Nitrogen and oxygen availabilities control water column nitrous oxide production during seasonal anoxia in the Chesapeake Bay

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Abstract. Nitrous oxide (N\textsubscript{2}O) is a greenhouse gas and an ozone depletion agent. Estuaries that are subject to seasonal anoxia are generally regarded as N\textsubscript{2}O sources. However, insufficient understanding of the environmental controls on N\textsubscript{2}O production results in large uncertainty about the estuarine contribution to the global N\textsubscript{2}O budget. Incubation experiments with nitrogen stable isotope tracer were used to investigate the geochemical factors controlling N\textsubscript{2}O production from denitrification in the Chesapeake Bay, the largest estuary in North America. The highest potential rates of water column N\textsubscript{2}O production via denitrification (7.5 ± 1.2 nmol-N L\textsuperscript{-1} h\textsuperscript{-1}) were detected during summer anoxia, during which oxidized nitrogen species (nitrate and nitrite) were absent from the water column. At the top of the anoxic layer, N\textsubscript{2}O production from denitrification was stimulated by addition of nitrate and nitrite. The relative contribution of nitrate and nitrite to N\textsubscript{2}O production was positively correlated with the ratio of nitrate to nitrite concentrations. Increased oxygen availability, up to 7 µmol L\textsuperscript{-1} oxygen, inhibited both N\textsubscript{2}O production and the reduction of nitrate to nitrite. In spring, high oxygen and low abundance of denitrifying microbes resulted in undetectable N\textsubscript{2}O production from denitrification. Thus, decreasing the nitrogen input into the Chesapeake Bay has two potential impacts on the N\textsubscript{2}O production: a lower availability of nitrogen substrates may mitigate short-term N\textsubscript{2}O emissions during summer anoxia; and, in the long-run (timescale of years), eutrophication will be alleviated and subsequent reoxygenation of the bay will further inhibit N\textsubscript{2}O production.

1 Introduction

Nitrous oxide (N\textsubscript{2}O) is a strong greenhouse gas with 298-fold higher global warming potential per mole than that of carbon dioxide. N\textsubscript{2}O is also a catalyst of ozone depletion in the stratosphere. Since the Industrial Revolution, the N\textsubscript{2}O atmospheric concentration has been increasing at an unprecedented rate, and the current concentration is the highest in the last 800 000 years of Earth’s history (Schilt et al., 2010). The contribution of N\textsubscript{2}O emissions to global warming and ozone depletion will increase because N\textsubscript{2}O is not as strictly regulated as are CO\textsubscript{2} and halocarbon compounds. With the successful mitigation of halocarbon compounds accomplished by the Montreal Protocol, N\textsubscript{2}O is likely to be the single most important anthropogenically emitted ozone-depleting agent in the 21st century (Ravishankara et al., 2009).

Microbial processes are responsible for the majority of N\textsubscript{2}O production, both in natural and anthropogenically impacted environments. These pathways include oxidative and reductive processes occurring at the full range of environmental oxygen concentrations. In the presence of oxygen, N\textsubscript{2}O can be produced as a byproduct during autotrophic aerobic ammonium (NH\textsubscript{4}\textsuperscript{+}) oxidation to nitrite (NO\textsubscript{2}\textsuperscript{-}) by bacteria (Arp and Stein, 2003) and archaea (Santoro et al., 2011). The production of N\textsubscript{2}O can also occur via NO\textsubscript{2}\textsuperscript{-} reduction by nitrifying organisms, termed nitrifier denitrification. This process was demonstrated in cultures (Poth and Focht, 1985; Frame and Casciotti, 2010) and in the water column of the subtropical North Pacific Ocean (Wilson et al., 2014). Under low-oxygen and anoxic conditions, denitrifying bacteria produce N\textsubscript{2}O via enzyme-mediated heterotrophic denitrification, which consists of the stepwise reduction of nitrate.
(NO$_3^-$), NO$_2^-$ and nitric oxide (NO), with organic matter as the electron donor. The nirS gene that encodes the genetic material for nitrite reductase (the enzyme mediating NO$_2^-$ reduction to NO) is often used as a proxy for abundance and diversity of denitrifying bacteria and is the gene in the denitrification sequence that is most reliably associated with a complete denitrification pathway (Graf et al., 2014). N$_2$O is not produced via anaerobic ammonium oxidation (anammox), another important nitrogen removal process in the natural environment (Kartal et al., 2011).

The increase in atmospheric N$_2$O is attributed to intensification of human activities (e.g., fossil fuel combustion, fertilizer application, human and animal waste disposal), which alter the microbial nitrogen cycle in the biosphere. Increased nitrogen supply from fertilizer and atmospheric deposition causes increased N$_2$O emission not only from agricultural land, but also in rivers, streams and coastal waters (Ciais et al., 2013; Thompson et al., 2014). Among these aquatic environments, intense N$_2$O efflux originates from estuaries and associated river networks, which occupy 0.3 % of global waters (Dürr et al., 2011) but could contribute up to 10 % of anthropogenic fluxes (Seitzinger and Kroeze, 1998; Ciais et al., 2013). Being the largest estuary in North America, the Chesapeake Bay and its tributaries have experienced eutrophication and expansion of summertime anoxia due to increased population, expansion of industrialization and land use changes since the 18th century (Cooper and Brush, 1993; Boesch et al., 2001). The Chesapeake tributary is a source of N$_2$O (indicated by surface N$_2$O oversaturation) in the summertime between June and September (Elkins et al., 1978; Kaplan et al., 1978; McElroy et al., 1978). The summertime water column is characterized by strong oxygen gradients (equilibrium with atmosphere at the surface and complete anoxia below $\sim 10$ m), depletion of NO$_3^-$ and NO$_2^-$, and accumulation of NH$_4^+$ in the deep water (Lee et al., 2015b). Increased microbial activities driving carbon assimilation and respiration have been demonstrated in the vicinity of the oxic–anoxic interface in the water column (Lee et al., 2015a). However, the N$_2$O production pathway and the associated environmental controlling factors have not been investigated in the Chesapeake Bay.

