Title: N2O accumulation from denitrification under different temperatures (Main article)

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Citation: Poh, L. S., Jiang, X., Zhang, Z., Liu, Y., Ng, W. J., & Zhou, Y. N2O accumulation from denitrification under different temperatures. Applied Microbiology and Biotechnology in press.

Date: 2015

URL: http://hdl.handle.net/10220/38691

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N$_2$O accumulation from denitrification under different temperatures

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Abstract

The effects of temperature on nitrous oxide (N$_2$O) accumulation during denitrification and denitritation were investigated. Batch experiments were performed to measure N$_2$O accumulation at 25 and 35 °C. More N$_2$O accumulation was observed during denitritation at the higher temperature as compared with full denitrification and low temperature tests. The highest
Nitrite concentration tested in this study (25 mg/L NO$_2^-$ N and pH 8.0) did not show inhibitory effect on N$_2$O reduction. It was found that the major cause of more N$_2$O accumulation during denitrification at higher temperature was due to higher N$_2$O production rate and lower N$_2$O solubility. Specific nitrate, nitrite, and N$_2$O reduction rates increased 62, 61, and 41 %, respectively, when temperature rose from 25 to 35 °C. The decrease of N$_2$O solubility in mixed liquor at 35 °C (when compared to 25 °C) resulted in faster diffusing rate of N$_2$O from liquid to gas phase. It was also more difficult for gas phase N$_2$O to be re-dissolved. The diffused N$_2$O was then accumulated in the headspace, which was not available for denitrification by denitrifiers. The results of this study suggest higher temperature may worsen N$_2$O emission from wastewater treatment plants (WWTPs).

**Keywords**
- Nitrous oxide (N$_2$O)
- Denitrification
- Temperature
- Greenhouse gas
- Wastewater treatment

**Introduction**

In the tropical regions, operating temperatures of wastewater treatment plants (WWTPs) are often 30 °C and above (Metcalf et al & Eddy, 2013). Temperature of wastewater is expected to gradually rise due to global warming.

Denitrification is a part of the nitrogen removal process. In this process, nitrate (NO$_3^-$) is sequentially reduced to nitrite (NO$_2^-$), nitric oxide (NO), nitrous oxide (N$_2$O), and nitrogen gas (N$_2$) by heterotrophic denitrifiers using organic carbon as electron donor (Metcalf et al & Eddy, 2013). As denitrification is a biochemical process, the reaction rate of each step is expected to change with rising temperature. However, the changes may not be proportional for each reduction step. Hence, accumulation of intermediates may occur due to possible “imbalanced” reduction rates.

N$_2$O accumulation and emission during the nitrogen removal process is a concern because it is a greenhouse gas (GHG) with a global warming potential
which is more than 300 times that of CO₂ (IPCC 2001). Therefore, the emission of N₂O must be minimized from wastewater treatment process. Denitrification has been reported to be a N₂O emission source (Ahn et al. 2010; Kampschreur et al. 2009). Competition for organic carbon (electron donor) and inhibition of N₂O reductase at high nitrite concentrations have been regarded as the causes of N₂O accumulation during denitrification (Ahn et al. 2010; Kampschreur et al. 2009; Pan et al. 2013a; Zhou et al. 2008). It is, however, currently unclear how temperature affects N₂O production, reduction, and accumulation during denitrification.

Most studies on N₂O emission from denitrification had been conducted at indoor room temperature of 21 to 25 °C (Baytshtok et al. 2009; Hu et al. 2013; Li et al. 2013; Lu and Chandran 2010; Pan et al. 2012, 2013a, b). More N₂O emission from an activated sludge process had once been reported at higher temperature (Gejlsbjerg et al. 1998), but it was not clear if the higher emission was due to temperature change or oxygen inhibition of N₂O reductase.

In this study, it is hypothesized that reaction kinetics and N₂O solubility may play significant roles in N₂O accumulation during denitrification at different temperatures. Biological denitrification rates are expected to increase when temperature rises. On the other hand, N₂O solubility in pure water decreases by 23 % when temperature increase from 25 to 35 °C (Weiss and Price 1980). N₂O solubility is an important factor affecting N₂O accumulation and emission because only dissolved N₂O can be degraded by N₂O reductase (Zumft 1997). A lower N₂O solubility at higher temperature will result in more N₂O escaping into the atmosphere before denitrification can be completed. The interactions between these factors are expected to influence N₂O accumulation rates at different temperatures. In this study, the effects of temperature on N₂O accumulation during denitrification were studied using three types of dissolved nitrogen oxides (nitrate, nitrite, and N₂O) as primary electrons acceptors. Specific reduction rates of these nitrogen oxides at two different temperatures (25 and 35 °C) were compared. The possible inhibitory effects of nitrite on N₂O reduction were also investigated. Temperature effects on N₂O distribution between liquid and gas phase in a closed reactor were also studied using computer simulation.

Materials and methods

Sludge source
The sludge used in this study was collected from a local municipal WWTP. Operating temperature of this WWTP varies from 24 to 33 °C, depending on day and night temperature variation, with an average temperature of 30.6 °C. The modified Ludzack–Ettinger (MLE) process is operated for nitrogen removal at this plant (Metcalf et al. & Eddy. 2013). Operating conditions of the plant and wastewater characteristics are summarized in Table 1. Prior to sludge collection, the performance of the plant was stable.

