1 **Variable contribution of wastewater treatment plant effluents to downstream nitrous**

2 **oxide concentrations and emissions**

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12 **Abstract**

13 Nitrous oxide (N_2O), a potent greenhouse gas and ozone-destroying agent, is produced during

14 nitrogen transformations in both natural and human-constructed environments. Wastewater

15 treatment plants (WWTPs) produce and emit N_2O into the atmosphere during the nitrogen removal

16 process. However, the impact of WWTPs on N_2O emissions in downstream aquatic systems

17 remains poorly constrained. By measuring N_2O concentrations at a monthly resolution over a year

18 in the Potomac River Estuary, a tributary of Chesapeake Bay in the eastern United States, we found

19 a strong seasonal variation in N_2O concentrations and fluxes: N_2O concentrations were larger in

20 fall and winter but the flux was larger in summer and fall. Observations at multiple stations across

21 the Potomac River Estuary revealed hotspots of N_2O emissions downstream of WWTPs. N_2O

22 concentrations were higher at stations downstream of WWTPs compared to other stations (median:

23 21.2 nM vs 16.2 nM) despite the similar concentration of dissolved inorganic nitrogen, suggesting

24 the direct discharge of N_2O from WWTPs into the aquatic system or a higher N_2O production yield

25 in waters influenced by WWTPs. Meta-analysis of N_2O measurements associated with WWTPs

26 globally revealed variable influence of WWTPs on downstream N_2O concentrations and

27 emissions. Since wastewater production has increased substantially with the growing population

28 and is projected to continue to rise, accurately accounting for N_2O emissions downstream of the

29 WWTPs is important for constraining and predicting future global N_2O emissions. Efficient N_2O

30 removal, in addition to dissolved nitrogen removal, should be an essential part of water quality

31 control in WWTPs.

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36 Summary: Wastewater treatment plants (WWTPs) are known to be hotspots of greenhouse gas
37 emissions. However, the impact of WWTPs on the emission of the greenhouse gas N_2O in
38 downstream aquatic environments is less constrained. We found spatially and temporally variable
39 but overall higher N_2O concentrations and fluxes in waters downstream of WWTPs, pointing to
40 the need for efficient N_2O removal in addition to treating nitrogen in WWTPs.

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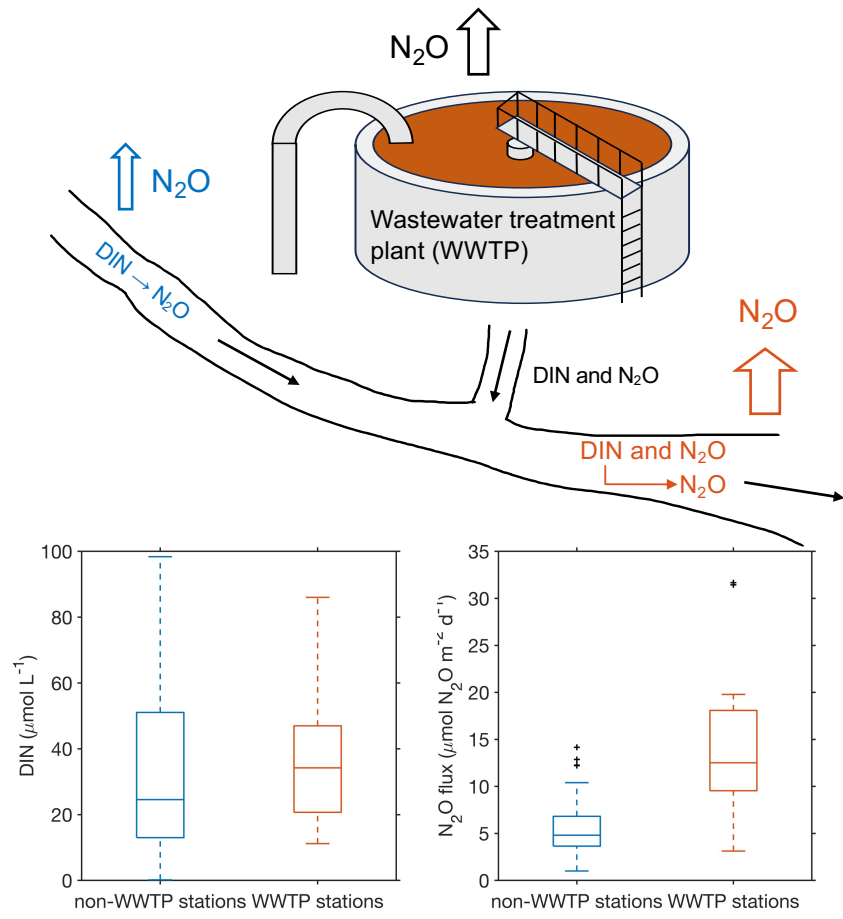
Introduction

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_2O – is produced (Quick et al., 2019). Aquatic systems are large but highly variable sources of N_2O to the atmosphere (Wang et al., 2023). For example, on a global basis, 0.04 - 0.291 Tg N yr^{-1} and 0.04 - 3.6 Tg N yr^{-1} of N_2O 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_2O emissions (2.5 - 4.3 Tg N yr^{-1} in Tian et al., 2020). The large uncertainty in the estimate of aquatic N_2O emission is partly due to high spatial and temporal variabilities of N_2O flux within/across rivers and estuaries and the lack of observations to capture such variability. Therefore, sampling and measurements of N_2O concentration at high spatial and temporal resolutions would be desirable to constrain aquatic N_2O emission.

The major factors that appear to correlate with N_2O 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^{-1} of N_2O (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^{-1} per decade (Tian et al., 2020). N_2O emission from WWTPs accounts for ~5.2% of total N_2O emission in 2021 in the United States (EPA, 2023). N_2O emissions from different WWTPs are highly variable, and are normally calculated as a function of DIN loading into the WWTPs, using an N_2O emission factor (Kampschreur et al., 2009). N_2O emission factors range from 0.16% to 4.5% (N_2O emitted/DIN loading) (Eggleston et al., 2006; De Haas and Andrews, 2022). In addition to direct emission from the WWTPs, N_2O 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_2O concentration is less studied and the downstream N_2O emission remains poorly constrained.

