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Novel sinks for the atmospherically potent gas nitrous oxide

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Abstract

Nitrous oxide (N_2O) is a potent climate gas, with its strong warming potential and ozone-depleting properties both focusing research on N_2O sources. While undersaturation in N_2O have been reported in natural waters indicating sinks for N_2O, most of these found in the surface ocean and shallow freshwaters remain unaccounted for. Although a sink for N_2O through biological fixation has been observed in the Pacific, the regulation of N_2O- compared to canonical N_2-fixation is unknown. Here I show that both N_2O and N_2 can be fixed by freshwater communities but with distinct seasonalities and temperature dependencies. N_2O fixation appears less sensitive to temperature than N_2 fixation, driving a strong sink for N_2O in winter. Moreover, by quantifying both N_2O and N_2 fixation I show that, rather than N_2O being first reduced to N_2 through denitrification, N_2O fixation is direct and could explain the widely reported N_2O sinks in natural waters. N_2O can be fixed into NH_4^+, which could then be further oxidised to NO_2^- and NO_3^- and being available to the wider community. In the cold, total N_2O reduction was higher and a higher proportion of the reduced N_2O was conserved. In addition, with activity of nitrification not detected in most of the ponds and anammox not detected in any pond, denitrification seem to be the primary process producing both N_2O and N_2. The availability of nitrate limits the temperature sensitivity of the production of N_2O and N_2 from denitrification, with production of both gases only sensitive to changes in temperature at high concentration of additional nitrate. With the high substrate, the net production ratio of N_2O to N_2 from denitrification increases at lower temperatures, which could provide more N_2O relative to N_2 for N fixation in the cold.

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This thesis is dedicated to my parents. Thank you for all your support for my studies and work throughout the years. Thank you for giving me the freedom to choose what I want, even though that's not always easy for you.

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Chapter 1 Established regulatory factors on the sources and sinks of N_2O in aquatic ecosystems

Abstract

Nitrous oxide (N_2O) is a potent climate gas, with its strong warming potential and ozone-depleting properties both focusing research on N_2O sources. While undersaturation in N_2O have been reported in natural waters indicating sinks for N_2O, most of these found in the surface ocean and shallow freshwaters remain unaccounted for. The canonical reduction of N_2O to N_2 occurs under oxygen-limited conditions and is typically known as the only pathway for N_2O reduction, which can not explain the undersaturation in N_2O found in these oxic waters. Although there is some evidence for an alternative N_2O reduction pathway in oxic conditions, i.e. N_2O fixation, the ecological benefit of N_2O-, compared to canonical N_2-fixation is unknown. Moreover, the occurrence and the regulatory factors of N_2O sinks are not clear. Here I compiled data from the literature that reported N_2O sinks and show that the availability of nitrate, ammonium, and temperature are important in regulating natural waters as either sinks or sources of N_2O.

1.1 Atmospheric potency of N_2O

Nitrous oxide (N_2O) is a potent climate gas, with approximately 265 times the global warming potential of carbon dioxide (CO_2) (Stocker, 2014) and strong ozone-depleting properties (Ravishankara et al., 2009). The atmospheric concentration of N_2O continues to rise through the use of nitrogen-based fertilizers, fossil fuel combustion, biomass burning, and sewage discharge (Davidson, 2009; Wuebbles, 2009) and has already increased by approximately 20%

since 1750 (Meinshausen et al., 2011). In addition, N_2O is a long-lived gas, with a residence time in the atmosphere of about 121 years (Stocker, 2014).

The solubility of N_2O is a function of temperature and salinity (Weiss and Price, 1980), with a 100% air saturation denoting the equilibrium between N_2O in the atmosphere and N_2O dissolved in the water. Accordingly, undersaturation (<100% air saturation) in N_2O means that the consumption exceeds the production of N_2O, i.e., the waters are sinks for N_2O, while oversaturation (>100% air saturation) denotes the waters as sources of N_2O.

Not surprisingly, given its atmospheric potency, research to date has focused on N_2O sources with N_2O sinks being largely ignored (Farías et al., 2013). The few studies reporting on both N_2O sources and sinks (Bange et al., 1998; Baulch et al., 2011; Lemon and Lemon, 1981; Whitfield et al., 2011) often simply document the sinks as concentrations below that expected for water (marine or freshwater) at equilibrium with the atmosphere and the true mechanism remains largely unknown.

In addition, the global budget of sources and sinks of N_2O is highly imbalanced. For example, the sinks for N_2O are minor compared to the sources for N_2O (less than 0.1 Tg N y^{-1} compared to ~18 Tg N y^{-1}) on the global scale (Syakila et al., 2010; Tian et al., 2020), with most N_2O sinks in natural waters not included in these budget estimates. To more fully understand atmospheric N_2O there is a pressing need to account for both the established sources and any novel sinks for N_2O.

1.2 Biological sources and sinks of N_2O

In aquatic environments, N_2O can be produced from both microbial nitrification (Codispoti, 2010; Goreau et al., 1980; Santoro et al., 2010) either via hydroxylamine oxidation ($NH_4^+ \rightarrow NH_2OH \rightarrow N_2O$), or hybrid formation ($NO_2^- + NH_2OH \rightarrow N_2O$) (Stieglmeier et al., 2014), and incomplete denitrification ($NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O[\rightarrow N_2]$) (Fig. 1) (Dalsgaard et al., 2012;

Naqvi et al., 2010). In soils, nitrifier-denitrification ($NH_4^+ \rightarrow NO_2^- \rightarrow N_2O$) can also produce N_2O (Wrage-Mönnig et al., 2018).

The reduction of N_2O, on the other hand, was generally attributed to its reduction to N_2 in the last step of microbial denitrification ($N_2O \rightarrow N_2$, Fig. 1) typically mediated by facultative anaerobic bacteria (Babbin et al., 2015; Dalsgaard et al., 2012; Knowles, 1982; Shapleigh, 2006), although recently it has been showed that ammonia-oxidizing archaea can also reduce N_2O to N_2 during NO dismutation (Kraft et al., 2022). During denitrification, microorganisms possessing N_2O-reductase can produce N_2, whereas others without the reductase may only generate N_2O as the end product (Cavigelli and Robertson, 2001; Philippot et al., 2011). The last step in the denitrification pathway generally occurs when oxygen is limiting or completely absent (e.g., O_2 < 1% saturation) (Codispoti, 2010; Naqvi et al., 2010), as N_2O-reductase can be inhibited by the presence of O_2 (Cavigelli and Robertson, 2001; Dalsgaard et al., 2014). Nevertheless, any undersaturation – indicating a sink for N_2O – as observed in natural waters has routinely been attributed to that last step in complete denitrification (Fig. 1).

