Variable contribution of wastewater treatment plant effluents to downstream nitrous oxide concentrations and emissions

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Abstract
Nitrous oxide (N₂O), a potent greenhouse gas and ozone-destroying agent, is produced during nitrogen transformations in both natural and human-constructed environments. Wastewater treatment plants (WWTPs) produce and emit N₂O into the atmosphere during the nitrogen removal process. However, the impact of WWTPs on N₂O emissions in downstream aquatic systems remains poorly constrained. By measuring N₂O concentrations at a monthly resolution over a year in the Potomac River Estuary, a tributary of Chesapeake Bay in the eastern United States, we found a strong seasonal variation in N₂O concentrations and fluxes: N₂O concentrations were larger in fall and winter but the flux was larger in summer and fall. Observations at multiple stations across the Potomac River Estuary revealed hotspots of N₂O emissions downstream of WWTPs. N₂O concentrations were higher at stations downstream of WWTPs compared to other stations (median: 21.2 nM vs 16.2 nM) despite the similar concentration of dissolved inorganic nitrogen, suggesting the direct discharge of N₂O from WWTPs into the aquatic system or a higher N₂O production yield in waters influenced by WWTPs. Meta-analysis of N₂O measurements associated with WWTPs globally revealed variable influence of WWTPs on downstream N₂O concentrations and emissions. Since wastewater production has increased substantially with the growing population and is projected to continue to rise, accurately accounting for N₂O emissions downstream of the WWTPs is important for constraining and predicting future global N₂O emissions. Efficient N₂O removal, in addition to dissolved nitrogen removal, should be an essential part of water quality control in WWTPs.
Summary: Wastewater treatment plants (WWTPs) are known to be hotspots of greenhouse gas emissions. However, the impact of WWTPs on the emission of the greenhouse gas N\textsubscript{2}O in downstream aquatic environments is less constrained. We found spatially and temporally variable but overall higher N\textsubscript{2}O concentrations and fluxes in waters downstream of WWTPs, pointing to the need for efficient N\textsubscript{2}O removal in addition to treating nitrogen in WWTPs.
Graphical abstract
Introduction

Nitrogen (N) enters the aquatic environment from agricultural and urban runoff, atmospheric deposition, and wastewater treatment plants (WWTPs), potentially leading to eutrophication, especially in densely populated regions (Galloway et al., 2008; Morée et al., 2013). During microbial transformations of N in aquatic systems (e.g., nitrification and denitrification), a powerful greenhouse gas and ozone depleting agent – N\textsubscript{2}O – is produced (Quick et al., 2019).

Aquatic systems are large but highly variable sources of N\textsubscript{2}O to the atmosphere (Wang et al., 2023). For example, on a global basis, 0.04 - 0.291 Tg N yr\textsuperscript{-1} and 0.04 - 3.6 Tg N yr\textsuperscript{-1} of N\textsubscript{2}O is estimated to outgas from rivers and estuaries, respectively (Murray et al., 2015; Maavara et al., 2019; Yao et al., 2019; Rosentreter et al., 2023). The high end of the estimates in these inland and coastal waters approaches the scale of the global marine N\textsubscript{2}O emissions (2.5 - 4.3 Tg N yr\textsuperscript{-1} in Tian et al., 2020). The large uncertainty in the estimate of aquatic N\textsubscript{2}O emission is partly due to high spatial and temporal variabilities of N\textsubscript{2}O flux within/across rivers and estuaries and the lack of observations to capture such variability. Therefore, sampling and measurements of N\textsubscript{2}O concentration at high spatial and temporal resolutions would be desirable to constrain aquatic N\textsubscript{2}O emission.

The major factors that appear to correlate with N\textsubscript{2}O concentration are dissolved inorganic nitrogen (DIN) and oxygen status (Hu et al., 2016; Zheng et al., 2022). Waste and wastewater release large amounts of DIN into the aquatic environment. In addition, waste and wastewater emit ~0.3 Tg N yr\textsuperscript{-1} of N\textsubscript{2}O (estimated from 2007-2016) into the atmosphere globally, an amount that is continuously increasing at a rate of 0.04±0.01 Tg N yr\textsuperscript{-1} per decade (Tian et al., 2020). N\textsubscript{2}O emission from WWTPs accounts for ~5.2% of total N\textsubscript{2}O emission in 2021 in the United States (EPA, 2023). N\textsubscript{2}O emissions from different WWTPs are highly variable, and are normally calculated as a function of DIN loading into the WWTPs, using an N\textsubscript{2}O emission factor (Kampechreur et al., 2009). N\textsubscript{2}O emission factors range from 0.16% to 4.5% (N\textsubscript{2}O emitted/DIN loading) (Eggleston et al., 2006; De Haas and Andrews, 2022). In addition to direct emission from the WWTPs, N\textsubscript{2}O can be discharged via WWTP effluent and produced due to DIN release from WWTP effluent into the creeks, rivers, and other downstream aquatic systems (McElroy et al., 1978; Beaulieu et al., 2010; Masuda et al., 2018). However, the impact of WWTPs on downstream N\textsubscript{2}O concentration is less studied and the downstream N\textsubscript{2}O emission remains poorly constrained.
Here we specifically compared the N$_2$O concentration upstream and downstream of the WWTPs in order to assess the impact of WWTPs on N$_2$O emission, which could help to constrain the emission factor associated with the WWTPs effluents.

