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# $N_2O$ emission during wastewater nitrification with enriched nitrifying bacteria

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ABSTRACT

Nitrifying bacteria were enriched in a sequencing batch reactor and used to quantify $N_2O$ emission specifically by wastewater nitrification while minimizing or preventing the chances of denitrification during the experiment. Batch wastewater nitrification was carried out in a reactor (working volume 0.5 L) at different aeration rates (10 and 20 mL/min) and $NH_4^+$-N concentrations (25, 50, and 100 mg/L). Wastewater nitrification efficiencies were more than 97% in all the cases except at 100 mg/L with 10 mL/min aeration rate (92%). Cumulative $N_2O$ emission reached 0.2, 1.15, and 2.67 mg (aeration: 10 mL/min) and 0.37, 2.18, and 3.08 mg (aeration: 20 mL/min) at the initial $NH_4^+$-N concentration of 25, 50, and 100 mg/L, respectively. $N_2O$ emission yields (kg $N_2O$-N per kg processed $NH_4^+$-N) were 0.016–0.058 and 0.029–0.088 at 10 and 20 mL/min aeration rate, respectively. The yields were higher than that of denitrification. The results showed that cumulative $N_2O$ emission and the yield increased at higher ammonium concentration and aeration rate. Accumulated nitrite during nitrification also contributed and played a pivotal role in $N_2O$ emission.

*Keywords:* Enriched nitrifiers; $N_2O$ emission; Nitrification; Nitrite

## 1. Introduction

Removal of wastewater nitrogen by nitrification and denitrification reduces oxygen depletion and provides better water quality of the receiving water bodies [1]. Nitrogen removal efficiency depends upon not only the characteristics of the influent wastewater but also the operational factors of the wastewater treatment plant. Inefficient or incomplete nitrogen removal from the wastewater often leads to undesirable products such as nitrous oxide ($N_2O$) which is one of the potential green house gases with global warming potential of 320 times greater than carbon dioxide on 100-year time horizon [2,3]. It also has the potential to disturb the stratospheric ozone layer by a photochemical reaction producing nitric oxide (NO) which catalyzes the damage of ozone layer [4]. It is well known that wastewater treatment plants produce and release substantial amounts of $N_2O$ into the atmosphere [5–7].

$N_2O$ is the outcome of biological activities of micro-organisms during the transformation of ammonium nitrogen ($NH_4^+$-N) to nitrogen gas ($N_2$) in wastewater treatment and soil. It is well documented in the literature that the principle processes involved in $N_2O$ emission are biological nitrification and denitrification

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[8–12], but the exact pathway, process (aerobic and anaerobic), and contributing factors toward its emission are not fully understood [9]. Dissolved oxygen (aeration) and nitrite are known as the important factors for $N_2O$ emission in nitrifying process [12,13].

Nitrification is sometimes linked to aerobic denitrification but these two can also be distinguished on the basis of different set of enzymes used for transformation of different forms of nitrogen [11]. Recent studies suggested that nitrifier denitrification is the dominant pathway of $N_2O$ emission from the aerobic step of wastewater treatment process [14,15]. It has been reported that low dissolved oxygen enhanced $N_2O$ emission in nitrifying activated sludge [12,16]. Similarly, it also has been known that more $N_2O$ is produced in aerobic zones when compared with anoxic zones [17,18]. Many inconsistent results have been reported about the role of aeration (dissolved oxygen) toward $N_2O$ emission that both high and low dissolved oxygen brought high emission of $N_2O$ [9,10,17]. The inconsistency of $N_2O$ emission could be resulted from the combined (or simultaneous) nitrification and denitrification in activated sludge because $N_2O$ can be produced by both nitrification and denitrification.

Therefore, the experiment needs to be carried out under the absence of heterotrophic denitrifying bacteria and organic compounds if we want to observe $N_2O$ emission by nitrification only while eliminating $N_2O$ emission by denitrification. For the purpose, we enriched nitrifiers in a sequencing batch reactor (SBR) by feeding autotrophic wastewater containing ammonium as the only energy source and used for the $N_2O$ emission experiment in a batch reactor under different ammonium and aeration conditions. The objective of this study is to investigate the effect of ammonium concentration and aeration on $N_2O$ emission during nitrification by using enriched nitrifiers grown in a laboratory SBR. $N_2O$ yields (based on processed $NH_4^+$-N) as well as cumulative $N_2O$ emissions were analyzed in order to correlate $N_2O$ emission with ammonium, nitrite, and aeration rate during the wastewater nitrification. This study provides valuable information for the environmentally cleaner design and operation of nitrogen removing wastewater treatment plant by reducing green house gas ($N_2O$) emissions.