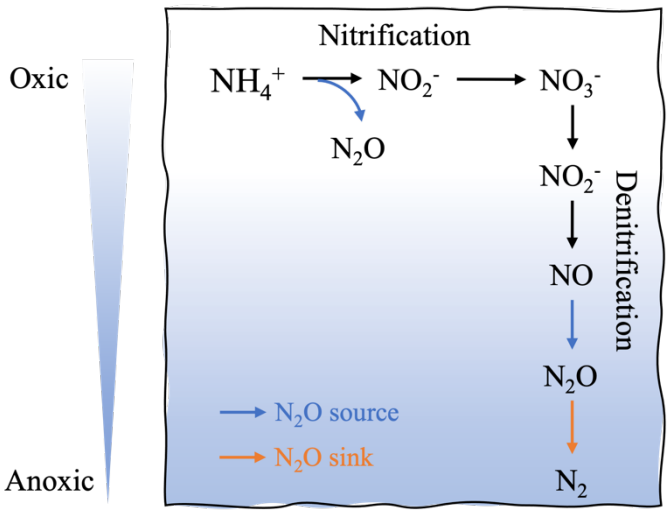

Fig. 1 | Recognised pathways of biological production and reduction of N_2O in natural waters. N_2O can be produced (i.e. sources, blue arrows) from nitrification under various oxygen conditions, or from denitrification typically under O_2 limited or absent conditions,

while it can be further reduced to N_2 (i.e. sinks, orange arrow) through complete denitrification under anoxic conditions. In addition, hybrid formation ($NO_2^- + NH_2OH \rightarrow N_2O$) (Stieglmeier et al., 2014) and nitrifier-denitrification ($NH_4^+ \rightarrow NO_2^- \rightarrow N_2O$) can also produce N_2O (Wrage-Mönnig et al., 2018), but are not known as common N_2O production pathways in natural waters.

1.3 Reported undersaturation in N_2O in oxic waters

N_2O undersaturation has been reported in many oxic (*see* Table 1 and the references cited therein), shallow freshwaters (down to 13% of air equilibration, typically ~ 70% to 100%) and surface-ocean-waters (down to 34%, typically ~90%), where canonical – oxygen limited – denitrification is unlikely to explain any undersaturation in N_2O in these oxic waters.

As such, the reasons for N_2O undersaturation in these waters remain poorly understood, with many instances of N_2O undersaturation remaining unaccounted for (Baulch et al., 2011; Diem et al., 2012; Guérin et al., 2008; Lemon and Lemon, 1981; Soued et al., 2016; Whitfield et al., 2011) or simply being dismissed as analytical artifacts (Butler et al., 1989; Cline et al., 1987). Further, as N_2O sources generally increase at higher concentrations of ammonium and nitrate (i.e., fixed, dissolved inorganic N) (Baulch et al., 2011; Walter et al., 2006), any potential undersaturation in N_2O could be masked by stronger production of N_2O from nitrification and denitrification. This might explain why many accounts of N_2O undersaturation have been reported in N limited environments (Diem et al., 2012; Farías et al., 2013; Lemon and Lemon, 1981; Maher et al., 2016; Soued et al., 2016; Verdugo et al., 2016). However, in these N limited environments, the reasons for N_2O undersaturation have often simply been attributed to low N_2O emission due to limited fixed N, without further discussion of the occurance of these N_2O sinks. Therefore, N_2O sinks under oxic conditions could be widespread and need more attention.

Table 1. Reported undersaturation and negative flux (consumption higher than production i.e., sinks) of N_2O into shallow freshwaters and surface ocean waters. These oxic waters are considered unsuitable for the reduction of N_2O to N_2 through denitrification, as this process occurs under O_2-limited or anoxic conditions. Data have been converted to the same unit across different studies and are presented as an average or a range where applicable.

Ecosystem	Region	N_2O sat. (%)	N_2O flux (μmol m^{-2} d^{-1})	Reference
Agricultural pond	China	84 – 745	-6.4 – 85.6	(Xia et al., 2013)
Aquaculture pond	China	28 – 460	< 5.45	(Yuan et al., 2021)
Boreal peatland	Canada	n.r.	-0.24 – 0.49	(Schiller and Hastie, 1994)
Boreal peatland	Finland	n.r.	-1.72 – 3	(Alm et al., 1999)
Boreal peatland	Finland	n.r.	-2 – 10.7	(Huttunen et al., 2002a; Huttunen et al., 2002b; Huttunen et al., 2002c)
Boreal peatland	Finland	n.r.	-26 – 200	(Regina et al., 1996)
Boreal reservoir	Canada	70 – 73	-0.05 – -0.03	(Hendzel et al., 2005)
Farm reservoir	Canada	74.2	-4.03	(Webb et al., 2019)[γ]
Lake, river, pond	Canada	n.r.	-23 – 116	(Soued et al., 2016)
Lake	Antarctica	n.r.	-3.1 – 14.5	(Liu et al., 2011b)
Lake	China	n.r.	-60 – 300	(Chen et al., 2011)
Lake	Ireland	n.r.	−3.8 – 6.9	(Whitfield et al., 2011)
Lake	USA	94 – 450	n.r.	(Lemon and Lemon, 1981)
Perialpine/alpine reservoir	Switzerland	>55	-0.6 – 1.6	(Diem et al., 2012)
Reservoir	French Guiana	n.r.	-135 – 219	(Guérin et al., 2008)
Reservoir	Panama	n.r.	-18 – 22	(Guérin et al., 2008)
Stream	Canada	13 – 119	-2.6 – 100	(Baulch et al., 2011)
Tundra wetland	Antarctica	n.r.	-0.47 – 1.95	(Zhu et al., 2008)

Coastal water	Baltic Sea	96 – 142	n.r.	(Bange et al., 1998)
Coastal wetland	China	n.r.	-0.18 – 0.15	(Yuan et al., 2015)
Coastal wetland	China	n.r.	-41.6 – 19.9	(Yu et al., 2012)*
Coastal wetland	USA	n.r.	-33	(Moseman-Valtierra et al., 2011)
Coastal wetland	USA	n.r.	-38.6±5.2	(Windham-Myers et al., 2018)
Cultivation pond	Hawaii	84.1	-3.4±3.5	(Ferrón et al., 2012)
Mangrove creek	Canada	77 – 106	-3.4 – 0.7	(Maher et al., 2016)
Ocean	Antarctica	94 – 101	n.r.	(Priscu et al., 1990)
Ocean	Antarctica	90 – 125	-0.06 – -0.09	(Rees et al., 1997)
Ocean	Antarctica	98 – 103	-1.2 – 1.8	(Law and Ling, 2001)
Ocean	Antarctica SW	90 – 98	-3.65±0.95	(Zhan et al., 2015)
Ocean	Arabian Sea	94 – 204	1.6 – 5.1	(Upstill-Goddard et al., 1999)
Ocean	Arctic Ocean	82 – 181	n.r.	(Kitidis et al., 2010)
Ocean	Arctic Ocean	42 - 111	n.r.	(Verdugo et al., 2016)
Ocean	Baltic Sea	79.3±10.7	n.r.	(Walter et al., 2006)
Ocean	East Indian Ocean	98.5 – 99	n.r.	(Butler et al., 1989)
Ocean	East China Sea	94 – 382	-0.7 – 97.5	(Zhang et al., 2008)
Ocean	Eastern South Pacific	34 – 132	n.r.	(Cornejo et al., 2015)
Ocean	Eastern South Pacific	n.r.	-100.6 – 0.4	(Farías et al., 2013)
Ocean	Eastern North Pacific	95	n.r.	(Cohen and Gordon, 1978)
Ocean	Eastern North Pacific	70 – 134	n.r.	(Fenwick and Tortell, 2018)
Ocean	North Atlantic	97 –140	-0.04 – 4.65	(Forster et al., 2009)Ψ

Ocean	Northwest Black Sea	96 – 149	1.6 – 5.2	(Amouroux et al., 2002)

n.r.: not reported for either N_2O saturation or N_2O flux.