75   Here we specifically compared the N_2O concentration upstream and downstream of the WWTPs

76   in order to assess the impact of WWTPs on N_2O emission, which could help to constrain the

77   emission factor associated with the WWTPs effluents.

78

79   The Potomac River is a major tributary of the Chesapeake Bay – the largest estuary in the United

80   States. The Potomac River Estuary is located in a highly populated area, mainly surrounded by

81   Washington, D.C., and the states of Virginia and Maryland in the eastern United States. The annual

82   mean discharge of Potomac River from 1895 to 2002 measured at Chain Bridge near Washington,

83   DC was 321 m^3 s^{-1} with a large interannual variability (Jaworski et al., 2007). The annual total

84   nitrogen loading was estimated to be around 27.7 $\times 10^6$ kg N $year^{-1}$ in 2008-2009 (Bricker et al.,

85   2014). The Potomac River Estuary has experienced ecological degradation for decades partly due

86   to excess nutrient inputs including from the effluents of WWTPs (Bricker et al., 2014; Jaworski et

87   al., 2007). For example, the Blue Plains Advanced WWTP in Washington, D.C. is one of largest

88   WWTPs in the world, treating an average of ~1454 million liters of water per day. Pioneering

89   work in 1978 showed that Blue Plains WWTP was a large source of nitrogen to the Potomac River

90   Estuary, triggering high N_2O production and concentration downstream (McElroy et al., 1978).

91   Thanks to higher standards mandated by governmental agencies (nitrogen concentration in

92   effluents below 7.5 mg L^{-1}) starting in 1980s and the technical improvements in N removal from

93   the wastewater, the nitrogen concentration in effluents of WWTPs in the Potomac River has

94   decreased substantially (Pennino et al., 2016). However, the concurrent effect on N_2O

95   concentration is largely unknown. The Department of Environmental Quality (DEQ) of Virginia

96   maintains an approximately monthly routine monitoring program for water quality (e.g., nitrogen

97   concentration, phosphorus concentration, chlorophyll concentration) and physical properties (e.g.,

98   temperature, salinity, pH, and dissolved oxygen concentration) in the Potomac River Estuary but

99   not for N_2O. Therefore, we collaborated with DEQ of Virginia to measure the spatial and temporal

100   variation of N_2O concentrations in the Potomac River Estuary.

101

102   **Materials and Methods**

103   **Sample collection for N_2O and nutrients**

104   Surface waters at ~0.5 m depth at eleven stations in the tidal Potomac River Estuary were sampled

105   monthly or bimonthly (depending on the weather) on a vessel (Grady White 208) for the analysis

106   of DIN concentration, and both concentration and nitrogen isotopes of N_2O from April 2022 to

107   May 2023 (Figure 1). The eleven stations are characterized into 3 groups: embayment downstream

108   of WWTPs, embayment not associated with WWTPs, and the central channel of the Potomac

109   River. Three embayment stations downstream of WWTPs are associated with three different

110   WWTPs: Noman Cole, Mooney and Aquia, all of which implement tertiary treatment of the

111   wastewater. We obtained the volume discharge and total N in treated water of each WWTP from

112   Discharge Monitoring Reporting required by Virginia Pollutant Discharge Elimination System

113   permit. Noman Cole WWTP discharges ~140.8 million liters of water and 370 kg N per day into

114   Pohick Creek. Mooney WWTP discharges ~54.9 million liters of water and 147 kg N per day into

115   the Neabsco Creek. Aquia WWTP discharges much less water and N into the Aquia Creek (~21.2

116   million liters per day and 35 kg N per day). The distances from the embayment stations

117   downstream of WWTPs to Noman Cole, Mooney, Aquia WWTPs were approximately 4, 1.8 and

118   5.8 km, respectively.

119

120   The embayment stations were 2-3 meters deep while the average depth of central channel stations

121   was around 8 meters. The embayment stations have been routinely sampled for water quality

122   analyses by the DEQ of Virginia since the early 1970's. The central channel stations were added

123   for this study. The purposes of this sampling design are to evaluate the impact of WWTPs on

124   downstream distribution of DIN and N_2O, and to compare DIN and N_2O concentrations between

125   edge and central channel of the river. The central channel is likely affected both by the Potomac

126   mainstem flow and by the input from tributaries, while the embayment stations may be mainly

127   affected by water flow from tributaries but also influenced by the tidal cycle (see the salinity

128   change in Supplementary Figure 1b). While estuarine N_2O concentrations could be affected by

129   tides (Gonçalves et la., 2015), sampling was not always conducted at the same tidal state due to

130   logistic difficulties. Triplicate water samples for N_2O concentrations and isotopes were collected

131   via a submersible pump into 60 mL serum bottles after overflowing three times the bottle's volume.

132   After removing 3 mL water to create a 3 mL air headspace via a syringe, the serum bottles were

133   immediately sealed with butyl stoppers and aluminum crimps and preserved with 0.5 mL of 10 M

134   NaOH solution to stop biological activities. NaOH has been shown to be an effective and less

135   environmentally hazardous preservative for N_2O and nutrient analysis (Frame et al., 2016; Wong

136   et al., 2017).

