Experimental Examination on Nitrous Oxide Accumulation during Nitrification in a Freshwater Lake

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Abstract

The $\text{N}_2\text{O}$ production during nitrification was studied in Lake Kizaki by vertical observations and two incubation experiments. The apparent $\text{N}_2\text{O}$ production ($\Delta\text{N}_2\text{O}$), the difference between observed and atmospheric-equilibrium concentration, was highly correlated with apparent oxygen consumption ($-\Delta \text{O}_2$), in situ $\text{NO}_3^-$ concentration and the amount of $\text{NH}_4^+$ decrease with time, indicating that $\text{N}_2\text{O}$ was produced by the nitrification process. In a long-term in situ incubation, simultaneous accumulations of $\text{NO}_3^-$ and $\text{N}_2\text{O}$ were observed, the amounts of which were comparable to the in situ changes, suggesting their in situ productions. Both $\text{NO}_3^-$ and $\text{N}_2\text{O}$ productions were greatly enhanced by an $\text{NH}_4^+$ enrichment after the emergence of nitrification activity.

From both vertical observations and long-term in situ incubations, the yield of $\text{N}_2\text{O}$ relative to $\text{NO}_3^-$ production was estimated to be about 0.1% during the active nitrification period. But thereafter, the obtained yields were appreciably higher. Another incubation experiment also showed that $\text{N}_2\text{O}$ production was delayed several days compared to $\text{NO}_3^-$ production, indicating that $\text{N}_2\text{O}$ production was accelerated in the late phase of nitrification. The physiological state of nitrifiers was suggested to be of some importance for such phenomena. Nitrapyrin, a specific inhibitor of nitrification, was found not to be very effective for $\text{N}_2\text{O}$ formations in contrast with $\text{NO}_3^-$ productions completely inhibited, suggesting a possibility of some $\text{N}_2\text{O}$ sources other than autotrophic nitrification.

Key words: nitrous oxide, nitrification, incubation experiments, freshwater lake

1. Introduction

The abundance of nitrous oxide ($\text{N}_2\text{O}$) in the atmosphere is thought to be significantly controlled by biological processes at the earth's surface (Crutzen, 1981). Many studies on the $\text{N}_2\text{O}$ distribution in a variety of aquatic environments have revealed that $\text{N}_2\text{O}$ concentrations are supersaturated with respect to the air. In the western Atlantic Ocean and the Caribbean Sea, Yoshinari (1976) found that the vertical distributions of $\text{N}_2\text{O}$ concentration were inversely correlated with those of dissolved oxygen concentration and that there was a linear relationship between apparent $\text{N}_2\text{O}$ production ($\Delta\text{N}_2\text{O}$) and apparent oxygen utilization (AOU). Similar dependences of $\text{N}_2\text{O}$ concentration upon oxygen concentration have also been obtained in other oceans (Elkins et al., 1978; Cohen and Gordon, 1978). On the basis of such relationships, these authors have suggested that $\text{N}_2\text{O}$ is produced through nitrification.

However, some uncertainties remain as to the role of nitrification in $\text{N}_2\text{O}$ formation in aquatic environments. AOU is merely a measure of the total biological oxygen utilization and oxidation of ammonium or organic amines accounts for only a minor part of the oxygen consumption (Pierotti and Rasmussen, 1980).
Close relationship between $\Delta N_2O$ and $NO_3^-$ concentration has never been shown. In addition, the ratio of $\Delta N_2O$ to AOU is reported to be higher in the Pacific Ocean than in the Atlantic Ocean and the active regions of $N_2$O production have been found to occur within the Pacific (Elkins et al., 1978; Cohen and Gordon, 1979; Hahn, 1981; Kaplan and Wofsy, 1985). On the other hand, Yoshida et al. (1989) have recently suggested from $^{15}N/^{14}N$ ratio of $N_2O$ in the western North Pacific that denitrification is the predominant $N_2O$ producing process, although $N_2O$ and oxygen concentrations inversely distributed, similar to other observations in oceans.