The Potomac River is a major tributary of the Chesapeake Bay – the largest estuary in the United States. The Potomac River Estuary is located in a highly populated area, mainly surrounded by Washington, D.C., and the states of Virginia and Maryland in the eastern United States. The annual mean discharge of Potomac River from 1895 to 2002 measured at Chain Bridge near Washington, DC was 321 m$^3$ s$^{-1}$ with a large interannual variability (Jaworski et al., 2007). The annual total nitrogen loading was estimated to be around 27.7 $\times 10^6$ kg N year$^{-1}$ in 2008-2009 (Bricker et al., 2014). The Potomac River Estuary has experienced ecological degradation for decades partly due to excess nutrient inputs including from the effluents of WWTPs (Bricker et al., 2014; Jaworski et al., 2007). For example, the Blue Plains Advanced WWTP in Washington, D.C. is one of largest WWTPs in the world, treating an average of ~1454 million liters of water per day. Pioneering work in 1978 showed that Blue Plains WWTP was a large source of nitrogen to the Potomac River Estuary, triggering high N$_2$O production and concentration downstream (McElroy et al., 1978). Thanks to higher standards mandated by governmental agencies (nitrogen concentration in effluents below 7.5 mg L$^{-1}$) starting in 1980s and the technical improvements in N removal from the wastewater, the nitrogen concentration in effluents of WWTPs in the Potomac River has decreased substantially (Pennino et al., 2016). However, the concurrent effect on N$_2$O concentration is largely unknown. The Department of Environmental Quality (DEQ) of Virginia maintains an approximately monthly routine monitoring program for water quality (e.g., nitrogen concentration, phosphorus concentration, chlorophyll concentration) and physical properties (e.g., temperature, salinity, pH, and dissolved oxygen concentration) in the Potomac River Estuary but not for N$_2$O. Therefore, we collaborated with DEQ of Virginia to measure the spatial and temporal variation of N$_2$O concentrations in the Potomac River Estuary.

**Materials and Methods**

**Sample collection for N$_2$O and nutrients**

Surface waters at ~0.5 m depth at eleven stations in the tidal Potomac River Estuary were sampled monthly or bimonthly (depending on the weather) on a vessel (Grady White 208) for the analysis.
of DIN concentration, and both concentration and nitrogen isotopes of N₂O from April 2022 to May 2023 (Figure 1). The eleven stations are characterized into 3 groups: embayment downstream of WWTPs, embayment not associated with WWTPs, and the central channel of the Potomac River. Three embayment stations downstream of WWTPs are associated with three different WWTPs: Noman Cole, Mooney and Aquia, all of which implement tertiary treatment of the wastewater. We obtained the volume discharge and total N in treated water of each WWTP from Discharge Monitoring Reporting required by Virginia Pollutant Discharge Elimination System permit. Noman Cole WWTP discharges ~140.8 million liters of water and 370 kg N per day into Pohick Creek. Mooney WWTP discharges ~54.9 million liters of water and 147 kg N per day into the Neabsco Creek. Aquia WWTP discharges much less water and N into the Aquia Creek (~21.2 million liters per day and 35 kg N per day). The distances from the embayment stations downstream of WWTPs to Noman Cole, Mooney, Aquia WWTPs were approximately 4, 1.8 and 5.8 km, respectively.