## 2. Materials and methods

### 2.1. SBR for nitrifier growth and enrichment

In this study, nitrifiers were grown and enriched in an SBR with a total volume of 11 L (height 40 cm, diameter 20 cm) and a working volume of 9 L. The schematic diagram of the laboratory SBR system is shown in Fig. 1. An SBR cycle took 6 h which consists of aeration of 325 min (including 180 min of feeding), settling of 25 min, and drawing of 10 min. Aeration rate was set at 1 L/min and mixing rate was set at 80 rpm. For every cycle 1.8 L of wastewater was pumped in and the same volume was removed from the reactor. 1 N HCl and $NaHCO_3$ solutions were used to control the reactor pH between 7.0 and 7.5. The composition of the synthetic wastewater for the SBR is given in Table 1. All the SBR operations (aeration, mixing, feeding and drawing, pH) were controlled by a computer program (Lab-view®) and the operational condition and cycle parameters are summarized in Table 2. Initially, activated sludge from a municipal wastewater treatment plant was used for inoculation and nitrifiers were enriched by feeding the ammonium containing synthetic wastewater. The SBR operation was carried out more than 3 months and the microbial communities of the enriched nitrifiers were regularly monitored by fluorescence *in situ* hybridization [19–22].

### 2.2. Batch reactor for the quantification of $N_2O$ emission during wastewater nitrification

In this study, a batch reactor (working volume: 0.5 L) was used for the quantification of $N_2O$ emission during nitrification with enriched nitrifiers from the SBR. The batch reactor was aerated for 2 h with 0.4 L of enriched nitrifiers from the SBR to remove the background ammonium. After 2 h of aeration, 100 mL of wastewater with different concentrations of $NH_4^+$-N such as 125, 250, and 500 mg/L was injected to the SBR to make 25, 50, and 100 mg $NH_4^+$-N/L. The synthetic wastewater used in this study does not contain any organic compounds and the composition is shown in Table 3. The batch reactor was mixed with a magnetic stirrer and 10 and 20 mL/min air flow rate was provided for nitrification. For the quantification of $N_2O$ emission from the batch reactor, off-gas samples were taken every 30 min and directly injected to a gas chromatograph (Agilent 6890, USA). GC ECD was calibrated with 1 ppm $N_2O$ in nitrogen standard (Supelco, Sigma–Aldrich, Cat. No. 501514) and also with $N_2O$ in air. Liquid samples were collected every hour for the measurement of $NH_4^+$-N, $NO_2^-$-N, and $NO_3^-$-N.

For measuring the dissolved $N_2O$, 5 mL sludge sample was taken from the batch reactor into a 20 mL serum vial containing 0.1 mL of 5% $HgCl_2$ to stop microbial activity. The vial was then shaken vigorously for 1 min. Vial containing sludge was purged by $N_2$ gas for 30 min. After purging the vial was left at room temperature for 1 h. The resulting off-gas (head space) in the vial was collected by a 0.5 mL gas syringe and analyzed by a gas chromatograph (Agilent

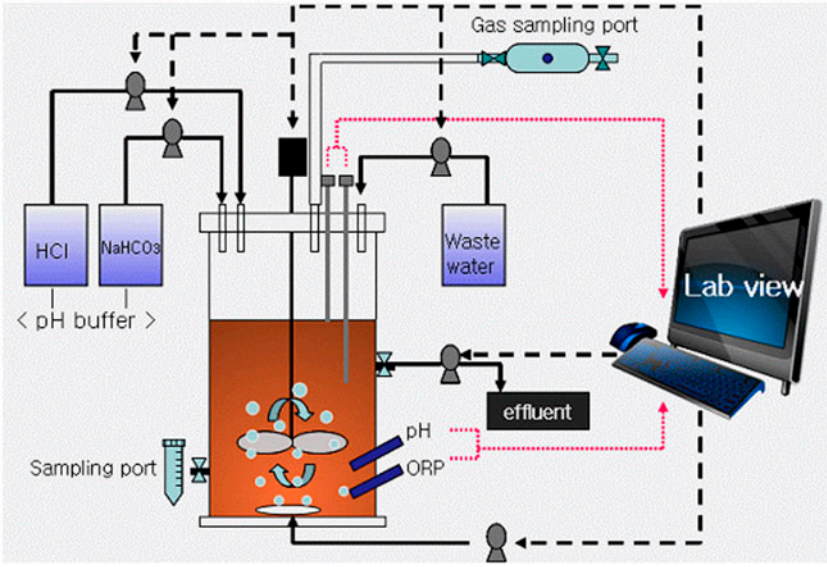


Fig. 1. Schematic diagram of the SBR setup for the growth and enrichment of nitrifying bacteria.