Here we report a pilot study using nitrogen stable isotope ($^{15}$N) incubation experiments to quantify N$_2$O production rates and their dependence on the availabilities of oxygen, NO$_3^-$ and NO$_2^-$ in the Chesapeake Bay. Because seasonal anoxia occurs at the study site in the central region of the Chesapeake Bay, reductive pathways of N$_2$O production (i.e., reduction of NO$_3^-$ and NO$_2^-$) are the main focus. Further understanding of the environmental controls on N$_2$O production in estuaries will facilitate the design of effective environmental engineering projects to mitigate N$_2$O emission.

2 Methods

2.1 Sample acquisition and processing

Sampling and incubation experiments were carried out on 19 July 2016, 17 November 2016 and 3 May 2017, corresponding to typical conditions of summer, autumn and spring, respectively. Samples were collected at 38.55° N, 76.43° W (bottom depth 26.5 m) close to the mouth of the Choptank River in the central region of the Chesapeake Bay. Conductivity–temperature–depth and dissolved oxygen ($\text{O}_2$) were measured with a YSI sonde package (Model 600XLM with a 650 MDS display logger) equipped with a diaphragm pump which was deployed for water sampling. The oxygen sensor had a detection limit of $\sim 5 \mu\text{mol L}^{-1}$. Samples for NO$_3^-$ and NO$_2^-$ concentration measurements were filtered (0.22 µm pore size, Sterivex-GP, EMD Millipore) and frozen at $-80$ °C until analysis. Discrete samples for N$_2$O concentration were collected directly from the pump outlet into the bottom of acid-washed, 60 mL glass serum bottles (catalog no. 223745, Wheaton, Millville, NJ, USA). Bottles were sealed with butyl rubber stoppers (catalog no. W224100-202, Wheaton, Millville, NJ, USA) and aluminium rings while submerged under water pumped from depth to avoid atmospheric N$_2$O and oxygen contamination. Samples for characterizing the N$_2$O concentration profile were preserved immediately after filling by injecting 0.1 mL saturated HgCl$_2$. Samples for N$_2$O incubation experiments (Sect. 2.2) were acquired from 12, 17 and 19.5 m during July 2016, November 2016 and May 2017, respectively; sealed the same way as described above for discrete N$_2$O concentration samples; and stored in the dark at 4 °C without adding HgCl$_2$. Samples for denitrifying nirS gene abundance were collected at 14, 17 and 19.5 m by filtering 600–2000 mL of water through a 0.22 µm filter (Sterivex-GP, EMD Millipore) and frozen at $-80$ °C until DNA extraction and analysis.

Samples for total dissolved inorganic carbon (DIC = \([\text{H}_2\text{CO}_3^-] + [\text{HCO}_3^-] + [\text{CO}_3^{2-}]\)) and community respiration rates were collected only in July 2016. The DIC samples were preserved with mercuric chloride (HgCl$_2$) for initial conditions, while biochemical oxygen demand (BOD) bottles were incubated in a temperature-controlled environmental chamber ($\pm 1 ^\circ\text{C}$ of in situ water temperatures). After 24 h, samples were siphoned from the vials, preserved with HgCl$_2$, and respiration rates were determined as the difference in DIC between initial and final samples divided by 24 h (Lee et al., 2015b).

2.2 $^{15}$N incubation experiments for N$_2$O production

Within 3 h of sampling, incubation experiments were initiated at the Horn Point Laboratory, Cambridge, Maryland. Samples were divided into three sets for control, nitrogen manipulation and oxygen manipulation experiments.
Table 1. Parameters for control, nitrogen manipulation and oxygen manipulation incubation experiments in July 2016, November 2016 and May 2017 sampling. In May 2017, only the control experiment was conducted. The unit “µmol L−1” is represented by “µM”. Bold columns highlight the concentrations for 15N tracers. In situ nitrate and nitrite concentrations in July 2016 were < 0.02 µmol L−1; in November 2016 the concentrations were 5.0 and 0.4 µmol L−1, respectively; in May 2017 the concentrations were 6.3 and 0.4 µmol L−1, respectively.