The embayment stations were 2-3 meters deep while the average depth of central channel stations was around 8 meters. The embayment stations have been routinely sampled for water quality analyses by the DEQ of Virginia since the early 1970’s. The central channel stations were added for this study. The purposes of this sampling design are to evaluate the impact of WWTPs on downstream distribution of DIN and N₂O, and to compare DIN and N₂O concentrations between edge and central channel of the river. The central channel is likely affected both by the Potomac mainstem flow and by the input from tributaries, while the embayment stations may be mainly affected by water flow from tributaries but also influenced by the tidal cycle (see the salinity change in Supplementary Figure 1b). While estuarine N₂O concentrations could be affected by tides (Gonçalves et la., 2015), sampling was not always conducted at the same tidal state due to logistic difficulties. Triplicate water samples for N₂O concentrations and isotopes were collected via a submersible pump into 60 mL serum bottles after overflowing three times the bottle’s volume. After removing 3 mL water to create a 3 mL air headspace via a syringe, the serum bottles were immediately sealed with butyl stoppers and aluminum crimps and preserved with 0.5 mL of 10 M NaOH solution to stop biological activities. NaOH has been shown to be an effective and less environmentally hazardous preservative for N₂O and nutrient analysis (Frame et al., 2016; Wong et al., 2017).
Figure 1. Sampling stations in the Potomac River Estuary including embayment stations associated with WWTPs (red circles) and without WWTPs (blue circles), and central channel stations (yellow circles). Locations of WWTPs (Noman Cole, Mooney and Aquia) are shown in red stars. Creeks/rivers with sampling stations are numbered in the map with names shown in the legend. Stream sampling sites upstream and downstream of WWTPs in creeks 4 – 7 are shown in Figure 4 below.

In addition to the routine sampling in the Potomac River Estuary, we also sampled its tributaries, some of which were associated with the WWTPs, on May 18, 2023 (Figure 1) to specifically evaluate the impact of WWTPs on downstream N₂O concentrations. Four creeks/rivers were sampled including Neabsco Creek (5 stations: 2 stations upstream and 3 stations downstream of Mooney WWTP), Occoquan River (3 stations, no WWTP), Pohick Creek (4 stations: 2 stations upstream and 2 stations downstream of Noman Cole WWTP), and Accotink Creek (2 stations, no WWTP). Because Aquia WWTP discharges much less water and N into the Aquia Creek, its impact was not specifically investigated. Since water depths of these creeks/rivers were shallow, the water samples were collected by directly submerging 60 mL serum bottles into the surface water (~0.1 m) and preserving them as described above.
Besides N₂O sampling, temperature, salinity, and dissolved O₂ concentrations were recorded via a YSI EXO1 sonde. Chlorophyll-a samples (300 mL) were filtered onto GF/F filters and kept on ice in a cooler. The filters were then kept frozen at -20°C in the lab until analysis within 3 months (Arar and Collins, 1997). One additional sample for total nitrogen and phosphorus (both particulate and dissolved) was collected into 250 mL HDPE bottles and kept in ice in a cooler until analysis within 48 hours on land (Rice et al., 2012; EPA, 1983). Total nitrogen is the sum of total Kjeldahl nitrogen and nitrite plus nitrate.

**Measurement of N₂O and nutrient concentrations**

N₂O in the serum bottles was stripped by helium carrier gas into a Delta V Plus mass spectrometer (Thermo) for the analyses of N₂O concentration and isotope ratio (m/z = 44, 45, 46) (Tang et al., 2022). The total amount of N₂O in the serum bottles was determined using a standard curve of N₂O peak area with N₂O standards containing a known amount of N₂O reference gas (0, 0.207, 0.415, 0.623, 0.831, 1.247 nmol N₂O). The total amount of N₂O dissolved in the water was calculated after subtracting the amount of N₂O in 3 mL air headspace. The amount of N₂O in 3 mL air headspace was generally less than 4% of the amount of N₂O dissolved in the 57 mL water samples. The N₂O concentration in samples was then calculated from the total amount of N₂O dissolved in the water divided by the volume of water in the serum bottles. The detection limit and precision of N₂O concentration measurement were 1.29 and 0.33 nM, respectively. We used N₂O produced from nitrate isotope standards (USGS34 = -1.8‰ and IAEA = 4.7‰) to calibrate for δ¹⁵N of N₂O samples. We then estimated N₂O saturation (%): \[ \text{N}_2\text{O}_{\text{measured}} / \text{N}_2\text{O}_{\text{equilibrium}} \times 100 \]. The equilibrium N₂O concentration (N₂O_{equilibrium}) was calculated based on the solubility of N₂O and atmospheric N₂O concentrations (Weiss and Price, 1980). The monthly atmospheric N₂O concentrations were obtained from the nearby atmospheric station in Brentwood, Maryland (https://gml.noaa.gov/) (Andrews et al., 2023).

After analyzing N₂O concentration, samples were neutralized to pH ~7 by adding 10% hydrochloric acid. NO₃⁻ + NO₂⁻ (NOₓ⁻) concentration in these samples was measured using the vanadium (III) reduction method by converting NOₓ⁻ to NO, which was then quantified by chemiluminescence analyzer (Braman and Hendrix, 1989). The detection limit of NOₓ⁻
concentration was 0.15 µM. NH$_4^+$ and NO$_2^-$ concentrations were measured at a few selected stations using the fluorometric orthophthalaldehyde method (Holmes et al., 1999) and the colorimetric method (Hansen and Koroleff, 1999), respectively. Their concentrations were much smaller than NO$_3^-$ alone, mostly accounting for less than 10% of the DIN concentration. Therefore, we only present NO$_x^-$ data in this study.