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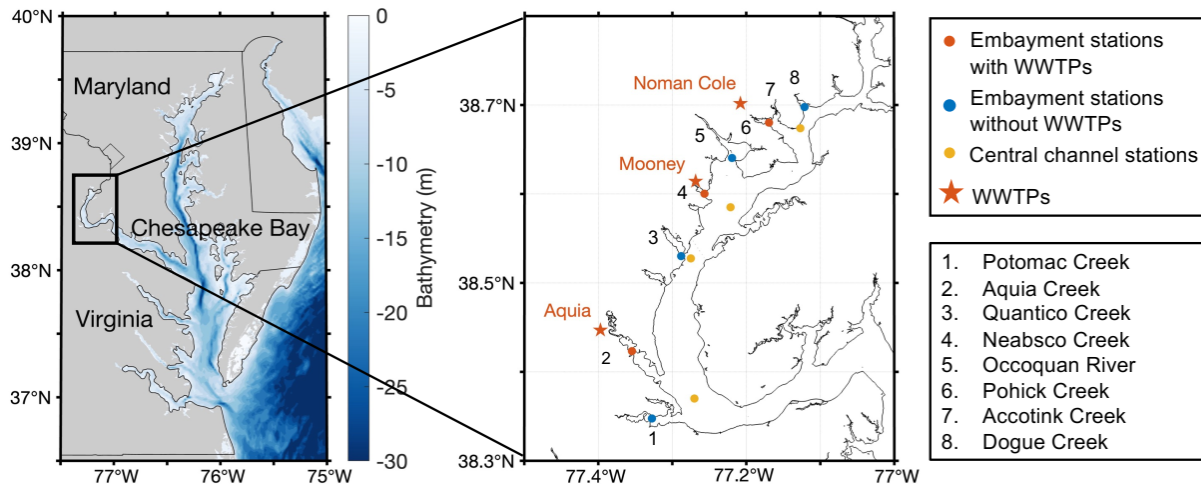
Figure 1. Sampling stations in the Potomac River Estuary including embayment stations associated with WWTPs (red circles) and without WWTPs (blue circles), and central channel stations (yellow circles). Locations of WWTPs (Noman Cole, Mooney and Aquia) are shown in red stars. Creeks/rivers with sampling 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 N_2O 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.

156

Besides N$_2$O sampling, temperature, salinity, and dissolved O$_2$ 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 N$_2$O and nutrient concentrations

N$_2$O in the serum bottles was stripped by helium carrier gas into a Delta V Plus mass spectrometer (Thermo) for the analyses of N$_2$O concentration and isotope ratio (m/z = 44, 45, 46) (Tang et al., 2022). The total amount of N$_2$O in the serum bottles was determined using a standard curve of N$_2$O peak area with N$_2$O standards containing a known amount of N$_2$O reference gas (0, 0.207, 0.415, 0.623, 0.831, 1.247 nmol N$_2$O). The total amount of N$_2$O dissolved in the water was calculated after subtracting the amount of N$_2$O in 3 mL air headspace. The amount of N$_2$O in 3 mL air headspace was generally less than 4% of the amount of N$_2$O dissolved in the 57 mL water samples. The N$_2$O concentration in samples was then calculated from the total amount of N$_2$O dissolved in the water divided by the volume of water in the serum bottles. The detection limit and precision of N$_2$O concentration measurement were 1.29 and 0.33 nM, respectively. We used N$_2$O produced from nitrate isotope standards (USGS34 = -1.8‰ and IAEA = 4.7‰) to calibrate for δ^{15}N of N$_2$O samples. We then estimated N$_2$O saturation (%): $\frac{N_2O_{measured}}{N_2O_{equilibrium}} \times 100$. The equilibrium N$_2$O concentration ($N_2O_{equilibrium}$) was calculated based on the solubility of N$_2$O and atmospheric N$_2$O concentrations (Weiss and Price, 1980). The monthly atmospheric N$_2$O concentrations were obtained from the nearby atmospheric station in Brentwood, Maryland (https://gml.noaa.gov/) (Andrews et al., 2023).

After analyzing N$_2$O concentration, samples were neutralized to pH ~7 by adding 10% hydrochloric acid. NO$_2^-$ + NO$_3^-$ (NO$_x^-$) concentration in these samples was measured using the vanadium (III) reduction method by converting NO$_x^-$ to NO, which was then quantified by chemiluminescence analyzer (Braman and Hendrix, 1989). The detection limit of NO$_x^-$

187   concentration was 0.15 µM. NH_4^+ and NO_2^- concentrations were measured at a few selected

188   stations using the fluorometric orthophthalaldehyde method (Holmes et al., 1999) and the

189   colorimetric method (Hansen and Koroleff, 1999), respectively. Their concentrations were much

190   smaller than NO_3^- alone, mostly accounting for less than 10% of the DIN concentration. Therefore,

191   we only present NO_x^- data in this study.

192

193   **N_2O flux calculation**

194   Surface N_2O flux was calculated using the following equation: $Flux = k \times (N_2O_{measured} -$

195   $N_2O_{equlibrium})$. The gas transfer velocity (k) was estimated based on three different

196   parameterizations: $k = 1.91 \times e^{0.35 \times U} \times (\frac{Sc}{600})^{-0.5}$ (Raymond and Cole, 2001); $k = (0.314 \times$

197   $U^2 - 0.436 \times U + 3.99) \times (\frac{Sc}{600})^{-0.5}$ (Jiang et al., 2008); $k = 0.251 \times U^2 \times (\frac{Sc}{660})^{-0.5}$

198   (Wanninkhof, 2014). U is the wind speed at the 10 m height obtained from the National Centers

199   for Environmental Prediction (NCEP) reanalysis (Kalnay et al., 1996;

200   https://psl.noaa.gov/data/gridded/data.ncep.reanalysis.html). Sc is the Schmidt number that could

201   be estimated as a function of temperature (Wanninkhof, 2014). Since our samples have salinity

202   close to 0, we used the parameterization of Sc for freshwater. Average values of the three N_2O flux

203   estimates are presented in the paper and N_2O fluxes estimated by different parameterizations are

204   provided in the associated dataset. We acknowledge large variations in estimating k values in the

205   riverine and estuarine systems by using different empirical models (Raymond and Cole, 2001;

206   Borges et al., 2004; Rosentreter et al., 2021). For instance, the effect of water velocity and water

207   depth on gas transfer velocity was not considered in the parameterizations above. Therefore, we

208   focus on evaluating the spatiotemporal variations in N_2O fluxes and their driving factors instead

209   of their absolute magnitude.