<table>
<thead>
<tr>
<th>Experiment ID</th>
<th>Experiment 1 A</th>
<th>15NO3− (µM)</th>
<th>15NO2− (µM)</th>
<th>14NO3− (µM)</th>
<th>14NO2− (µM)</th>
<th>NO2− : NO3−</th>
<th>15N fraction label (species)</th>
<th>O2 (µM)</th>
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</thead>
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<tr>
<td>Control (July 2016)</td>
<td>1-A</td>
<td>5</td>
<td>5</td>
<td>1 : 1</td>
<td>0.99 (NO3−)</td>
<td>0.6</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>1-B</td>
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<td>5</td>
<td>1 : 1</td>
<td>0.99 (NO3−)</td>
<td>0.6</td>
<td></td>
<td></td>
</tr>
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<td>Nitrogen manipulation (July 2016)</td>
<td>2-A</td>
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<td>0.16 (NO3−)</td>
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</tr>
<tr>
<td></td>
<td>2-B</td>
<td>0.2</td>
<td>1</td>
<td>10</td>
<td>1 : 10</td>
<td>0.016 (NO3−)</td>
<td>0.6</td>
<td></td>
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<td></td>
<td>2-C</td>
<td>0.2</td>
<td>1</td>
<td>3</td>
<td>1.2 : 3</td>
<td>0.16 (NO3−)</td>
<td>0.6</td>
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<tr>
<td></td>
<td>2-D</td>
<td>0.2</td>
<td>1</td>
<td>3</td>
<td>1 : 3</td>
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<td>0.6</td>
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<tr>
<td></td>
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<td>3 : 1</td>
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<tr>
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<td>2-G</td>
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<tr>
<td></td>
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<td>1</td>
<td>10 : 1</td>
<td>0.16 (NO3−)</td>
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<tr>
<td>Oxygen manipulation (July 2016)</td>
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<td>0.99 (NO3−)</td>
<td>0.6</td>
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<td></td>
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<tr>
<td></td>
<td>3-B</td>
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<td>1 : 1</td>
<td>0.99 (NO3−)</td>
<td>0.6</td>
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<td></td>
<td>3-C</td>
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<td>0.99 (NO3−)</td>
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<td>5</td>
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<td>0.99 (NO3−)</td>
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<td></td>
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<td>5</td>
<td>1 : 1</td>
<td>0.99 (NO3−)</td>
<td>0.6</td>
<td></td>
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<td></td>
<td>3-J</td>
<td>5</td>
<td>5</td>
<td>1 : 1</td>
<td>0.99 (NO3−)</td>
<td>0.6</td>
<td></td>
<td></td>
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<tr>
<td>Control (November 2016)</td>
<td>4-A</td>
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<td>0.4</td>
<td>10</td>
<td>0.54 : 1</td>
<td>0.93 (NO3−)</td>
<td>0.6</td>
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<tr>
<td></td>
<td>4-B</td>
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<td>5.4</td>
<td>5</td>
<td>0.54 : 1</td>
<td>0.50 (NO3−)</td>
<td>0.6</td>
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<tr>
<td>Oxygen manipulation (November 2016)</td>
<td>5-A</td>
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<td>0.4</td>
<td>10</td>
<td>0.54 : 1</td>
<td>0.93 (NO3−)</td>
<td>0.6</td>
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<tr>
<td></td>
<td>5-J</td>
<td>5</td>
<td>5.4</td>
<td>5</td>
<td>0.54 : 1</td>
<td>0.50 (NO3−)</td>
<td>0.6</td>
<td></td>
</tr>
<tr>
<td>Control (May 2017)</td>
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<td>0.4</td>
<td>11.3</td>
<td>0.48 : 1</td>
<td>0.93 (NO3−)</td>
<td>0.6</td>
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</tr>
<tr>
<td></td>
<td>6-B</td>
<td>5</td>
<td>5.4</td>
<td>6.3</td>
<td>0.48 : 1</td>
<td>0.44 (NO3−)</td>
<td>0.6</td>
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</table>

Control experiment. The control experiment was conducted in July 2016, November 2016 and May 2017. A small (3 mL) headspace was created in the serum bottles, which were subsequently flushed with helium for 10 min to minimize oxygen contamination from sampling and transportation. Two suites of 15N tracer solutions (15NO2− plus 14NO3−, 15NO3− plus 14NO2−, 0.1 mL) were injected to achieve final concentrations of 5 µmol L−1 NO3− and NO2− (see conditions for experiment 1-A and 1-B, 4-A and 4-B, and 6-A and 6-B in Table 1). Tracer solutions were made from deionized water and were flushed with helium prior to addition to incubation experiments. In order to have enough mass to detect N2O production, ~ 1.2 nmol of natural abundance N2O was injected to each bottle, reaching a concentration of ~ 20 nmol L−1 in the water phase (calculated equilibrium concentration according to Weiss and Price, 1980, with 3 mL headspace and 57 mL water). Initial conditions (one bottle for each time course) were sampled within 30 min of tracer
addition by injecting 0.1 mL saturated HgCl$_2$. Incubations lasted $\sim$ 2 h at in situ temperature ($\pm 0.5^\circ$C), during which duplicate bottles were preserved with a saturated HgCl$_2$ solution every 40 to 60 min, totalling seven bottles over four time points, including the initial for a time course analysis.

**Dissolved inorganic nitrogen (DIN) manipulation.** The DIN manipulation experiment was conducted only in July 2016 because NO$_2^-$ and NO$_3^-$ were absent from the water column (see Sect. 3.1). A 3 mL headspace was created before flushing with helium for 10 min to establish anoxic condition. Then, $\sim$ 1.2 nmol N$_2$O was injected to reach a concentration of $\sim$ 20 nmol L$^{-1}$ in the water phase. Two suites of $^{15}$N tracer solutions ($^{15}$NO$_2^-$ plus $^{14}$NO$_3^-$, $^{15}$NO$_3^-$ plus $^{14}$NO$_2^-$, 0.1 mL of total volume of tracer addition) were injected to designated bottles to achieve ratios of NO$_2^-$ : NO$_3^-$ $\approx$ 1 : 10, 1 : 3, 3 : 1 and 10 : 1, with $^{15}$N fraction labeled between 0.016 and 0.16 (Table 1, experiment 2-A to 2-H). This allowed simultaneous detection of N$_2$O production from NO$_2^-$ and NO$_3^-$ at different ratios of NO$_2^-$ to NO$_3^-$ concentration. Incubations lasted $\sim$ 2 h with the same sampling strategy as the control experiment.

**Oxygen manipulation.** The oxygen manipulation experiment was conducted in July 2016 and November 2016. Headspace (3–8 mL) was created before flushing with helium for 10 min. Oxygen-saturated site water was made by air equilibration at in situ temperature. To achieve different oxygen levels, 0.2, 0.5, 1.0, 2.0 or 5.0 mL of oxygen-saturated site water was injected. With a final volume of $\sim$ 3 mL of headspace during the course of the incubation, the oxygen concentrations in the water phase were 0.3 to 6.4 µmol L$^{-1}$ in July 2016 (Table 1, experiment 3-A–3-J) and were 0.2 to 7.3 µmol L$^{-1}$ in November 2016 (Table 1, experiment 5-A–5-J) after the calculated equilibration between headspace and water (Garcia and Gordon, 1992). In addition, an optical sensor was used to measure oxygen concentrations directly in a parallel experimental setup and the agreement between calculated target concentration and measured concentration was excellent (data not shown). After oxygen adjustment, $\sim$ 1.2 nmol N$_2$O was injected into each bottle, and two suites of $^{15}$N tracer solutions ($^{15}$NO$_2^-$ plus $^{14}$NO$_3^-$, $^{15}$NO$_3^-$ plus $^{14}$NO$_2^-$, 0.1 mL) were injected to achieve a final concentration of 5 µmol L$^{-1}$ NO$_2^-$ and NO$_3^-$. The $^{15}$N fraction for NO$_2^-$ or NO$_3^-$ during the incubation experiments is shown in Table 1. Incubations lasted $\sim$ 2 h with the same sampling strategy as the control experiment.