$^{\gamma}$: Data for saturation and flux are medians. The range of N_2O flux reported here is -11.91 to 165.77 μmol m^{-2} d^{-1}.

*: The range of N_2O flux are monthly averages from May to October 2004, measured with a chamber positioned within the zone dominated by the wetland plant *Scirpus mariqueter*.

$^{\Psi}$: Data are from the mixed layer depth.

1.4 N_2O fixation - a possible explanation for undersaturation in N_2O in oxic waters?

In recent years, a few pieces of evidence have been presented for a novel pathway for N_2O reduction - N_2O dependent N fixation. N_2O fixation has been reported for pure cultures of the marine isolates *Trichodesmium* sp. and *Crocosphaera* sp. (Farías et al., 2013). N_2O fixation also occurred in the surface waters of the Eastern Tropical South Pacific (Cornejo et al., 2015; Farías et al., 2013), where the measured N_2O fixation activity could contribute some (0.2 – 60%) of the total N_2O reduction (Farías et al., 2013). As long ago as the 1954, it was shown (Mozen and Burris, 1954) that $^{15}N_2O$ could be assimilated by soybean root nodules with activity comparable to $^{15}N_2$ assimilation. Interestingly, while the authors presented no clear explanation, N_2O was consumed by some wetland plants, with a positive correlation between N_2O undersaturation and net primary production (Windham-Myers et al., 2018; Yu et al., 2012).

These findings show that N_2O fixation (e.g. $N_2O \rightarrow NH_4^+$, if it functions similarly to N_2 fixation i.e. $N_2 \rightarrow NH_4^+$) represents an alternative N_2O reduction pathway to the terminal step in denitrification ($N_2O \rightarrow N_2$) that may explain some of the unexplained undersaturation reported for N_2O. Yet, within the widespread accounts of N_2O undersaturation found in oxic waters,

only a few studies mentioned the possibility of N_2O fixation (Cornejo et al., 2015; Farías et al., 2013; Verdugo et al., 2016) and it is not widely acknowledged.

1.5 The temperature dependence of N_2 fixation: a metadata analysis

The fixation of N_2 gas to NH_3 or NH_4^+ is crucial in supporting primary production in N-limited ecosystems (Falkowski, 1997). Although N_2 forms 78% of the atmosphere, only a few types of microbes can fix N_2, partly due to the breaking of the highly stabilized triple bond in the N_2 molecule is very energy-demanding (Howard and Rees, 1996). Moreover, N_2 fixation generally has high activation energies, e.g., ~0.8 to 1.6 eV (Rinne-Garmston et al., 2019; Welter et al., 2015), which are considerably higher than ~0.65 eV and ~0.32 eV for respiration and photosynthesis, respectively (Allen et al., 2005).

Activation energy (AE) has often been used to characterise the temperature sensitivities of biological processes (Allen et al., 2005; Yvon-Durocher et al., 2014; Zhu et al., 2020). To see if the temperature dependence of N_2 fixation is universal, here I compiled a list of studies that reported N_2 fixation under different temperatures in both aquatic and terrestrial ecosystems (Table 2). I estimated the apparent AE of biological N_2 fixation ($\overline{E_{BNF}}$) by fitting the natural log-transformed rate of N_2 fixation against the centered temperature term $\left(\frac{1}{kT_C} - \frac{1}{kT_i}\right)$ (Yvon-Durocher et al., 2014; Zhu et al., 2020):

$$\ln F(T_i) = (\overline{E_{BNF}} + a_i)\left(\frac{1}{kT_c} - \frac{1}{kT_i}\right) + \overline{\ln F(T_c)} + b_i \quad (1)$$

where k is the Boltzmann constant (8.62×10^{-5} eV K^{-1}, 1 eV = 96.485 kJ mol^{-1}). Temperature was centred as T_c = (max+min)/2 of the dataset (T_c = 289.6 K i.e. 16.4 °C), and T_i the absolute temperature from study i (i =1, 2, … ,9). Thus, $F(T_i)$ represents the rate of N_2

fixation at the median temperature T_c. Further, to account for variances across the different studies, I included random slope (a_i) and random intercept (b_i) terms in the equation.

Statistical analysis and plotting were performed in R (Team, 2021) using RStudio (Version 1.3.1093). As the studies used different normalization methods, e.g., μmol C_2H_4 (mg Chl a)$^{-1}$ h^{-1} in (Zappa et al., 2007), μmol N d^{-1} L^{-1} in (Avnimelech et al., 2001), and μmol C_2H_4 h^{-1} (g dry wt)$^{-1}$ in (Lehtimaki et al., 1997; Waughman, 1977), I standardised the data by subtracting the study-specific intercept from the rate of N_2 fixation for each study (Yvon-Durocher et al., 2014; Zhu et al., 2020). Data were fitted using linear mixed-effect models (Bates et al., 2014), and the models then ranked by the small sample-size corrected Akaike Information Criterion (AICc) using the 'MuMIn' package. The best-fitting model was determined by the lowest AICc score (*see* Table 3) and, AE, in the unit of eV, was derived from the slope of the best-fitting model – regardless of the *y*-axis units (Fig. 2). In addition, data with temperatures higher than 30°C were excluded from the analysis, as the rate of N_2 fixation often reached a plateau or had started to decline i.e., deactivate (Breitbarth et al., 2007; Staal et al., 2003; Waughman, 1977).

Table 2. A summary of the studies used for the meta-analysis to explore the effect of temperature on N_2 fixation in aquatic or terrestrial ecosystems. Number of measurements (*n*). In total, the dataset for the meta-analysis consists of 151 measurements from 10 studies.

Ecosystem	*n*	Studies
Aquatic	62	(Breitbarth et al., 2007; Falcón et al., 2005; Lehtimaki et al., 1997; Staal et al., 2003)
Terrestrial	89	(Andersen and Shanmugam, 1977; Rainbird et al., 1983; Rao, 1977; Ryle et al., 1989; Smith and Hayasaka, 1982; Waughman, 1977)

From the meta-analysis, N_2 fixation activity is indeed sensitive to increasing temperature (Table 3, likelihood ratio test comparing best-fitting model M0 and null model M3: $\chi^2 = 9.18$, $p < 0.01$), with a consistent temperature sensitivity between aquatic and terrestrial ecosystems (Table 3, likelihood ratio test comparing M0 and M2: $\chi^2 = 0.35$, $p = 0.84$). The estimated average activation energy of biological N_2 fixation was 1 eV, with a 95% confidence level of 0.48 to 1.6 eV for both aquatic and terrestrial ecosystems (Fig. 2). This high activation energy shows that N_2 fixation is very energy-demanding and fixing N_2 in the cold is ecologically challenging. As a consequence, the abundance of diazotrophs has been shown to decrease as temperatures decline (Welter et al., 2015; Williamson et al., 2016).

