), and aerobic tank (3) were 0.19, 3.92, 3.01, 0.19, 1.98, respectively. Therefore, it was concluded that DO concentration did not have a significant impact on the change of the relative distribution ratio of microorganisms in this study.

**Thiobacillus denitrificans** in anoxic tanks

*Thiobacilli* are found in various ecosystems and they play an important role in the conversion of inorganic sulphur compounds (Lane et al. 1992). Among them, *T. denitrificans* is found in soil, domestic sewage, industrial water treatment lagoons and digestion tanks (Kelly & Wood 2000). *T. denitrificans* also play an important role in the nitrate-dependent anaerobic oxidation of reduced sulphur compounds on a global scale, linking the biogeo-

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**Table 6** | Heterotrophic and autotrophic denitrification efficiency in each phase

<table>
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<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
</tr>
</thead>
<tbody>
<tr>
<td>TN removal efficiency (%)</td>
<td>33.9 ± 13.5†</td>
<td>52.4 ± 10.6</td>
<td>68.0 ± 4.84</td>
<td>77.5 ± 2.85</td>
<td>74.4 ± 7.61</td>
</tr>
<tr>
<td>TN removal by heterotrophic denitrification (%)</td>
<td>33.9</td>
<td>33.9</td>
<td>34.8</td>
<td>36.5</td>
<td>33.9</td>
</tr>
<tr>
<td>TN removal by autotrophic denitrification (%)</td>
<td>0</td>
<td>18.5</td>
<td>33.2</td>
<td>41.0</td>
<td>40.5</td>
</tr>
</tbody>
</table>

*Mean value. †Standard deviation.
To investigate the distribution ratio of *T. denitrificans*, FISH was applied using probe Td626. Figure 4 shows the digital images of the aggregates.

The relative distribution ratio of *T. denitrificans* to eubacteria in anoxic tank (1) and anoxic tank (2) was 2.61 and 2.76% when no SSC was injected. It increased to about 5% with the increase of SSC dosage, and the maximum value was 5.89% as shown in Figure 5. Even though this increase of the relative distribution ratio is not significant considering the typical analytical error of FISH method, the pattern was quite similar to our previous study, which investigated the distribution ratio of *T. denitrificans* in a lab. scale MLE process fed with synthetic wastewater (Park et al. 2008). Through these results, it was suggested that *T. denitrificans* could perform the autotrophic denitrification using SSC without the increase of their population, or there might be possibility of the existence of other autotrophic denitrifier. Therefore, it was thought that the studies for elucidating the relationship between microorganisms (diversity and distribution) and the additional TN removal caused by SSC injection have to be further investigated.

**CONCLUSIONS**

Biological nitrogen removal by applying autotrophic denitrification has drawn a great attention due to its lower sludge production and maintenance costs. As SSC from petrochemical plant harbors a high concentration of sulfide and alkalinity, it could be utilized as an electron donor for autotrophic denitrification. As pH of SSC is usually over 13.0, neutralization is necessary for stable process performance and discharge water quality. In this study, pollutant removal performances and microbial community were investigated when pH-controlled SSC was applied to pilot scale reactor of Bardenpho process.

Through these results, it was suggested that *T. denitrificans* could perform the autotrophic denitrification using SSC without the increase of their population, or there might be possibility of the existence of other autotrophic denitrifier. Therefore, it was thought that the studies for elucidating the relationship between microorganisms (diversity and distribution) and the additional TN removal caused by SSC injection have to be further investigated.

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