### 2.3 Analytical procedures

For water column nutrients, dissolved NO$_2^-$ was measured using a colorimetric method (Hansen and Koroleff, 2007) and NO$_3^-$ + NO$_2^-$ was measured using a hot (90°C) acidified vanadium(III) reduction column coupled to a chemiluminescence NO/NO$_3^-$ analyzer (Teledyne API, San Diego, CA, USA) (Garside, 1982; Braman and Hendrix, 1989). DIC was measured with an automated infrared analyzer (Apollo SciTech, Newark, DE, USA) as previously reported (Lee et al., 2015b). Preserved N$_2$O samples were stored in the dark at room temperature ($\sim$ 22°C) for less than 3 weeks before analysis. Dissolved N$_2$O was extracted by flushing with helium for 40 min at a rate of 37 mL min$^{-1}$ (extraction efficiency 99±2%) and subsequently cryo-trapped by liquid nitrogen and isolated from interfering compounds (H$_2$O, CO$_2$) by gas chromatography (Weigand et al., 2016). Pulses of purified N$_2$O were injected into an isotope ratio mass spectrometer (Delta Plus, Thermo Fisher Scientific, Waltham, MA) for mass (m/z = 44, 45, 46) and isotope ratio (m$_1$/m$_2$ = 45/44, 46/44) measurements. The amount of N$_2$O was calibrated with standard N$_2$O vials, which were made by injecting 1, 2 or 5 nmol N$_2$O into 20 mL glass vials (catalog no. C4020-25, Thermo Fisher Scientific, Waltham, MA).

After N$_2$O analysis, samples incubated with $^{15}$NO$_2^-$ were also assayed for $^{15}$NO$_3^-$ to determine rates of NO$_2^-$ reduction. Two milliliters of each sample were transferred from the 60 mL serum bottle to a 20 mL glass vial and then flushed with helium for 10 min. Dissolved $^{14}$NO$_2^-$ was converted to N$_2$O using the acetic-acid-treated sodium azide solution for quantitative conversion (McIlvin and Altabet, 2005). Resulting N$_2$O was measured for nitrogen isotope ratio ($^{15}$N/$^{14}$N) so as to determine the $^{15}$N enrichment of NO$_3^-$.

For the analysis of nirS gene abundance, DNA extraction and qPCR for the nirS gene using SYBR Green were performed as previously described (Jayakumar et al., 2009, 2013). Extracted DNA was quantified using PicoGreen fluorescence (Molecular Probes, Eugene, OR) prior to the qPCR assay. Samples for qPCR were run in triplicates including a no-template control, a no-primer control and five different dilutions of a nirS standard. Threshold cycle (Ct) values were obtained using automatic analysis settings of the quantitative PCR and further used to calculate the gene copy numbers as described in Jayakumar et al. (2013).

### 2.4 Data analysis

N$_2$O concentration was calculated from the amount of N$_2$O detected by mass spectrometry divided by the volume of water in the serum bottles. N$_2$O production ($R$) was calculated from the progressive increase in $^{45}$N$_2$O and $^{46}$N$_2$O concentrations in each serum bottle over the time course experiments.

$$R = \frac{1}{F} \times \left( \frac{d^{45}N_2O}{dt} + 2 \times \frac{d^{46}N_2O}{dt} \right).$$

where $d^{45}N_2O/dt$ and $d^{46}N_2O/dt$ represent the production rates (nmol-N L$^{-1}$ h$^{-1}$) of mass 45 and 46 N$_2$O during incubation. $F$ represents the $^{15}$N fraction in the initial substrate (NO$_2^-$ or NO$_3^-$). Rates were considered significant based on the linear regression of the time course data having $p < 0.05$ ($n = 7$, Student’s $t$ test). The detection limit for N$_2$O production was 0.002 nmol-N L$^{-1}$ h$^{-1}$. The $^{15}$N incubation experiments can identify the pathway but cannot distinguish the
relative contributions of two or more functioning microbial groups to a single N\textsubscript{2}O production pathway (i.e., N\textsubscript{2}O production via NO\textsubscript{2}\textsuperscript{-} reduction by nitrifier denitrification and/or heterotrophic denitrification).

The rate of NO\textsubscript{3}\textsuperscript{-} reduction to NO\textsubscript{2}\textsuperscript{-} was calculated as

\[
\text{NO}_2^{-} \text{production} = \frac{(d\text{NO}_2^{15}/dt)}{F},
\]

where \(d\text{NO}_2^{15}/dt\) represents the production rate of \(\text{NO}_2^{15}\) (nmol-N L\textsuperscript{-1} h\textsuperscript{-1}). \(F\) represents initial \(\text{NO}_3^{-}\) enrichment of substrate NO\textsubscript{3}\textsuperscript{-}. Rates were considered significant based on linear regression of the time course data having \(p < 0.05\) (Student’s \(t\) test). The detection limit for NO\textsubscript{2}\textsuperscript{-} production was 0.05 nmol-N L\textsuperscript{-1} h\textsuperscript{-1}.