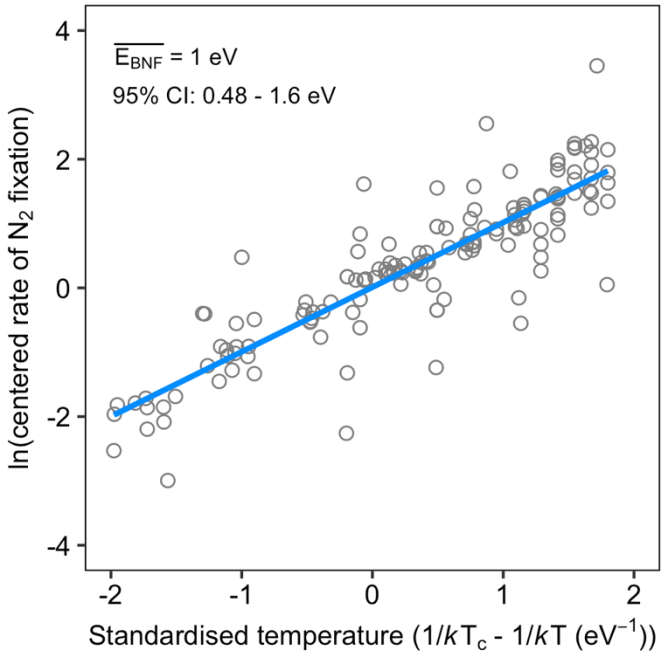

Fig. 2 | Meta-analysis fitting published rates of biological N_2 fixation as a function of temperature in incubations with biomass from both aquatic and terrestrial communities. Biological N_2 fixation clearly increases at higher temperatures with an average activation energy of 1 eV, which is consistent for both aquatic and terrestrial communities. I visualized the data using the "Visreg" package (Breheny and Burchett, 2017) in R showing the best-fitting linear mixed-effect model (blue line, Table 2) and partial residuals (grey circles). Temperature

was centered as Tc = (max+min)/2, i.e. 16.4°C, while the rate of N_2 fixation was natural log (ln) transformed and then centered by subtracting each study-specific intercept. n = 151 measurements from 10 studies.

Table 3 | Meta-analysis for published rates of biological N_2 fixation (BNF) as a function of incubation temperature for biomass from both terrestrial and aquatic ecosystems (Fig. 2). Linear mixed-effects model selection included centered temperature (Tc) and the interaction between Tc and ecosystem type as fixed effects, with a random intercept (1|Study) and slope (0+Tc|Study) to account for variation across the different studies (Table 2). Here, the best-fitting model (**M0**) showed that the rate of N_2 fixation increased at higher temperatures, but that the temperature sensitivity for N_2 fixation was not different between aquatic and terrestrial ecosystems. Models were ranked by the small sample-size corrected Akaike Information Criterion (AICc) with the better models (in **bold**) having lower AIC values. M3 is the null model which only included an intercept, denoted by 1. lnBNF is the natural log (ln) transformed rate of biological N_2 fixation, Temp is temperature and Ecosystem is ecosystem type (terrestrial or aquatic). Each model was compared to the best model using the Log-likelihood ratio test (LogLik, d.f. degrees of freedom) showing χ^2 (Chi-squared statistic) and p (the corresponding p-value).

Model	d.f.	AICc	LogLik	χ^2	p
M0: lnBNF~Tc	5	356.6	-173.1		
M1: lnBNF~Tc+Ecosystem	6	358.4	-172.9	0.37	0.54
M2: lnBNF~Tc*Ecosystem	7	360.5	-172.9	0.47	0.79
M3: lnBNF~1	4	364.0	-177.9	9.49	**<0.01**
M4: lnBNF~Ecosystem	5	365.8	-177.7	9.19	**<0.001**

1.6 The ecological advantage of N_2O fixation compared to N_2 fixation

Some early studies (1952-1986) showed that N_2O is a competitive inhibitor for N_2 fixation and it could also be a substrate for the enzymatic nitrogenase complex (Jensen and

Burris, 1986; Repaske and Wilson, 1952; Rivera-Ortiz and Burris, 1975; Wilson and Roberts, 1954), indicating that N_2O fixation (e.g. $N_2O \rightarrow NH_4^+$) may be related to N_2 fixation (e.g. $N_2 \rightarrow NH_4^+$). Further, as the dissociation energy of the N bond in N_2O (N≡N−O) is only half of that for N_2 (476 kJ per mol of N_2O vs. 940 kJ per mole of N_2) (Shestakov and Shilov, 2001), being able to fix N_2O could confer an ecological advantage to microbes in the cold compared to fixing N_2. Indeed, if, as for canonical N_2 fixation, N_2O is fixed by a nitrogenase using ferredoxin as the electron carrier, N_2O fixation is more energetically feasible (Alberty, 2005) ($\Delta_rG^0<0$, Eq. 2 and Eq. 3, *see* below). Therefore, being able to fix N_2O could confer an ecological advantage to some microbes either in the cold, e.g., when N_2-fixation is limited, or when resources (light or reduced substrates) in general are limiting.

As canonical N_2 fixation is mediated by nitrogenase using ferredoxin as a reductant, the biochemical equation can be expressed as (Alberty, 2005):

$$N_2(g) + 8Fd_{red} + 10H^+ \rightarrow 2NH_4^+ + 8Fd_{ox} + H_2(g) \quad (\Delta_rG^0 = -463 \text{ kJ mol}^{-1} N_2) \qquad (2)$$

The standard Gibbs free energy of N_2 fixation, Δ_rG^0, at 25°C, 1 bar was calculated as the sum of the standard Gibbs free energy from the formation of each component Δ_fG^0, i.e., $\Delta_rG^0 = \sum n\Delta_fG^0(\text{products}) - \sum n\Delta_fG^0(\text{reactants})$, where n is the number of molecules (Alberty, 2005).

If we assume that N_2O fixation uses the common nitrogenase complex for canonical N_2 fixation, we can replace N_2 in Eq. 2 with N_2O to obtain the equation for N_2O fixation. Further, to allow stoichiometric balance, H_2 in Eq. 2 is replaced with H_2O. The biochemical equation of N_2O fixation is therefore:

$$N_2O(g) + 8Fd_{red} + 10H^+ \rightarrow 2NH_4^+ + 8Fd_{ox} + H_2O(l) \quad (\Delta_rG^0 = -805 \text{ kJ mol}^{-1} N_2O) \quad (3)$$

The Δ_rG^0 of N_2O fixation can then be calculated from the Δ_fG^0 of each component from Eq. 3. As Δ_fG^0 for $N_2O(g)$ and H_2O were not included in Alberty, R. (2005), the values of these two components reported elsewhere (Hanselmann, 1991) were applied.

1.7 Regulatory factors on the sources and sinks of N_2O: a metadata analysis

1.7.1 Methods for data compilation and analysis

Despite the numerous reports of N_2O sinks in natural waters, the occurrence and the possible regulatory factors of these are not so clear. For example, as the high availability of fixed-N, such as NO_3^-, often promotes the production of N_2O, N_2O sinks often occurred in pristine environments (Farías et al., 2013; Hendzel et al., 2005; Maher et al., 2016; Soued et al., 2016; Whitfield et al., 2011). However, N_2O sinks have also been found in N-rich environments, such as fertilised land (Flechard et al., 2005) and farm reservoirs (Webb et al., 2019; Xia et al., 2013). Therefore, to gain a better understanding of where N_2O sinks typically occur, the scale of them, and to explore the possible regulatory factors, I compiled data from studies that reported undersaturation and/or negative flux of N_2O (Table 4).

