The effect of S/N ratio on NO and N₂O accumulation during sulfide autotrophic denitrification

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Abstract. Nitric oxide (NO) and Nitrous oxide (N₂O) are inevitable intermediates of sulfide autotrophic denitrification (SAD) process, and have huge environmental hazard. This study investigated the produce mechanism and accumulation characteristic of NO and N₂O in SAD process under different S/N ratios. The results showed that the lower S/N ratio, the higher NO accumulation. Which was due to the strong reducing effect of S²⁻ and the electronic competition between nitrogen reductase. And under S/N ratio = 2, N₂O level reached its peak, which was caused by electronic competition. When the S/N ratio = 1, although the electronic competition was more intense, the total amount of electrons was less, thus the N₂O produced is lower than that produced when S/N=2. Microbial community analysis showed that Thiothrix sp. (62.44%) was the predominant genus, and conducted the sulfide-oxidizing autotrophic denitrification process.

1. Introduction
Sulfide autotrophic denitrification (SAD) process could simultaneous removal nitrate/nitrite and sulfide without carbon source by sulfide-oxidizing autotrophic denitrification bacteria [1]. SAD has been extensive used in wet flue gas desulfurization and denitrification [2]. In SAD process, S²⁻ is oxidized to SO₄²⁻, and provide electron for denitrification (Equation (1)). Nitrate and nitrite work as electron acceptor and transformed to nitric oxide (NO), nitrous oxide (N₂O) and finally to N₂ (Equation (2)) [3].

\[
\begin{align*}
S^{2-} - 2e^- & \rightarrow S^0 - 2e^- \rightarrow 1/2 S_{2}O_{3}^{2-} - 4e^- \rightarrow SO_{4}^{2-} \\
NO_3^- + 2e^- & \rightarrow NO_2^- + e^- \rightarrow NO + e^- \rightarrow 1/2 N_2O + e^- \rightarrow 1/2 N_2
\end{align*}
\]

NO and N₂O are unavoidable intermediate products of denitrification, and have environmental harm. NO could cause the hole in the ozone layer, and N₂O has an extremely strong greenhouse effect [4]. Many researches have been focused on the production of N₂O to minimize its emission. Auguet et al. found that in sulfide sewage, N₂O production increased with nitrite or nitrate added [5]. Klok et al. reported a kinetic model for SAD process to describe product formation for halophilic biomass
under dynamic conditions [6]. And Liu et al. used electron completion model to predict N$_2$O accumulation in SAD [7].

The major purpose of this study is to investigate the production characteristic of NO and N$_2$O in SAD process. And by changing the S/N ratios, monitoring the NO, N$_2$O, pH, oxidation-reduction potential (ORP), S$^2-$, NO$_3^-$ and NO$_2^-$ to further understanding the metabolic characteristic of the SAD process.

2. Material and methods

2.1. SBBR reactor and operation

A lab scaled SBBR reactor filled with fibrous carriers was used in this study with a working volume of 2 L (Figure 1). Reactor operated for two cycles each day, and each cycle contained 5 min feeding, 660 min stirring, 30 min settling, 10 min decanting and 15 min idle. The temperature was controlled at 25 ± 0.5°C, and the stirring speed was as 600 r/min by a magnetic stirrer. And the reactor was covered with aluminized paper to protect form light. At the beginning of each cycle, N$_2$ was blown into the reactor for at least 10 min to drive out oxygen.

![Figure 1. The schematic diagram of the SBBR.](image)

2.2. Synthetic wastewater

The SBBR was fed with Na$_2$S·9H$_2$O as electron donor, NaNO$_3$ as electron acceptors. The operation was divided into 4 phases with different S/N ratios by changing Na$_2$S·9H$_2$O concentration (Table 1). NaHCO$_3$ was provided for inorganic carbon source. K$_2$HPO$_4$ and CaCl$_2$ was added for buffer. 1mL trace elements solution was added. The trace elements contained as follows (per liter): 30 mg CuCl$_2$, 50 mg ZnCl$_2$, 50 mg (NH$_4$)$_6$Mo$_7$O$_2$·4H$_2$O, 50mg CoCl$_2$·6H$_2$O, 50 mg H$_3$BO$_3$, 50mg NiCl$_2$·2H$_2$O, and 50 mg EDTA [8].