Table 1
Characteristics of MLE process at the WWTP

<table>
<thead>
<tr>
<th></th>
<th>Anoxic tank</th>
<th>Aerobic tank</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature (°C)</td>
<td>30.6</td>
<td>30.8</td>
</tr>
<tr>
<td>pH</td>
<td>7.5</td>
<td>6.8</td>
</tr>
<tr>
<td>DO (mg/L)</td>
<td>0.02</td>
<td>0.5</td>
</tr>
<tr>
<td>Influent</td>
<td>Effluent</td>
<td></td>
</tr>
<tr>
<td>COD (mg/L)</td>
<td>520</td>
<td>25</td>
</tr>
<tr>
<td>NH$_4^+$ (mg/L NH$_4^+$-N)</td>
<td>43</td>
<td>0.5</td>
</tr>
<tr>
<td>NO$_3^-$ (mg/L NO$_3^-$-N)</td>
<td>Not detected</td>
<td>16</td>
</tr>
</tbody>
</table>

The sludge was washed with a mineral solution twice to remove dissolved organics and nitrogen compounds. The mineral solution was formulated to be similar to domestic wastewater with trace organic matter and nitrogen compounds. It was made by adding to tap water the following compounds (mg/L): CaCl$_2$·2H$_2$O (47), MgSO$_4$·7H$_2$O (27), KH$_2$PO$_4$ (16), FeCl$_3$·6H$_2$O (0.6), H$_3$BO$_3$ (0.06), CuSO$_4$·5H$_2$O (0.012), KI (0.072), MnCl$_2$·4H$_2$O (0.048), Na$_2$MoO$_4$·2H$_2$O (0.048), CoCl$_2$·6H$_2$O (0.06), EDTA-Na·2H$_2$O (5.1), bactopeptone (3.5), and yeast extract (3.5). The biomass was then diluted to approximately 2500 mg/L volatile suspended solids (VSS) with the mineral solution and prepared for the batch experiments. Washed and diluted sludge was acclimatized to required temperature in a water bath for at least 2 h before the start of each experiment to minimize the effects of temperature shock.

Batch experiments

Batch experiments were carried out to determine the specific reduction rates
of three types of dissolved nitrogen oxides (NO$_3^-$, NO$_2^-$, and N$_2$O) at two temperatures (25 and 35 °C). The temperatures at the two extreme ends of the temperature range of the WWTP were selected to better illustrate the effects of temperature on N$_2$O accumulation. The effect of nitrite on N$_2$O accumulation at different temperatures was studied with two nitrite concentrations (15 and 25 mg/L NO$_2^-$-N). Batch experiments were repeated with and without headspace to investigate the effect of N$_2$O solubility on N$_2$O emission at different temperatures. In total, 14 sets of batch experiments were performed in this study (Table 2).

**Table 2**

Summary of all batch experiments

<table>
<thead>
<tr>
<th>Set</th>
<th>Temperature (°C)</th>
<th>Primary NO$_x$ concentration (mg/L - N)</th>
<th>Headspace volume (L)</th>
<th>Liquid volume (L)</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>25</td>
<td>NO$_3^-$, 25</td>
<td>0.75</td>
<td>1.5</td>
<td>NO$_3^-$ with headspace</td>
</tr>
<tr>
<td>2</td>
<td>35</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>25</td>
<td>NO$_2^-$, 25</td>
<td>0.75</td>
<td>1.5</td>
<td>NO$_2^-$ with headspace</td>
</tr>
<tr>
<td>4</td>
<td>35</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>25</td>
<td>NO$_2^-$, 25</td>
<td>0</td>
<td>2.25</td>
<td>NO$_2^-$ without headspace</td>
</tr>
<tr>
<td>6</td>
<td>35</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>25</td>
<td>NO$_2^-$, 15</td>
<td>0.75</td>
<td>1.5</td>
<td>Low NO$_2^-$ with headspace</td>
</tr>
<tr>
<td>8</td>
<td>35</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>25</td>
<td>NO$_2^-$, 15</td>
<td>0</td>
<td>2.25</td>
<td>Low NO$_2^-$ without headspace</td>
</tr>
<tr>
<td>10</td>
<td>35</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>25</td>
<td>N$_2$O, 25</td>
<td>0</td>
<td>2.25</td>
<td>N$_2$O without headspace</td>
</tr>
<tr>
<td>12</td>
<td>35</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>25</td>
<td>N$_2$O, 25</td>
<td>0.75</td>
<td>1.5</td>
<td>N$_2$O with headspace</td>
</tr>
</tbody>
</table>
A 2.25-L gas tight, water-jacketed reactor was used for all experiments. Temperature of the mixed liquor was controlled at 25.0 or 35.0 ± 0.5 °C by continuously circulating chilled or warm water through the water jacket from a water bath (PolyScience, IL, USA). The mixed liquor was continuously stirred with a magnetic stirrer at 300 RPM. For experiments with headspace, the liquid volume was 1.5 L and headspace volume was 0.75 L. The liquid volume was 2.25 L for experiments without headspace.

Before each experiment, the biomass was sparged with N\(_2\) gas for 5 min to remove residual oxygen. The reactor was sealed immediately after sparging. Sodium acetate was used as the dissolved organic carbon source for denitrification in all sets. Organic carbon concentration, in terms of chemical oxygen demand (COD), was controlled at a COD/N ratio of 10 (250 or 150 mg/L) at the start of each set. The high COD/N ratio was selected to avoid electron donor limitation for denitrification. pH of the mixed liquor was maintained at 8.0 ± 0.1 by manual dosing of 0.25 M HCl or NaOH. Every batch experiment was carried out in duplicate. The details of all batch experiments are summarized in Table 2.