N_2O saturation (normally in % of air equilibration) is an indicator of whether biological processes are net producing or consuming N_2O. Accordingly, undersaturation in N_2O means that the reduction of N_2O was greater than its rate of delivery such as from exchange with the atmosphere or biological sources, which shows that the study area is a sink for N_2O. As the equilibrial concentration for N_2O in water and air is a function of temperature and salinity (Weiss and Price, 1980), with studies that did not report N_2O saturation directly, I calculated N_2O saturation if they reported temperature, salinity, and N_2O concentration in the water. Where numerical data were not reported directly in the studies, data points were extracted from figures using the software WebPlotDigitizer 4.5 (Rohatgi, 2021).

Statistical analysis and plotting were performed in R (Team, 2021) using RStudio (Version 1.3.1093). I used generalized additive mixed effects models (GAMMs) (Zuur et al., 2009) to characterise any relationships between different environmental factors and N_2O

saturation (Fig. 4). Data compiled from different studies were converted to the same unit. In each model, the environmental factor e.g. temperature, nitrate etc., was treated as a fixed effect, with each study treated as a random effect. Models were ranked by the small sample-size corrected Akaike Information Criterion (AICc) using the 'MuMIn' package and the best-fitting model was determined by the lowest AICc score (*see* Table 5).

Table 4. A summary of the studies used for the meta-analysis to explore the existence of undersaturation and negative fluxes of N_2O in shallow freshwaters and surface-ocean-waters, and the regulatory factors on the saturation of N_2O in these oxic waters. Number of studies (*n*). In total, the dataset for the meta-analysis consists of 12 environment types from 46 studies.

Environment	*n*	Studies
Agriculture	2	(Hasegawa et al., 2000; Lemon and Lemon, 1981)
Aquaculture	1	(Ferrón et al., 2012)
Brackish water	1	(Bange et al., 1998)
Lake	6	(Lemon and Lemon, 1981; Liu et al., 2011b; McCrackin and Elser, 2011; Soued et al., 2016; Whitfield et al., 2011)
Mangrove	1	(Maher et al., 2016)
Surface ocean	13	(Amouroux et al., 2002; Cohen and Gordon, 1978; Cornejo et al., 2015; Elkins et al., 1978; Farías et al., 2013; Fenwick et al., 2017; Fenwick and Tortell, 2018; Kitidis et al., 2010; Ma et al., 2019; Priscu et al., 1990; Rees et al., 1997; Walter et al., 2006; Zhan et al., 2015)
Peatland	4	(Alm et al., 1999; Arsenault et al., 2018; Huttunen et al., 2002a; Regina et al., 1996)

Pond	4	(Huttunen et al., 2002b; Outram and Hiscock, 2012; Soued et al., 2016; Xia et al., 2013)
Reservoir	6	(Deemer et al., 2016; Diem et al., 2012; Hendzel et al., 2005; Huttunen et al., 2002c; Webb et al., 2019; Xia et al., 2013)
River	4	(Lemon and Lemon, 1981; Outram and Hiscock, 2012; Soued et al., 2016; Xia et al., 2013)
Stream	6	(Baulch et al., 2011; Beaulieu et al., 2008; Harrison and Matson, 2003; Hasegawa et al., 2000; Stow et al., 2005)
Wetland	6	(Søvik and Kløve, 2007; Ueda et al., 2000; Windham-Myers et al., 2018; Yu et al., 2012; Yuan et al., 2015; Zhu et al., 2008)

1.7.2 Saturation and flux of N_2O in oxic waters

From the meta-analysis, undersaturation or negative N_2O flux occurs across diverse environments (*see* Table 4 and the references cited therein). Reported undersaturation in N_2O in oxygenated waters (e.g., shallow freshwaters and surface-ocean-waters) was 77.2%, on average, ranging from 13.4% to 99.7% (Fig 3**a**). Whereas the negative flux of N_2O, i.e. net N_2O sink, was -13.6 μmol m^{-2} d^{-1}, on average, median -3.2 μmol m^{-2} d^{-1}, with a broad range of -145.3 to -0.03 μmol m^{-2} d^{-1} (Fig 3**b**).

The scale of N_2O saturation and flux varied greatly across different environments, generally with a highly skewed distribution (Fig 3), which leads to a lot of uncertainties in estimating the global N_2O budget (Syakila et al., 2010; Tian et al., 2020). Freshwater streams and ponds seem to be the most undersaturated in N_2O, with the lowest N_2O saturation reported at 13% (Fig 3**a**). Whereas rivers and agricultural waters are generally oversaturated in N_2O, despite a few studies also documenting undersaturation in N_2O in these waters (Hasegawa et al., 2000; Soued et al., 2016). Rivers with high loads of fixed N, such as those draining

agricultural catchments are well-known net sources of N_2O through the combination of denitrification and nitrification (Fig. 1) (Cole and Caraco, 2001; Laursen and Seitzinger, 2004).

The magnitude of negative N_2O flux, on the other hand, is overall larger in salty than fresh waters, with significant N_2O sinks found in wetlands and surface oceans (Fig 3**b**). Overall, for these studies that reported undersaturation in N_2O, the scale of oversaturation is still more pronounced than that of undersaturation, which could be one of the reasons that studies often focus on sources, rather than sinks of N_2O.