**N$_2$O flux calculation**

Surface N$_2$O flux was calculated using the following equation: $\text{Flux} = k \times (N_2O_{\text{measured}} - N_2O_{\text{equilibrium}})$. The gas transfer velocity ($k$) was estimated based on three different parameterizations: $k = 1.91 \times e^{0.35 \times U} \times \left(\frac{Sc}{600}\right)^{-0.5}$ (Raymond and Cole, 2001); $k = 0.314 \times U^2 - 0.436 \times U + 3.99$ (Jiang et al., 2008); $k = 0.251 \times U^2 \times \left(\frac{Sc}{600}\right)^{-0.5}$ (Wanninkhof, 2014). U is the wind speed at the 10 m height obtained from the National Centers for Environmental Prediction (NCEP) reanalysis (Kalnay et al., 1996; https://psl.noaa.gov/data/gridded/data.ncep.reanalysis.html). Sc is the Schmidt number that could be estimated as a function of temperature (Wanninkhof, 2014). Since our samples have salinity close to 0, we used the parameterization of Sc for freshwater. Average values of the three N$_2$O flux estimates are presented in the paper and N$_2$O fluxes estimated by different parameterizations are provided in the associated dataset. We acknowledge large variations in estimating $k$ values in the riverine and estuarine systems by using different empirical models (Raymond and Cole, 2001; Borges et al., 2004; Rosentreter et al., 2021). For instance, the effect of water velocity and water depth on gas transfer velocity was not considered in the parameterizations above. Therefore, we focus on evaluating the spatiotemporal variations in N$_2$O fluxes and their driving factors instead of their absolute magnitude.

**Results and discussion**

**Spatial and temporal variations of N$_2$O concentrations in the Potomac River Estuary**

Along the roughly 50 km sampling transect in the Potomac River Estuary, NO$_x^-$ concentration decreased from 98 to <1 µM from upstream to downstream (Figure 2a). NO$_x^-$ concentration showed a clear seasonal pattern: higher in winter and spring while lower in summer and fall. The spatial and temporal patterns were likely attributable to the distribution of nutrient sources into the...
Potomac River, DIN uptake and other removal processes along the river (Glibert et al., 1995; Carstensen et al., 2015). For example, the maximum N loading into the Chesapeake Bay occurs in winter and spring (Da et al., 2018). Meanwhile, N$_2$O concentration decreased from approximately 40 to 10 nM along the sampling transect and was higher in the fall and winter (Figure 2b). Since temperature decreased from ~31°C in summer to 4°C in winter (Supplementary Figure 1a), the increase in N$_2$O solubility in colder water during winter partly explained the seasonal change. In contrast, N$_2$O saturation had higher values in summer and fall (Figure 2c), suggesting a higher N$_2$O production in summer and fall. It is worth noting that N$_2$O saturation was above 100% at all sampling stations with a maximum reaching 500%, indicating the Potomac River Estuary was a consistent and strong source of N$_2$O to the atmosphere. N$_2$O fluxes ranged from 1 to 31.7 μmol N$_2$O m$^{-2}$ d$^{-1}$, generally decreasing from upstream to downstream (Figure 2d). N$_2$O fluxes showed a similar seasonal pattern to N$_2$O saturation: higher in summer and fall. N$_2$O concentrations (median: 18.2 nM) and fluxes (median: 5.6 μmol N$_2$O m$^{-2}$ d$^{-1}$) in the Potomac River Estuary were substantially higher than in the mainstem of the Chesapeake Bay (2.6 to 20.9 nM N$_2$O with a median value at 10.6 nM and -0.3 to 4.3 μmol N$_2$O m$^{-2}$ d$^{-1}$ with a median at 0.5 μmol N$_2$O m$^{-2}$ d$^{-1}$ (Tang et al., 2022; Laperriere et al., 2019)). Therefore, the tributaries (i.e., Potomac River) are more intense sources of N$_2$O to the atmosphere than mainstem of the bay.
Figure 2. Spatial and temporal variations of NO$_x$ concentration (a), N$_2$O concentration (b), N$_2$O saturation (c), N$_2$O flux (d) and $\delta^{15}$N of N$_2$O (e). The distance shows from upstream to downstream stations in the Potomac River. Embayment stations associated with WWTPs (red circles and lines) and without WWTPs (blue circles and lines), and central channel stations (yellow circles and lines). For the boxplots, the red line in each box is the median. The bottom and top of each box are the 25th and 75th percentiles of the observations, respectively. The error bars represent 1.5 times the interquartile range away from the bottom or top of the box, with black + signs showing outliers beyond that range. Embayment stations associated with WWTPs had significantly higher N$_2$O concentration, N$_2$O saturation, N$_2$O flux and $\delta^{15}$N values compared to other stations ($p<0.01$, t-test) but not significantly different NO$_x$ concentration.
Stations close to each other had similar NO\(_x\) concentrations (e.g., upstream stations > downstream stations), regardless of station category (i.e., with WWTP, without WWTP, central channel of the Potomac River). In contrast, N\(_2\)O concentrations and fluxes varied within locations according to the station category: N\(_2\)O concentrations and fluxes were substantially higher at stations downstream of WWTPs (p<0.01, t-test). N\(_2\)O concentrations and fluxes were similar between stations in embayments without WWTPs and the central channel (Figure 2). This suggests these WWTPs are efficient in removing DIN from sewage and other sources but WWTPs may discharge N\(_2\)O directly into the effluent or enhance downstream N\(_2\)O production (e.g., higher N\(_2\)O production yield from the same amount of DIN). This effect extended to our sampling stations ~1.8-4 km downstream of the WWTPs. However, the effect of WWTPs on downstream N\(_2\)O varied among stations. For example, elevated N\(_2\)O concentrations were observed downstream from Noman Cole and Mooney WWTPs but not downstream from Aquia WWTP. This difference may be related to the different N removal processes of WWTPs that produce N\(_2\)O at different yields (de Haas and Andrews. 2022; Zhao et al., 2024). However, we don’t have detailed information about the three WWTPs other than that they all implement tertiary treatment. In addition, the different dilution factors by riverine discharges also matter. For example, the volume of effluent from Mooney WWTP was higher than the discharge of Neabsco Creek while the volume of effluent from Aquia WWTP were generally lower than the discharge of Aquia Creek (Supplementary Figure 2a-b).