Table 1
Composition of the autotrophic synthetic wastewater used for the growth and enrichment of nitrifying bacteria in the SBR. Tap water is used for the wastewater preparation

| Contents | Concentration (mg/L) |
|---|---|
| $N((NH_4)_2SO_4)$ | 240 |
| $NaHCO_3$ | 1,700 |
| $MgSO_4{\cdot}H_2O$ | 25 |
| KCl | 35 |
| $NaHPO_4{\cdot}12H_2O$ | 145 |
| $CaCl_2{\cdot}2H_2O$ | 35 |
| $KH_2PO_4$ | 55 |
| $FeCl_3{\cdot}6H_2O$ | 5 |

Table 2
Operational cycle and condition of the SBR for the growth and enrichment of nitrifying bacteria

| Operation cycle (min) | | Operation condition | |
|---|---|---|---|
| Aeration (feeding) | 325 (180) | DO | 2.0–4.0 mg/L |
| Settling | 25 | MLSS | 1,525 mg/L |
| Decanting | 10 | SRT | 20 days |
| Total | 360 | Air | 1 L/min |
| | | Temperature | 26–29 ℃ |

6890, USA) using HP-FFTP column and electron capture detector (ECD).

The dissolved $N_2O$ concentration was calculated by the solubility equation used by Kimochi et al. [23]. The value of Ostwald's solubility coefficient of $N_2O$ ($\beta$) used in this study was 0.785 which was taken from the study of Molstad et al. [24]. All the wastewater nitrification and N2O emission measurements were carried out in duplicate and the average values were used for the analysis. Detailed batch reactor setup and other experimental/analytical conditions can be found elsewhere [22].

Table 3
Composition of the autotrophic synthetic wastewater used for the nitrification in the batch reactor. Tap water is used for the wastewater preparation

| Contents | Concentration (mg/L) |
|---|---|
| $N((NH_4)_2SO_4)$ | 10–100 |
| $NaHCO_3$ | 72–720 |
| $MgSO_4{\cdot}H_2O$ | 5 |
| KCl | 7 |
| $NaHPO_4{\cdot}12H_2O$ | 29 |
| $CaCl_2{\cdot}2H_2O$ | 7 |
| $KH_2PO_4$ | 11 |
| $FeCl_3{\cdot}6H_2O$ | 1 |

## 3. Results and discussion

### 3.1. SBR for enrichment of nitrifiers

An SBR was used as the mother reactor to grow and enrich nitrifiers for the quantification of $N_2O$ emission during nitrification in the batch reactor. Fig. 2 shows the profiles of nitrogen compounds of a typical SBR cycle. Input $NH_4^+$-N (1.8 L of 240 mg N/L, 432 mg per a cycle) was completely oxidized into

$NO_3^-$-N via $NO_2^-$-N in a cycle (6 h). Maximum $NH_4^+$-N and $NO_2^-$-N concentrations were less than 5.0 and 2.3 mg/L, respectively, due to wastewater nitrification during the feeding time (3 h). The total nitrogen (TN) and $NO_3^-$-N in the SBR increased by the feeding of $NH_4^+$-N, and in the last it showed almost the same nitrogen mass present at the start of the cycle, and it reflects that all the incoming $NH_4^+$-N of the cycle was nitrified and removed (decanted) from the SBR.

### 3.2. $N_2O$ emission from batch nitrification by enriched nitrifying sludge

For the nitrification in a batch reactor, 400 mL of enriched nitrifying sludge was taken from the SBR after the aeration cycle and transferred to the batch reactor. Batch reactor was aerated for 2 h before the wastewater addition in order to remove any residual ammonium present. After 2 h of aeration $NH_4^+$-N concentration in the batch reactor was near to zero for all experiments as shown in nitrogen profiling figures (Fig. 3(A)–(F)). After then, 100 mL of fresh synthetic wastewater was injected into the batch reactor and the $N_2O$ in the off-gas was measured for the analysis.