3 Results and discussion

3.1 Water column features

The physical and chemical properties of the water column in the central Chesapeake Bay showed seasonal variation (Fig. 1). Temperature and salinity differed among the three seasons but were essentially constant in the top 7 m of the water column on the three sampling dates. In July, the water column was stratified because of lower salinity (∼ 16 PSU) and higher temperature (∼ 28.5 °C) in the top ∼ 10 m, resulting in a pronounced halocline and thermocline (Fig. 1a and b). Less pronounced stratification in May and November was due to a weaker temperature difference between the top 10 m and below. The July oxygen profile showed a significant concentration decrease between 3 and 10 m (Fig. 1c), with a sharp oxycline (∼ 30 µmol L\textsuperscript{-1} m\textsuperscript{-1}). Below 10 m, the oxygen concentration was below detection of the sensor (∼ 5 µmol L\textsuperscript{-1}) and was likely anoxic. The water samples were free of any hydrogen sulfide odor, so we conclude that sulfide was either absent or was present at a very low level (∼ 0.02 µmol L\textsuperscript{-1}). No anoxic layer was observed in May and November (Fig. 1c), and previous studies showed that the water column of the Chesapeake Bay was reoxygenated following summertime anoxia during winter and spring (Lee et al., 2015a).

The surface N\textsubscript{2}O saturation concentrations in July, November and May were 6.6, 10.4 and 12.0 nmol L\textsuperscript{-1}, respectively. In July, N\textsubscript{2}O concentration was close to air-saturation level (6.6 nmol L\textsuperscript{-1}) at the surface layer. In the low oxygen layer (below 12 m), N\textsubscript{2}O was apparently undersaturated (2.0–3.7 nmol L\textsuperscript{-1}, 20 %–50 % air saturation, Fig. 1d). In November, the surface N\textsubscript{2}O concentration was slightly oversaturated (11.3 nmol L\textsuperscript{-1}, 108 % air saturation). N\textsubscript{2}O concentrations at depth were oversaturated; the concentrations varied between 11.0 and 11.5 nmol L\textsuperscript{-1}, corresponding to 109 %–115 % air saturation. In May, both the surface and water column N\textsubscript{2}O concentrations were air undersaturated; the surface concentration was 9.1 nmol L\textsuperscript{-1}, 76 % air saturation; concentrations between 8 and 17 m ranged from 9.4 to 11.0 nmol L\textsuperscript{-1}, corresponding to 82 %–97 % air saturation. As the surface and water column N\textsubscript{2}O saturation levels vary greatly between seasons, the assessment of the N\textsubscript{2}O dynamics of the Chesapeake Bay requires expanding the temporal and spatial coverage of the field sampling. In the following, we focus on N\textsubscript{2}O production and its environmental controlling factors.

The concentrations of NO\textsubscript{3}\textsuperscript{-} and NO\textsubscript{2}\textsuperscript{-} (Fig. 1e and f) in July were below 0.02 µmol L\textsuperscript{-1} within the sampling depth interval (top 17 m of water column). Measurable levels of NO\textsubscript{3}\textsuperscript{-} and NO\textsubscript{2}\textsuperscript{-} species were found in May and November. The surface concentrations of NO\textsubscript{3}\textsuperscript{-} and NO\textsubscript{2}\textsuperscript{-} in May were 20 and 0.5 µmol L\textsuperscript{-1}, respectively; and the concentrations decreased with depth. In November, NO\textsubscript{3}\textsuperscript{-} and NO\textsubscript{2}\textsuperscript{-} were depleted at the surface (∼ 3 m) and their concentrations increased with depth; at 17 m the concentrations of NO\textsubscript{3}\textsuperscript{-} and NO\textsubscript{2}\textsuperscript{-} were 5.0 and 0.4 µmol L\textsuperscript{-1}, respectively. The increase in water column...
umn NO$_3^-$ and NO$_2^-$ concentrations in May and November can be attributed to increased runoff from the anthropogenically influenced watershed. Water column depletion of NO$_3^-$ and NO$_2^-$ in the summer is the result of denitrification (Baird et al., 1995; Boynton et al., 1995), which indicates potential water column N$_2$O production via denitrification (discussed in Sect. 3.2).

As a proxy for the size of the denitrifying community, the abundance of the nirS gene was (5.91 ± 0.1) × 10$^4$ copy mL$^{-1}$ at 14 m in July, which was the highest among the three sampling trips (Fig. 1g). The lowest nirS gene abundance (9.1 ± 1.3) × 10$^3$ copy mL$^{-1}$ was observed in May at 19.5 m. The abundance of nirS was measured only at the depths at which incubations were performed, and the nirS abundance increased with increasing rates of N$_2$O production (see Sect. 3.2). In July 2016, water column DIC concentrations ranged from 1377 to 1831 µmol L$^{-1}$, with the highest concentrations below 10 m. Average community respiration rates at 3 and 14 m depth were 2.01 and 0.63 µmol L$^{-1}$ h$^{-1}$, respectively.

### 3.2 Active water column N$_2$O Production

The anoxic control experiment (anoxic condition with 5 µmol L$^{-1}$ 15NO$_3^-$ or 15NO$_2^-$) was used to demonstrate active N$_2$O production: in July 2016, at the top of the anoxic layer (~ 12.3 m), rates of N$_2$O production from NO$_2^-$ and NO$_3^-$ reduction were 5.42 ± 0.35 and 2.04 ± 0.86 nmol-NL$^{-1}$ h$^{-1}$, respectively (Fig. 2). In November 2016, at 17 m within the oxygenated water column ([O$_2$] > 180 µmol L$^{-1}$), rates of N$_2$O production were 0.33 ± 0.01 and 0.95 ± 0.35 nmol-NL$^{-1}$ h$^{-1}$, respectively. In May 2017, no N$_2$O production was detected at 19.5 m.