### Nitrate as primary electron acceptor (set 1–2)
Sodium nitrate (25 mg/L NO\(_3^-\)\(_N\)) was used in experiment sets 1 and 2 with headspace. Each test lasted for 90 min; gas and liquid samples were taken at 15-min intervals. The specific reduction rates of nitrate, intermediate nitrite, and N\(_2\)O at 25 and 35 °C were determined. N\(_2\)O accumulation in dissolved and gas phase were also monitored.

### Nitrite as primary electron acceptor (set 3–10)
Sodium nitrite was used in sets 3 to 10. In these eight sets of experiments, the specific reduction rates of nitrite and N\(_2\)O at different temperatures were determined. The effects of temperature, nitrite, and N\(_2\)O solubility on gaseous and dissolved N\(_2\)O accumulation during denitrification (nitrite reduction) were also analyzed. The initial concentration of sodium nitrite was 25 mg/L NO\(_2^-\)\(_N\) for sets 3 to 6, while that was 15 mg/L NO\(_2^-\)\(_N\) in sets 7 to 10. The
experiments lasted for 90 min and gas and liquid samples were taken at 15-min intervals for sets 3 to 6. In sets 7 to 10 where lower nitrite was applied, the experiments lasted for 60 min with samples taken at 10-min intervals. Sets 3, 4, 7, and 8 were operated with headspace and the rest were without headspace.

**N₂O as primary electron acceptor (set 11–14)**

Saturated N₂O solution was used as the source of dissolved N₂O for sets 11 to 14. In these sets of experiment, the specific N₂O reduction rates under different temperatures were determined. As a part of the study of the effects of N₂O solubility on specific N₂O reduction rates at different temperatures, headspace and dissolved N₂O concentration data from sets 11 to 14 were used in computer simulation to evaluate N₂O distribution in a closed reactor system. Saturated N₂O solution was prepared by sparging deionized water with pure N₂O gas for 5 min (Zhou et al. 2008). When the saturated N₂O solution was ready, a measured volume was injected into the mixed liquor for a final dissolved concentration of 25 mg/L N₂O-N. Sets 11 and 12 were performed without headspace, while sets 13 and 14 had headspace. The 25 °C experiments (sets 11 and 13) lasted for 90 min with gas sampling (set 13) at 15-min intervals. The 35 °C sets (12 and 14) were operated for 60 min with gas sampling (set 14) at 10-min intervals.

**Sampling and analytical methods**

Liquid samples (8 mL) were collected at regular intervals as described above (except for sets 11–14). Collected mixed liquor samples were filtered through 0.45-μm disposable syringe filters and stored at 4 °C before measurement. In order to balance the headspace gas pressure, equal volume of washed biomass was replaced into the reactor immediately after each liquid sample was taken. NO₃⁻ and NO₂⁻ concentrations were measured according to methods 4500-NO₃⁻ B and 4500-NO₂⁻ described in standard methods (APHA 2012). Measurements of nitrogen oxide compounds are expressed as mass-nitrogen (NOₓ-N) for easier comparison. Soluble COD concentration was measured using low range COD measurement kit (HACH, Loveland, CO, USA). Soluble COD concentrations decreased proportionally according to denitrification rates in all experiments of this study (data not shown).

Dissolved N₂O concentration in the mixed liquor was continuously read and logged with an online N₂O sensor at 1 s intervals (N2O-R, Unisense A/S,
Aarhus, Denmark). The online N\textsubscript{2}O sensor was calibrated strictly according to the manufacturer’s instructions. Gas phase samples (0.2 mL) were collected from the headspace before each liquid sampling for N\textsubscript{2}O measurements. Gaseous N\textsubscript{2}O concentration was measured using GC-\textmu ECD (7890A GC, Agilent, Santa Clara, CA, USA) equipped with a capillary column (GS-Gaspro, Agilent J&W, Santa Clara, CA, USA).

Gas pressure in the headspace was measured with a handheld manometer before each gas sampling. Gas pressure and volume were used for calculation of gaseous N\textsubscript{2}O quantity. The amount of N\textsubscript{2}O in the headspace was calculated with the ideal gas law equation.

\[ PV = nRT \]

**AQ3**

where \( P \) is the measured absolute gas pressure in the headspace (Pa), \( V \) is measured volume of N\textsubscript{2}O in the headspace (m\textsuperscript{3}), \( n \) is amount of N\textsubscript{2}O (moles), \( R \) is the universal gas constant (8.314 J/mol. K), and \( T \) is the absolute temperature (298 or 308 K). The amount of N\textsubscript{2}O in moles was then multiplied by 0.028 to convert to mass in terms of nitrogen (mg N).

Mixed liquor samples were collected in triplicates before and at the end of all batch experiment for mixed liquor volatile suspended solids (MLVSS) measurements according to Standard Methods 2540E (APHA 2012).

**Calculations**

**Specific reduction rates of nitrogen oxides**

The specific nitrogen oxide reduction rate of primary nitrogen oxides (nitrate in sets 1 and 2, nitrite in sets 3 to 10, N\textsubscript{2}O in sets 11 to 14) was calculated by linear regression of the measured amount of nitrate, nitrite, and N\textsubscript{2}O and then dividing the gradient from linear regression by the measured amount of biomass.