#### 3.2.1. Effect of ammonium concentration and aeration rate on $N_2O$ emission during wastewater nitrification

Batch wastewater nitrification experiments were carried out at two different aeration rates (10 and 20 mL/min) and $NH_4^+$-N concentrations of 25, 50, and 100 mg/L. Fig. 3 presents the profiles of nitrogen compounds and cumulative $N_2O$ emission from the batch reactor. Higher aeration rate (20 mL/min) showed better nitrification performance than lower aeration rate (10 mL/min) in all the cases. Injected ammonium (25 mg N/L) was completely removed at both of 10 and 20 mL/min aeration rates as shown in Fig. 3(A) and (B). In the meantime, $NO_2^-$-N was accumulated up to 10 mg/L in both cases which were caused by the imbalance of the activities of ammonium oxidation and nitrite oxidation. $N_2O$ started to emit after the injection of ammonium, and it clearly indicates that $N_2O$ comes from the ammonium in the wastewater. Cumulative $N_2O$-N emission reached 0.2 and 0.37 mg, respectively, when ammonium (25 mg N/L) was completely nitrified at 10 and 20 mL/min aeration rates. Fig. 3(C) and (D) shows the profiles of nitrogen compounds during nitrification with 50 mg/L $NH_4^+$-N. $NO_2^-$-N was accumulated up to 20 mg/L, and the levels were higher than those of 25 mg/L $NH_4^+$-N in both aeration rates. Cumulative $N_2O$-N emission reached 1.15 and 2.18 mg at 10 and 20 mL/min aeration, respectively. Nitrification efficiencies reached 92 and 97% with 100 mg/L $NH_4^+$-N and maximum $NO_2^-$-N concentration reached up to 35 and 40 mg/L at 10 and 20 mL/min aeration rates, respectively, as shown in Fig. 3(E) and (F). And the cumulative $N_2O$-N emission reached 2.67 and 3.08 mg, respectively.

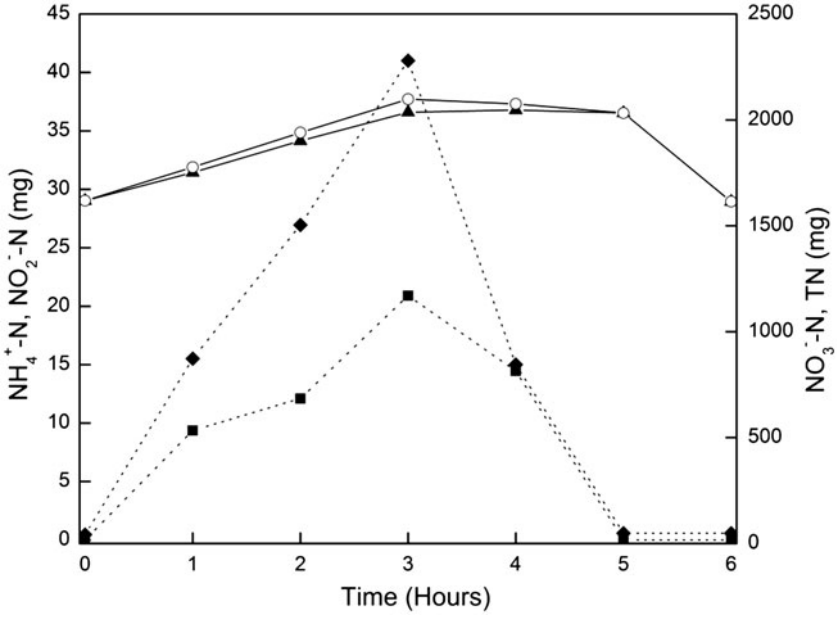


Fig. 2. Nitrogen profiles of a typical cycle of the SBR. All the nitrogen compounds ($NH_4^+$-N, $NO_2$-N, $NO_3^-$-N and TN) were given in mass (mg) (◆: $NH_4^+$-N; ■: $NO_2^-$-N; ▲: $NO_3^-$-N; ○: total N (TN)).

