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Selective Inhibition of Ammonia Oxidation and Nitrite Oxidation Linked to N_2O Emission with Activated Sludge and Enriched Nitrifiers

Ali, Toor Umair[1], Minwook Kim[1], and Dong-Jin Kim[1,2]*

[1]*Department of Environmental Sciences and Biotechnology, Hallym University, Chunchon 200-702, Korea*
[2]*Institute of Energy and Environment, Hallym University, Chunchon 200-702, Korea*

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Nitrification in wastewater treatment emits a significant amount of nitrous oxide (N_2O), which is one of the major greenhouse gases. However, the actual mechanism or metabolic pathway is still largely unknown. Selective nitrification inhibitors were used to determine the nitrification steps responsible for N_2O emission with activated sludge and enriched nitrifiers. Allylthiourea (86 μM) completely inhibited ammonia oxidation and N_2O emission both in activated sludge and enriched nitrifiers. Sodium azide (24 μM) selectively inhibited nitrite oxidation and it led to more N_2O emission than the control experiment both in activated sludge and enriched nitrifiers. The inhibition tests showed that N_2O emission was mainly related to the activity of ammonia oxidizers in aerobic condition, and the inhibition of ammonia monooxygenase completely blocked N_2O emission. On the other hand, N_2O emission increased significantly as the nitrogen flux from nitrite to nitrate was blocked by the selective inhibition of nitrite oxidation.

Key words: Activated sludge, ammonia oxidation, enriched nitrifiers, inhibition, nitrite oxidation, N_2O emission

Nitrous oxide (N_2O) is a greenhouse gas, and the nitrogen removal process of wastewater treatment is an important source of N_2O emission. Literature survey showed that N_2O emission during wastewater treatment differs significantly depending on the characteristics of the wastewater, treatment process, and operating condition [9]. However, the relative contribution of the metabolic pathways to N_2O emission is difficult to determine when mixed culture (activated sludge) is used in wastewater treatment [3, 10, 11, 13, 18, 20]. Identifying the dominant metabolic pathways of N_2O emission and their relative contribution is critical to mitigate N_2O emission from wastewater treatment plants, as the developed countries need to reduce greenhouse gas emissions by the United Nations Framework Convention on Climate Change (UNFCCC) [19].

Most of the N_2O from a wastewater treatment plant is produced through nitrification and denitrification. Biological nitrification consists of two sequential reactions: ammonia (NH_3) is oxidized to nitrite (NO_2^-) *via* hydroxylamine (NH_2OH), and this is followed by NO_2^- oxidation into nitrate (NO_3^-). Many metabolic pathways have been reported to produce N_2O during nitrification. It has been known that N_2O can be produced by the chemical decomposition of intermediates such as NH_2OH or NO_2^- during the oxidation of NH_3 [20]. Incomplete oxidation of NH_2OH can also lead to N_2O production [8]. Nitrifier denitrification by ammonia oxidizers, which reduces NO_2^- to N_2O with hydrogen or ammonia as the electron donor when oxygen is limited, is also regarded as a significant pathway for N_2O emission [17]. Furthermore, heterotrophic denitrification is also an important pathway of N_2O emission in the presence of electron donors (organic components), which involves a biochemical reduction of NO_3^-/NO_2^- to N_2 *via* NO and N_2O as the intermediates. The emission of N_2O by incomplete denitrification is mostly attributed to the inhibition of N_2O reductase or the limitation of electron donors [11,20].

Specific and selective nitrification inhibitors can be helpful to study nitrifying activities of many environmental samples [2, 9, 12]. It can also be used to investigate the responsible metabolic pathways involved in N_2O emission in aerobic and anoxic conditions [4, 16]. Allylthiourea (ATU) is known to specifically inhibit ammonia oxidation at 86 μM [6], whereas sodium azide inhibits nitrite oxidation specifically at 24 μM [5,6]. Based on literature survey, ATU and sodium azide were chosen to study the effect of specific inhibition of ammonia and nitrite oxidation on N_2O emission. The result is directly related to identifing

**Corresponding author*
Phone: +82-33-248-2154; Fax: +82-33-256-3420;
E-mail: dongjin@hallym.ac.kr

the responsible metabolic pathways of N_2O emission during nitrification. A previous study of specific nitrification inhibition experiments in a continuously operated activated sludge showed that N_2O response was followed by an increase in effluent NH_3 and/or effluent NO_2^- [4]. They observed increase of N_2O emission by the addition of ATU and azide in both cases. The result seems to be contradictory to the existing hypothesis, since N_2O emission was not expected when the first step of nitrification was blocked. It is speculated that N_2O was probably produced through denitrification by heterotrophic denitrifying bacteria, since they had a denitrification unit as well as a nitrification unit in their experimental set-up. Therefore, it needs further study to find out which nitrifying activities are responsible for N_2O emission during nitrification in wastewater treatment in a precisely controlled environment.