In Lake Kizaki, a Japanese freshwater lake, it is known that nitrification takes place actively in early summer (Takahashi et al., 1982; Yoshikawa et al., 1985). Yoh et al. (1988a) have observed a linear relationship between the amount of apparent $N_2O$ production ($\Delta N_2O$) and that of apparent oxygen consumption ($-\Delta O_2$) in this lake, similar to that in oceans, suggesting $N_2O$ production by nitrification. However, there appears to be a considerable difference between the nitrification in Lake Kizaki and that in the ocean, from the viewpoint of substrate concentration, pH and so on. Thus, further detailed observations of $N_2O$ concentration were made in Lake Kizaki to elucidate the role of nitrification for $N_2O$ production and its characteristics. The relationship between nitrification and $N_2O$ production was examined on the basis of the change in its vertical distribution and incubation experiments of lake water employing a nitrification inhibitor, nitrapyrin, and an $NH_4^+$ supplement.

2. Materials and methods

2-1. Sampling of water

Water samples for chemical analysis were collected with a Van-Dorn sampler. They were filtered using Whatman GF/C filter in the field laboratory and kept frozen until analysis. Samples for a long-term in situ incubation and a time course experiment were collected with a pump and a Van-Dorn sampler, respectively, after allowing some volume to overflow. During sampling, bottles were covered with black sheets to avoid direct sunlight.

2-2. Long-term in situ incubation

The aims of this experiment were to determine whether $N_2O$ is formed in lake water in the course of nitrification and is inhibited by the nitrification inhibitor, nitrapyrin. Waters were incubated in situ to provide incubation conditions analogous to those of lake.

Ground-glass-stoppered bottles of 10 liter volume filled with samples were suspended at several depths with a buoy. Samples were taken from 14, 19 and 24 m depths and incubated generally at identical depths for about three weeks with or without nitrapyrin (Dow Chemical Co., Ltd., emulsifiable concentrate, “N-serve 24E”). Nitrapyrin was used as a specific inhibitor for autotrophic nitrification at a final concentration of 10 ppm (Yoshikawa et al., 1985). The incubation was carried out twice: from 6 June to 26 June and from 28 June to 18 July, 1988. In the first incubation, to test the effect of light on nitrification, some bottles were wrapped with black sheets and a sample collected from 24 m depth was suspended at 14 m depth.

After incubations, subsamples were siphoned out for the subsequent determinations of $N_2O$, dissolved oxygen and nitrogeneous nutrient concentrations.

2-3. Time course experiment

Water samples collected from 16 m depth were tested seven times for $NO_3^-$ and $N_2O$ production activities before and after active nitrification in 1986. Samples in 1.25-liter glass bottles with teflon-coated plastic caps were incubated with or without nitrapyrin (final concentration of 10 ppm) and an $NH_4^+$ enrichment (as $(NH_4)_2SO_4$, final concentration of 20 $\mu$g atom $N\cdot L^{-1}$) at 7°C under dark for seven days. After incubations, subsamples were siphoned out for the subsequent determinations of $N_2O$ and nitrogeneous nutrient concentrations. Samples for 0 day were treated in the same way without incubation.

2-4. Chemical analysis

Ammonium concentration was determined by the method of Sagi (1966). The concentrations...
of nitrate and nitrite were determined with a Technicon Autoanalyzer (Technicon Industrial Method, 1972). Dissolved oxygen and dissolved nitrous oxide were determined by the Winkler-method and by the procedure described previously (YÖH et al., 1988b).

3. Results

3-1. Change in $N_2O$ concentration during nitrification

Figure 1 shows vertical distributions of $NH_4^+$ and $NO_3^-$ in the hypolimnion of Lake Kizaki in 1988. A rapid increase in $NO_3^-$ concentration with a concomitant decrease in $NH_4^+$ concentration was observed from 5 June to 27 June, showing the active nitrification as has been observed in this lake (Takahashi et al., 1982; YoshioKA et al., 1985). After the exhaustion of $NH_4^+$ throughout the water column (except for upper and lowest hypolimnion) on June 27, there was a further increase in $NO_3^-$ concentration afterward (19 July).