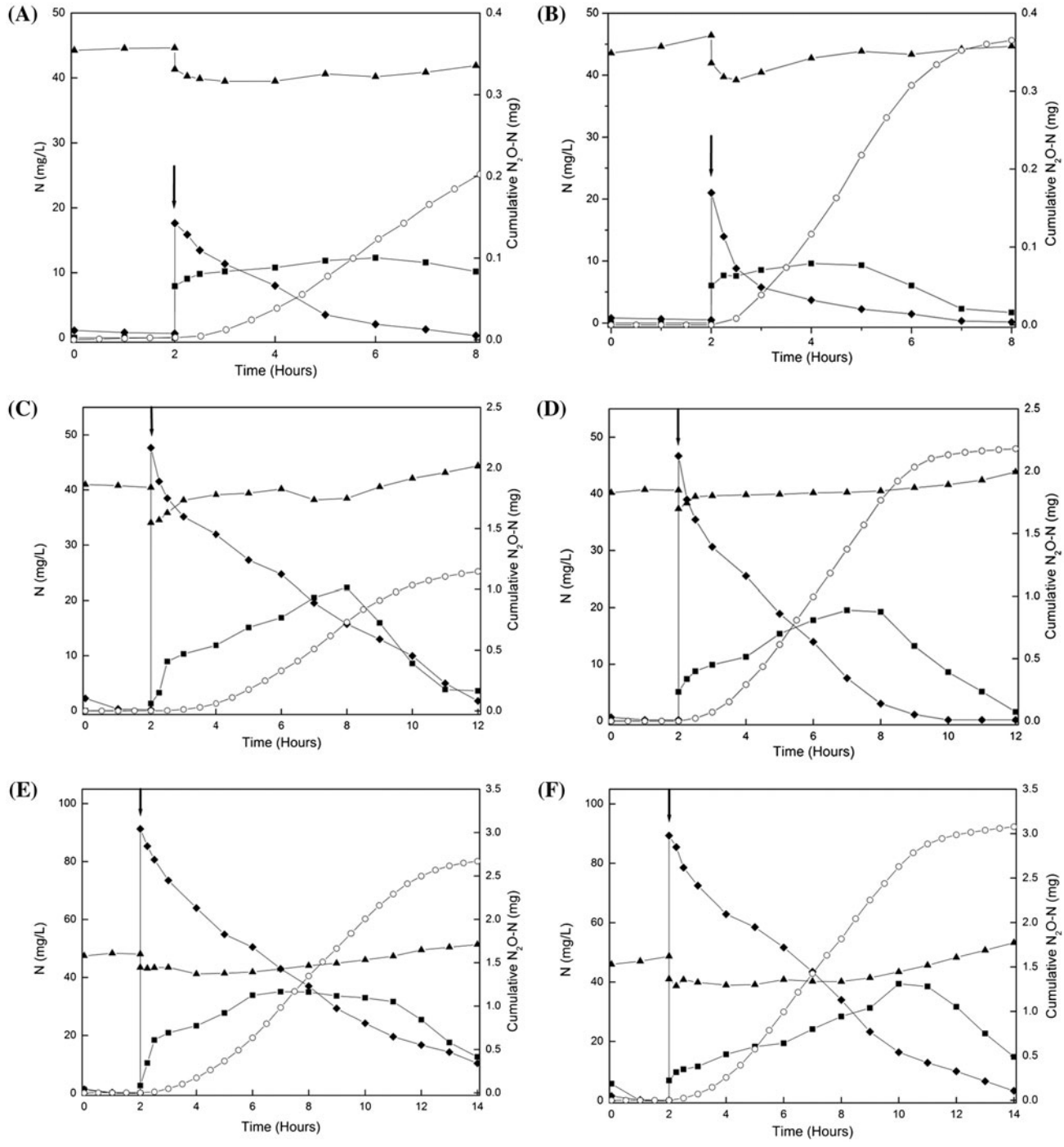


Fig. 3. Profiles of nitrogen compounds for different concentrations of $NH_4^+$-N and aeration rates in the batch reactor. (A) 25 mg/L $NH_4^+$-N and 10 mL/min aeration rate; (B) 25 mg/L $NH_4^+$-N and 20 mL/min aeration rate; (C) 50 mg/L $NH_4^+$-N and 10 mL/min aeration rate; (D) 50 mg/L $NH_4^+$-N and 20 mL/min aeration rate; (E): 100 mg/L $NH_4^+$-N and 10 mL/min aeration rate; (F) 100 mg/L $NH_4^+$-N and 20 mL/min aeration rate (◆: $NH_4^+$-N; ■: $NO_2^-$-N; ▲: $NO_3^-$-N; ◯: cumulative $N_2O$-N). The arrows indicate wastewater injection for nitrification.

The experimental results showed that higher ammonium concentration and aeration rate emitted more $N_2O$ (Fig. 4(A)). As we used enriched nitrifying bacteria and ammonium as the only energy source in the batch reactor, it can be assumed that nitrification was the dominant microbial activity and it is also