| Phases   | S/N ratio | S$^2-$   | NO$_3^-$ |
|----------|-----------|----------|----------|
| Phase I  | 1         | 60mg/L   | 60mg/L   |
| Phase II | 2         | 120mg/L  | 60mg/L   |
| Phase III| 3         | 180mg/L  | 60mg/L   |
| Phase IV | 4         | 240mg/L  | 60mg/L   |
2.3. Analytical methods
NO$_3^-$-N, NO$_2^-$-N and S$^2-$ was analyzed according to standard methods [9]. NO$_3^-$-N was analyzed through ultraviolet spectrophotometry, NO$_2^-$-N was analyzed by N-1 naphthalene ethylenediamine photometric method, and S$^2-$ was detected through P-aminodiphenylamine photometric method. All samples were filtered by filter paper before analysis. pH value was measured by PHS-3C pH meter (Leici, China). ORP was measured by ORP sensor (DZB-718, Leici, China). NO and N$_2$O was monitored by NO and N$_2$O online microsensors (Unisense A/S, Aarhus, Denmark).

2.4. Microbial community analysis
Microbial community was analyzed through high-throughput sequencing by Sangon Biotech Co., Ltd. (Shanghai, China). The V3-V4 region of 16S rRNA gene were selected by polymerase chain reactor (PCR) with forward primer 338F: ACTCCTACGGGAGGCAGCA and a revise primer of 806R: GGACTACHVGGGTWTCTAAT [10].

3. Results and discussion
3.1. The denitrification process under different S/N ratios
Figure 2 showed the changes of pH, ORP, S$^2-$, NO$_3^-$ and NO$_2^-$ under different S/N ratios. During SAD process, S$^2-$ was oxidized to S$^0$ and then to SO$_4^{2-}$, and the oxidation rate of S$^2-$ to S$^0$ (0.245-0.317) was much higher than that of S$^0$ to SO$_4^{2-}$ (0.021-0.037) [7]. Thus, at the beginning, the concentration of S$^2-$ showed a sharply decrease, companied with nitrite declined, and nitrite accumulated simultaneous. And compared the four different S/N ratios, it was found that the nitrite accumulation peak decreased with S/N ratios growing. And under S/N ratio = 4, only a small amount of nitrite accumulated. For that nitrate was a better electron acceptor than nitrite under SAD process [11]. Under lower S/N ratios, nitrate reductase (Nar) was easier to get electron than nitrite reductase (Nir), thus caused the nitrite accumulation [3]. Under high S/N ratio, with sufficient S$^2-$ provide, Nar and Nir could get enough electron synchronously, thus showed little nitrite accumulation [12]. In the middle and late stage of the reaction, without S$^2-$ residual, nitrate and nitrite decreased slowly. The tardy reaction rate of nitrate and nitrite showed that the reducibility of S$^0$ to SO$_4^{2-}$ were lower than that of S$^2-$to S$^0$.

ORP value showed the redox state of the system. The lower ORP indicated more reducing substances in the reactor. The increase in ORP indicates that the reaction is continuing, and the faster the increase, the faster the reaction. As shown in Figure 2, the ORP value decreased fast after feeding, and then showed an increase, and the value tended to a stable state at the end of reaction. For that with S$^2-$ fed and mixed, the reducing power of the reactor reached peak. And along with the oxidation of sulfide, the reducing power in the reactor was gradually reduced. When the nitrogen oxide was basically reduced to nitrogen, the reaction was extremely slow, so the ORP was in a stable state. And under S/N ratio = 4, ORP value was stable at -300 mV, while in other S/N ratios, the ORP value was stable at 0 mV. This might due to S$^0$ has a higher reducibility than SO$_4^{2-}$. Under S/N ratio = 4, there was some S$^0$ in the reactor that had not reacted completely.

The pH value showed a rapidly increase and the decreased slowly. The rapid rose might be due to the NOx reduction process of S$^2-$ was an alkali-producing process, and the reaction was fast under the strong reduction of S$^2-$ . And the growth of cell would consume CO$_2$ in the liquid and cause the increase in pH. The slow drop in pH was due to the neutralization of denitrification and alkali production by CO$_2$ produced by the buffer in the liquid phase and cell respiration.
Figure 2. The changes of pH, ORP, S$^2$-, NO$_3^-$ and NO$_2^-$ under different S/N ratios.