The objective of this study was to clarify which metabolic pathway (ammonia oxidation, nitrite oxidation) is responsible for N_2O production by the application of selective and specific inhibitors (ATU and sodium azide) during nitrification in wastewater treatment in a batch reactor. Both activated sludge and enriched nitrifiers were used for the nitrification experiment in order to eliminate the possibility of N_2O emission by heterotrophic denitrification. As far as the authors understand, this is the first report that specifically explores the effect of specific inhibition of nitrification steps on N_2O emission with activated sludge and enriched nitrifiers.

The experiment was carried out in a gas-tight glass reactor (total volume: 1 L; working volume: 0.5 L) in a batch mode. The batch reactor was mixed by a magnetic stirrer and compressed air was supplied for the aeration (50 ml/min). Synthetic wastewater was used for the experiment. Initial ammonium concentration in the batch nitrification reactor was adjusted at 20 mg NH_4^+-N/L by the supplement of $(NH_4)_2SO_4$, and the mineral composition of the wastewater was as follows: $MgSO_4 \cdot H_2O$, 5 mg/l; KH_2PO_4, 11 mg/l; $NaHPO_4 \cdot 12H_2O$, 29 mg/l; KCl, 7 mg/l; $CaCl_2 \cdot 2H_2O$, 7 mg/l; $FeCl_3 \cdot 6H_2O$, 1 mg/l. ATU (86 μM) and sodium azide (24 μM) were provided for the specific inhibition of ammonia oxidation and nitrite oxidation, respectively. Activated sludge and enriched nitrifiers were used for the experiments. Activated sludge was taken from a municipal wastewater treatment plant (Chuncheon City, Korea), which has a nitrification/denitrification process for nitrogen removal. Enriched nitrifiers were grown in a laboratory sequencing batch reactor (SBR) by feeding ammonium as the only energy source for more than 3 months [14]. The synthetic wastewater (100 ml) and 400 ml of activated sludge or enriched nitrifiers were mixed in the reactor for the nitrification experiments. All the batch nitrification and N_2O emission measurements were carried out in duplicate and the average values were used for the analysis. Details of the experimental condition can be found elsewhere [13]. For the measurement of N_2O emission, off-gas from the batch reactor was taken every 30 min and injected directly into a gas chromatograph (6890; Agilent, USA) using an HP-FFTP column and electron capture detector (ECD), and N_2 gas was used as the carrier gas. The temperatures of the oven, injection, and detector were set at 50°C, 100°C, and 250°C, respectively. Cumulated N_2O-N in the off-gas was calculated from the N_2O concentration and the off-gas flow rate. Liquid samples were taken every hour from the batch reactor for the measurement of NH_4^+-N, NO_2^--N, and NO_3^--N by the Standard Methods [1].

N_2O Emission Under Selective Nitrification Inhibition with Activated Sludge

The N_2O emission characteristics of activated sludge (Fig. 1) and enriched nitrifiers (Fig. 2) during nitrification were investigated and compared under the selective nitrification inhibition. The activated sludge contained nitrification bacteria as well as other heterotrophic bacteria, as it was taken from a nitrogen removal wastewater treatment plant. Fig. 1A shows the profiles of nitrogen compounds (NH_4^+-N, NO_2^--N, NO_3^--N) during nitrification and N_2O emission in the batch reactor with activated sludge when no nitrification inhibitors were added (control). The 20 mg/l of NH_4^+-N was completely nitrified in 5 h, and the concentrations of nitrite and nitrate were gradually increased by nitrification. Cumulative N_2O-N in the off-gas reached up to 0.3 mg during the nitrification. Fig. 1B shows the profiles of nitrification and N_2O emission under the presence of 86 μM ATU, which specifically inhibits ammonia monooxygenase for the production of hydroxylamine (NH_2OH) from ammonia [2]. During the ATU inhibition, NH_4^+-N was slightly decreased whereas residual nitrite was oxidized to nitrate as the sum of nitrite and nitrate remained constant. NH_4^+-N decreased 1 mg in 7 h and its loss seems to be due to the microbial assimilation and stripping of ammonia. It confirms that ATU selectively inhibited ammonia oxidation, but it did not inhibit nitrite oxidation. During the experiment, N_2O was hardly produced and almost no N_2O was emitted. The results indicate that N_2O production during nitrification is directly linked to ammonia oxidation to hydroxylamine, which is governed by ammonia oxidizers.