The apparent reduction rates of intermediate nitrogen oxides (NO\textsubscript{2}\textsuperscript{−} and N\textsubscript{2}O) were calculated by regression of profiles of the intermediate nitrogen oxides. The real reduction rates of intermediate nitrogen oxides were then calculated by adding the apparent reduction rates to the reduction rates of nitrogen oxide immediately upstream (Pan et al. 2013a).

\[ \tau_{\text{NO}_3} = \tau_{\text{NO}_3,a} \]
where $r_{NO_2, a}$ are apparent reduction rates and $r_{NO_2}$ are real reduction rates.

NO was assumed to be quickly broken down after formation and of negligible quantity (Richardson et al. 2009; Schreiber et al. 2009; Zeng et al. 2003).

Simulation of $N_2O$ distribution between gas and liquid phase

The model was developed to simulate the $N_2O$ degradation and transfer between gas–liquid phases under anoxic condition based on ASMN model (Hiatt and Grady 2008). Carbon removal and four-step denitrification involving $N_2O$ generation and degradation were incorporated in the model. Biomass decay and hydrolysis of organic nitrogen in biomass were also considered in this model. Aerobic growth and nitrification were not included.

In order to simulate the transfer of $N_2O$ between gas–liquid phases, the following formula was employed:

$$F_{N_2O} = V_{N_2O} \left( \frac{P_{N_2O} * 28}{K'_H} - C_{N_2O} \right)$$

where $F_{N_2O}$ is the $N_2O$ transfer rate between mixed liquor and headspace in the reactor (mg N/L·day), $V_{N_2O}$ is the specific mass transfer coefficient of $N_2O$ in the system (day$^{-1}$), $P_{N_2O}$ is the partial pressure of $N_2O$ in the headspace (atm), $K'_H$ is the Henry’s Law constant (atm·l/mmole) and $C_{N_2O}$ is the concentration of dissolved $N_2O$ in the mixed liquor (mg N/L).

The mass transfer coefficient and Henry’s Law constant of $N_2O$ in the system were calibrated with the measured concentrations of $N_2O$ in headspace and mixed liquor (sets 13 and 14) using the Monte Carlo method in Matlab®. The flux of $N_2O$ between liquid and gas phase was then calculated with the following equation:

$$N_2O_{\text{flux}} = F_{N_2O} \times V$$

where $N_2O_{\text{flux}}$ is the flux of $N_2O$ between gas–liquid phases (mg N/d), $V$ is the
volume of liquid phase (l). A positive flux indicates N\textsubscript{2}O transfer from liquid to gas phase and conversely, dissolution of N\textsubscript{2}O from the headspace into the liquid phase is indicated by a negative flux. When the flux is 0, it indicates that N\textsubscript{2}O in the gas and liquid phases are in equilibrium.

The N\textsubscript{2}O specific mass transfer coefficient is influenced by factors such as the gas–liquid interfacial area, liquid depth, agitation speed, and temperature, of which gas–liquid interfacial area, liquid depth, and agitation speed were kept identical in all batch experiments. Henry’s Law constant is dependent on temperature and concentration of dissolved solids in the liquid phase. Contributions from these factors were consolidated into these two terms (\(V_{\text{N}_2\text{O}}\) and \(K'_H\)) for simplicity.

**Results**

**Nitrate as primary electron acceptor**

Denitrification started immediately after the addition of sodium nitrate and sodium acetate at both temperatures (25 and 35 °C). Nitrate decreased in linear trends (Fig. 1). The specific denitrification rates for nitrate were 5.8 and 9.4 mg N/g VSS·h at 25 and 35 °C respectively, which was a 62 % increase in specific nitrate reduction rates from 25 to 35 °C.

**Fig. 1**

Denitrification of 25 mg/L NO\textsubscript{3}--N; **a** set 1, 25 °C; **b** dissolved and gas phase N\textsubscript{2}O accumulation of set 1; **c** set 2, 35 °C; **d** dissolved and gas phase N\textsubscript{2}O accumulation of set 2
Nitrite was accumulated under both temperature conditions. At 25 °C, nitrite was accumulated in a gradual and linear trend across the 90-min test period. At 35 °C, nitrite was accumulated in the first 60 min before decreasing as nitrate was depleted in the final 30 min. The specific reduction rates for intermediate nitrite were 3.8 and 6.8 mg N/g VSS·h for 25 and 35 °C, respectively, increasing by 79 % with the increase in temperature.

$\text{N}_2\text{O}$ accumulation displayed different trends at the two temperatures. At 25 °C, dissolved $\text{N}_2\text{O}$ was below 0.10 mg $\text{N}_2\text{O}$-N throughout the experiment. $\text{N}_2\text{O}$ in the gas phase was below detection limits in the first 60 min and increased to 0.10 and 0.06 mg $\text{N}_2\text{O}$-N at the 75th and 90th minute, respectively. At 35 °C, most $\text{N}_2\text{O}$ accumulation occurred in the gas phase. The highest amount of total $\text{N}_2\text{O}$ accumulation (gaseous and dissolved) was recorded at the 90th minute at 2.7 mg $\text{N}_2\text{O}$-N, of which 1.9 mg $\text{N}_2\text{O}$-N was in the gas phase. The specific reduction rates for intermediate $\text{N}_2\text{O}$ were 3.8 and 6.3 mg N/g VSS·h at 25 and 35 °C, respectively, which increased by 66 %.