The total N$_2$O production rate of 7.5 ± 1.2 nmol-NL$^{-1}$ h$^{-1}$ in July 2016 is lower than the measurements (18–77 nmol-NL$^{-1}$ h$^{-1}$) made 40 years ago in the Potomac River (McElroy et al., 1978), a tributary to the Chesapeake Bay. This difference could be due to much higher water column nutrients in the Potomac River (NO$_2^-$ plus NO$_3^-$ concentration > 30 µmol L$^{-1}$) at that time and presumably denser microbial populations because of sediment resuspension (4–10 m water depth). With added substrates (NO$_2^-$ and NO$_3^-$) being more than an order of magnitude higher than in situ levels in July 2016, and the anoxic conditions being used in the November 2016 experiments (in situ [O$_2$] > 180 µmol L$^{-1}$), N$_2$O production rates reported here are potential rates, which nevertheless highlight the potential for N$_2$O production in anoxic waters responding rapidly (within hours) to pulses of NO$_2^-$ or NO$_3^-$.

Based on the nirS gene abundance, the denitrifying population was more abundant in July than in November and was the smallest in May in the lower water column (14–19.5 m) of the Chesapeake Bay (Fig. 1g). In July the highest N$_2$O production rates from denitrification co-occurred with the highest nirS abundances (Fig. 2). While the water column oxygen rate of N$_2$O production from NO$_2^-$ reduction vs. N$_2$O production from NO$_3^-$ reduction positively correlates with the ratio of NO$_2^-$ : NO$_3^-$ concentrations (Fig. 3).

<table>
<thead>
<tr>
<th>Month</th>
<th>N$_2$O production (µmol L$^{-1}$ h$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>July 2016</td>
<td>7.5 ± 1.2</td>
</tr>
<tr>
<td>November 2016</td>
<td>0.33 ± 0.01</td>
</tr>
</tbody>
</table>

Figure 2. Abundances of nirS gene and rates of N$_2$O production from nitrate plus nitrite reduction at three sampling times. The nirS gene abundances were analyzed at 14.1, 17.0 and 19.5 m during July 2016, November 2016 and May 2017, respectively. The N$_2$O production rates were measured in the control experiment (helium-flushed anoxic incubation) at 12.3, 17.0 and 19.5 m during July 2016, November 2016 and May 2017, respectively.

### 3.3 N$_2$O production pathways regulated by availability of nitrogen substrate

The ratio of the rates of N$_2$O production from NO$_2^-$ reduction vs. N$_2$O production from NO$_3^-$ reduction positively correlates with the ratio of NO$_2^-$ : NO$_3^-$ concentrations (Fig. 3). This suggests increasing NO$_2^-$ or NO$_3^-$ availability favors N$_2$O production from the reduction of the respective substrate. At concentration ratios of NO$_2^-$ : NO$_3^-$ < 0.5, the ratios of rates were similar to the concentration ratio, 0.3 ± 0.2. At a concentration ratio of NO$_2^-$ : NO$_3^-$ = 1 : 1, the ratio of rates of N$_2$O production from respective substrates measured from replicate experiments varied from 0.6 to 2.6. At NO$_2^-$ : NO$_3^-$ = 10, the ratio of rates was greater than 10. Therefore, the primary nitrogen source of N$_2$O production...
via denitrification depends in part on the relative availability of the substrate (NO$_2^-$ or NO$_3^-$).

As denitrification is a stepwise enzymatic reduction from NO$_3^-$, NO$_2^-$, NO, N$_2$O to N$_2$, the pathway can be somewhat modular (Graf et al., 2014); i.e., many organisms possess only one or a few steps, rather than the complete pathway. In complete denitrifiers (organisms capable of reducing NO$_3^-$ to N$_2$), the degree to which intermediates (i.e., NO$_2^-$) exchange across cellular membranes with the ambient environment is unknown (Moir and Wood, 2001). We use data from the DIN manipulation experiment (conducted in July 2016) to show that full exchange between intracellular and ambient NO$_2^-$ during NO$_3^-$ reduction to N$_2$O is unlikely, as explained below.

The conditions and results from experiment 2-H (Table 1) were used because this experiment had the highest ambient NO$_2^-$ pool; an exchange between the pools could be easily detected. During NO$_3^-$ reduction to N$_2$O, if denitrifiers reduce $^{15}$NO$_3^-$ (total 1.2 µmol L$^{-1}$, $^{15}$N fraction labeled 0.16) to $^{15}$NO$_2^-$ at the maximal rate (0.2 µmol-NL$^{-1}$h$^{-1}$; see Sect. 3.4) and the product fully exchanges with the ambient $^{14}$NO$_3^-$ (10 µmol L$^{-1}$, $^{15}$N fraction labeled 0.0037), after 2 h, the $^{15}$N addition to the total NO$_2^-$ pool will be 0.064 µmol L$^{-1}$:

$$\text{(Rate of NO}_2^-\text{ production from NO}_3^-\times \text{incubation time} \times \text{initial } ^{15}\text{N fraction of NO}_3^- )$$

$$= (0.2 \text{µmol-NL}^{-1} \text{h}^{-1} \times 2 \text{h} \times 0.16) = 0.064 \text{µmol L}^{-1} ;$$

and the resulting $^{15}$N fraction (unitless) of NO$_2^-$ will be 0.01:

$$\left( ^{15}\text{N addition to NO}_2^- + \text{inital } ^{15}\text{N fraction of NO}_2^- \times \text{initial concentration of NO}_2^- \right) / (\text{total concentration of NO}_2^- )$$

$$= (0.064 \text{µmol L}^{-1} + 0.0037 \times 10 \text{µmol L}^{-1}) / (10 + 0.064) \text{µmol L}^{-1} \approx 0.01 .$$

