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 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 (\textsuperscript{15}N) were used to investigate the geochemical factors controlling N\textsubscript{2}O production in the Chesapeake Bay, the largest estuary in North America. The highest potential rates of water column N\textsubscript{2}O production (7.5±1.2 nmol-N L\textsuperscript{-1} hr\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 µM 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 (time scale of years), eutrophication will be alleviated and subsequent re-oxygenation 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 21\textsuperscript{st} century (Ravishankara et al., 2009).
Microbial processes are responsible for the majority of $\text{N}_2\text{O}$ 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, $\text{N}_2\text{O}$ can be produced as a by-product during autotrophic aerobic ammonium ($\text{NH}_4^+$) oxidation to nitrite ($\text{NO}_2^-$) by bacteria (Arp and Stein, 2003) and archaea (Santoro et al., 2011). The production of $\text{N}_2\text{O}$ can also occur via $\text{NO}_2^-$ 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 $\text{N}_2\text{O}$ via enzyme-mediated heterotrophic denitrification, which consists of the stepwise reduction of nitrate ($\text{NO}_3^-$), $\text{NO}_2^-$ and nitric oxide (NO), with organic matter as the electron donor. The $\text{nirS}$ gene that encodes the genetic material for nitrite reductase (the enzyme mediating $\text{NO}_2^-$ 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). $\text{N}_2\text{O}$ is not produced via anaerobic ammonium oxidation (anammox), another important nitrogen removal process in the natural environment (Kartal et al., 2011).

The increase of atmospheric $\text{N}_2\text{O}$ 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 $\text{N}_2\text{O}$ 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 $\text{N}_2\text{O}$ 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 the 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 $\text{N}_2\text{O}$ (indicated by surface $\text{N}_2\text{O}$ 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 ~ 10 m), depletion of $\text{NO}_3^-$ and $\text{NO}_2^-$, and accumulation of $\text{NH}_4^+$ 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 N₂O production pathway and the associated environmental controlling factors have not been investigated.

Here we report a pilot study using nitrogen stable isotope (¹⁵N) incubation experiments to quantify N₂O production rates and their dependence on the availabilities of oxygen, NO₃⁻ and NO₂⁻ in the Chesapeake Bay. Because seasonal anoxia occurs at the study site in the central region of the Chesapeake Bay, reductive pathways of N₂O production (i.e. reduction of NO₃⁻ and NO₂⁻) are the main focus. Further understanding of the environmental controls on N₂O production in estuaries will facilitate the design of effective environmental engineering projects to mitigate N₂O emission.

2 Methods

2.1 Sample acquisition and processing

Sampling and incubation experiments were carried out on July 19, 2016, November 17, 2016 and May 3, 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 oxygen 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 ~ 5 μmol L⁻¹. Samples for NO₂⁻ and NO₃⁻ concentration measurements were filtered (0.22 μm poresize, Sterivex-GP, EMD Millipore) and frozen at -80 °C until analysis. Discrete samples for N₂O concentration were collected directly from the pump outlet into the bottom of acid washed, 60 mL glass serum bottles (Catalog # 223745, Wheaton, Millville, NJ). Bottles were sealed with butyl rubber stoppers (Catalog # W224100-202, Wheaton, Millville, NJ) and aluminium rings while submerged under water pumped from depth to avoid atmospheric N₂O and oxygen contamination. Samples for characterizing N₂O concentration profile were preserved immediately after filling by injecting 0.1 mL saturated HgCl₂. Samples for N₂O incubation experiments (section 2.2) were acquired from 12 m, 17 m and 19.5 m during July 2016, November 2016 and May 2017, respectively, and sealed the same way as described above for discrete N₂O concentration samples, and stored in the dark at 4°C without adding HgCl₂. Samples for denitrifying nirS gene abundance were collected at 14, 17 and 19.5 m by filtering
600mL - 2000mL of water through 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=[H₂CO₃]+[HCO₃⁻]+[CO₃²⁻]) and community respiration rates were collected only in July 2016. The DIC samples were preserved with mercuric chloride (HgCl₂) for initial conditions, while biochemical oxygen demand (BOD) bottles were incubated in a temperature-controlled environmental chamber (±1 °C of in situ water temperatures). After 24 h, samples were siphoned from the vials, preserved with HgCl₂, and respiration rates were determined as the difference in DIC between initial and final samples divided by 24 hours (Lee et al., 2015b).