Particularly, the highest N\(_2\)O concentration of up to 40 nM was found at two stations downstream of the Noman Cole and Mooney WWTPs on August 23, 2022 when the river discharge was low (Supplementary Figure 2). Thus, the effect of WWTPs on downstream N\(_2\)O concentrations also varies seasonally (Schult et al., 2023; Murray et al., 2020), with a relatively more important role in the dry season. Repeated spatial and temporal sampling allowed us to capture these N\(_2\)O hotspots. Previous studies have shown the impact of WWTPs on downstream N\(_2\)O concentrations and emissions in aquatic environments. For example, the highest N\(_2\)O concentration ~675 nM in the Potomac River was measured near the discharge of the Blue Plains WWTP in 1977 (McElroy et al., 1978). Highest N\(_2\)O emissions in the Ohio River near Cincinnati were attributed to direct input of N\(_2\)O from WWTPs’ effluents (Beaulieu et al., 2010).

In addition, a higher nitrogen isotopic signature (\(\delta^{15}N\)) of N\(_2\)O associated with WWTPs (median \(\delta^{15}N\) at 13‰) also suggests the distinct sources or cycling processes of N\(_2\)O compared to stations...
of the central channel and without the influence of WWTPs (median δ^{15}N of N_2O at 6‰, Figure 2e) in the Potomac River Estuary. In comparison, the average δ^{15}N of N_2O in the tropospheric air is around 6.55‰ (Snider et al., 2015). δ^{15}N of N_2O for stations with the influence of WWTPs showed a clear seasonal variation: higher in summer than winter (Figure 2e). This seasonal difference may be related to the seasonal change in the relative importance of WWTPs’ effluents versus riverine discharge (Supplementary Figure 2c). For example, a relatively larger WWTPs’ effluent volume compared to the riverine discharge led to a larger δ^{15}N of N_2O in summer. However, no clear seasonal pattern of δ^{15}N of N_2O was seen for stations without the influence of WWTPs. δ^{15}N of N_2O produced in WWTPs depends on the treatment stages and aeration conditions (Toyoda et al., 2011; Tumendelger et al., 2014). For example, the average δ^{15}N values were reported to be -24.5‰ and 0‰ respectively for N_2O produced from nitrification during oxic treatment versus N_2O produced from anaerobic denitrification in a California WWTP (Townsend-Small et al., 2011). The δ^{15}N values of N_2O in these urban WWTPs were lower than those found in waters downstream of WWTPs in the Potomac River (median δ^{15}N at 13‰). One of the reasons for the increased δ^{15}N of N_2O may be partial N_2O reduction via denitrification in the WWTPs, in downstream creeks, or in sediments; this denitrification effect has been seen in the marine oxygen minimum zones (Kelly et al., 2021). Denitrification as the cause of the elevated δ^{15}N is partly supported by the higher δ^{15}N of N_2O when NO_3^- was reduced to less than 40 μM, suggesting the occurrence of N_2O reduction when the concentration of other denitrification substrates became low (Supplementary Figure 3). However, we do not know the exact locations where denitrification occurred (e.g., WWTPs, anoxic niches in suspended particles, sediments), which deserves further investigations. The influence of denitrification on unique isotopic signatures of N_2O produced from WWTPs has also been observed in Tama River in Japan (Toyoda et al., 2009).