Assuming 6 nmol-NL$^{-1}$ h$^{-1}$ as the rate of N$_2$O production from NO$_2^-$ reduction (the NO$_2^-$ → N$_2$O rate shown in Fig. 3; $^{15}$N fraction of NO$_2^-$ = 0.01), and the initial N$_2$O concentration as 20 nmol L$^{-1}$ (described in Sect. 2.2; $^{15}$N fraction of N$_2$O = 0.0037), after 2 h, the resulting $^{15}$N fraction of N$_2$O will be 0.0052:

$$\left( ^{15}\text{N fraction of NO}_2^- \times \text{rate of N}_2\text{O production from NO}_2^- \times \text{incubation time} \right) + (\text{initial } ^{15}\text{N fraction of N}_2\text{O} \times \text{initial concentration of N}_2\text{O} \times \text{molar nitrogen in molar N}_2\text{O} ) / (\text{rate of N}_2\text{O production from NO}_2^- \times \text{incubation time} ) + (\text{initial concentration of N}_2\text{O} \times \text{molar nitrogen in molar N}_2\text{O} )$$

$$= ((0.01 \times 6 \text{nmol-NL}^{-1} \text{h}^{-1} \times 2 \text{h}) + (0.0037 \times 20 \text{nmol-N}_2\text{O} \text{L}^{-1} \times 2 \text{N}/\text{N}_2\text{O} ) ) / (6 \times 2 + 20 \times 2) \text{nmol-NL}^{-1} = 0.0052 .$$

The calculated $^{15}$N fraction of N$_2$O (0.0052) is much lower than the measured $^{15}$N fraction of N$_2$O (> 0.02) in experiment 2H. This means that full exchange of NO$_2^-$ during NO$_3^-$ reduction to N$_2$O, at maximum possible rates of NO$_3^-$ reduction to NO$_2^-$ and N$_2$O, would yield a rate of N$_2$O production from NO$_3^-$ much lower than observed in the experimental results. Thus, we concluded that the intracellular exchange of NO$_2^-$ during NO$_3^-$ reduction to N$_2$O by the denitrifying community in the Chesapeake Bay is limited. Such a tight coupling among nitrate reduction, nitrite reduction and nitric oxide reduction suggests the co-occurrence of the respective functional genes and enzymes in the cell of nitrate reducers. Both dissimilatory nitrate and nitrite reducers are able to produce N$_2$O independently, so total N$_2$O production can be quantified accurately by separate measurement of NO$_3^-$ and NO$_2^-$ reduction.

3.4 Oxygen inhibits N$_2$O production by denitrification

The sensitivities to increasing [O$_2$] of NO$_2^-$ reduction and NO$_3^-$ reduction to N$_2$O were evaluated in samples from July and November 2016 (Fig. 4). The control experiments (anoxic incubation; see Sect. 3.2) in July 2016 and November 2016 showed rates of N$_2$O production from denitrification of 7.5 ± 1.2 and 1.28 ± 0.35 nmol-NL$^{-1}$ h$^{-1}$, respectively. Increasing [O$_2$] generally decreased N$_2$O production rates from denitrification. In July 2016, under
[O₂] = 0.3 µmol L⁻¹, N₂O production from NO₃⁻ reduction decreased from 5.4 to 2.5 nmol-N L⁻¹ h⁻¹, whereas the rate of NO₃⁻ reduction to N₂O increased from 2.0 to 3.5 nmol-N L⁻¹ h⁻¹. Further increase in [O₂], up to 6.4 µmol L⁻¹, did not fully inhibit N₂O production from NO₂⁻ reduction, the rate of which was 0.08 nmol-N L⁻¹ h⁻¹. However, N₂O production from NO₃⁻ reduction was completely inhibited when [O₂] > 0.6 µmol L⁻¹ (Fig. 4a). In November 2016, increasing [O₂] gradually decreased rates of NO₃⁻ reduction to N₂O; no rates were detected when [O₂] > 2 µmol L⁻¹. Rates of NO₃⁻ reduction to N₂O were not detected at [O₂] > 0 µmol L⁻¹ (Fig. 4b).

Rates of NO₃⁻ reduction to NO₂⁻ under increasing [O₂] were also measured in July 2016 to supplement the sensitivity analysis of denitrification to oxygen. The rate of NO₃⁻ reduction to NO₂⁻ was 100 nmol-N L⁻¹ h⁻¹ under anoxic condition. At [O₂] = 0.3 µmol L⁻¹, the rate doubled to 200 nmol-N L⁻¹ h⁻¹ (Fig. 4). Further increase in [O₂] significantly decreased the rate of NO₃⁻ reduction to NO₂⁻. However, at [O₂] = 6.4 µmol L⁻¹ NO₃⁻ reduction to NO₂⁻ was still detectable at 0.82 ± 0.06 nmol-N L⁻¹ h⁻¹ (Fig. 5).

These results suggest that oxygenation of the water column in the Chesapeake Bay, even micro-molar level oxygen, would significantly mitigate N₂O production from denitrification. Both July 2016 and November 2016 data showed the difference in the effect of oxygen on N₂O production from NO₂⁻ vs. NO₃⁻ reduction. Samples from July 2016 showed 98 % and complete inhibition on N₂O production from NO₂⁻ and NO₃⁻ reduction at [O₂] = 6 µmol L⁻¹, respectively. The November 2016 samples showed 94 % and complete inhibition on N₂O production from NO₂⁻ and NO₃⁻ reduction at [O₂] = 0.4 µmol L⁻¹, respectively. Furthermore, N₂O production in the Chesapeake Bay was likely attributed to both heterotrophic denitrification and nitrifier denitrification. Studies have shown that both nitrifiers and denitrifiers are present in the Chesapeake Bay (Bouskill et al., 2012; Hong et al., 2014) and they are capable of NO₂⁻ reduction to N₂O, whereas NO₃⁻ reduction to N₂O is solely mediated by heterotrophic denitrifiers. N₂O production via nitrifier denitrification occurs under the full range of oxygen environments in agricultural soil (Zhu et al., 2013) and the open ocean (Wilson et al., 2014). Partial denitrification (NO₃⁻ reduction to N₂O), however, is moderately oxygen sensitive. Thus, increasing oxygen inhibits the activities of denitrifiers, as demonstrated in decreasing rates of NO₃⁻ reduction to N₂O (Fig. 5) and NO₃⁻ reduction to NO₂⁻ (Fig. 5). Increasing oxygen does not completely inhibit N₂O production activity of nitrifiers but probably lowers the N₂O production rates by nitrifier denitrification.
4 Conclusion and outlook