210

211   **Results and discussion**

212   **Spatial and temporal variations of N_2O concentrations in the Potomac River Estuary**

213   Along the roughly 50 km sampling transect in the Potomac River Estuary, NO_x^- concentration

214   decreased from 98 to <1 µM from upstream to downstream (Figure 2a). NO_x^- concentration

215   showed a clear seasonal pattern: higher in winter and spring while lower in summer and fall. The

216   spatial and temporal patterns were likely attributable to the distribution of nutrient sources into the

217 Potomac River, DIN uptake and other removal processes along the river (Glibert et al., 1995;

218 Carstensen et al., 2015). For example, the maximum N loading into the Chesapeake Bay occurs in

219 winter and spring (Da et al., 2018). Meanwhile, N_2O concentration decreased from approximately

220 40 to 10 nM along the sampling transect and was higher in the fall and winter (Figure 2b). Since

221 temperature decreased from ~31°C in summer to 4°C in winter (Supplementary Figure 1a), the

222 increase in N_2O solubility in colder water during winter partly explained the seasonal change. In

223 contrast, N_2O saturation had higher values in summer and fall (Figure 2c), suggesting a higher

224 N_2O production in summer and fall. It is worth noting that N_2O saturation was above 100% at all

225 sampling stations with a maximum reaching 500%, indicating the Potomac River Estuary was a

226 consistent and strong source of N_2O to the atmosphere. N_2O fluxes ranged from 1 to 31.7 μmol

227 N_2O m^{-2} d^{-1}, generally decreasing from upstream to downstream (Figure 2d). N_2O fluxes showed

228 a similar seasonal pattern to N_2O saturation: higher in summer and fall. N_2O concentrations

229 (median: 18.2 nM) and fluxes (median: 5.6 μmol N_2O m^{-2} d^{-1}) in the Potomac River Estuary were

230 substantially higher than in the mainstem of the Chesapeake Bay (2.6 to 20.9 nM N_2O with a

231 median value at 10.6 nM and -0.3 to 4.3 μmol N_2O m^{-2} d^{-1} with a median at 0.5 μmol N_2O m^{-2} d^{-1}

232 (Tang et al., 2022; Laperriere et al., 2019)). Therefore, the tributaries (i.e., Potomac River) are

233 more intense sources of N_2O to the atmosphere than mainstem of the bay.

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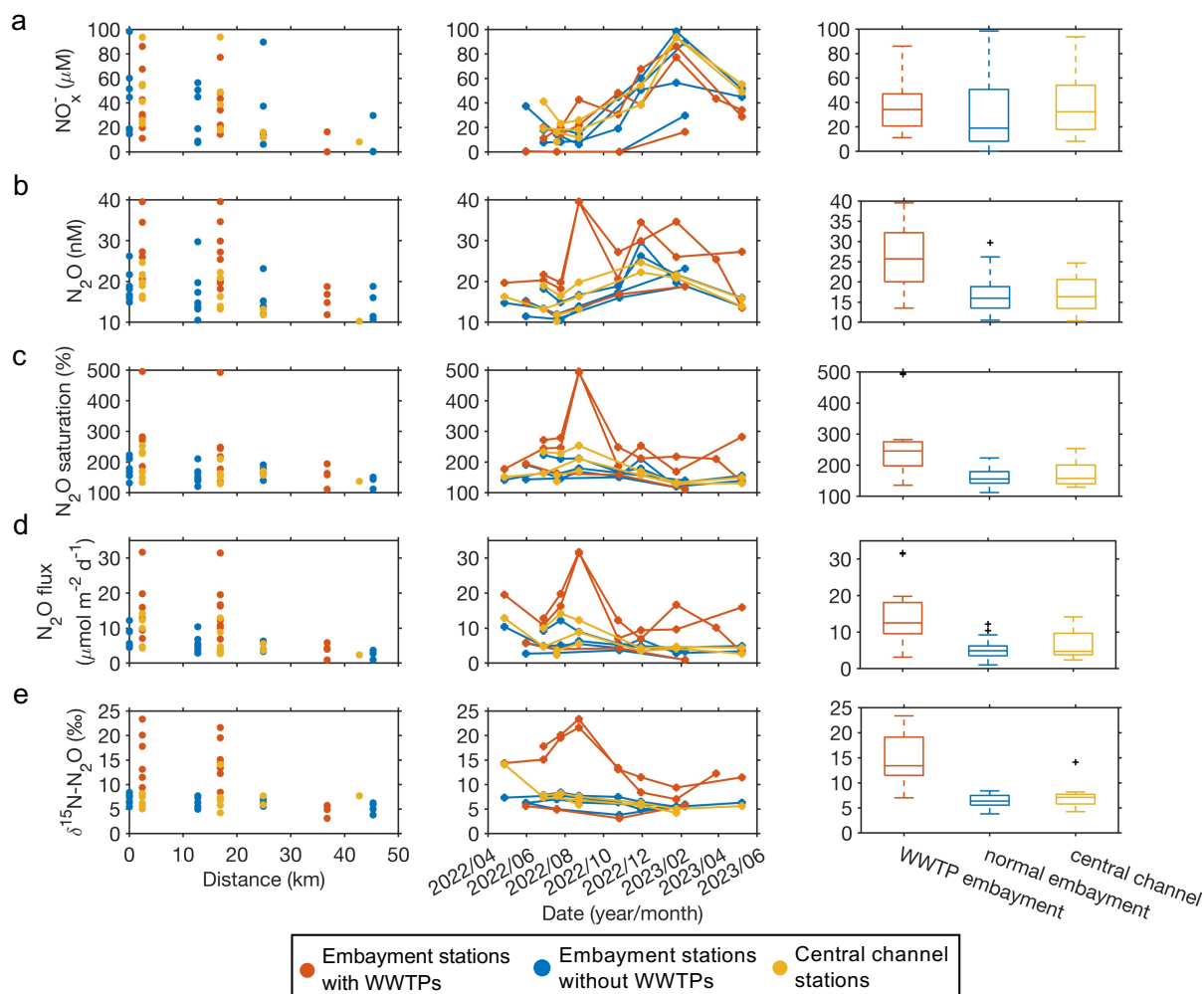
Figure 2. Spatial and temporal variations of NO_x^- concentration (a), N_2O concentration (b), N_2O saturation (c), N_2O flux (d) and $\delta^{15}N$ of N_2O (e). The distance shows from upstream to downstream stations in the Potomac River. Embayment stations associated with WWTPs (red circles and lines) 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_2O concentration, N_2O saturation, N_2O flux and $\delta^{15}N$ values compared to other stations (p<0.01, t-test) but not significantly different NO_x^- concentration.