On June 27, just after the active nitrification, the profile of $N_2O$ concentration showed an increase with depth (Fig. 2). A similar vertical trend of $N_2O$ concentration was also obtained on 19 July. These distributions were inversely correlated with those of dissolved oxygen concentration, similar to the observations in the pelagic ocean (Yoshinari, 1976). There was a considerable increase in $N_2O$ concentration throughout the hypolimnion during the interval between 27 June and 19 July, concurrent with oxygen consumption (Fig. 2) and $NO_3^-$ production (Fig. 1). Larger $N_2O$ increases were observed in the deeper layers.

In Figure 3, the amount of apparent $N_2O$ production ($\Delta N_2O$) on 27 June is plotted versus $NH_4^+$ consumption from 5 June to 27 June at corresponding depths. The $\Delta N_2O$ is defined as the difference between observed concentration and atmospheric-equilibrium concentration at in situ temperature (YÖH et al., 1988a, b). ($\Delta N_2O$ is substituted for the increase with time here, because the $N_2O$ data on 5 June was not available.) Just after the vernal circulation (April 23), $N_2O$ was nearly equilibrated with the air throughout the water column (105±2%, n=7). The amount of $N_2O$ produced during the active nitrification could be hence represented by the excess $N_2O$ ($\Delta N_2O$) on 27 June, when the active nitrification ended. In

\[ (\mu g \text{at. N})^{-1} \]

\[ (\mu g \text{at. N})^{-1} \]

\[ \text{Depth} \]

\[ 6/27 \quad 4/23 \quad 7/19 \quad \text{6/5} \]

\[ 0 \quad 5 \quad 10 \quad 15 \quad 20 \]

\[ 10 \]

\[ (a) \]

\[ NH_4^+ \]

\[ (b) \]

\[ NO_3^- \]

\[ 0 \quad 5 \quad 10 \quad 15 \quad 20 \quad 25 \]

\[ 20 \]

\[ 30 \]

Fig. 1. Vertical distribution of $NH_4^+$ (a) and $NO_3^-$ (b) concentrations in the hypolimnion of Lake Kizaki; on 23 April, 5 June, 27 June and 19 July, 1988. Dates are shown in the figure.
Figure 3, both variables are linearly correlated with an excellent correlation coefficient of 0.97, clearly indicating that a constant amount of N$_2$O was released concomitant with an NH$_4^+$ consumption during the active nitrification. From the slope of the regression line, the yield of N$_2$O relative to NH$_4^+$ consumption can be estimated to be 0.14%. The regression line shown in the figure has a y-intercept, where ΔN$_2$O is 3.5 ng at. N·1$^{-1}$, suggesting that a small amount of N$_2$O had been already accumulated on 5 June. However, it is evident from the figure that most N$_2$O accumulated on 27 June was formed after 5 June (shown by the N$_2$O accumulation correlated with NH$_4^+$ consumption).

Figures 4 and 5 show relationships between ΔN$_2$O and apparent oxygen consumption (−ΔO$_2$) and between ΔN$_2$O and the in situ NO$_3^-$ concentration, respectively. The apparent oxygen consumption, −ΔO$_2$, is also defined as the difference between observed concentration and the atmospheric-equilibrium concentration at in situ temperature. The ΔN$_2$O was found to be highly correlated not only with the apparent oxygen consumption but with the in situ NO$_3^-$ concentration. Since oxygen was abundant throughout the depths from which data were collected and N$_2$O and NO$_3^-$ concentrations increased with depth except in the deepest layer (designated in parentheses in Fig. 5), any reductive processes would be insignificant in these periods. Hence, these correlations suggest that N$_2$O was formed in proportion to both the oxygen consumption and the NO$_3^-$ production. The regression lines in Fig.

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ure 4 pass through the origin, indicating that little N$_2$O is produced unless oxygen is consumed. This is in agreement with the actual observation just after the vernal circulation (23 April). In Figure 5, the regression lines have a common x-intercept of about 10 μg atom N • 1$^{-1}$ of N$\text{O}_3$-, which also nearly corresponded to the N$\text{O}_3$- concentration on 23 April (Fig. 1).

The gradients of regression lines were larger on 19 July than on 27 June in both Figures 4 and 5. This implies that N$_2$O production might take place actively after the active nitrification period. From the slope of regressions in Figure 5, the N$_2$O yields are estimated to be 0.15% and 0.26% on 27 June and 19 July, respectively. The yield on 27 June, 0.15%, was almost identical to that obtained from Figure 3 (0.14%).