Environmental controls on N_2O concentrations

N_2O concentrations showed positive correlations with total N (r=0.62, p<0.01) and NO_3^- concentrations (r=0.51, p<0.01) (Figure 3a). Correlation analyses done separately for stations with or without WWTPs had similar patterns (Supplementary Figure 4). A better correlation between the N_2O concentration and total N may indicate the contribution of other N sources besides NO_3^- to N_2O production. N_2O could be produced from nitrification in the process of oxidizing NH_4^+ to NO_3^- in the oxic environment as previously shown in the oxygenated mainstem of the Chesapeake


Bay (Tang et al., 2022). However, we can’t exclude the possibility of N₂O production from denitrification associated with anaerobic microsites in particles or in sediment (Beaulieu et al., 2011; Wan et al., 2023). Future investigations with ¹⁵N tracers should be conducted to differentiate N₂O production pathways around the WWTPs. Furthermore, N₂O concentration was negatively correlated with temperature since higher temperature reduced the N₂O solubility. Although previous studies have showed dissolved oxygen to be an important driver of N₂O concentrations or fluxes in rivers and estuaries (Rosamond et al., 2012; Wang et al., 2015; Zheng et al., 2022), we did not find a strong dependence of N₂O on oxygen concentrations in the Potomac River Estuary (Figure 3a). This lack of strong dependence is probably because of the overall oxygenated conditions (Supplementary Figure 1c), and opposite correlations found in stations without WWTPs (positive) versus in stations with WWTPs (negative) (Supplementary Figure 4), which may be influenced by the different N₂O production pathways.

![Figure 3](image.png)

Figure 3. (a) Correlation coefficients among different environmental factors and N₂O concentrations. (b) Relationship between N₂O and NOₓ concentrations at different categories of sampling stations.

The significant positive relationship between N₂O and NOₓ concentration existed for samples collected at stations from all three different categories (Figure 3b). N₂O concentrations at stations downstream of WWTPs were notably higher than at other stations not associated with WWTPs even under the similar range of NOₓ concentration. The larger slope of N₂O concentration versus
NO$_3^-$ concentration at stations downstream of WWTPs may be related to the direct input of N$_2$O from WWTPs into the downstream waters or different N$_2$O production pathways and production yields that deserve further investigations. The DIN concentration has been found to be a good predictor of N$_2$O concentration and emission in many other rivers and estuaries (Murray et al., 2015; Reading et al., 2020; Zheng et al., 2022). However, the correlation varied spatially, which may be affected by the variable N$_2$O emission factors from DIN cycling. The emission factors are affected by temperature, concentration and forms of N, oxygen, organic carbon concentration and many other factors (Hu et al., 2016). The external N$_2$O input (e.g., input from WWTPs) could also affect the relationship between N$_2$O and DIN concentrations (Dong et al., 2023). Compared to DIN (~28 to 71 μM) and N$_2$O concentrations (~16 to 61 nM) measured approximately 45 years ago in the same section of the Potomac River (McElroy et al., 1978), current DIN and N$_2$O concentrations have slightly decreased. Thus, an additional benefit of nutrient regulation is the reduction of greenhouse gas - N$_2$O - emissions, beyond improving water quality.

Since N$_2$O concentrations had the strongest correlation with total N concentrations (reflecting the N$_2$O production potential) and temperature (affecting N$_2$O solubility), we developed a predictive model of N$_2$O concentration based on total N and temperature. Predictions were performed separately for stations with WWTPs ($N_2O$ concentration = 0.115 × total N − 0.241 × temperature + 17.185, n=18, r=0.78; p<0.01) and without WWTPs including central channel stations ($N_2O$ concentration = 0.049 × total N − 0.298 × temperature + 18.888, n=23, r=0.81, p<0.01). The observed N$_2$O variability was generally captured by these simple linear models (Supplementary Figure 5) but there were variabilities in the observations remaining to be explained. Addition of other predictors did not significantly improve the model performance, so we chose the simple predictive model that is mechanistically understandable. We then applied the two predictive models separately to estimate N$_2$O concentrations at the embayment station in the Pohick Bay (with WWTP) and the embayment station in the Occoquan Bay (without WWTP) using total N concentration and temperature that were measured since 2008 by the DEQ of Virginia monitoring program (Supplementary Figures 6 and 7). Predicted N$_2$O concentrations showed a clear seasonality: higher in winter and lower in summer. N$_2$O concentrations in the Pohick Bay decreased substantially (-0.9 nM/year) possibly due to the nutrient reduction (total N concentration decreasing at 8.8 μM/year) over the last 14 years (Supplementary Figure 6). However, N$_2$O
concentrations in the Occoquan Bay only decreased slightly (-0.1 nM/year, not statistically significant) along with the minor nutrient reduction (total N concentration decreasing at non-statistically significant rate of 0.5 μM/year) (Supplementary Figure 7). Continuation of environmental monitoring in the Potomac River (e.g., N nutrients and temperature), which is much easier than sampling and measuring N₂O gas, could be used to indirectly estimate the changes in N₂O concentrations in the future. These predictors are likely to be important in other estuaries, but the weighting would vary among locations.