247   Stations close to each other had similar NO_x^- concentrations (e.g., upstream stations > downstream

248   stations), regardless of station category (i.e., with WWTP, without WWTP, central channel of the

249   Potomac River). In contrast, N_2O concentrations and fluxes varied within locations according to

250   the station category: N_2O concentrations and fluxes were substantially higher at stations

251   downstream of WWTPs (p<0.01, *t*-test). N_2O concentrations and fluxes were similar between

252   stations in embayments without WWTPs and the central channel (Figure 2). This suggests these

253   WWTPs are efficient in removing DIN from sewage and other sources but WWTPs may discharge

254   N_2O directly into the effluent or enhance downstream N_2O production (e.g., higher N_2O production

255   yield from the same amount of DIN). This effect extended to our sampling stations ~1.8-4 km

256   downstream of the WWTPs. However, the effect of WWTPs on downstream N_2O varied among

257   stations. For example, elevated N_2O concentrations were observed downstream from Noman Cole

258   and Mooney WWTPs but not downstream from Aquia WWTP. This difference may be related to

259   the different N removal processes of WWTPs that produce N_2O at different yields (de Haas and

260   Andrews. 2022; Zhao et al., 2024). However, we don't have detailed information about the three

261   WWTPs other than that they all implement tertiary treatment. In addition, the different dilution

262   factors by riverine discharges also matter. For example, the volume of effluent from Mooney

263   WWTP was higher than the discharge of Neabsco Creek while the volume of effluent from Aquia

264   WWTP were generally lower than the discharge of Aquia Creek (Supplementary Figure 2a-b).

265   Particularly, the highest N_2O concentration of up to 40 nM was found at two stations downstream

266   of the Noman Cole and Mooney WWTPs on August 23, 2022 when the river discharge was low

267   (Supplementary Figure 2). Thus, the effect of WWTPs on downstream N_2O concentrations also

268   varies seasonally (Schult et al., 2023; Murray et al., 2020), with a relatively more important role

269   in the dry season. Repeated spatial and temporal sampling allowed us to capture these N_2O

270   hotspots. Previous studies have shown the impact of WWTPs on downstream N_2O concentrations

271   and emissions in aquatic environments. For example, the highest N_2O concentration ~675 nM in

272   the Potomac River was measured near the discharge of the Blue Plains WWTP in 1977 (McElroy

273   et al., 1978). Highest N_2O emissions in the Ohio River near Cincinnati were attributed to direct

274   input of N_2O from WWTPs' effluents (Beaulieu et al., 2010).

275

276   In addition, a higher nitrogen isotopic signature ($\delta^{15}N$) of N_2O associated with WWTPs (median

277 $\delta^{15}N$ at 13‰) also suggests the distinct sources or cycling processes of N_2O compared to stations

278 of the central channel and without the influence of WWTPs (median δ^{15}N of N_2O at 6‰, Figure

279 2e) in the Potomac River Estuary. In comparison, the average δ^{15}N of N_2O in the tropospheric air

280 is around 6.55‰ (Snider et al., 2015). δ^{15}N of N_2O for stations with the influence of WWTPs

281 showed a clear seasonal variation: higher in summer than winter (Figure 2e). This seasonal

282 difference may be related to the seasonal change in the relative importance of WWTPs' effluents

283 versus riverine discharge (Supplementary Figure 2c). For example, a relatively larger WWTPs'

284 effluent volume compared to the riverine discharge led to a larger δ^{15}N of N_2O in summer.

285 However, no clear seasonal pattern of δ^{15}N of N_2O was seen for stations without the influence of

286 WWTPs. δ^{15}N of N_2O produced in WWTPs depends on the treatment stages and aeration

287 conditions (Toyoda et al., 2011; Tumendelger et al., 2014). For example, the average δ^{15}N values

288 were reported to be -24.5‰ and 0‰ respectively for N_2O produced from nitrification during oxic

289 treatment versus N_2O produced from anaerobic denitrification in a California WWTP (Townsend-

290 Small et al., 2011). The δ^{15}N values of N_2O in these urban WWTPs were lower than those found

291 in waters downstream of WWTPs in the Potomac River (median δ^{15}N at 13‰). One of the reasons

292 for the increased δ^{15}N of N_2O may be partial N_2O reduction via denitrification in the WWTPs, in

293 downstream creeks, or in sediments; this denitrification effect has been seen in the marine oxygen

294 minimum zones (Kelly et al., 2021). Denitrification as the cause of the elevated δ^{15}N is partly

295 supported by the higher δ^{15}N of N_2O when NO_x^- was reduced to less than 40 μM, suggesting the

296 occurrence of N_2O reduction when the concentration of other denitrification substrates became

297 low (Supplementary Figure 3). However, we do not know the exact locations where denitrification

298 occurred (e.g., WWTPs, anoxic niches in suspended particles, sediments), which deserves further

299 investigations. The influence of denitrification on unique isotopic signatures of N_2O produced

300 from WWTPs has also been observed in Tama River in Japan (Toyoda et al., 2009).