2.2 ¹⁵N incubation experiments for N₂O production

Within 3 hours 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.

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 minutes to minimize oxygen contamination from sampling and transportation. Two suites of ¹⁵N tracer solutions (¹⁵NO₂⁻ plus ¹⁴NO₃⁻, ¹⁵NO₃⁻ plus ¹⁴NO₂⁻, 0.1mL) were injected to achieve final concentrations of 5 μmol L⁻¹ NO₂⁻ and NO₃⁻ (see conditions for experiment 1-A and 1-B, 4-A and 4-B, 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 (Weiss and Price, 1980) with 3 mL headspace and 57 mL water). Initial conditions (one bottle for each time course) were sampled within 30 minutes of tracer addition by injecting 0.1 mL saturated HgCl₂. Incubations lasted ~2 hours at in situ temperature (±0.5 °C), during which duplicate bottles were preserved with saturated HgCl₂ solution every 40 to 60 minutes, 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 NO₂⁻ and NO₃⁻ were absent from the water column (see section 3.1). A 3 mL headspace was created before flushing with helium for 10 min to establish anoxic condition. Then, ~1.2 nmol N₂O was injected to reach a concentration of ~20 nmol L⁻¹ in the water phase. Two suites of ¹⁵N tracer solutions (¹⁵NO₂⁻ plus ¹⁴NO₃⁻, ¹⁵NO₃⁻ plus ¹⁴NO₂⁻, 0.1 mL of total volume of
tracer addition) were injected to designated bottles to achieve ratios of NO$_2^-$ : NO$_3^-$ ≈ 1:10, 1:3, 3:1 and 10:1, with $^{15}$N fraction labelled between 0.016 and 0.16 (Table 1, experiment 2-A to 2-H). This allows 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 ~ 2 hours 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 minutes. 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 ~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 seawater (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, ~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$_2^-$, 0.1mL) were injected to achieve 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 are shown in Table 1. Incubations lasted ~ 2 hours 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/NOx Analyzer (Teledyne API, San Diego, CA) (Garside, 1982; Braman and Hendrix, 1989). DIC was measured with an automated infrared analyzer (Apollo SciTech, Newark, DE) as previously reported (Lee et al., 2015b). Preserved N$_2$O samples were stored in the dark at room temperature (~22 °C) for less than three 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 a Delta V$^+$ mass spectrometer (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 \(\text{N}_2\text{O}\) was calibrated with standard \(\text{N}_2\text{O}\) vials, which were made by injecting 1, 2, or 5 nmol \(\text{N}_2\text{O}-\text{N}\) into 20 mL glass vials (Catalog # C4020-25, Thermo Fisher Scientific, Waltham, MA).

After \(\text{N}_2\text{O}\) analysis, samples incubated with \(^{15}\text{NO}_3^-\) were also assayed for \(^{15}\text{NO}_2^-\) to determine rates of \(\text{NO}_3^-\) reduction. Two millilitres 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 \(^{15}\text{NO}_2^-\) was converted to \(\text{N}_2\text{O}\) using the acetic acid-treated sodium azide solution for quantitative conversion (McIlvin and Altabet, 2005). Resulting \(\text{N}_2\text{O}\) was measured on the Delta V\(^{\text{Plus}}\) for nitrogen isotope ratio so as to determine the \(^{15}\text{N}\) enrichment of \(\text{NO}_2^-\).