responsible for $N_2O$ emission. Therefore, the more nitrification, the more $N_2O$ emission is expected. It has been reported that higher aeration rate in the presence of $NH_4^+$-N resulted in transient accumulation of nitrite, and subsequently $N_2O$ was emitted during wastewater nitrification [10,25,26]. Recent reports showed that $N_2O$ emission is positively correlated to the concentration of ammonium and nitrite because both compounds act as the substrates for nitrification and nitrifier denitrification from the pure culture studies of ammonia oxidizing bacteria [17,27]. In Fig. 4(A), it has clearly shown that $N_2O$ emission increased with the increase in $NH_4^+$-N concentration for both aeration rates (10 and 20 mL/min). Similarly, it can be observed that $N_2O$ yield also increased by increasing $NH_4^+$-N concentration from 25 to 100 mg/L in Fig. 4(B). These results inferred that $N_2O$ emission directly proportional to the $NH_4^+$-N concentration and recently. Toor et al. found that $N_2O$ emission has a positive linear correlation with logarithm of $NH_4^+$-N concentration by performing regression analysis [6]. Medium to higher concentration of $NH_4^+$-N also increases the nitrification activities by increasing the ammonium oxidation rate and $N_2O$ production rate reported recently by Avrahami et al. [28]. Moreover, Law et al. through their metabolic model based on $N_2O$ production proposed that increased ammonia oxidation could emit more $N_2O$ by unstable break down of nitrosyl radical NOH during $NH_2OH$ oxidation (an intermediate of nitrification), but this suggestion has not yet been confirmed with other studies [29]. $N_2O$ yield (kg $N_2O$-N per kg processed $NH_4^+$-N) was determined to find out the effect of aeration rate on $N_2O$ emissions (Fig. 4(B)). The yields were 0.016, 0.047, and 0.058 at 10 mL/min aeration rate while they were 0.029, 0.088, and 0.060 at 20 mL/min with $NH_4^+$-N concentrations of 25, 50, and 100 mg/L, respectively. The results also showed that higher aeration rate gave higher $N_2O$ yields in all the $NH_4^+$-N concentrations. Toor et al. reported $N_2O$-N yield of 0.027 with activated sludge from wastewater nitrification at similar condition ($NH_4^+$-N concentration: 22.5 mg/L, aeration: 20 mL/min), and it is very close to 0.029 of this study [6]. It can be explained that as $N_2O$ emission is caused by the metabolic activity of nitrifying bacteria, activated sludge and nitrifying bacteria could have the same $N_2O$ yield if the experimental conditions are the same.

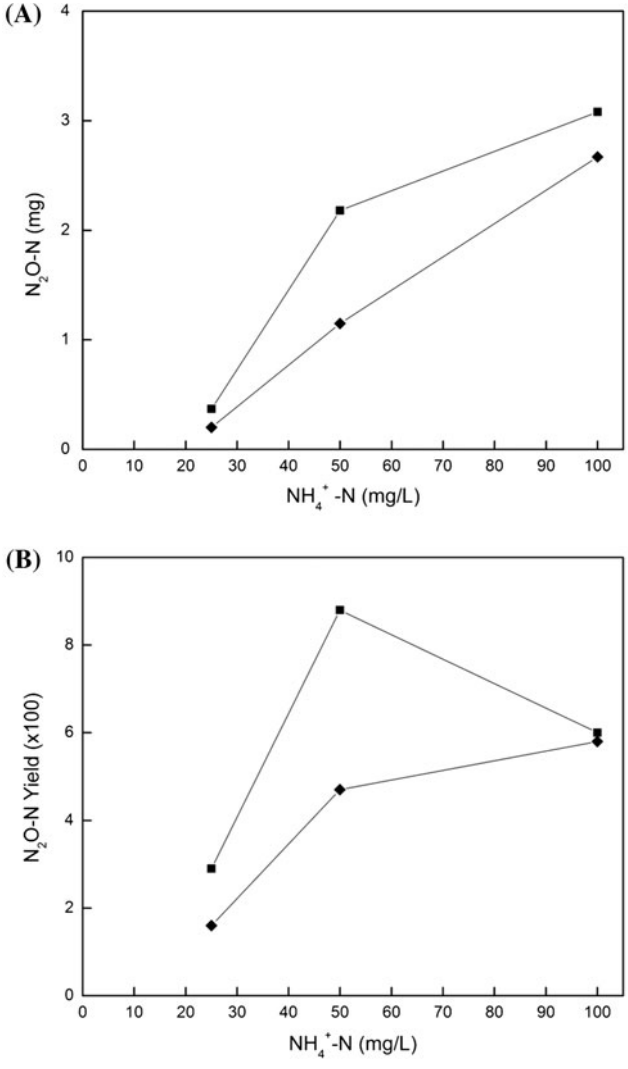


Fig. 4. Effect of $NH_4^+$-N concentration and aeration rate (10 and 20 mL/min) on $N_2O$ emission. (A) Cumulative $N_2O$-N in the off-gas; (B) $N_2O$-N yields (◆: 10 mL/min aeration rate; ■: 20 mL/min aeration rate).