301

302 **Environmental controls on N_2O concentrations**

303 N_2O concentrations showed positive correlations with total N (r=0.62, p<0.01) and NO_x^-

304 concentrations (r=0.51, p<0.01) (Figure 3a). Correlation analyses done separately for stations with

305 or without WWTPs had similar patterns (Supplementary Figure 4). A better correlation between

306 the N_2O concentration and total N may indicate the contribution of other N sources besides NO_x^-

307 to N_2O production. N_2O could be produced from nitrification in the process of oxidizing NH_4^+ to

308 NO_x^- 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 N_2O production from denitrification associated with anaerobic microsites in particles or in sediment (Beaulieu et al., 2011; Wan et al., 2023). Future investigations with ^{15}N tracers should be conducted to differentiate N_2O production pathways around the WWTPs. Furthermore, N_2O concentration was negatively correlated with temperature since higher temperature reduced the N_2O solubility. Although previous studies have showed dissolved oxygen to be an important driver of N_2O 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 N_2O 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 N_2O production pathways.
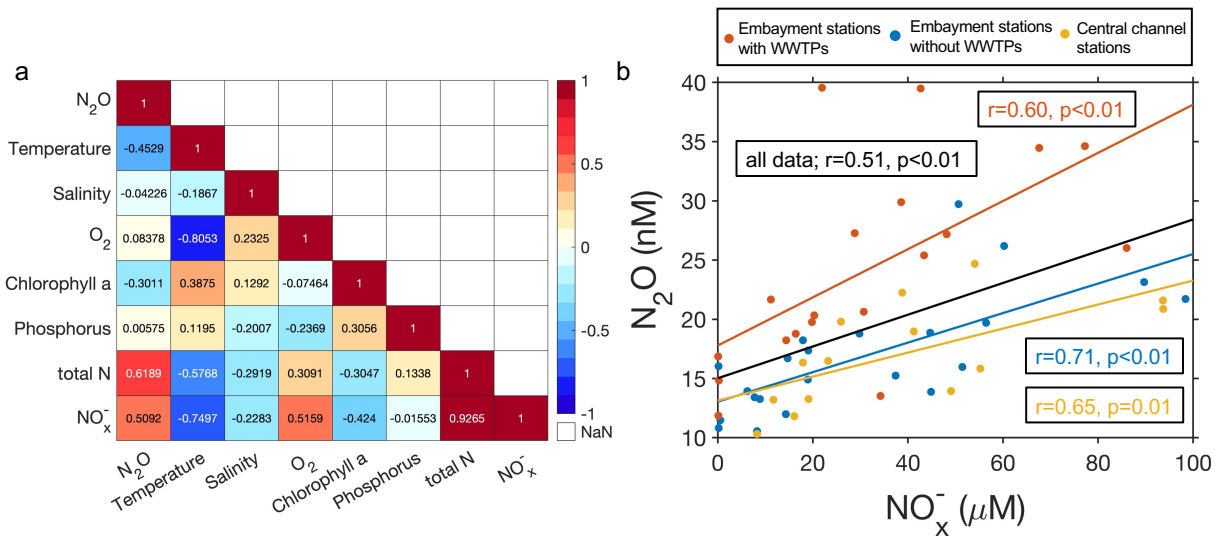
Figure 3. (a) Correlation coefficients among different environmental factors and N_2O concentrations. (b) Relationship between N_2O and NO_x^- concentrations at different categories of sampling stations.

The significant positive relationship between N_2O and NO_x^- concentration existed for samples collected at stations from all three different categories (Figure 3b). N_2O concentrations at stations downstream of WWTPs were notably higher than at other stations not associated with WWTPs even under the similar range of NO_x^- concentration. The larger slope of N_2O concentration versus

331   NO_x^- concentration at stations downstream of WWTPs may be related to the direct input of N_2O
332   from WWTPs into the downstream waters or different N_2O production pathways and production
333   yields that deserve further investigations. The DIN concentration has been found to be a good
334   predictor of N_2O concentration and emission in many other rivers and estuaries (Murray et al.,
335   2015; Reading et al., 2020; Zheng et al., 2022;). However, the correlation varied spatially, which
336   may be affected by the variable N_2O emission factors from DIN cycling. The emission factors are
337   affected by temperature, concentration and forms of N, oxygen, organic carbon concentration and
338   many other factors (Hu et al., 2016). The external N_2O input (e.g., input from WWTPs) could also
339   affect the relationship between N_2O and DIN concentrations (Dong et al., 2023). Compared to DIN
340   (~28 to 71 μM) and N_2O concentrations (~16 to 61 nM) measured approximately 45 years ago in
341   the same section of the Potomac River (McElroy et al., 1978), current DIN and N_2O concentrations
342   have slightly decreased. Thus, an additional benefit of nutrient regulation is the reduction of
343   greenhouse gas - N_2O - emissions, beyond improving water quality.

344

345   Since N_2O concentrations had the strongest correlation with total N concentrations (reflecting the
346   N_2O production potential) and temperature (affecting N_2O solubility), we developed a predictive
347   model of N_2O concentration based on total N and temperature. Predictions were performed
348   separately for stations with WWTPs ($N_2O\ concentration = 0.115 \times total\ N - 0.241 \times$
349   $temperature + 17.185$, n=18, r=0.78; p<0.01) and without WWTPs including central channel
350   stations ($N_2O\ concentration = 0.049 \times total\ N - 0.298 \times temperature + 18.888$, n=23,
351   r=0.81, p<0.01). The observed N_2O variability was generally captured by these simple linear
352   models (Supplementary Figure 5) but there were variabilities in the observations remaining to be
353   explained. Addition of other predictors did not significantly improve the model performance, so
354   we chose the simple predictive model that is mechanistically understandable. We then applied the
355   two predictive models separately to estimate N_2O concentrations at the embayment station in the
356   Pohick Bay (with WWTP) and the embayment station in the Occoquan Bay (without WWTP)
357   using total N concentration and temperature that were measured since 2008 by the DEQ of Virginia
358   monitoring program (Supplementary Figures 6 and 7). Predicted N_2O concentrations showed a
359   clear seasonality: higher in winter and lower in summer. N_2O concentrations in the Pohick Bay
360   decreased substantially (-0.9 nM/year) possibly due to the nutrient reduction (total N concentration
361   decreasing at 8.8 μM/year) over the last 14 years (Supplementary Figure 6). However, N_2O

362   concentrations in the Occoquan Bay only decreased slightly (-0.1 nM /year, not statistically
363   significant) along with the minor nutrient reduction (total N concentration decreasing at non-
364   statistically significant rate of 0.5 μM/year) (Supplementary Figure 7). Continuation of
365   environmental monitoring in the Potomac River (e.g., N nutrients and temperature), which is much
366   easier than sampling and measuring N_2O gas, could be used to indirectly estimate the changes in
367   N_2O concentrations in the future. These predictors are likely to be important in other estuaries, but
368   the weighting would vary among locations.