**Nitrite as primary electron acceptor**

In denitrification experiments, sets 3 to 10, nitrite decreased in linear patterns at both temperatures. Examples of the linear nitrite reduction patterns (sets 3 and 4) are illustrated in Fig. 2. Specific nitrite reduction rate increased 61 % between 25 and 35 °C when initial nitrite concentration was 25 mg/L $\text{NO}_2^-$ N.
The specific nitrite and N\textsubscript{2}O reduction rates of sets 3 to 10 are presented in Table 3. At 35 °C, specific nitrite reduction rates were 55 to 72 % faster than those at 25 °C. In sets with higher initial nitrite concentrations (sets 3 to 6), specific nitrite and N\textsubscript{2}O reduction rates were higher, ranging from 4.3 to 7.9 mg NO\textsubscript{2} \textsuperscript{-} N/g VSS·h and 4.3 to 7.2 mg N\textsubscript{2}O-N/g VSS·h as compared to the specific nitrite and N\textsubscript{2}O reductions rates in sets 7 to 10 with lower initial nitrite concentrations at 4.0–7.0 mg NO\textsubscript{2} \textsuperscript{-} N/g VSS·h and 3.7–6.3 mg N\textsubscript{2}O-N/g VSS·h, respectively. Specific nitrite reduction rate is dependent on the initial nitrite concentration.

**Fig. 2**

Denitrification of 25 mg/L NO\textsubscript{2} \textsuperscript{-} N; a set 3, 25 °C; b set 4, 35 °C

**Table 3**

Specific nitrite reduction rates (rNO\textsubscript{2} \textsuperscript{-}), specific N\textsubscript{2}O reduction rates (rN\textsubscript{2}O), and total N to 10

<table>
<thead>
<tr>
<th>Set</th>
<th>Temperature (°C)</th>
<th>Initial nitrite concentration (mg/L NO\textsubscript{2} N)</th>
<th>Headspace</th>
<th>rNO\textsubscript{2} \textsuperscript{-} (mg N/g VSS·h)</th>
<th>Increase (%)</th>
<th>rN\textsubscript{2}O (mg N/g VSS·h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>25</td>
<td>25</td>
<td>Yes</td>
<td>4.9</td>
<td>61</td>
<td>4.7</td>
</tr>
<tr>
<td>4</td>
<td>35</td>
<td>25</td>
<td>Yes</td>
<td>7.9</td>
<td>61</td>
<td>6.4</td>
</tr>
<tr>
<td>5</td>
<td>25</td>
<td>25</td>
<td>No</td>
<td>4.3</td>
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<td>8</td>
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<td>Yes</td>
<td>7.0</td>
<td>75</td>
<td>4.6</td>
</tr>
</tbody>
</table>
Specific N\textsubscript{2}O reduction rates between 25 and 35 °C increased by a smaller magnitude when compared to specific nitrite reduction rates. These different increments of specific nitrite and N\textsubscript{2}O reduction rates were observed in both denitritation experiments with and without headspace. In sets with headspace (sets 3, 4, 7, and 8), increment of specific nitrite reduction rate was two to three times of the increment of specific N\textsubscript{2}O reduction rate when temperature increased from 25 to 35 °C. However, in experiments without headspace (sets 5, 6, 9, and 10), increment of specific nitrite and N\textsubscript{2}O reduction rate differed less significantly when temperature was raised (Table 3). Nitrite and N\textsubscript{2}O reduction occurred at identical rates in experiments without headspace at 25 °C (4.3 mg N/g VSS·h for both specific nitrite and N\textsubscript{2}O reduction rates in set 5 and 4.2 mg N/g VSS·h for both specific nitrite and N\textsubscript{2}O reduction rates in set 9). In sets 6 and 10 (35 °C without headspace), N\textsubscript{2}O reduction occurred at slightly slower rates (7.2 and 6.3 mg N\textsubscript{2}O-N/g VSS·h respectively) compared to nitrite reduction (7.4 and 6.5 mg NO\textsubscript{2} -N/g VSS·h respectively), leading to accumulations of dissolved N\textsubscript{2}O (Fig. 3 d, h).