$N_2O$ emission during denitrification differs significantly depending on the wastewater treatment process, characteristics of the wastewater, and operation condition of the wastewater treatment plant. However, Kampschreur et al. reported that $N_2O$ emission yield during denitrification was lower than nitrification in their review [14]. Ahn et al. also reported that aerobic zones (nitrification) emitted about two times more $N_2O$ than anoxic zones (denitrification) from their full-scale wastewater treatment plant survey in the US [17].

For getting more insight of aeration rate impact on $N_2O$ emission further batch nitrifications were carried out with 10 mg/L of $NH_4^+$-N at different aeration rates (10, 20, 40, and 50 mL/min). Similar results were found that $N_2O$ emission increased at higher aeration rate (Fig. 5). Toor et al. also obtained higher $N_2O$-N yields of 0.092–0.124 during nitrification by activated sludge with 25–120 mg $NH_4^+$-N/L at higher aeration rate (50 mL/min) than this study [6]. It can be thought that the more $N_2O$ emission in the off-gas at the higher aeration rate

may be due to the air stripping effect of $N_2O$ [17]. As the solubility of $N_2O$ in water is not very low (1.02 g/L at 25°C and standard pressure), high aeration rate during nitrification may accelerate the transfer of $N_2O$ from water to gas phase. As we measured the dissolved $N_2O$ in the batch reactor, less dissolved $N_2O$ was found at higher aeration rate and the dissolved $N_2O$ levels were far below the saturation level in all the aeration rates (data not shown). However, the contribution by stripping is relatively negligible when considering the amounts of dissolved $N_2O$ in the reactor at different aeration conditions and the $N_2O$ emitted during nitrification. Our estimation revealed that the dissolved $N_2O$ in the reactor was less than 2% of the cumulative $N_2O$ emission in all the experiments as shown in Fig. 5. The result shows that stripping of $N_2O$ at higher aeration rate does not exert significant contribution on $N_2O$ emission during nitrification, and it also coincides with the result of Jian et al. [9]. Instead, higher $N_2O$ production by enriched nitrifiers at higher aeration condition caused higher $N_2O$ emissions.

### 3.2.2. *Effect of nitrite on $N_2O$ emission during wastewater nitrification*

The results of batch nitrification showed that $N_2O$ emission increased with wastewater $NH_4^+$-N concentration and aeration rate which is due to higher nitrification. Moreover, nitrite accumulation increased at higher ammonium concentration (Fig. 3) and it is well recognized that nitrite directly contributes $N_2O$ emission during wastewater nitrification [11,14,22]. Kim and Kim found similar results i.e. by increasing higher ammonium more $N_2O$ accumulated (3.0 and 5.7 for 50 and 100 mg/L $NH_4^+$-N concentration), which is higher than our results and reason could be the different apparatus used as in literature we also found variable results based on different conditions and experimental scales used [22]. It has been reported that $N_2O$ is mainly produced during ammonium oxidation to nitrite from the selective inhibition experiments of ammonia oxidation and nitrite oxidation [30]. We also found that $N_2O$ was mainly emitted in the early or active stage of batch ammonium oxidation other than late stage of batch ammonium oxidation as observed in Fig. 3.

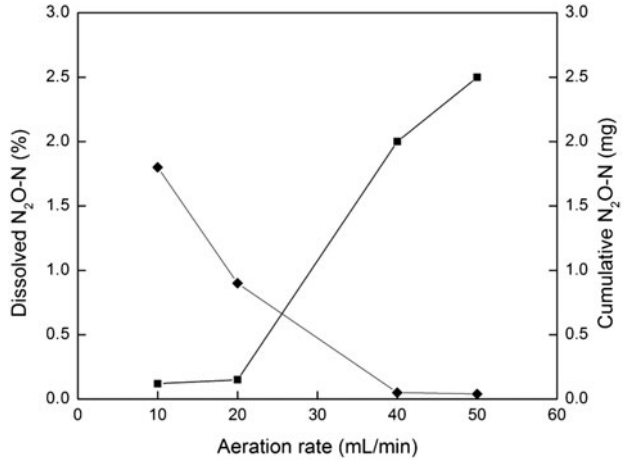


Fig. 5. Effect of aeration rate on the dissolved $N_2O$-N (% of the cumulative $N_2O$-N emission) and the cumulative $N_2O$-N emission during wastewater nitrification with 10 mg/L $NH_4^+$-N (♦: dissolved $N_2O$-N (%); ■: cumulative $N_2O$-N emission (mg)).