The Chesapeake Bay is a potential N2O source via denitrification when NO3− and NO2− are present under anoxic conditions. Relative rates of NO3− and NO2− reduction to N2O were positively correlated with relative concentrations of NO3− and NO2−. Increased oxygen, either by natural water column oxygenation or by experimental manipulation, decreased N2O production rates via denitrification. The size of the denitrifying community increased with increasing rates of N2O production via denitrification. The potential N2O production in the summertime suggests that intermittent N2O efflux to the atmosphere could occur when a shallow oxic–anoxic interface (typically 10–15 m) is present (Taft et al., 1980; Kemp et al., 1992; Lee et al., 2015a), as well as frequent disturbance of water column stratification by storm events, boat traffic and surface cooling. The seasonal variation of surface and water column N2O saturation levels (air undersaturated in May and air oversaturated in November) and the detection of significant N2O production in July (summer) when N2O concentrations were the lowest imply that N2O consumption was also occurring in the Chesapeake Bay and probably minimizing N2O efflux to the atmosphere. A long-term, comprehensive survey with wide spatial coverage will help (i) assess if the Chesapeake Bay is a net N2O source or sink on an annual scale and (ii) to investigate the physical, chemical and biological controls of N2O emission in the Chesapeake Bay.

Denitrification is critical for complete removal of fixed nitrogen so as to mitigate eutrophication in natural waters. The N2O production rates could serve as a proxy for estimating nitrogen loss. It is estimated that 1% of total denitrified nitrogen is converted to N2O in river networks (Beaulieu et al., 2011) so the ratio of N2O : N2 during denitrification is 1 : 100. Assuming that N2O production occurs at a rate of 7 nmol-N L−1 h−1 within 0.2 m of the oxic–anoxic interface in summertime (based on the July 2016 control data, N2O production from NO3− plus NO2−), denitrification yields a potential water column nitrogen removal rate of 140 µmol-N m−2 h−1, or 0.24 mg-N m−2 d−1. In addition, the sediment in the bay is capable of anaerobic ammonia oxidation (Rich et al., 2008) and denitrification (Kemp et al., 1990; Kana et al., 2006). Total sedimentary N2 production, measured by the acetylene block reduction method (Kemp et al., 1990) and N2 accumulation method (Kana et al., 2006), recorded areal rates of 50–70 µmol-N m−2 h−1. Therefore, expansion of anoxia in the Chesapeake Bay could increase the potential of biological nitrogen removal by the sediment–water system that counteracts the increase in nitrogen loading from anthropogenic activities.

The oxidation of NH4+, although not the focus of this study, is a possible pathway for N2O production under low-oxygen conditions (Anderson, 1964). The yield of N2O (molar ratio of N2O production to NH4+ oxidation) increases with decreasing oxygen (Goreau et al., 1980). Culture (Qin et al., 2017) and field studies (Bristow et al., 2016; Peng et al., 2016) have shown high affinity of oxygen (≤ 5 µmol L−1) during NH4+ oxidation. The main sources of NH4+ in the Chesapeake Bay include remineralization of organic matter in the oxygenated water column and sediments (Kemp et al., 1990) and atmospheric deposition (Larsen et al., 2001). Onset of NH4+ oxidation is viable at NH4+ concentration < 100 nmol L−1 by the natural ammonia-oxidizing community (Horak et al., 2013). Thus, N2O production from NH4+ oxidation might be stimulated under low-oxygen condition by influx of ammonium near the oxic–anoxic interface, which deserves future research efforts.

The inhibition of N2O production by oxygen highlights the positive outcomes of reoxygenation of the Chesapeake Bay. Since the late 20th century, the Chesapeake Bay has received increased anthropogenic nitrogen loading from various sources including fertilizer (Groffman et al., 2009), untreated sewage (Kaplan et al., 1978) and atmospheric deposition (Russell et al., 1998; Loughner et al., 2016). Fueled by increased nitrogen input, elevated primary production in the surface layer stimulates aerobic remineralization at depth, which consumes oxygen rapidly. In summertime, water column stratification restricts influx of oxygen to depth, creating seasonal anoxia/hypoxia in the bay. The documented eutrophication and expansion of anoxia/hypoxia in the Chesapeake Bay in the late 20th century attracted public attention because of increasing mortality of organisms with high commercial and recreational value (Cooper and Brush, 1993). Moreover, expansion of the volume of low-oxygen waters will result in more “hot spots” for N2O production. The key factor for mitigating anoxia is to control the nitrogen input to the bay (Hagy et al., 2004; Zhou et al., 2014). Effective fertilizer application, sewage treatment, natural nitrogen removal by denitrification and anammox and plant uptake have been successfully enforced to control the nitrogen runoff into the bay from the tributaries (Boesch et al., 2001). The near absence of summertime water column NO3− + NO2− concentrations close to the central Chesapeake Bay as shown in this study and others (Lee et al., 2015a) could prevent N2O production. Reducing the nitrogen input into the Chesapeake Bay will help mitigate N2O efflux: In the short term (timescale of days to months), nitrogen sources (NH4+, NO3− and NO2−) for N2O production will be decreased. In the long run (inter-annual timescale), eutrophication will be alleviated, which will reoxygenate the water column, and inhibit N2O production.

Data availability. All data presented in this manuscript can be found in the Supplement.

The Supplement related to this article is available online at: https://doi.org/10.5194/bg-15-6127-2018-supplement
Author contributions. QJ, CF, AJ, JCC and BBW developed the experimental design. QJ, CF, XS, MJ, YSL, AJ and JCC conducted the field experiments. QJ, CF, XS, MJ and AJ conducted the sample analysis. QJ, CF, XS, MJ, AJ, JCC and BBW wrote the paper.

Competing interests. The authors declare that they have no conflict of interest.

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