Nitrification and N_2O emission of activated sludge were also monitored for comparison when nitrite oxidation was selectively inhibited. Fig. 1C shows the profiles of nitrification and N_2O emission under the presence of 24 μM sodium azide, which specifically inhibits nitrite oxidoreductase for the oxidation of nitrite to nitrate. Ammonium could be oxidized to nitrite without inhibition, and NO_2^--N was accumulated up to 6.4 mg by the selective inhibition of nitrite oxidation to nitrate. Therefore, the selective inhibition of azide on nitrite oxidation could also be confirmed.

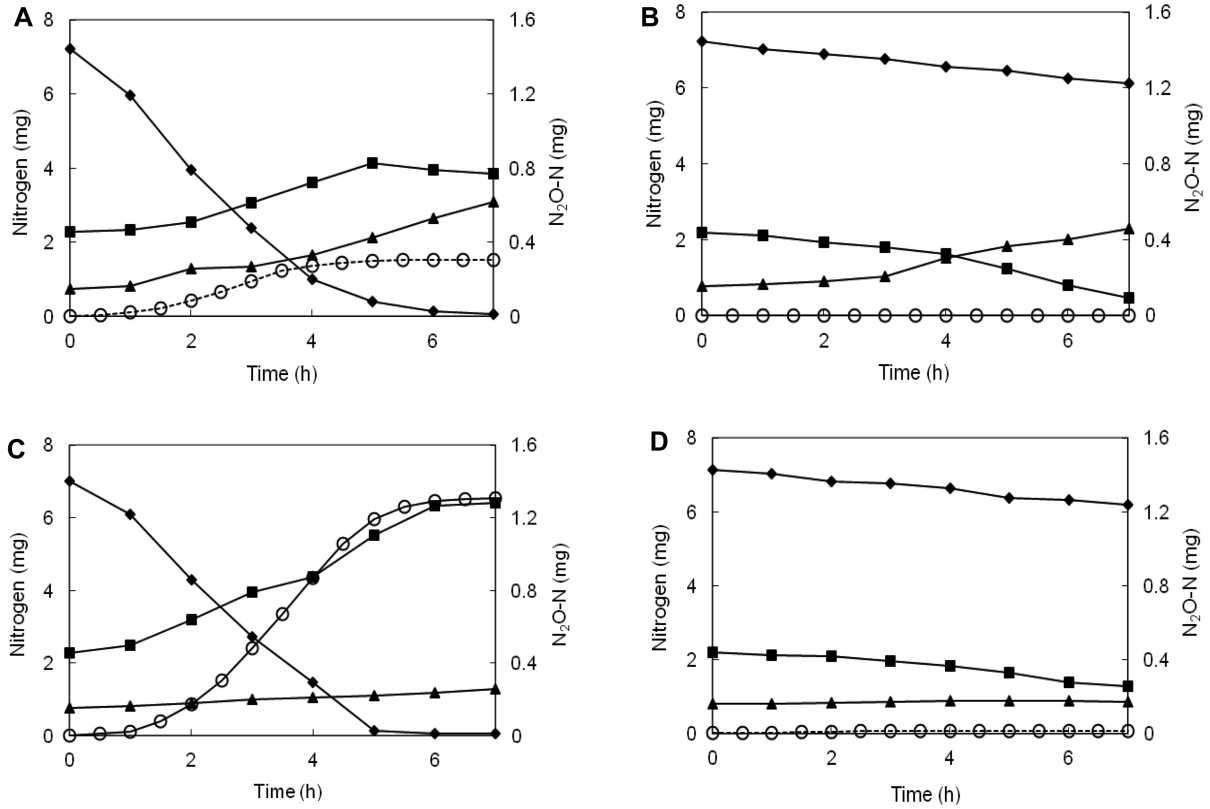

Fig. 1. Effect of selective nitrification inhibition on nitrification in wastewater treatment and N_2O emission of activated sludge in the batch reactor: (**A**) without inhibition (control), (**B**) ammonia oxidation inhibition by ATU (86 μM), (**C**) nitrite oxidation inhibition by sodium azide (24 μM), and (**D**) simultaneous inhibition of ammonia oxidation and nitrite inhibition by ATU (86 μM) and sodium azide (24 μM).
○: accumulated N_2O-N; ◆: NH_4^+-N; ■: NO_2^--N; ▲: NO_3^--N.