369

370   **Impact of wastewater treatment plants on N_2O concentrations and emissions**

371   To further evaluate how WWTPs affect the N_2O distribution in the Potomac River, we measured
372   N_2O concentrations upstream and downstream of the two WWTP effluents (Mooney and Noman
373   Cole in Neabsco Creek and Pohick Creek, respectively) and compared them to N_2O concentrations
374   measured in two creeks that do not have WWTPs (Figure 4a). Interestingly, the N_2O concentration
375   and flux at the station downstream of Mooney WWTP in Neabsco Creek were lower than the N_2O
376   concentration and flux at the station upstream of Mooney WWTP (15.0 nM vs 20.1 nM; 14.6 μmol
377   m^{-2} d^{-1} vs 24.7 μmol m^{-2} d^{-1}). The exact mechanisms were not clear but one of the potential reasons
378   could be the influence by tidal cycles: high tide during the sampling time (salinity was 0.17 instead
379   of 0) may have reversed the water flow and diluted the WWTP effluent with low N_2O
380   concentration Potomac water (12.1 nM at the outflow of Neabsco Creek into the Potomac River
381   Estuary). In contrast, we found substantially a higher N_2O concentration and flux downstream of
382   the Noman Cole WWTP than the upstream station (30.8 nM vs 16.7 nM; 55 μmol m^{-2} d^{-1} vs 17.6
383   μmol m^{-2} d^{-1}) in the Pohick Creek, which is less affected by the tidal cycle due to its semi-closed
384   geography (salinity was 0.12). The high downstream N_2O concentration and flux may suggest the
385   direct addition of N_2O from WWTP effluent to the downstream environment. Furthermore, $\delta^{15}N$
386   of N_2O in stations downstream of WWTPs were generally higher than the other two creeks that do
387   not have WWTPs (Figure 4b), confirming the distinct source of N_2O production by WWTPs found
388   in the Potomac River Estuary. Overall, the influence of WWTP effluents on downstream
389   distribution of N_2O is variable, and could be affected by the physical movement of water.

390
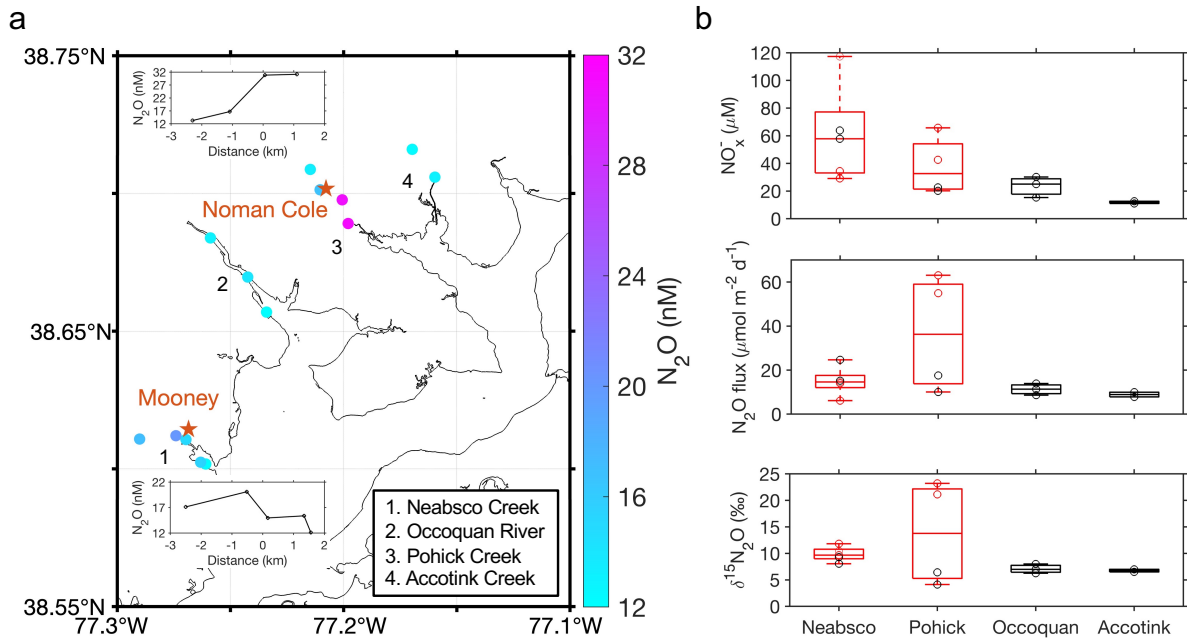
Figure 4. (a) Color-coded N_2O concentration at creek sampling stations on May 18, 2023. WWTPs (Mooney and Noman Cole) are shown in red stars. The insert figures show the change in N_2O concentrations as a function of distance up or down stream from the WWTPs. Creeks/rivers with sampling stations are numbered in the map with names shown in the legend. (b) Box plots of NO_x^-, N_2O flux and $\delta^{15}N$ of N_2O 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_x^-, N_2O flux and $\delta^{15}N$ of N_2O 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_2O emissions globally. Here we compiled data from previous studies with direct N_2O measurements in aquatic systems associated with WWTPs (not included in Dong et al., 2023) to assess the global impact of WWTPs on aquatic N_2O 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_2O concentrations and fluxes observed in the aquatic system (Table 1 and Supplementary Figure 8). For example, up to 12,411.4% saturation of N_2O was measured in the effluent of WWTPs in the Tama River in Japan (Toyoda et al., 2009). In addition, N_2O flux up to 40,800 µmol N_2O-N m^{-2} d^{-1} was found