In this study, nitrite seemed to play a main role in $N_2O$ emission as the cumulative $N_2O$ emission increased with the nitrite concentration during nitrification (Fig. 3). Even though nitrite is known as an intermediate of denitrification, heterotrophic denitrification can be excluded in this experimental condition because enriched nitrifiers were used and ammonium was provided as the only energy source in aerobic condition. In nitrifier denitrification, nitrite is reduced to $N_2O$ and $N_2$, and it is mostly carried out by autotrophic ammonia oxidizers [11,31]. Further study showed that nitrate cannot be reduced but only nitrite can be reduced to $N_2O$ because *Nitrosomas europaea*, one of the most representative ammonia oxidizers, has denitrification enzyme from nitrite to $N_2O$ but lacks the enzyme responsible for the reduction of nitrate and $N_2O$ in their genomes [32]. The enzyme required for the reduction of nitrite to $N_2O$ is believed to be the same for ammonia oxidation and denitrification present in ammonia oxidizers [11]. Hydroxylamine ($NH_2OH$) is used as the electron donor for nitrifier denitrification [31].

In this study, the average $NO_2^-$-N concentration during nitrification was used to examine its effect on $N_2O$ emission since $NO_2^-$-N concentration varies during the batch nitrification. Fig. 6 shows that $N_2O$ emission increased with the average $NO_2$-N concentration from the batch nitrification results. Higher aeration with 20 mL/min emitted more $N_2O$ than 10 mL/min aeration. The results are also in agreement with previous study with activated sludge [6]. The cause of nitrite accumulation during nitrification has been investigated. Free ammonia ($NH_3$) and free nitrous acid ($HNO_2$) have been known to inhibit nitrite oxidizers selectively to accumulate nitrite during nitrification [19,29]. In this experiment, the two factors are coupled together to accumulate nitrite. Nitrification at higher ammonium concentration accumulates more nitrite, and higher nitrite (nitrous acid) concentration further

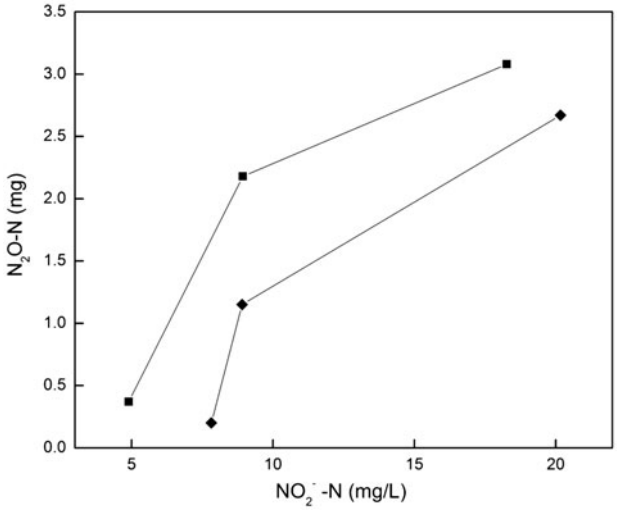


Fig. 6. Effect of the average $NO_2^-$-N concentration on $N_2O$ emission during wastewater nitrification (◆: 10 mL/min aeration rate; ■: 20 mL/min aeration rate).

accelerates nitrite accumulation by selectively inhibiting nitrite oxidizers [21]. Wastewater nitrification with enriched nitrifying bacteria indicates that $N_2O$ emission increase with $NO_2^-$-N concentration which is in agreement with previous study with activated sludge [6]. From the above results, it can be thought that nitrification rate (specifically ammonium oxidation rate) accelerates $N_2O$ emission more than by the single operational factors like aeration rate and the concentration of ammonium and nitrite because nitrite accumulation is caused by combined effects of ammonium and aeration. Further research is needed to clearly and quantitatively identify the effects of operational parameters of wastewater treatment plant for $N_2O$ emission during nitrification.

## 4. Conclusions

$N_2O$ emission during wastewater nitrification was investigated using autotrophic synthetic wastewater with enriched nitrifying sludge. Results showed that $N_2O$ emission largely depends upon aeration rate, ammonium and nitrite concentration during nitrification. Higher aeration rate and ammonium concentration increased $N_2O$ emission and the yield (kg $N_2O$-N per kg processed $NH_4^+$-N). Nitrite also accelerated $N_2O$ emission. In order to mitigate $N_2O$ emission during wastewater nitrification, appropriate aeration rate which could completely nitrify ammonium and maintain low nitrite accumulating process conditions are required.

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