**Fig. 3**

N\textsubscript{2}O accumulation during denitritation sets 3 to 10; a 25 mg/L NO\textsubscript{2} -N, 25 °C, with headspace; b 25 mg/L NO\textsubscript{2} -N, 35 °C, with headspace; c 25 mg/L NO\textsubscript{2} -N, 25 °C, no headspace; d 25 mg/L NO\textsubscript{2} -N, 35 °C, no headspace; e 15 mg/L NO\textsubscript{2} -N, 25 °C, with headspace; f 15 mg/L NO\textsubscript{2} -N, 35 °C, with headspace; g 15 mg/L NO\textsubscript{2} -N, 25 °C, no headspace; h 15 mg/L NO\textsubscript{2} -N, 35 °C, no headspace
\( \text{N}_2\text{O} \) accumulation was observed in all denitrification experiments and more \( \text{N}_2\text{O} \) accumulation occurred at 35 °C compared to 25 °C. In sets with headspace, gas phase \( \text{N}_2\text{O} \) made up most of the accumulation, consisting of...
33–98 % of total N$_2$O accumulated (Fig. 3). The accumulation of N$_2$O in headspace is more severe when denitritation was performed at 35 °C compared to 25 °C (Fig. 3a, b, e, f).
Figure 4 presents the net flux of N$_2$O between liquid and gas phases (sets 3 and 4) from the simulation study. At 35 °C, N$_2$O flux peaked mildly at around 0.2 mg N/min while the maximum N$_2$O flux at 25 °C was close to 0.02 mg N/min. The flux then decreased as dissolved N$_2$O was removed by biological reduction in the liquid phase (Fig. 4). The decrement indicated less N$_2$O was diffusing to gas phase until the flux reached the equilibrium point (flux = 0 mg N/min). The time taken for N$_2$O flux reached the equilibrium is around 44 and 54 min when temperature was at 25 and 35 °C, respectively. After the equilibrium point, flux remained in the negative zone indicating that N$_2$O in the headspace was re-dissolving into the mixed liquor. Removal of N$_2$O by biological denitrification and diffusion from liquid phase were calculated based on mass balance. Upon reaching the equilibrium point, 15.6 and 28.0 mg of N$_2$O-N were derived from upstream NO$_2^-$ reduction at 25 and 35 °C. From the N$_2$O produced, 93 % (15.0 mg N$_2$O-N) was removed from liquid phase through biological reduction at 25 °C. At higher temperature (35 °C), only 73 % (20.5 mg N$_2$O-N) of the N$_2$O produced was biologically denitrified.

**Fig. 4**

Net N$_2$O transfer rate in sets 3 (25 °C, with headspace) and 4 (35 °C, with headspace) with NO$_2^-$ as electron acceptor

![Net N$_2$O transfer rate graph](image)

Nitrous oxide as primary electron acceptor

Four sets (11 to 14) of experiments were conducted using dissolved N$_2$O as
the primary electron acceptor. In sets 11 (25 °C) and 12 (35 °C) without headspace, dissolved N\textsubscript{2}O concentration expressed a linearly decreasing pattern (Fig. 5a, b). Specific N\textsubscript{2}O reduction rate increased 41% from 8.0 to 11.3 mg N/g VSS·h from 25 to 35 °C. In sets 13 (25 °C) and 14 (35 °C) with headspace, dissolved N\textsubscript{2}O concentrations decreased in a non-linear pattern (Fig. 5c, d). Specific N\textsubscript{2}O reduction rates for total N\textsubscript{2}O (gaseous and dissolved) were 7.9 and 9.4 mg N/g VSS·h for 25 and 35 °C, respectively, increasing by 19%.

**Fig. 5**

N\textsubscript{2}O reduction sets 11 to 14; a 25 °C, no headspace; b 35 °C, no headspace; c 25 °C, with headspace; d 35 °C, with headspace

In the initial period of sets 13 and 14, dissolved N\textsubscript{2}O depleted quickly through both biological reduction and conversion into the gaseous form, the amount of dissolved N\textsubscript{2}O decreased with a steeper gradient. Gaseous N\textsubscript{2}O built up rapidly in the headspace after saturated N\textsubscript{2}O solution was injected into the mixed liquor. In later period of the experiments when dissolved N\textsubscript{2}O concentrations were lower, gaseous N\textsubscript{2}O re-dissolved into the mixed liquor as biological reduction continued, resulting in a more gradual dissolved N\textsubscript{2}O
concentration gradient towards the end of the experiment. The interference of 
N₂O phase exchanges resulted in specific N₂O reduction rates falling by 1.3 %
at 25 °C and 20 % at 35 °C when compared with sets without headspace (Fig. 5).

Henry’s Law constant (K’₇) and specific mass transfer coefficient (V_N₂O) of 
N₂O at 25 and 35 °C were calibrated using specific N₂O reduction rates from 
sets 11 and 12 and N₂O concentration data from sets 13 and 14. The 
calibration results were summarized in Table 4.

**Table 4**

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Henry’s Law constant, K’₇ (atm·l/mmole)</th>
<th>Specific mass transfer coefficient, V_N₂O (day⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>25</td>
<td>0.061</td>
<td>0.051</td>
</tr>
<tr>
<td>35</td>
<td>0.087</td>
<td>0.420</td>
</tr>
</tbody>
</table>

Higher specific mass transfer coefficient in the system indicated a more active 
gas–liquid exchange at higher temperature. The specific mass transfer 
coefficient was eight times at 35 °C of the value at 25 °C (0.42 vs. 
0.051 day⁻¹).

The flux of N₂O from liquid phase to headspace in sets 13 and 14 were 
simulated using the calibrated values and concentration data and illustrated in 
Fig. 6a. N₂O flux from liquid to headspace peaked rapidly upon the 
introduction of N₂O into the mixed liquor. The net N₂O transfer rate 
decreased at a slower pace at 35 °C (Fig. 6a). At 25 °C (set 13), the net N₂O flux reached equilibrium after 20 min while it reached equilibrium at the 26th 
minute when temperature was 35 °C (set 14). The cumulative amount of N₂O 
diffused from liquid to gas phase is presented in Fig. 6b. In the first 20 min, 
when N₂O mainly diffused from liquid to gas phase, 27 % more of N₂O 
diffused to headspace at 35 °C compared with the amount diffused at 25 °C.