For the analysis of \(\text{nirS}\) gene abundance, DNA extraction and qPCR for the \(\text{nirS}\) 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 5 different dilutions of a \(\text{nirS}\) 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

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

\[
R = \frac{1}{F} \times \left( \frac{d^{45}\text{N}_2\text{O}}{dt} + 2 \times \frac{d^{46}\text{N}_2\text{O}}{dt} \right)
\]  

where \(d^{45}\text{N}_2\text{O}/dt\) and \(d^{46}\text{N}_2\text{O}/dt\) represent the production rates (nmol-N L\(^{-1}\) hr\(^{-1}\)) of mass 45 and 46 \(\text{N}_2\text{O}\) during incubation. \(F\) represents the \(^{15}\text{N}\) fraction in the initial substrate (\(\text{NO}_2^-\) or \(\text{NO}_3^-\)). Rates were considered significant based on the linear regression of the time course data \((p < 0.05, n=7, \text{student t-test})\). The detection limit for \(\text{N}_2\text{O}\) production is 0.002 nmol-N L\(^{-1}\) hr\(^{-1}\). The \(^{15}\text{N}\) incubation experiments can identify the pathway but cannot distinguish the relative contributions of two or more
functioning microbial groups to a single N₂O production pathway (i.e. N₂O production via NO₂⁻ reduction by nitrifier
denitrification and/or heterotrophic denitrification).

The rate of NO₃⁻ reduction to NO₂⁻ was calculated as

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

(2)

where \( d^{15}\text{NO}_2^-/dt \) represents the production rate of \(^{15}\text{NO}_2^- \) (nmol L\(^{-1}\) hr\(^{-1}\)), which is calculated as the slope of \(^{15}\text{NO}_2^- \) concentrations versus time. \( F \) represents initial substrate \(^{15}\text{NO}_3^- \) enrichment. Rates were considered significant based on linear regression of the time course data (\( p<0.05 \), student’s t-test). The detection limit for NO₂⁻ production is 0.05 nmol N L\(^{-1}\) hr\(^{-1}\).

3 Results and discussion

3.1 Water column features

The physical and chemical properties of the water column in central Chesapeake Bay experience 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 1b). 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 to 10 m (Fig. 1c), with a sharp oxycline (~ 30 μmol L\(^{-1}\) m\(^{-1}\)). Below 10 m, the oxygen concentration was below detection of the sensor (~ 5 μmol L\(^{-1}\)) and was likely anoxic. However, sulphide compounds were most likely not present in July at depth; the water samples were free of any hydrogen sulphide odor. 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 N₂O saturation values in July, November and May were 6.6, 10.4 and 12.0 nmol L\(^{-1}\), respectively. In July, N₂O concentration was close to air-saturation level (6.6 nmol L\(^{-1}\)) at the surface layer. In the low oxygen layer (below 12 m), N₂O was apparently undersaturated (2.0 – 3.7 nmol L\(^{-1}\), 20 – 50 % air-saturation, Fig. 1d). In November, the surface N₂O concentration was slightly oversaturated (11.3 nmol L\(^{-1}\), 108 % air-saturation). N₂O concentrations at depth were oversaturated; the concentrations varied between 11.0 and 11.5 nmol L\(^{-1}\), 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 1f) in July were below 0.02 μmol L\textsuperscript{−1} within the sampling depth interval (top 17 m of water column). Measureable 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 of water column NO\textsubscript{3}\textsuperscript{−} and NO\textsubscript{2}\textsuperscript{−} concentrations was likely due to increased runoff from the anthropogenically influenced watershed. Water column depletion of NO\textsubscript{3}\textsuperscript{−} and NO\textsubscript{2}\textsuperscript{−} in the summer is the result of denitrification (Baird et al., 1995; Boynton et al., 1995), which indicates potential water column N\textsubscript{2}O production via denitrification (discussed in section 3.2).