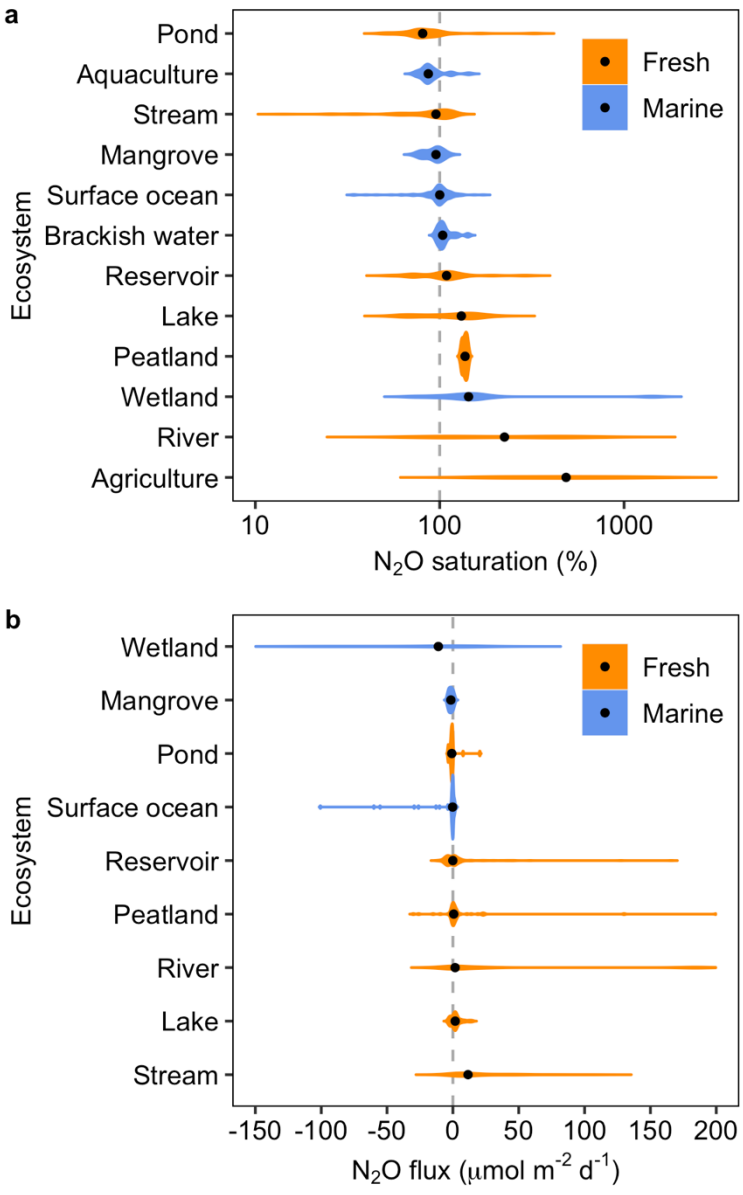

Fig. 3 | Saturation and fluxes of N_2O in shallow freshwaters and surface-ocean-waters where undersaturation and/or negative flux of N_2O have been reported. a, Violin plots of N_2O saturation across different fresh or marine ecosystems in rank order of median undersaturation. Note the log10 scale on the *x*-axis. *n* = 655 measurements in 12 different environments from 28 studies. **b**, Violin plots of N_2O flux across different fresh or marine ecosystems. *n* = 503 measurements, 9 environment types from 31 studies. The solid black points within each violin plot in **a** and **b** are the medians. The grey dashed line in **a** represents 100% atmospheric equilibrium for N_2O, while it denotes zero net N_2O flux in **b**, both indicating a complete balance between the consumption and production of N_2O.

1.7.3 Relationship between dissolved inorganic N and the sources and sinks of N_2O

Dissolved inorganic nitrogen (DIN) is important in regulating the production and consumption of both N_2 and N_2O. The production of N_2O has often been found to increase with higher concentrations of nitrate (NO_3^-) in both fresh and marine waters (Baulch et al., 2011; Beaulieu et al., 2008; Cornejo et al., 2007; Deemer et al., 2016; Forster et al., 2009; Liu et al., 2011a; Liu et al., 2011b; McCrackin and Elser, 2011; Reay et al., 2003; Stow et al., 2005; Xia et al., 2013), as higher availability of NO_3^- usually promotes the production of N_2O from denitrification (Garcia-Ruiz et al., 1998; Richardson et al., 2004). Similar to the effect of NO_3^-, higher availability of nitrite (NO_2^-) can also promote N_2O production (Cornejo et al., 2007; Nicholls et al., 2007). Accordingly, N_2O saturation can increase with the higher availability of NO_3^- (Fig. 4**a**, 4**d**, Table 5, M0 *vs*. null model M4: $\boldsymbol{p} < 0.001$) or NO_x^- (NO_2^- plus NO_3^-, Fig. 4**b**, 4**e**, Table 5, M0 *vs*. null model M4: $\boldsymbol{p} < 0.001$). For example, undersaturation in N_2O in streams is generally found at NO_x^- concentrations below 2.7 μM (Baulch et al., 2011).

In addition, higher availability of ammonium (NH_4^+) could also promote N_2O production (Barnes and Upstill-Goddard, 2011; Harrison and Matson, 2003; Xia et al., 2013) either through nitrification or coupled nitrification and denitrification, with most undersaturation in N_2O found at lower concentrations of NH_4^+ (Fig. 4**c**, 4**f**, Table 5, M0 *vs*. null model M4: $\boldsymbol{p} < 0.01$).

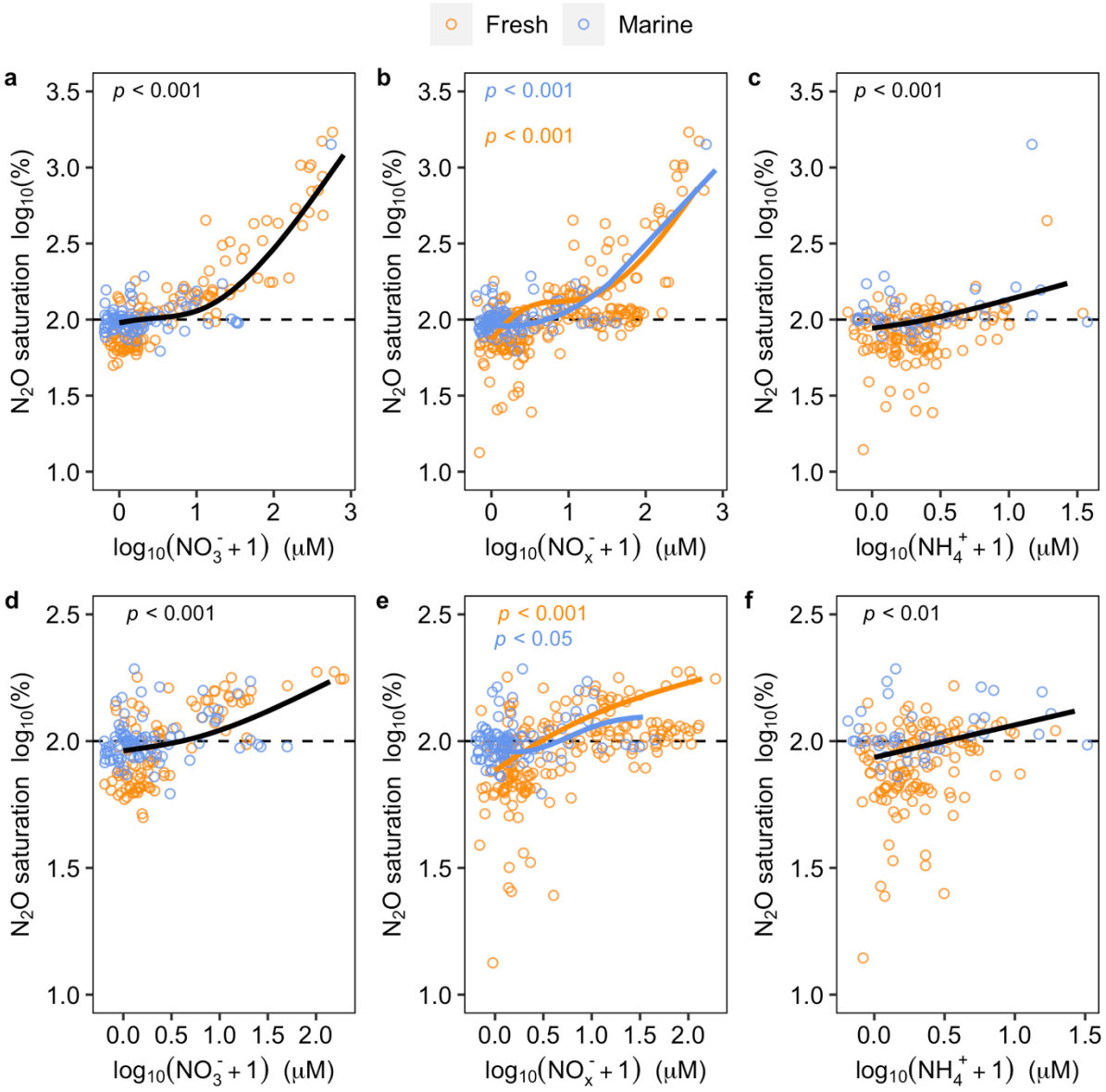

Fig. 4 | The relationship between N_2O saturation and dissolved inorganic nitrogen (DIN) species in fresh and marine oxic waters from the whole (a-c) or part of the dataset (d-f).