Figure 3. The changes of dissolve NO and N$_2$O concentration under different S/N ratio.
3.2. The production characteristic of NO and N₂O under different S/N ratios

3.2.1. The production characteristic of NO. Figure 3 showed the dissolve NO and N₂O concentration under different S/N ratio. In general, a small amount of NO was produced in the early stage of the reaction, which was manifested as a rapid rise in the early stage, and declined after reaching its peak. In the early stage of the reaction, S²⁻ was used as the reducing substance to reduce nitrate to other nitrogen substances, including a small amount of NO, which may be caused by the different electron affinity of nitrogen reductase of denitrifying bacteria to S²⁻. The affinity of Nar to S²⁻ was slightly greater than that of Nir, and the production rate of NO was lower than the reduction rate of NO, leading to the accumulation of NO during this period [13]. And NO level reached the peak when S²⁻ was oxidized completely. Subsequently, NO is reduced to N₂O under the action of other sulfur reducing substances (S⁰, SO₄²⁻), and with its own spillover effect, NO level gradually reduced to the minimum monitoring threshold. The absence of NO production in the middle and back stages of the reaction (120-660 min) indicated that the reduction of the material with the excluded S²⁻ does not lead to NO production. Above that, the production process of NO might be caused by the strong reducing power of S²⁻.

Comparing with the NO production under different S/N ratios, it was found with S/N ratios increase, the dissolved NO decrease. For that higher S/N ratios provided more electron for nitrogen reduction, according to the calculation of electron transfer equilibrium, S/N ratio should reach at least 1.43 for nitrate converted to nitrogen completely (without cell growth). Under S/N ratio = 1, the insufficient electron supply led to intense electron competition, which amplified the low affinity characteristic of NO reductase for S²⁻, leading to the accumulation of more NO. Although NO is an essential intermediate product of sulfide autotrophic denitrifying bacteria, the cells only produce a small amount of NO rather than excessively high concentration because of the toxic effect of excessive NO on the cells.

3.2.2. The production characteristic of N₂O. As shown in Figure 3, when S/N ratio = 1, N₂O was produced at the beginning of the reaction and reached peak at 120 min, and then followed by a slow decline. With the increase of S/N ratio, the peak value of N₂O was gradually post-set. When S/N=4, almost no N₂O was produced. When S/N ratio was low, the supply of electrons was insufficient, and nitrogen reductase of sulfur autotrophic denitrifiers would compete for electrons. In this competition process, N₂O reductase was often in a weak position because it was vulnerable to the influence of external environment (nitrite, NO, etc.), thus leading to a large amount of N₂O accumulation. At S/N ratio = 4, when the supply of electrons is sufficient, the bacteria would convert the nitrate and nitrite completely into nitrogen.

According to that the lower the S/N ratio, the more intense the electronic competition, it should be that the lower the S/N, the higher the N₂O produced. While in fact the most N₂O is produced when S/N=2. This was because when S/N= 1, due to the lack of sufficient electrons, there were still more nitrates and nitrites remaining, making the final N₂O lower than S/N=2. Under higher S/N ratios, with sufficient electrons, and less N₂O produced.

3.3. Microbial community analysis in the SAD system
Figure 4 and Figure 5 showed the microbial community structure of the SBBR in phylum and genus level, respectively. *Proteobacteria* was the main phylum in the system and accounted for 95.12%, followed by *Bacteroidetes* (2.19%), *Acidobacteria* (1.79%) and *Firmicutes* (0.14%). And *Thiobacillus* sp. (62.44%) was the predominant genus. *Stappia* sp. (3.42%), *Aquamicrobium* sp. (1.27%), *Thauera* sp. (0.78), *Paracoccus* sp. (0.47%), *Shinella* sp. (0.47%), *Chiayiivirga* sp. (0.41) and *Thiobacter* sp. (0.40%) were detected in the SBBR system.

*Thiobacillus* sp. was a typical sulfdie-oxidizing autotrophic denitrification bacteria [14], *Stappia* sp. was important denitrifying microorganism [15]. *Thauera* sp. was autotrophic denitrifier which was common in denitrifying sulfide removal process [16]. Therefore, *Thiobacillus* sp., *Stappia* sp., *Thauera* sp. were the main functional microbial group conducted sulfide oxide and denitrification.
4. Conclusions
This work studied the producing mechanism and accumulation characteristic of NO and N₂O in SAD process under different S/N ratios. It was found that the production of NO and N₂O was mainly caused by the electronic competition between nitrogen reductase of sulfide autotrophic denitrifying bacteria. The low S/N ratios caused an intense electron competition, and the level of electron donor also decided the production of NO and N₂O. And the results showed that under S/N = 4, almost no N₂O production in the system. High-throughput sequencing showed that *Thiobacillus* sp. (62.44%) was the main sulfide autotrophic denitrifying bacterium in the system.

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