As a proxy for the size of the denitrifying community, the abundance of the nir\textsubscript{S} gene was (5.91 ± 0.1) × 10\textsuperscript{4} copy mL\textsuperscript{−1} at 14 m in July, which was the highest among the three sampling trips (Fig. 1g). Lowest nir\textsubscript{S} gene abundance (9.1 ± 1.3) × 10\textsuperscript{3} copy mL\textsuperscript{−1} was observed in May at 19.5 m. The abundance of nir\textsubscript{S} was measured only at the depths at which incubations were performed, and the nir\textsubscript{S} abundance increased with increasing rates of N\textsubscript{2}O production (see section 3.2). In July 2016, water column DIC concentrations ranged from 1,377 to 1,831 μmol L\textsuperscript{−1}, with the highest concentrations below 10 m. Average community respiration rates at 3 m and 14 m depth were 2.01 and 0.63 μmol L\textsuperscript{−1} hr\textsuperscript{−1}, respectively.

### 3.2 Active water column N\textsubscript{2}O production

The anoxic control experiment (anoxic condition with 5 μmol L\textsuperscript{−1} NO\textsubscript{2}\textsuperscript{−} or NO\textsubscript{3}\textsuperscript{−}) was used to demonstrate active N\textsubscript{2}O production: In July 2016, at the top of anoxic layer (~ 12.3 m), rates of N\textsubscript{2}O production from NO\textsubscript{2}\textsuperscript{−} and NO\textsubscript{3}\textsuperscript{−} reduction were 5.42±0.35 and 2.04±0.86 nmol-N L\textsuperscript{−1} hr\textsuperscript{−1}, respectively (Fig. 2). In November 2016, at 17 m within the oxygenated water column ([O\textsubscript{2}] > 180 μmol L\textsuperscript{−1}), rates of N\textsubscript{2}O production were 0.33±0.01 and 0.95±0.35 nmol-N L\textsuperscript{−1} hr\textsuperscript{−1}, respectively. In May 2017, no N\textsubscript{2}O production was detected at 19.5 m.
The total N\textsubscript{2}O production rate of 7.5±1.2 nmol-N L\textsuperscript{-1} hr\textsuperscript{-1} in July 2016 is lower than the measurements (18 – 77 nmol-N L\textsuperscript{-1} hr\textsuperscript{-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 (\textit{NO}\textsubscript{2}\textsuperscript{-} plus \textit{NO}\textsubscript{3}\textsuperscript{-} concentration > 30 \textmu mol L\textsuperscript{-1}) at that time, and presumably denser microbial populations because of sediment resuspension (4 – 10 m water depth). With added substrates (\textit{NO}\textsubscript{2}\textsuperscript{-} and \textit{NO}\textsubscript{3}\textsuperscript{-}) being more than an order of magnitude higher than \textit{in situ} levels in July 2016, and the anoxic conditions being used in the November 2016 experiments (\textit{in situ} [\textit{O}\textsubscript{2}] > 180 \textmu mol L\textsuperscript{-1}), \textit{N}\textsubscript{2}O production rates reported here are potential rates, which nevertheless highlight the potential for \textit{N}\textsubscript{2}O production in anoxic waters responding rapidly (within hours) to pulses of \textit{NO}\textsubscript{2}\textsuperscript{-} or \textit{NO}\textsubscript{3}\textsuperscript{-}.