Impact of wastewater treatment plants on N₂O concentrations and emissions
To further evaluate how WWTPs affect the N₂O distribution in the Potomac River, we measured N₂O concentrations upstream and downstream of the two WWTP effluents (Mooney and Noman Cole in Neabsco Creek and Pohick Creek, respectively) and compared them to N₂O concentrations measured in two creeks that do not have WWTPs (Figure 4a). Interestingly, the N₂O concentration and flux at the station downstream of Mooney WWTP in Neabsco Creek were lower than the N₂O concentration and flux at the station upstream of Mooney WWTP (15.0 nM vs 20.1 nM, 14.6 μmol m⁻² d⁻¹ vs 24.7 μmol m⁻² d⁻¹). The exact mechanisms were not clear but one of the potential reasons could be the influence by tidal cycles: high tide during the sampling time (salinity was 0.17 instead of 0) may have reversed the water flow and diluted the WWTP effluent with low N₂O concentration Potomac water (12.1 nM at the outflow of Neabsco Creek into the Potomac River Estuary). In contrast, we found substantially a higher N₂O concentration and flux downstream of the Noman Cole WWTP than the upstream station (30.8 nM vs 16.7 nM, 55 μmol m⁻² d⁻¹ vs 17.6 μmol m⁻² d⁻¹) in the Pohick Creek, which is less affected by the tidal cycle due to its semi-closed geography (salinity was 0.12). The high downstream N₂O concentration and flux may suggest the direct addition of N₂O from WWTP effluent to the downstream environment. Furthermore, δ¹⁵N of N₂O in stations downstream of WWTPs were generally higher than the other two creeks that do not have WWTPs (Figure 4b), confirming the distinct source of N₂O production by WWTPs found in the Potomac River Estuary. Overall, the influence of WWTP effluents on downstream distribution of N₂O is variable, and could be affected by the physical movement of water.
Figure 4. (a) Color-coded N\textsubscript{2}O concentration at creek sampling stations on May 18, 2023. WWTPs (Mooney and Noman Cole) are shown in red stars. The insert figures show the change in N\textsubscript{2}O concentrations as a function of distance up or down stream from the WWTPs. Creeks/rivers with sampling stations are numbered in the map with names shown in the legend. (b) Box plots of NO\textsubscript{x}, N\textsubscript{2}O flux and \(\delta^{15}\)N of N\textsubscript{2}O comparing four creeks. Neabsco and Pohick Creeks with WWTPs are displayed with red color boxes. Red and black circles in the boxplots show the data points of stations downstream and upstream/or without WWTPs, respectively. NO\textsubscript{x}, N\textsubscript{2}O flux and \(\delta^{15}\)N of N\textsubscript{2}O were clearly higher at stations downstream from the WWTP in Pohick Creek.

Dong et al. (2023) evaluated the potential impact of wastewater nitrogen discharge on estuarine N\textsubscript{2}O emissions globally. Here we compiled data from previous studies with direct N\textsubscript{2}O measurements in aquatic systems associated with WWTPs (not included in Dong et al., 2023) to assess the global impact of WWTPs on aquatic N\textsubscript{2}O concentrations or emissions (McElroy et al., 1978; Hemond and Duran, 1989; Toyoda et al., 2009; Beaulieu et al., 2010; Rosamond et al., 2012; Chun et al., 2020; Masuda et al., 2021; Masuda et al., 2018; Dylla, 2019). WWTP effluents or water downstream of the WWTPs contain some of the highest N\textsubscript{2}O concentrations and fluxes observed in the aquatic system (Table 1 and Supplementary Figure 8). For example, up to 12,411.4% saturation of N\textsubscript{2}O was measured in the effluent of WWTPs in the Tama River in Japan (Toyoda et al., 2009). In addition, N\textsubscript{2}O flux up to 40,800 \(\mu\)mol N\textsubscript{2}O-N m\textsuperscript{-2} d\textsuperscript{-1} was found.
downstream of the Regina WWTP in the Wascana Creek in Canada (Dylla, 2019). The downstream N$_2$O flux was >300 times higher than the N$_2$O flux upstream of the Regina WWTP. In comparison, the maximum N$_2$O saturation and flux previously reported in a global riverine N$_2$O dataset were around 2,500% and 12,754 $\mu$mol N$_2$O-N m$^{-2}$ d$^{-1}$ (Hu et al., 2016). Across the sites listed in Table 1, N$_2$O concentration/saturation/flux downstream of the WWTPs was 1.45 to 374-fold of the upstream waters. The only exception was our observed decrease in N$_2$O concentrations downstream of Mooney WWTP on May 18, 2023, which was likely influenced by the tidal cycle.