### 3-2. Long-term in situ incubation

Results of the first incubation and the second incubation are shown in Table 1 (a) and (b), respectively. During the first incubation (Table 1 a), from 6 June to 26 June, N$\text{O}_3$- + N$\text{O}_2$- productions in the absence of nitrapyrin were observed at rates from 6.1 to 9.3 μg at. N$\cdot$1$^{-1}$20 days$^{-1}$ concomitant with NH$_4^+$ consumptions of 3.7 to 7.3 μg at. N$\cdot$1$^{-1}$20 days$^{-1}$. The increases in N$\text{O}_3$- + N$\text{O}_2$- concentration slightly exceeded decreases in NH$_4^+$ concentration, due possibly to an NH$_4^+$ supply through organic matter decomposition. The N$\text{O}_3$- + N$\text{O}_2$- productions during the incubation increased with increasing depth, in accord with lake water samples (designated as “in situ” in the table). During the second incubation (Table 1 b), from 28 June to 18 July, there were also appreciable N$\text{O}_3$- + N$\text{O}_2$- productions in samples from 14 m and 24 m depths despite the exhaustion of NH$_4^+$ at the start of the incubation (Fig. 1). (The incubation bottle at 19 m was lost.) In fact, little NH$_4^+$ was consumed during this incubation (Table 1 b), indicating that nitrification depended mostly on the decomposition of organic matter contained in waters at the start of incubation. The N$\text{O}_3$- + N$\text{O}_2$- production at the 24 m-sample, 6.3 μg at. N$\cdot$1$^{-1}$20 days$^{-1}$, was considerably larger than that of the 14 m-sample, 1.9 μg at. N$\cdot$1$^{-1}$20 days$^{-1}$. Such a difference may reflect the difference in initial content of available organic matter, since the oxygen consumption in the 24 m-sample was several times as high as in the 14 m-sample.

Increases in N$_2$O concentration were invariably observed in these incubations. During the first incubation, N$_2$O accumulation ranged from 3.9 to 12.8 ng at. N$\cdot$1$^{-1}$. The amounts of N$_2$O accumulation in the 19 m sample and in the 24 m sample were 2-3 times larger than that of the 14 m-sample, 1.9 μg at. N$\cdot$1$^{-1}$20 days$^{-1}$. Such a difference may reflect the difference in initial content of available organic matter, since the oxygen consumption in the 24 m-sample was several times as high as in the 14 m-sample.

In contrast, when the sample of 24 m was incubated at 14 m depth, each component showed a rather slight increase, due presumably to the elevated temperature from 6.7°C (24 m) to 8.1°C.
These results indicate that the nitrification process was little affected by the light penetration, at least below 14 m.

Table 1. (a, b) Changes in N₂O, NO₂⁻ + NO₃⁻, NH₄⁺ and oxygen concentration during long-term *in situ* incubations and the ratio of N₂O relative to NO₂⁻ + NO₃⁻ production:

(a) First incubation from 6 June to 26 June;
(b) Second incubation from 28 June to 18 July.