downstream of the Regina WWTP in the Wascana Creek in Canada (Dylla, 2019). The downstream N_2O flux was >300 times higher than the N_2O flux upstream of the Regina WWTP. In comparison, the maximum N_2O saturation and flux previously reported in a global riverine N_2O dataset were around 2,500% and 12,754 μmol N_2O-N m^{-2} d^{-1} (Hu et al., 2016). Across the sites listed in Table 1, N_2O 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_2O 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_2O 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_2O emissions (Brown et al., 2022). Overall, failing to account for N_2O emissions downstream of the WWTPs and their variability would substantially bias estimates of aquatic N_2O 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_2O emission via efficient N_2O reduction in the WWTPs considering the projected increase in future wastewater production (Qadir et al., 2020).

Table 1. Global N_2O observations in aquatic systems associated with wastewater treatment plants. N_2O data are presented in concentration (nM), saturation (%) or flux (μmol N_2O-N m^{-2} d^{-1}) according to how they are reported in different studies. *downstream vs upstream.

River/location	WWTP	N_2O upstream or in tributaries without WWTPs	N_2O in WWTP effluents	N_2O downstream or in tributaries with WWTPs	*Average fold change	Reference
Potomac River/ Washington, D.C., USA	Blue Plains WWTP	11-34 nM		147-318 nM	9.3	McElroy et al., 1978
Assabet River/ Massachusetts, USA	Westborough WWTP	~10 nM	1045 nM	163 nM	16.3	Hemond and Duran. 1989
Tama River/ Tokyo, Japan	Plant 1 Plant 2	350.7% 219.3%	12411.4% 3326.2%	3454.8% 1029.6%	9.8 4.7	Toyoda et al., 2009
Ohio River/ Cincinnati, USA		27.9 μmol N_2O-N m^{-2} d^{-1}		1068 μmol N_2O-N m^{-2} d^{-1}	38.2	Beaulieu et al., 2010

Grand River/ Ontario, Canada	e.g., Kitchener WWTP	4-12 μmol N$_2$O-N m^{-2} d^{-1}		9-113 μmol N$_2$O-N m^{-2} d^{-1}	9.4	Rosamond et al., 2012
Wascana Creek/ Saskatchewan, Canada	Regina WWTP	–32.5 to 109 μmol N$_2$O-N m^{-2} d^{-1}	227 to 72800 μmol N$_2$O-N m^{-2} d^{-1}	398 to 40800 μmol N$_2$O-N m^{-2} d^{-1}	374	Dylla. 2019
Han River/ Seoul, Korea	JNW	39.7 nM	602.1 nM	441.6 nM	11.1	Chun et al., 2020
A-river	A-WWPT	61 nM	493 nM	180 nM	3	Masuda et al., 2021
B-river	B-WWTP	95 nM	246 nM	286 nM	3	Masuda et al., 2018
C-river/Miyagi, Japan	C-WWTP	100 nM	319 nM	145 nM	1.45	
Potomac River Estuary /Virginia, USA	Noman Cole Mooney Aquia	10.8-29.7 nM 1-12.2 μmol N$_2$O-N m^{-2} d^{-1}		11.87-39.5 nM 0.95-31.7 μmol N$_2$O-N m^{-2} d^{-1}	1.6 2.2	This study
Neabsco Creek/ Virginia, USA	Mooney	20.1 nM 24.7 μmol N$_2$O-N m^{-2} d^{-1}		15.0 nM 14.6 μmol N$_2$O-N m^{-2} d^{-1}	0.75 0.59	This study
Pohick Creek/ Virginia, USA	Noman Cole	16.7 nM 17.6 μmol N$_2$O-N m^{-2} d^{-1}		30.8 nM 55 μmol N$_2$O-N m^{-2} d^{-1}	1.84 3.12	This study

Conclusion

Taking advantage of the routine water monitoring program by the DEQ of Virginia, we detected strong spatial and temporal variabilities of N$_2$O 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 N$_2$O emissions associated with WWTPs effluents. Higher N$_2$O concentrations downstream of WWTPs compared to regions with similar nitrogen nutrient concentrations suggested the direct discharge of dissolved N$_2$O from WWTPs and/or intense N$_2$O production. The influence of WWTPs on downstream N$_2$O concentrations and emissions is largely affected by volumes of river discharges versus WWTPs effluents. A survey of globally available data shows N$_2$O concentrations or emissions are consistently elevated in waters downstream from WWTPs. Future ^{15}N tracer incubations would help to explain the high N$_2$O concentration downstream of WWTPs by disentangling the N$_2$O production pathways. In addition, concurrent measurements of the N flux and N$_2$O concentration downstream of WWTPs will help to constrain overall N$_2$O 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 N$_2$O and other greenhouse gases along with many regularly monitored

451 environmental factors like temperature and nutrients. Such efforts may identify previously
452 overlooked sources of N_2O emission and help to better estimate N_2O emissions from aquatic
453 systems.
454

Data availability

456 Data presented in this study has been deposited in Zenodo repository:
457 https://doi.org/10.5281/zenodo.10775250.
458

Author contribution

460 W.T. conceived the study. J.T., T.J., and W.T. collected N_2O samples from the Potomac River
461 Estuary. W.T. analyzed samples and interpreted data with other coauthors. W.T. wrote the first
462 draft of the manuscript with input from B.B.W. All coauthors contributed to the result discussion
463 and manuscript writing.
464

Competing interests

466 The authors declare that they have no conflict of interest.
467

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