Based on the \textit{nirS} gene abundance, the denitrifying population was more abundant in July (summer) than November (autumn), and was the smallest in May (spring) in the lower water column (14 – 19.5 m) of the Chesapeake Bay (Fig. 1g). In July highest \textit{N}\textsubscript{2}O production rates co-occurred with the highest \textit{nirS} abundances (Fig. 2). While the water column oxygen in November was > 180 \textmu mol L\textsuperscript{-1}, the \textit{nirS} gene abundance supported potential denitrification at a \textit{N}\textsubscript{2}O production rate of 1.28 ± 0.35 nmol-N L\textsuperscript{-1} hr\textsuperscript{-1} in anoxic incubation experiments. In May when hypoxic conditions had not yet developed, no \textit{N}\textsubscript{2}O production was detected, and the \textit{nirS} abundance (9.1 \times 10\textsuperscript{3} copies mL\textsuperscript{-1}) was the lowest among three sample dates. It is likely that the denitrifying community did not recover from oxygen inhibition during the 2-hour anoxic incubation. A metatranscriptome analysis showed that the transcript ratios for denitrification were the lowest in June before the onset of hypoxia, and highest ratios in August when anoxia was most pronounced (Eggleston et al., 2015).

### 3.3 \textit{N}\textsubscript{2}O production pathways regulated by availability of nitrogen substrate

The ratio of the rates of \textit{N}\textsubscript{2}O production from \textit{NO}\textsubscript{2}\textsuperscript{-} reduction vs. \textit{N}\textsubscript{2}O production from \textit{NO}\textsubscript{3}\textsuperscript{-} reduction positively correlates with the ratio of \textit{NO}\textsubscript{2}\textsuperscript{-} : \textit{NO}\textsubscript{3}\textsuperscript{-} concentrations (Fig. 3). This suggests increasing \textit{NO}\textsubscript{2}\textsuperscript{-} or \textit{NO}\textsubscript{3}\textsuperscript{-} availability favours \textit{N}\textsubscript{2}O production from the reduction of the respective substrate. At concentration ratios of \textit{NO}\textsubscript{2}\textsuperscript{-} : \textit{NO}\textsubscript{3}\textsuperscript{-} < 0.5, the ratios of rates were similar to the concentration ratio, 0.3±0.2. At a concentration ratio of \textit{NO}\textsubscript{2}\textsuperscript{-} : \textit{NO}\textsubscript{3}\textsuperscript{-} = 1 : 1, the ratio of rates of \textit{N}\textsubscript{2}O production from respective substrates measured from replicate experiments varied from 0.6 to 2.6. At \textit{NO}\textsubscript{2}\textsuperscript{-} : \textit{NO}\textsubscript{3}\textsuperscript{-} = 10, the ratio of rates was greater than 10. Therefore, the primary nitrogen source of \textit{N}\textsubscript{2}O production via denitrification depends in part on the relative availability of the substrate (\textit{NO}\textsubscript{2}\textsuperscript{-} or \textit{NO}\textsubscript{3}\textsuperscript{-}).
As denitrification is a step-wise 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 and 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 maximal rate (0.2 µmol-N L$^{-1}$ hr$^{-1}$, see section 3.4) and the product fully exchanges with the ambient $^{14}$NO$_2^-$ (10 µmol L$^{-1}$, $^{15}$N fraction labeled 0.0037), after 2 hours, the $^{15}$N addition to the total NO$_2^-$ pool will be 0.064 µmol L$^{-1}$:

\[ \text{Rate of NO}_2^- \text{ production from NO}_3^- \times \text{incubation time} \times \text{initial fraction labelled of NO}_3^- \]

\[ = (0.2 \ \mu\text{mol-N L}^{-1} \times 2 \ \text{hr} \times 0.16) = 0.064 \ \mu\text{mol L}^{-1}, \]

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

\[ (^{15}\text{N addition to NO}_2^- \times \text{initial fraction labelled of NO}_2^- \times \text{initial concentration of NO}_2^-) / (\text{total concentration of NO}_2^-) \]

\[ = (0.064 \ \mu\text{mol L}^{-1} + 0.0037 \times 10 \ \mu\text{mol L}^{-1}) / (10 + 0.064) \ \mu\text{mol L}^{-1} \approx 0.01. \]