<table>
<thead>
<tr>
<th>First incubation</th>
<th>Change in concentration</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N₂O ng at. N·l⁻¹</td>
<td>NO₃⁻ + NO₂⁻ μg at. N·l⁻¹</td>
</tr>
<tr>
<td>14 m</td>
<td>(in situ)</td>
<td>0.2</td>
</tr>
<tr>
<td></td>
<td>no treatment</td>
<td>3.9</td>
</tr>
<tr>
<td></td>
<td>shading</td>
<td>5.3</td>
</tr>
<tr>
<td></td>
<td>nitrapyrin</td>
<td>1.6</td>
</tr>
<tr>
<td>19 m</td>
<td>(in situ)</td>
<td>2.1</td>
</tr>
<tr>
<td></td>
<td>no treatment</td>
<td>7.9</td>
</tr>
<tr>
<td></td>
<td>shading</td>
<td>4.4</td>
</tr>
<tr>
<td></td>
<td>nitrapyrin</td>
<td>4.3</td>
</tr>
<tr>
<td>24 m</td>
<td>(in situ)</td>
<td>7.7</td>
</tr>
<tr>
<td></td>
<td>no treatment</td>
<td>12.8</td>
</tr>
<tr>
<td></td>
<td>nitrapyrin</td>
<td>5.1</td>
</tr>
<tr>
<td></td>
<td>at 14 m</td>
<td>13.8</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Second incubation</th>
<th>Change in concentration</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N₂O ng at. N·l⁻¹</td>
<td>NO₃⁻ + NO₂⁻ μg at. N·l⁻¹</td>
</tr>
<tr>
<td>14 m</td>
<td>(in situ)</td>
<td>10.3</td>
</tr>
<tr>
<td></td>
<td>no treatment</td>
<td>14.2</td>
</tr>
<tr>
<td></td>
<td>nitrapyrin</td>
<td>13.3</td>
</tr>
<tr>
<td>19 m</td>
<td>(in situ)</td>
<td>12.8</td>
</tr>
<tr>
<td></td>
<td>no treatment</td>
<td>13.3</td>
</tr>
<tr>
<td></td>
<td>nitrapyrin</td>
<td>13.3</td>
</tr>
<tr>
<td>24 m</td>
<td>(in situ)</td>
<td>17.2</td>
</tr>
<tr>
<td></td>
<td>no treatment</td>
<td>21.9</td>
</tr>
<tr>
<td></td>
<td>nitrapyrin</td>
<td>13.8</td>
</tr>
</tbody>
</table>

All sample waters were incubated at depths identical to those at which they were collected, except when expressed as "at 14 m". All bottles were incubated without wrapping, except those expressed as "shading". As initial concentrations of incubation, data of lake water samples at corresponding depths are employed: 5 June and 27 June, respectively. For N₂O in the first incubation, the data on 23 April are used, because data on 5 June were lacking.

* in situ: observed change in lake water at corresponding layers.
* no treatment: incubated without treatment.
* shading: incubated with wrapping.
* nitrapyrin: incubated in the presence of nitrapyrin at 10 ppm.
* at 14 m: 24 m sample incubated at 14 m depth.
* : complete consumption of NH₄⁺.

(14 m). These results indicate that the nitrification process was little affected by the light penetration, at least below 14 m. As shown in Table 1 (a) and (b), it was found that there was larger N₂O productions in the second incubation compared with the first one.
suggesting an accelerated \(N_2O\) production after the active nitrification period. The yield of \(N_2O\) relative to \(NO_3^- + NO_2^-\) production is also shown in Table 1. It ranged from 0.06 to 0.15 \% (average, 0.12\%) in the first incubation, while 0.75\% at 14 m layer and 0.35\% at 24 m layer in the second incubation. Evidently, the yields were larger in the second incubation. In addition, during the first incubation, the yields from the 14 m-incubation, 0.06 (without shading) and 0.08\% (with shading), were consistently smaller than those from the deeper layers (0.12- 0.15\%). The yield estimated from lake water samples from 27 June to 19 July at corresponding depths also exhibited an increasing tendency with depth from 0.1 to 1.0\% (Table 1 a).

Little \(NO_3^- + NO_2^-\) was accumulated in each bottle incubated with nitrapyrin during both incubations, suggesting that nitrification was almost completely inhibited by nitrapyrin. On the other hand, although \(N_2O\) productions in the presence of nitrapyrin were lower than those without treatment, there were substantial \(N_2O\) accumulations in the bottles treated with nitrapyrin, suggesting that nitrapyrin inhibited \(N_2O\) production only partially. These indicate a different response to nitrapyrin between \(NO_3^- + NO_2^-\) production and \(N_2O\) production. Table 2 shows the efficiency of inhibition by nitrapyrin in inhibiting \(NO_3^- + NO_2^-\) and \(N_2O\) productions. It reduced the rate of \(N_2O\) production by a half to two-thirds in the first incubation, but much less in the second incubation.