Interestingly, N_2O-N emission accumulated up to 1.3 mg, which was much higher than that of the control experiment. The reason seems to be due to the blockage of the nitrogen flux *via* nitrite to nitrate by the specific inhibition, and it diverted the nitrogen flux from nitrite to N_2O in nitrification. It is supported by the fact that nitrite has been known as the key intermediate compound to enhance N_2O emission in nitrification and denitrification [11]. Fig. 1D shows the profiles of nitrification and N_2O emission under the presence of ATU (86 μM) and sodium azide (24 μM), together which simultaneously inhibit both ammonia monooxygennase and nitrite oxidoreductase. NH_4^+-N was decreased very slowly as in Fig. 1B, probably due to the bacterial assimilation and stripping. Meanwhile, nitrite also decreased slightly whereas the nitrate level remained constant. As expected, almost no N_2O-N was found in the off-gas. The possibility of N_2O emission by heterotrophic denitrification could be excluded from the results of Figs. 1B and 1D, as N_2O was hardly detected in both cases. The results also do not guarantee that N_2O is solely emitted by the oxidation of ammonia (decomposition of hydroxylamine) even though no N_2O emission was observed when ammonia oxidation was selectively inhibited. Nitrifier denitrification can also reduce nitrite to N_2O by the oxidation of ammonia (donation of electron). The relative contributions of nitrification (hydroxylamine) and nitrifier denitrification in actual N_2O emissions are still in dispute and need further investigation. Summarizing the results of activated sludge, N_2O emission is directly related to ammonia oxidation to hydroxylamine; however, nitrite oxidation has no direct effect on N_2O emission. N_2O emission by heterotrophic denitrification was also negligible with activated sludge, based on the N_2O emission results of control (Fig. 1A) and ammonia oxidation inhibition (Figs. 1B and 1D).

N_2O Emission Under Selective Nitrification Inhibition with Enriched Nitrifiers

Nitrification and N_2O emission experiments under the selective nitrification inhibition were also carried out with the enriched nitrifiers (Fig. 2). Heterotrophic denitrification can hardly occur with enriched nitrifiers in the absence of organics, especially in aerobic condition. Ammonia oxidizers and nitrite oxidizers occupied 30–35% of the total bacteria in the enriched nitrifiers based on fluorescence *in situ*