Assuming 6 nmol-L$^{-1}$ hr$^{-1}$ as the rate of N$_2$O production from NO$_2^-$ reduction (the NO$_2^-$ $\rightarrow$ N$_2$O rate shown in fig. 3; $^{15}$N fraction labeled of NO$_2^-$ = 0.01), and the initial N$_2$O concentration as 20 nmol L$^{-1}$ (described in section 2.2; $^{15}$N fraction labeled of N$_2$O = 0.0037), after 2 hours, the resulting $^{15}$N fraction of N$_2$O will be 0.0052:

\[ (^{15}\text{N fraction labelled of NO}_2^- \times \text{rate of N}_2\text{O production from NO}_2^- \times \text{incubation time}) + (\text{initial fraction labelled of N}_2\text{O} \times \text{initial concentration of N}_2\text{O} \times \text{molar nitrogen in molar N}_2\text{O})) / ((\text{rate of N}_2\text{O production from NO}_2^- \times \text{incubation time}) + (\text{initial concentration of N}_2\text{O} \times \text{molar nitrogen in molar N}_2\text{O})) \]
\[(0.01 \times 6 \text{ nmol-N L}^{-1} \text{ hr}^{-1} \times 2 \text{ hr}) + (0.0037 \times 20 \text{ nmol-N}_2\text{O L}^{-1} \times 2N/N_2\text{O}) / (6 \times 2 + 20 \times 2) \text{ nmol-N L}^{-1} = 0.0052\]

The calculated \(^{15}\text{N}\) fraction of \(\text{N}_2\text{O}\) (0.0052) is much lower than the measured \(^{15}\text{N}\) fraction of \(\text{N}_2\text{O}\) (> 0.02) in experiment 2H. This means that full exchange of \(\text{NO}_2^-\) during \(\text{NO}_3^-\) reduction to \(\text{N}_2\text{O}\), at maximum possible rates of \(\text{NO}_3^-\) reduction to \(\text{NO}_2^-\) and \(\text{N}_2\text{O}\), would yield a rate of \(\text{N}_2\text{O}\) production from \(\text{NO}_3^-\) much lower than observed in the experimental results. Thus, we concluded that the intracellular exchange of \(\text{NO}_2^-\) during \(\text{NO}_3^-\) reduction to \(\text{N}_2\text{O}\) by the denitrifying community in 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 \(\text{N}_2\text{O}\) independently, so total \(\text{N}_2\text{O}\) production can be quantified accurately by separate measurement of \(\text{NO}_3^-\) and \(\text{NO}_2^-\) reduction.

### 3.4 Oxygen inhibits \(\text{N}_2\text{O}\) production by denitrification

The sensitivities to increasing \([\text{O}_2]\) of \(\text{NO}_2^-\) reduction and \(\text{NO}_3^-\) reduction to \(\text{N}_2\text{O}\) were evaluated in samples from July and November 2016 (Fig. 4). The control experiment (anoxic incubation, see Section 3.2) showed a total \(\text{N}_2\text{O}\) production rate (from \(\text{NO}_2^-\) plus \(\text{NO}_3^-\) reduction) of 7.5±1.2 and 1.28 ± 0.35 nmol-N L\(^{-1}\) hr\(^{-1}\) during July 2016 and November 2016, respectively. Increasing \([\text{O}_2]\) generally decreased \(\text{N}_2\text{O}\) production rates from denitrification. In July 2016, under \([\text{O}_2] = 0.3 \ \mu\text{mol L}^{-1}\), \(\text{N}_2\text{O}\) production from \(\text{NO}_2^-\) reduction decreased from 5.4 to 2.5 nmol-N L\(^{-1}\) hr\(^{-1}\), whereas the rate of \(\text{NO}_3^-\) reduction to \(\text{N}_2\text{O}\) increased from 2.0 to 3.5 nmol-N L\(^{-1}\) hr\(^{-1}\). Further increase in \([\text{O}_2]\), up to 6.4 \ \mu\text{mol L}^{-1}, significantly inhibited the rate of \(\text{N}_2\text{O}\) production from both \(\text{NO}_2^-\) and \(\text{NO}_3^-\) reduction (Fig. 4a). Note that 6 \ \mu\text{mol L}^{-1} \ [\text{O}_2] did not fully inhibit \(\text{N}_2\text{O}\) production from \(\text{NO}_2^-\) reduction, the rate of which was 0.08 nmol-N L\(^{-1}\) hr\(^{-1}\). However, \(\text{N}_2\text{O}\) production from \(\text{NO}_3^-\) reduction was completely inhibited when \([\text{O}_2] > 0.6 \ \mu\text{mol L}^{-1}\). Similar to results from July 2016, in November 2016, increasing \([\text{O}_2]\) gradually decreased rates of \(\text{NO}_2^-\) reduction to \(\text{N}_2\text{O}\); no rates were detected when \([\text{O}_2] > 2 \ \mu\text{mol L}^{-1}\). Rates of \(\text{NO}_3^-\) reduction to \(\text{N}_2\text{O}\) were not detected at \([\text{O}_2] > 0 \ \mu\text{mol L}^{-1}\) (Fig. 4b).