The changes in \(N_2O\) and \(NO_3^-\) concentrations and the decreases in \(NH_4^+\) concentration were comparable to or larger in bottles than those observed in situ. Although it is not easy to compare closely the data of this incubation with those from lake water because of some losses of these components by diffusion in lake and possible bottling effect, these results suggest that nitrification and \(N_2O\) production proceeded in lake water per se and that sediment, sinking particles or inflow contributed little. This is in agreement with the previous observation in Lake Kizaki (YOSHIOKA et al., 1985), in which nitrifying bacteria attached to rapidly sinking particles proved relatively inactive.

### Table 2. The efficiency of inhibition by nitrapyrin of \(N_2O\) and \(NO_3^- + NO_2^-\) productions during long-term incubation (%).

<table>
<thead>
<tr>
<th></th>
<th>(N_2O)</th>
<th>(NO_3^- + NO_2^-)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1)st</td>
<td></td>
<td></td>
</tr>
<tr>
<td>14 m</td>
<td>59, 69*</td>
<td>93, 95*</td>
</tr>
<tr>
<td>19 m</td>
<td>46, 54*</td>
<td>98, 98*</td>
</tr>
<tr>
<td>24 m</td>
<td>60</td>
<td>92</td>
</tr>
<tr>
<td>(2)nd</td>
<td></td>
<td></td>
</tr>
<tr>
<td>14 m</td>
<td>6</td>
<td>100</td>
</tr>
<tr>
<td>24 m</td>
<td>37</td>
<td>100</td>
</tr>
</tbody>
</table>

The efficiency \(E\) is calculated as:

\[
E = \frac{(A - B)}{A} \times 100
\]

where \(A\) and \(B\) are the increases in concentration in the absence and in the presence of nitrapyrin, respectively.

* : "no treatment" and "shading", respectively.

### 3-3. Time course experiment

Results of an incubation experiment done seven times in 1986 are shown in Figure 6. The \(NO_3^-\) production was low in early June, reached maximum in late June and diminished by degrees through July (Fig. 6 a). Such a change in nitrifying activity with time is almost identical to the previous result in this lake (YOSHIOKA and SAITO, 1985). As in the long-term incubation, \(NO_3^-\) production was completely prevented by addition of nitrapyrin. The additions of \(NH_4^+\) to the water stimulated \(NO_3^-\) production several-fold only after the emergence of the nitrifying activity. Afterward, this stimulation diminished with time.

The change in \(N_2O\) production with time generally coincided with that in the \(NO_3^-\) production. The \(N_2O\) production during incubation was low through June, reached maximum in early July and then decreased (Fig. 6 b). Addition of \(NH_4^+\) enhanced \(N_2O\) production several-fold in late June and afterward, similar to the results for \(NO_3^-\).

However, the patterns of \(NO_3^-\) production and \(N_2O\) production were not the same. There appeared to be a delay of some days in the \(N_2O\) production relative to the \(NO_3^-\) production. For instance, in late June, \(NO_3^-\) production was
maximum, whereas N₂O production was still at a low level. On the other hand, in mid July, though the nitrification activity fell substantially, the N₂O production activity was almost maximum. Calculated from data of Figure 6, the N₂O/NO₃⁻ ratio was 0.3% in the active nitrification phase (late June); but increased to 2.1% and 4.0% in the late phase of nitrification (early and mid July, respectively). Although these values are apparently high compared to those from the long-term incubation, due possibly to an increased bottling effect (1.25-1 volume), the results of this experiment confirm the difference of N₂O fraction during nitrification among the nitrification phases.

Nitrapyrin was again unable to completely

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Fig. 6. (a), (b) Activities of NO₃⁻ production (a) and N₂O production (b) in lake water from June to September in 1986, and the effects of nitrapyrin and ammonium enrichment on them. The location of columns corresponds to the time when sample was collected.

Open column (with dotted line): no addition.
Closed column: with nitrapyrin (10 ppm).
Hatched column: with ammonium enrichment ((NH₄)₂SO₄, +20 μg at. N·L⁻¹).
ND: not determined.
inhibit N₂O production; in general, nitrapyrin reduced it by half. In contrast, it inhibited NO₃⁻ production effectively. The nitrapyrin-insensitive N₂O production (closed column) became maximal in early July when the total N₂O production was also active. Such inhibition efficiencies of nitrapyrin for NO₃⁻ and N₂O were quite similar to the results of long-term incubation experiment.