a, N_2O saturation increases with higher concentrations of NO_3^- and is consistent between fresh and marine waters. *n* = 213 measurements, 10 environment types from 18 studies. **b**, N_2O saturation increases with higher concentrations of NO_x^- (NO_2^- + NO_3^-), with different models denoting different responses between fresh and marine waters. *n* = 301 measurements, 11 environment types from 22 studies. **c**, N_2O saturation increases with higher concentrations of NH_4^+ and is consistent between fresh and marine waters. *n* = 174 measurements, 9 environment types from 13 studies. **d**, **e**, and **f** were plotted with part of the datasets that were used in **a**, **b**, and **c**, respectively, to show the effect of DIN when N_2O saturation was lower than 200% (log10 = 2.3). The dashed line in all plots denote 100% atmospheric equilibration for N_2O. The solid lines represent the best fitting generalized additive mixed effects models (GAMM, Table 5), where separate lines showing significant different pattern between fresh and marine waters, and one black solid line showing a consistent pattern. Note the log10 scale on the *x*-axes and *y*-axes of all plots. Data in all plots were mainly compiled from different studies that reported undersaturation in N_2O in shallow freshwater or surface ocean, where most of the undersaturation could not be explained by the reduction of N_2O to N_2 through canonical denitrification (typically occur in anoxic waters).

Table 5 | Multi-GAMM selection for exploring the effect of dissolved inorganic nitrogen (DIN) on N_2O saturation in fresh and marine waters (Fig. 4a-c). Here, I treated concentrations of DIN species (NO_3^-, NO_x^-, or NH_4^+) as fixed effects and fitted different smooth terms s() i.e., shape or pattern, and the interaction between DIN and ecosystem type (Marine or Fresh waters) as fixed effects, along with a random intercept (1|Study) to account for variation across the different studies (*see* Table 4). The term 'by=Ecosystem' within s() denotes a different shape for the smooth term describing the effect of DIN on N_2O saturation, whereas '+Ecosystem' denotes a different intercept (i.e., median) for either ecosystem. Models were ranked by the small sample-size corrected Akaike Information Criterion (AICc) with the better models (in **bold**) having the lowest AIC values. The null models only included an intercept, denoted by 1. Each model was compared to the best model in each panel using the Log-

likelihood ratio test (LogLik, d.f. degrees of freedom) showing χ^2 (Chi-squared statistic) and *p* (the corresponding *p*-value).

Model	d.f.	AICc	LogLik	χ^2	*p*
NO_3^-					
M0: log10(Sat)~s(NO_3^-)	**5**	**-319.0**	**164.7**		
M1: log10(Sat)~s(NO_3^-)+Ecosystem	6	-314.8	163.6	2.1	0.15
M2: log10(Sat)~s(NO_3^-,by=Ecosystem)	7	-311.0	162.8	3.8	0.15
M3: log10(Sat)~s(NO_3^-,by=Ecosystem)+Ecosystem	8	-307	161.9	5.6	0.13
M4: log10(Sat)~1	3	-106.5	56.3	217	**<0.001**
M5: log10(Sat)~Ecosystem	4	-103.2	55.7	218	**<0.001**
NO_x^-					
M0: log10(Sat)~s(NO_x^-, by=Ecosystem)	**7**	**-360.3**	**187.4**		
M1: log10(Sat)~s(NO_x^-)	5	-356.3	183.2	8.2	**<0.05**
M2: log10(Sat)~s(NO_x^-,by=Ecosystem)+Ecosystem	8	-354.8	185.7	3.4	0.07
M3: log10(Sat)~s(NO_x^-)+Ecosystem	6	-350.7	181.5	11.7	**<0.001**
M4: log10(Sat)~1	3	-138.6	72.4	230	**<0.001**
M5: log10(Sat)~Ecosystem	4	-134.7	71.4	231	**<0.001**
NH_4^+					
M0: log10(Sat)~s(NH_4^+)	**5**	**-115.1**	**62.7**		
M1: log10(Sat)~s(NH_4^+)+Ecosystem	6	-110.6	61.6	2.4	0.12
M2: log10(Sat)~s(NH_4^+,by=Ecosystem)	7	-108.9	61.8	2.0	0.37
M3: log10(Sat)~1	3	-108.7	57.4	10.7	**<0.01**
M4: log10(Sat)~s(NH_4^+,by=Ecosystem)+Ecosystem	8	-104.7	60.8	4.0	0.26
M5: log10(Sat)~Ecosystem	4	-104.1	56.2	13.1	**<0.001**

As assimilating already fixed-N is less energetically expensive than N_2 fixation, the rate of N_2 fixation often decreases with higher availability of fixed-N. While this inhibition effect of fixed-N on N_2 fixation is widely acknowledged, the thresholds of the inhibition, however, is

not so clear to date. N_2 fixation in anaerobic cultures of marine diazotrophs can be inhibited by NH_4^+ around 2 μM (Darnajoux et al., 2022). While 1 or 2 μM NH_4^+ did not inhibit N_2 fixation in cultures of *Trichodesmium* (Fu and Bell, 2003; Mulholland et al., 2001), N_2 fixation was inhibited by ~ 10 μM NH_4^+ in pure cultures of *Crocosphaera watsonii* (Dekaezemacker and Bonnet, 2011) and *Trichodesmium* (Mulholland and Capone, 2001; Mulholland et al., 2001). Thresholds higher than a few micromolar have also been reported - the proportion of nitrogenase-containing cells in cultures of *Trichodesmium sp.* significantly decreased after 48 h of 500 μM NH_4^+ addition, while no effect was found at lower dosings of NH_4^+ addition or shorter incubation periods (Lin et al., 1998).

The inhibition threshold of fixed N on N_2 fixation may vary for different diazothoph species. The inhabition on N_2 fixation occurred when NH_4^+ was less than 2 μM in pure cultures of sulfate reducing bacteria, while it was around 20 μM for fermenting bacteria (Darnajoux et al., 2022). Micromolar NO_3^- additions inhibited N_2 fixation in cultures of *Trichodesmium* spp. (Holl and Montoya, 2005; Mulholland et al., 2001), whereas the inhibition was not found in *Crocosphaera watsonii* (Dekaezemacker and Bonnet, 2011). Moreover, the exposure time could also be important - in cultures of *Trichodesmium sp.*, 10 μM of either NH_4^+ or NO_3^- did not inhibit N_2 fixation in the first generation, while significant inhibition occurred on that of the fifth generation (Fu and Bell, 2003).