Rate of \(\text{NO}_3^-\) reduction to \(\text{NO}_2^-\) was also measured in July 2016 to supplement the sensitivity analysis of denitrification to oxygen. The rate of \(\text{NO}_3^-\) reduction to \(\text{NO}_2^-\) was 100 nmol L\(^{-1}\) hr\(^{-1}\) under anoxic condition. At \([\text{O}_2] = 0.3 \ \mu\text{mol L}^{-1}\), the rate
doubled, to 200 nmol-N L⁻¹ hr⁻¹ (Fig. 4). Further increase of [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 hr⁻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. 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. 3) 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 availabilities, either by natural water column oxygenation or by experimental manipulation, caused 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), and 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 N\textsubscript{2}O production in July (summer) when N\textsubscript{2}O concentrations were the lowest imply that N\textsubscript{2}O consumption was also occurring in the Chesapeake Bay and probably minimizing N\textsubscript{2}O efflux to the atmosphere. A long-term, comprehensive survey with wide spatial coverage will help assess if the Chesapeake Bay is a net N\textsubscript{2}O source or sink on an annual scale, and to investigate the physical, chemical and biological controls of N\textsubscript{2}O emission in the Chesapeake Bay.

Denitrification is critical for complete removal of fixed nitrogen so as to mitigate eutrophication in natural waters. The N\textsubscript{2}O production rates could serve as a proxy for estimating nitrogen loss. It is estimated that 1% of total denitrified nitrogen is converted to N\textsubscript{2}O in river networks (Beaulieu et al., 2011) so the ratio of N\textsubscript{2}O : N\textsubscript{2} during denitrification = 1 : 100. Assuming that N\textsubscript{2}O production occurs at a rate of 7 nmol-N L\textsuperscript{-1} hr\textsuperscript{-1} within 0.2 m of the oxic-anoxic interface in summertime (based on the July 2016 control data, N\textsubscript{2}O production from NO\textsubscript{3}\textsuperscript{-} plus NO\textsubscript{2}\textsuperscript{-}), denitrification yields a potential water column N removal rate of 140 μmol-N m\textsuperscript{-2} hr\textsuperscript{-1}, or 0.24 mg-N m\textsuperscript{-2} d\textsuperscript{-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 N\textsubscript{2} production, measured by the acetylene block reduction method (Kemp et al., 1990) and N\textsubscript{2} accumulation method (Kana et al., 2006) recorded areal rates of 50 – 70 μmol-N m\textsuperscript{-2} hr\textsuperscript{-1}. Therefore, the sediment-water system in the Chesapeake Bay is effective in biological nitrogen removal.