4. Discussion

Nitrification has been suggested to be the major source of N₂O in well-oxygenated zones of oceans (Yoshinari, 1976; Elkins et al., 1978; Cohen and Gordon, 1978; 1979) and a freshwater lake (Yosh et al., 1988 a), based mainly on the linear N₂O-AOU relationship. However, no close relationship of N₂O concentration with NO₃⁻ concentration has been presented, in contrast to the N₂O-AOU relationship commonly obtained. In the present investigation, apparent N₂O production (ΔN₂O) was found to be highly correlated not only with apparent oxygen consumption (−ΔO₂) but with NO₃⁻ concentration and the amount of NH₄⁺ decrease with time (Figs. 3, 4 and 5). The lake water incubation experiments showed simultaneous accumulations of NO₃⁻ and N₂O (Table 1). In addition, it was found that both productions were several-fold enhanced by NH₄⁺ enrichments after the emergence of nitrification (Fig. 6) and inhibited, at least partly, by the addition of the specific inhibitor of nitrification, nitrapyrin (Table 1 and Fig. 6). These relationships and experimental results clearly indicate that N₂O was produced during the process of nitrification in Lake Kizaki.

The process of nitrification in Lake Kizaki can be divided into two phases: the first is the rapid NO₃⁻ formation accompanied by the oxidation of an accumulating NH₄⁺ from 5 June to 27 June (active nitrification); the second is the slower NO₃⁻ formation after the exhaustion of NH₄⁺, observed from 27 June to 19 July. The higher gradients on 19 July than on 27 June in the linear relationships between ΔN₂O and oxygen deficiency (−ΔO₂) and in situ NO₃⁻ concentration (Figs. 4 and 5) indicate that, although N₂O was produced during both phases of nitrification, the fraction of N₂O was larger during the latter phase of nitrification. The results in the long-term in situ incubation experiment (Table 1) and in the time course experiment (Fig. 6) also show accelerated N₂O production after the active nitrification period.

The N₂O yields estimated from the vertical observation were 0.14% (Fig. 3) or 0.15% (Fig. 5) on 27 June, just after the active nitrification, and increased to 0.26% on 19 July (Fig. 5), when the subsequent nitrification had taken place. The yields obtained from the first long term incubation, 0.06%–0.15% (average, 0.12%) (Table 1 a), were very similar to the above values on 27 June. In addition, the values of 0.75% and 0.35% obtained from the second incubation (Table 1 b) could account for the increase in N₂O yield found on 19 July in the lake. These consistencies between the lake observation and the incubation experiment corroborate the difference of N₂O yield between nitrification phases.

In the western Atlantic Ocean, Yoshinari (1976) estimated the N₂O yield of 0.12%, on the basis of the stoichiometric N/O ratio. The values obtained in the present investigation during active nitrification phase as mentioned above are almost identical to this value. On the other hand, the yields with respect to the late phase of nitrification are similar to those of 0.2% to 0.7% obtained from the field experiments in the Chesapeake Bay (McCarthy et al., 1984).

In the aerobic culture of Nitrosomonas europaea, Hynes and Knowles (1984) have presented the yield of 0.05 to 0.15% over the wide range of NH₄⁺ concentrations, while Goreau et al. (1980) have observed a slightly higher value around 0.3% in the fully-aerated cultures of Nitrosomonas sp. It is observed in well-aerated soil that the N₂O evolution is equivalent to 0.1–0.2% of the nitrified N (Goodroad and Keeney, 1984) or 0.04 to 0.45% of added (NH₄)₂SO₄ or urea (Bremer and Blackmer, 1978). Thus, the yields of 0.06 to 0.15% obtained in the present study during the active nitrification period are in good agreement with
those obtained in not only laboratory cultures but other environments. It is noteworthy that similar percentages have been observed despite the great differences in environmental conditions (e.g., structure, fertility, pH) as well as in nitrification rates among these systems and that the present values are almost at the lowest level.