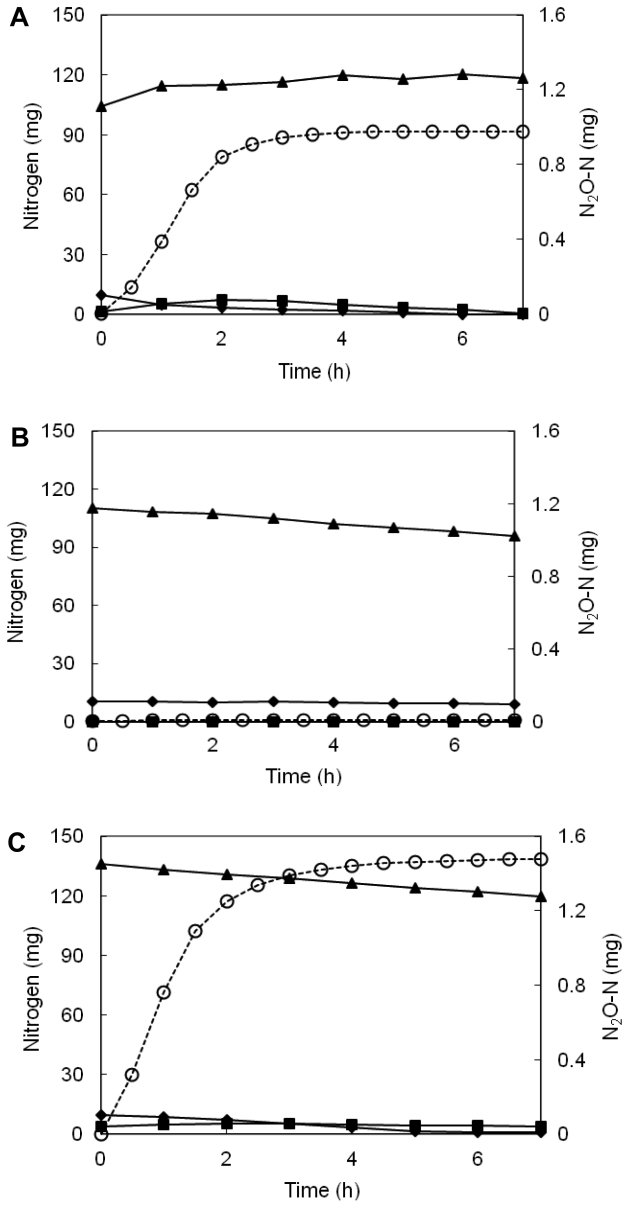

Fig. 2. Effect of selective nitrification inhibition on nitrification in wastewater treatment and N_2O emission of enriched nitrifiers in the batch reactor: (**A**) without inhibition (control), (**B**) ammonia oxidation inhibition by ATU (86 μM), and (**C**) nitrite oxidation inhibition by sodium azide (24 μM).
○: accumulated N_2O-N; ◆: NH_4^+-N; ■: NO_2^--N; ▲: NO_3^--N.

hybridization analysis. Fig. 2A shows the profiles of the nitrogen compounds (NH_4^+-N, NO_2^--N) during the nitrification with enriched nitrifiers when no nitrification inhibitor was added (control). NO_3^--N was not shown because of its high background nitrate level (100–130 mg NO_3^--N/L). It was due to the mixed liquors of enriched nitrifiers from the sequencing batch reactor, as a high concentration of ammonium (150 mg NH_4^+-N/L) was provided as the substrate of enriched nitrifiers. Ammonium (20 mg NH_4^+-N/L) was completely nitrified in 5 h, and the nitrate level was increased as a result of the nitrification. Interestingly, cumulative N_2O-N in the off-gas reached up to 1.0 mg during the nitrification, which was significantly higher than that of the activated sludge. The reason is not clear yet, but it seems that increased nitrifying bacteria of the enriched nitrifers might contribute to the N_2O emission. Fig. 2B shows the profiles of nitrification and N_2O emission with enriched nitrifers in the presence of 86 μM ATU. During the ATU inhibition experiment, NH_4^+-N did not decrease and remained constant because of the inhibition by ATU. In the meanwhile, very little N_2O was produced and emitted. The result matches up with that of the activated sludge experiment. It is thought that nitrifiers do not produce N_2O under the inhibition of ammonia monooxygenase. The result also indicates that heterotrophic denitrification could also be excluded as a source of N_2O emission. The contributions from ammonia oxidation (hydroxylamine) and nitrifier denitrification are not clear, as discussed before. Even though the possibility of N_2O emission by a direct chemical decomposition of hydroxylamine is low, hydroxylamine can react with other intermediates to produce N_2O [20].

When 24 μM of sodium azide was added to the enriched nitrifiers, N_2O-N emission accumulated up to 1.5 mg when ammonium was completely oxidized (Fig. 2C), and it was also higher than that of the control as in the case of activated sludge. Nitrification with enriched nitrifiers showed similar N_2O emission trends as activated sludge, in that inhibition by ATU completely blocked N_2O emission and inhibition by azide emitted more N_2O than that of the control experiment. The behavior of N_2O emission during nitrification (ammonia oxidation) inhibition by ATU of this study was different from the result of Butler *et al*. [4]. As was mentioned before, heterotrophic denitrification could be a source of N_2O in their study. Shock loading of the inhibitor to the continuous-flow activated sludge might contribute to the different N_2O emission characteristics. More importantly, the concentration of the inhibitor (ATU) applied to the continuous reactor was much different from the literature. The estimated concentration of ATU applied to the reactor was 0.76 mg/l, and it was 7.6% of the inhibition concentration [6]. The application of lower ATU concentration could not fully inhibit ammonia oxidation, and hydroxylamine and nitrite could be produced and utilized for N_2O emission.

The above results indicate that a wastewater treatment plant that removes nitrogen by nitrification and denitrification needs to maintain the nitrite level as low as possible to reduce N_2O emission. Special attention needs to be paid to the novel nitrogen removal processes, like Anammox (Anaerobic Ammonium Oxidation) and SHARON (Single

reactor system for High activity Ammonium Removal Over Nitrite), because the processes utilize nitrite accumulation in the reactions for economic reasons [7, 15].

Summarizing the above results, it is confirmed that N_2O emission is mainly related to the activity of ammonia oxidizers in aerobic condition, and the inhibition of ammonia monooxygenase completely blocked N_2O emission. Hydroxylamine and/or nitrite play(s) a key role in N_2O production. Nitrogen flux blockage from nitrite to nitrate by selectively inhibiting nitrite oxidation increased N_2O emission significantly. Therefore, nitrite accumulation in nitrification in wastewater treatment is an undesirable situation to mitigate N_2O emission.

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