The wide range of apparent WWTP effect is related to many factors including the variable N$_2$O emission factors in the WWTPs, the ratio of WWTP effluent volume to riverine discharge, the distance from the WWTPs where measurements were conducted, and the direction of water flow (e.g., tidal cycle). In addition, the estuarine type, mixing regime, and stratification are also important factors controlling N$_2$O emissions (Brown et al., 2022). Overall, failing to account for N$_2$O emissions downstream of the WWTPs and their variability would substantially bias estimates of aquatic N$_2$O emissions. This uncertainty is increased by the fact that only a few observations are available (all in the northern hemisphere) (Supplementary Figure 8) compared to >58,000 WWTPs present globally (Ehalt Macedo et al., 2022). It is also important to restrict the N$_2$O emission via efficient N$_2$O reduction in the WWTPs considering the projected increase in future wastewater production (Qadir et al., 2020).

Table 1. Global N$_2$O observations in aquatic systems associated with wastewater treatment plants. N$_2$O data are presented in concentration (nM), saturation (%) or flux ($\mu$mol N$_2$O-N m$^{-2}$ d$^{-1}$) according to how they are reported in different studies. *downstream vs upstream*
Grand River/ Ontario, Canada
Kitchener WWTP 4-12 µmol N₂O-N m⁻² d⁻¹ 9-113 µmol N₂O-N m⁻² d⁻¹ 9.4 Rosamond et al., 2012

Wascana Creek/ Saskatchewan, Canada
Regina WWTP 32.5 to 100 µmol N₂O-N m⁻² d⁻¹ 227 to 3200 µmol N₂O-N m⁻² d⁻¹ 398 to 40800 µmol N₂O-N m⁻² d⁻¹ Dylla, 2019

Han River/ Seoul, Korea
JNW 39.7 nM 602.1 nM 441.6 nM 11.1 Chun et al., 2020

Potomac River
Noman Cole/Neabsco Creek/ Virginia, USA
Mooney Aquia
Aquia 20.1 nM 15.0 nM 0.75 This study

Neabsco Creek/ Virginia, USA
Mooney Aquia
Aquia 16.7 nM 30.8 nM 1.84 This study

Pohick Creek/ Virginia, USA
Noman Cole
Aquia 16.7 nM 24.6 nM 0.95-31.7 2.2 This study

Conclusion
Taking advantage of the routine water monitoring program by the DEQ of Virginia, we detected strong spatial and temporal variabilities of N₂O concentrations and emissions in the Potomac River Estuary, a major tributary of Chesapeake Bay. Observations across the Potomac River Estuary also allowed us to identify hotspots of N₂O emissions associated with WWTPs effluents. Higher N₂O concentrations downstream of WWTPs compared to regions with similar nitrogen nutrient concentrations suggested the direct discharge of dissolved N₂O from WWTPs and/or intense N₂O production. The influence of WWTPs on downstream N₂O concentrations and emissions is largely affected by volumes of river discharges versus WWTPs effluents. A survey of globally available data shows N₂O concentrations or emissions are consistently elevated in waters downstream from WWTPs. Future ¹⁵N tracer incubations would help to explain the high N₂O concentration downstream of WWTPs by disentangling the N₂O production pathways. In addition, concurrent measurements of the N flux and N₂O concentration downstream of WWTPs will help to constrain overall N₂O emission factors associated with WWTPs. Our work could encourage potential collaborations between scientific community and governmental agencies/the public to better observe the environmental pollution or quality, e.g., increasing the frequency and resolution of observations for N₂O and other greenhouse gases along with many regularly monitored...
environmental factors like temperature and nutrients. Such efforts may identify previously
overlooked sources of N$_2$O emission and help to better estimate N$_2$O emissions from aquatic
systems.

**Data availability**

Data presented in this study has been deposited in Zenodo repository:

**Author contribution**

W.T. conceived the study. J.T., T.J., and W.T. collected N$_2$O samples from the Potomac River
Estuary. W.T. analyzed samples and interpreted data with other coauthors. W.T. wrote the first
draft of the manuscript with input from B.B.W. All coauthors contributed to the result discussion
and manuscript writing.

**Competing interests**

The authors declare that they have no conflict of interest.

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