Although several factors have been found to affect N₂O production by nitrifiers in culture experiments (Yoshida and Alexander, 1970), little is known of the factors actually controlling the N₂O production in natural environments. One of the most important parameters of such kinds is the ambient oxygen concentration. Goreau et al. (1980) have presented data showing that when oxygen partial pressure decreases from 20% (fully aerobic) to 5%, the N₂O yield increases from about 0.3% to nearly 1%. However, this would not be the case in field observation such as in Lake Kizaki and in marine ecosystems, both of which show linear relationships between apparent N₂O production and apparent oxygen consumption over wide ranges of oxygen concentration (Fig. 4).

Yoshida and Alexander (1970) demonstrated that the aged cells of *Nitrosomonas europaea* release larger fractions of N₂O relative to NO₂⁻ than the younger cells. In previous studies on the nitrification process in Lake Kizaki, Takahashi et al. (1982) and Yoshioka et al. (1985) have reported that the potential activity of nitrification (with NH₄⁺ supplements) drops concurrent with the termination of active nitrification (i.e., the consumption of pre-existing NH₄⁺). (This was not so clear in the present study, possibly because of the longer incubation time of 7 days in the time course experiment.) The above authors also observed that there was nevertheless little change in the nitrifier population before and after the active nitrification period. Thus, the drop of nitrifying activity in water could be attributed to change in the metabolisms of nitrifiers *per se*, possibly caused by the substrate starvation (Takahashi et al., 1982). It is therefore highly likely that such a physiological change of the nitrifier population might be associated with the accelerated N₂O production observed in the late phase of nitrification.

In addition, there appeared to be a vertical difference of the N₂O yield: the estimated yields were consistently lower in the shallower layer than in the deeper layers during the first long-term incubation (Table 1a). This tendency could be also found in the ratio of N₂O and NO₃⁻ increases in lake water during the corresponding interval (Table 1a). It has been also observed in the equatorial Pacific that the ΔN₂O/AOU ratio in the shallower zone was about half of that in the deeper zone (Cline et al., 1987). Besides oxygen concentration, N₂O production in oceans is proposed to be a function of temperature (Elkins et al., 1978; Cline et al., 1987) and water pressure (Butler et al., 1989). However, such a vertical difference in Lake Kizaki, if this was the case, may be also explained by the same mechanism described above: because there is a considerable downward flux of nitrifying bacteria in Lake Kizaki (Yoshioka et al., 1985) and NH₄⁺ is first exhausted in the lower hypolimnion (Takahashi et al., 1982; Yoshioka et al., 1985), nitrifying bacteria in the deeper layer are presumed to be older or starved longer, which could elevate N₂O fraction during nitrification.

On the other hand, it was found in the present study that nitrapyrin was not very effective for N₂O production despite the complete inhibition of NO₃⁻ production (Tables 1 and 2). This is in contrast to the observations in fertilized soil (Bremner and Blackmer, 1978; Blackmer et al., 1980) showing that N₂O evolution is greatly reduced by nitrapyrin. According to Shattuck and Alexander (1963), nitrapyrin is the specific inhibitor for autotrophic nitrification. Thus, the incomplete inhibition of N₂O production by nitrapyrin may imply that some N₂O was released by alternative processes other than autotrophic nitrification. As shown in Table 3, the NO₃⁻ concentrations in some of nitrapyrin-treated bottles were rather high compared with the non-treated bottles, especially after the second long-term incubation. This could favor the above view, since nitrapyrin inhibits NH₄⁺ oxidizers more effectively than NO₃⁻.
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oxidizers (SHATTUCK and ALEXANDER, 1963; YOSHIOKA et al., 1985). Most studies ever made in natural environments have attributed N2O source to nitrification or denitrification. However, ROBERTSON and TIEDIE (1987) have recently presented evidence suggesting that there may be some biological sources of N2O other than denitrification or nitrification in forest soils. In addition, GOREAU et al. (1980) have noted that large quantities of N2O was produced by the culture of Nitrosomonas sp. when contaminated by Fusarium sp., a fungus that releases N2O (BOLLAG and TUNG, 1972). Thus, the possibility that some heterotrophs may contribute to the N2O production, especially in the late phase of nitrification, can not be excluded.

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