More N₂O was thus emitted into headspace at higher temperature (35 °C). 
Upon reaching the equilibrium point at 25 °C, the amount of N₂O degraded by 
denitrifiers was 8.3 mg N₂O-N whereas 9.3 mg N₂O-N diffused to headspace. 
The amount of N₂O diffused to gas phase was 12.1 mg N₂O-N and 12 mg 
N₂O-N was removed through denitrification at 35 °C.

**Fig. 6**
a Flux of N$_2$O from liquid phase to headspace in set 13, 25 °C, and set 14, 35 °C. b Cumulative N$_2$O transfer from liquid to gas phase in set 13, 25 °C, and set 14, 35 °C

AQ4

Discussion

The results indicated denitrification rates, nitrite concentrations, headspace, and N$_2$O solubility at different temperatures had varied effects on N$_2$O accumulation during denitrification. The contribution of each mechanism on N$_2$O accumulation is discussed in this section.

Imbalanced denitrification rates increment and electron competition

The specific nitrate, nitrite, and N$_2$O reduction rates did not increase uniformly when temperature increased from 25 to 35 °C (Table 5). Data
collected in this study is in agreement with previous reports that denitrification rates increase with rising temperature (Dawson and Murphy 1972; Lewandoswki 1982). However, the extents of increase observed in this study are lower than that of earlier studies.

**Table 5**

Specific reduction rates of sets 1, 2, 3, 4, 11, and 12 (initial dissolved NO$_x$ = 25 mg/L NO$_x$-N).

<table>
<thead>
<tr>
<th>Set</th>
<th>Temperature</th>
<th>Nitrate</th>
<th>Nitrite</th>
<th>N$_2$O</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>25 °C</td>
<td>5.8</td>
<td>3.8</td>
<td>3.8</td>
<td>25 mg/L NO$_3$-N with headspace</td>
</tr>
<tr>
<td>2</td>
<td>35 °C</td>
<td>9.4</td>
<td>6.8</td>
<td>6.3</td>
<td></td>
</tr>
<tr>
<td>% increase</td>
<td>62</td>
<td>79</td>
<td>66</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>25 °C</td>
<td>4.9</td>
<td>4.7</td>
<td></td>
<td>25 mg/L NO$_2$-N with headspace</td>
</tr>
<tr>
<td>4</td>
<td>35 °C</td>
<td>7.9</td>
<td>6.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>% increase</td>
<td>61</td>
<td>36</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>25 °C</td>
<td></td>
<td>8.0</td>
<td></td>
<td>25 mg/L N$_2$O-N without headspace</td>
</tr>
<tr>
<td>12</td>
<td>35 °C</td>
<td></td>
<td>11.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>% increase</td>
<td></td>
<td>41</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The differences between upstream and downstream specific reduction rates between nitrate, nitrite, and N$_2$O lead to accumulation of intermediate nitrite and N$_2$O during denitrification and denitritation (sets 1 to 10). Accumulation of intermediate NO$_x$ was higher when there were larger differences in specific reduction rates at the higher temperature (35 °C).

Intracellular competition for electrons is a possible cause for the lower specific N$_2$O reduction rate during denitrification. It was reported that nitrate reduction receives electrons from the ubiquinone/ubiquinol (UQ/UQH$_2$) pool, while nitrite, nitric oxide, and nitrous oxide reductions receive electrons from...
the cytochrome c550 pool which in turn obtains electrons from the UQ/UQH₂ pool (Richardson et al. 2009). From this configuration of intracellular electrons distribution, it can be seen that nitrate reductase receives its electrons nearer to the electron source (UQ/UQH₂ pool), without direct competition from other nitrogen oxide reductases. While nitrite, NO, and N₂O reductases receive their electrons from a shared source after an additional step (cytochrome c550 pool), the competition for electrons between nitrite, NO, and N₂O reductases is therefore higher than that for nitrate reductase. It was reported that electrons competition occurred despite carbon abundant conditions in a methanol fed denitrification process at 23 °C (Pan et al. 2013a). Ribera-Guardia et al. (2014) also demonstrated that a preference in electron flow, which gives priority to nitrate/nitrite reduction in their work. The authors suggested that N₂O reduction rate could be affected more significantly compared to nitrate and nitrite reduction when electron competition occurred. The preference of electron flow would also be the reason, which led to the difference of denitrification rate increment. Higher specific reduction rates at warmer temperature (35 °C), shown in this study, might induce a tighter competition for electrons, leading to higher accumulation of N₂O. Further investigation is required to understand the true cause of both higher nitrite and N₂O accumulation at high temperature.

Nitrite inhibition

N₂O accumulation was previously linked to high nitrite concentration during biological wastewater treatment. It was later revealed that free nitrous acid (FNA) was the true inhibitor of N₂O reductase (Zhou et al. 2011). In this study, the highest nitrite concentration was 25 mg/L NO₂⁻-N (sets 3 and 4) and FNA concentrations at pH 8.0 were 0.56 and 0.44 µg/L N₂O-N at 25 and 35 °C, respectively. According to the equilibrium equation, a higher temperature will result in lower FNA concentration (Anthonisen et al. 1976). These FNA concentrations were much lower than 10 µg/L N, which was the threshold found to cause inhibition of anoxic metabolism (Zhou et al. 2010).