The oxidation of NH\textsubscript{4}\textsuperscript{+}, although not the focus of this study, is a possible pathway for N\textsubscript{2}O production under low oxygen conditions (Anderson, 1964). The yield of N\textsubscript{2}O (molar ratio of N\textsubscript{2}O production to NH\textsubscript{4}\textsuperscript{+} 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\textsuperscript{-1}) during NH\textsubscript{4}\textsuperscript{+} oxidation. The main sources of NH\textsubscript{4}\textsuperscript{+} 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 NH\textsubscript{4}\textsuperscript{+} oxidation is viable at NH\textsubscript{4}\textsuperscript{+} concentration < 100 nmol L\textsuperscript{-1} by the natural ammonia oxidizing community (Horak et al., 2013). Thus, N\textsubscript{2}O production from NH\textsubscript{4}\textsuperscript{+} oxidation might be stimulated under low oxygen conditions by influx of ammonium near the oxic-anoxic interface, which deserves future research efforts.

The inhibition of N\textsubscript{2}O production by oxygen highlights the positive outcomes of re-oxygenation of the Chesapeake Bay. Since the late 20\textsuperscript{th} century, 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 of 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/anammox, and plant uptake have been successfully enforced to control the nitrogen runoff into the bay from the tributaries (Boesch et al., 2001; Program, 2017). The near absence of summertime water column NO₂⁻ + NO₃⁻ concentrations near the middle of 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 (time scale of days to months), nitrogen sources (NH₄⁺, NO₂⁻ and NO₃⁻) for N₂O production will be decreased. In the long run (inter-annual time scale), eutrophication will be alleviated, which will re-oxygenate the water column, and inhibit N₂O production.
Figure 1: Depth profiles on three sampling dates, July 19, 2016 (filled square), November 17, 2016 (cross), May 3, 2017 (grey circle) of a) salinity, b) temperature, c) oxygen, d) nitrous oxide, e) nitrate, f) nitrite. Analysis of nirS gene abundance (g) was only conducted at one depth, at which incubations were also performed, during each trip.

Figure 2: Abundances of nirS gene and total N$_2$O production rates (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 total 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.
Figure 3: Ratio of rates of N$_2$O production from NO$_2^-$ reduction and NO$_3^-$ reduction plotted with the respective ratio of NO$_2^-$ to NO$_3^-$ concentration in the DIN manipulation experiment from July 2016 sampling. Log scale on both axes is for clarity at the low values.

Figure 4: Rates of N$_2$O production from NO$_2^-$ reduction (orange circles), NO$_3^-$ reduction (green squares) and combined NO$_2^-$ and NO$_3^-$ reduction (black diamonds) under increasing oxygen concentrations in July 2016 (a) and November 2016 (b). The standard deviation of rates in most of the samples were small so that error bars are not visible. Note the scale break at 2 μmol L$^{-1}$ O$_2$ on x-axis.
Figure 5: Rates of NO$_2^-$ production from NO$_3^-$ reduction under increasing oxygen concentrations. Error bar indicates the standard deviation of rates from linear regression of three time points (n=7).
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<td>5</td>
<td>0.54:1</td>
<td>0.50 (NO$_3^-$)</td>
<td>7.3</td>
</tr>
<tr>
<td>Control</td>
<td>6-A</td>
<td>5</td>
<td></td>
<td>0.4</td>
<td>11.3</td>
<td>0.48:1</td>
<td>0.93 (NO$_2^-$)</td>
<td>0</td>
</tr>
<tr>
<td>(May 2017)</td>
<td>6-B</td>
<td></td>
<td></td>
<td>5.4</td>
<td>6.3</td>
<td>0.48:1</td>
<td>0.44 (NO$_3^-$)</td>
<td>0</td>
</tr>
</tbody>
</table>

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 control experiment was conducted. The unit “μmol L$^{-1}$” is represented by “μM”. Shaded columns highlight the concentrations for $^{15}$N 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.
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6 References