Comparing the specific N₂O reduction rates in sets 3 (25 mg/L NO₂⁻-N, 25 °C) and 7 (15 mg/L NO₂⁻-N, 25 °C) (Table 2 and Fig. 3), it can be observed that N₂O reduction occurred at rather similar rates. Higher initial nitrite concentration did not cause lower specific N₂O reduction rate. Same observations were also found in experiments at 35 °C (Set 4 and 8). Based on the similarities in specific N₂O reduction rates, it can be concluded that nitrite
up to 25 mg/L NO₂⁻-N at pH 8.0 did not significantly affect N₂O reduction during denitrification between 25 and 35 °C. This suggested that inhibitory effect of FNA was not the major cause of N₂O accumulation in this study.

**Headspace and effects of N₂O solubility**

N₂O accumulation was recorded in all denitrification experiments (sets 3 to 10, Fig. 3). The highest N₂O accumulation occurred in sets with headspace and warm temperature (sets 4 and 8, Fig 3b, f). In these sets, most of the accumulated N₂O occurred in the headspace. At 35 °C, the solubility of N₂O in mixed liquor is 30 % lower than 25 °C (Table 5). The presence of headspaces in sets 4 and 8 allowed N₂O to diffuse and accumulate in the gas phase due to the lower solubility at warmer temperature. The accumulation of more N₂O in gas phase resulted in less-dissolved N₂O available for denitrification. In sets without headspace (sets 5, 6, 9, and 10), N₂O produced from nitrite reduction were unable to diffuse into the gas phase. Dissolved N₂O was quickly reduced by denitrifiers resulting in higher specific N₂O reduction rates that were closer to its production rates and hence, less N₂O accumulations.

The calibrated Henry’s constants of N₂O developed in this study (Table 4) were in similar range to the Henry’s constant of N₂O in pure water (0.04 and 0.053 atm·l/mmole at 25 and 35 °C, respectively) (Haynes 2013). The higher Henry’s constant at 35 °C reflected a lower N₂O solubility at higher temperature. N₂O solubility had been reported to decrease by 20.4 to 28.2 % when temperature increased from 25 to 35 °C (Clarke 1964; Haimour and Sandall 1984; Versteeg and Van Swaaij 1988; Yaghi and Houache 2008). The effects of solubility on N₂O accumulation could be more obvious when N₂O was used as primary electron acceptor with headspace in sets 13 and 14. The simulation results demonstrated that the lower solubility of N₂O could lead to higher mass transfer coefficient at 35 °C. The mass transfer coefficient represented how fast N₂O was transferred between liquid and gas phases. N₂O exchange between headspace and mixed liquor is expected to occur faster and be more responsive to changes in gaseous and dissolved N₂O concentration at higher temperature. As a result, cumulative N₂O diffused from liquid to headspace was higher when temperature rose. In addition, lower solubility hindered N₂O re-dissolve back to liquid phase after soluble N₂O was reduced by microorganisms. It took longer time for net N₂O transfer rate to achieve equilibrium at 35 °C. More N₂O diffused to the headspace and slower re-dissolution of N₂O caused higher accumulation of N₂O in gas phase. The
gaseous form of N\textsubscript{2}O was not available to be removed by biological denitrification even though the potential specific N\textsubscript{2}O reduction is higher at 35 °C (11.3 mg N/g VSS·h) compared with the rate at 25 °C (8.0 mg N/g VSS·h). The lower solubility at higher temperature thus resulted in more N\textsubscript{2}O emission.

Implications to denitrification process in wastewater treatment plant operation

This study described higher operating temperature may increase denitrification kinetics, which can potentially reduce capital costs of treatment systems. However, imbalanced enhancement of different reactions may also cause accumulation of intermediates. Care should be taken to ensure the proper management of these accumulated intermediate nitrogen oxides to avoid release into the environment.

More gaseous N\textsubscript{2}O accumulation occurred at higher temperature due to higher N\textsubscript{2}O production rate and lower N\textsubscript{2}O solubility. Thus, a significant amount of N\textsubscript{2}O may be emitted from the WWTP under high-temperature condition. Operators running anoxic denitrification processes at warm temperature may need to consider covering their reactors so as to encourage the re-dissolution of gaseous N\textsubscript{2}O and minimize unintentional release of N\textsubscript{2}O into the atmosphere. It is also suggested that denitrification off-gas be re-circulated to prolong the retention time of N\textsubscript{2}O in the system to enhance N\textsubscript{2}O reduction and reduce emissions. This study would also suggest warmer temperatures may worsen GHG emission from WWTPs. The case could be more severe in tropical countries. It would be interesting to investigate if the reaction kinetics can adapt to temperature changes and establish a new equilibrium following long term operation.

In summary, the impact of temperature on nitrate, nitrite, and N\textsubscript{2}O reduction was investigated in this study. It was found that denitrification rates of different nitrogen oxide compounds do not respond in similar magnitudes to temperature changes. When temperature increased from 25 to 35 °C, specific nitrate, nitrite, and N\textsubscript{2}O reduction rates were increased by 62, 61, and 41 %, respectively. The imbalanced changes in specific reduction rates resulted in more intermediate nitrogen oxides accumulations. Higher N\textsubscript{2}O production rates and lower N\textsubscript{2}O solubility were found to cause more N\textsubscript{2}O accumulation at higher temperature.

Acknowledgments
This project is supported by funding from Ministry of Environment and Water Resources, Singapore (MEWR), Environment and Water Industry Programme Office (EWI) through project “Energy +: A novel integrated concept for retrofitting and optimizing existing wastewater treatment plants into energy self-sufficiency,” project reference number 2P 10004/95.

Conflict of interest The authors declare that they have no competing interests.

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