Since studies on N_2O fixation are very limited, we know little about the regulating factors on N_2O fixation. (Cornejo et al., 2015) showed that the addition of NO_2^- (800 μM) or NH_4^+ (500 μM) can greatly suppress N_2O fixation in the laboratory experiments. However, the concentrations added are unrealistically high and greatly exceed the typical concentrations of fixed-N found in natural waters, e.g., NO_2^- in seawater typically ranges from undetectable to a few micromolar. As N_2O emissions often increase at higher DIN concentration, N_2O fixation, if it exists alongside a high availability of DIN, would likely be masked by N_2O emission.

Therefore, we are likely to find N_2O sinks in natural waters where fixed N is limited (Farías et al., 2013; Hendzel et al., 2005; Maher et al., 2016; Soued et al., 2016; Whitfield et al., 2011).

1.7.4 Relationship between dissolved oxygen and the sources and sinks of N_2O

In aquatic ecosystems, the production of N_2O is highly dependent on the availability of dissolved oxygen (Codispoti, 2010; Liss and Johnson, 2014). In oxic waters, N_2O concentration can increase with higher availability of dissolved oxygen, probably due to the stronger activity of N_2O production from nitrification as oxygen increases (Fenwick and Tortell, 2018; Walter et al., 2006). In waters where O_2 becomes physiologically limiting, e.g., $O_2 < 62$ μM (Naqvi et al., 2010) or $O_2 < 30\%$ air saturation (Codispoti, 2010), the reduction of NO_2^- to N_2O can also be facilitated through either canonical denitrification or nitrifier-denitrification (Farías et al., 2009; Naqvi et al., 2010; Yoshinari et al., 1997). As such, O_2-limited seawater often acts as a strong source of N_2O, with a negative correlation between N_2O production and the availability of O_2 (Cohen and Gordon, 1978; Rosamond et al., 2012; Trimmer et al., 2016). Therefore, with the expanding sub-oxic and hypoxic area in the oceans (Stramma et al., 2008), the budgets of N_2O could be greatly enhanced.

The reduction of N_2O, on the other hand, has also been found in environments over a wide range of dissolved O_2 (e.g. from 0 % to ~ 270% of air saturation) (Webb et al., 2019; Xia et al., 2013). The near-anoxic conditions (e.g., < 4.5 μM or near the core of the oxygen minimum zone) could favour the further reduction of N_2O to N_2 from canonical denitrification (Dalsgaard et al., 2012), resulting in a sharp drop in N_2O concentration (Codispoti and Christensen, 1985; Farías et al., 2009). Therefore, if canonical denitrification is the only pathway for N_2O reduction in natural waters, we would expect N_2O undersaturation to exist solely in low O_2 waters and that the activity of N_2O reduction to increase with decreasing O_2.

However, many accounts of N_2O reduction have been found under highly oxic conditions (*see* Table 1, Fig. 5**a**), which indicate that other process, e.g., N_2O fixation, could be responsible for N_2O reduction.

Although significant correlation between N_2O production and dissolved O_2 occurred in some studies (Mengis et al., 1997; Naqvi et al., 2010; Trimmer et al., 2016), due to the complicated effect of O_2 on the production and consumption of N_2O, the universal pattern is not so clear when I gathered different studies together (Fig. 4**d**). Interestingly, the effect of dissolved O_2 on N_2O saturation can also be bell-shape, with N_2O concentration decreasing with either low or high availability of dissolved O_2. This biphasic response of dissolved O_2 on N_2O saturation has been reported in both fresh (Webb et al., 2019) and marine waters (Walter et al., 2006) with a breakpoint of ~125 % and ~19% O_2 saturation, respectively, and many accounts of N_2O undersaturation occurring in O_2 oversaturated waters. This shows that net N_2O sinks can happen under both low and high O_2 conditions (Fig. 5**a**), and indicates that different pathways could be responsible or dominating for N_2O reduction under fully oxic or O_2 limiting conditions. For example, similar to N_2 fixation, N_2O fixation might also be tightly coupled to primary production due to the energy required to break the N-N bond. Therefore, while undersaturation in N_2O found in low O_2 waters is probably coupled to the dissimilatory reduction of N_2O to N_2, those found in waters with ample O_2 (e.g., shallow freshwaters and ocean surface waters) could be due to assimilatory N_2O fixation.

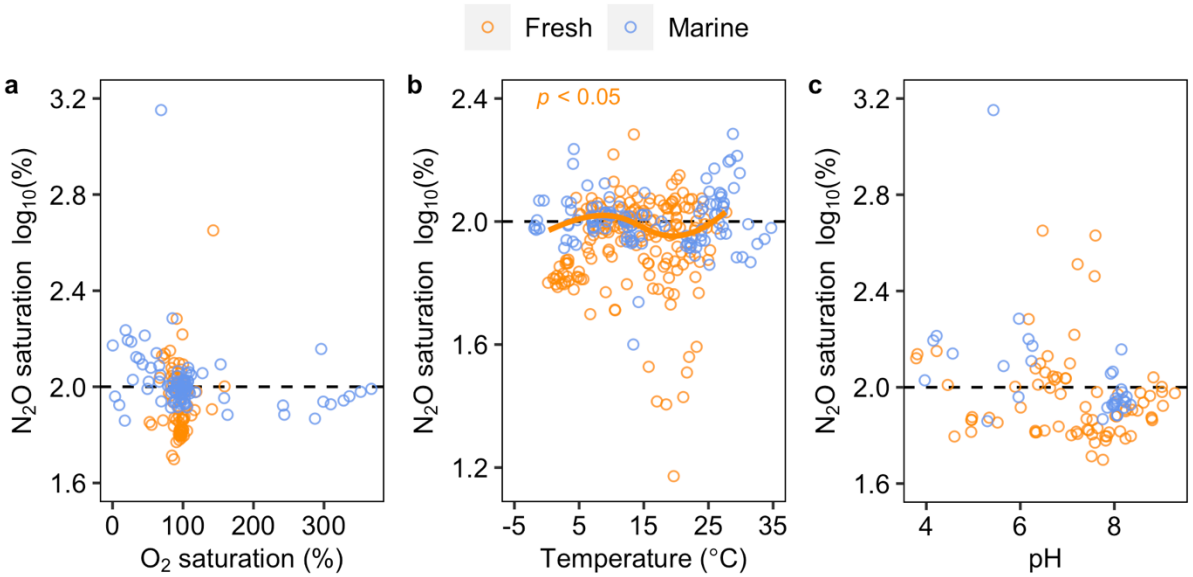

Fig. 5 | The relationship between N_2O saturation and dissolved O_2, temperature, and pH in fresh and marine waters. a, Overall, N_2O saturation seems not related to the availability of dissolved O_2, with N_2O undersaturation occurring under many O_2 conditions. n = 169 measurements, 9 environment types from 15 studies. **b**, Relationship between temperature and N_2O saturation in the freshwaters is statistically significant ($p < 0.05$), with N_2O being more undersaturated at around 5ºC and 20ºC degrees. While N_2O saturation was consistent across different temperatures in marine waters ($p = 0.72$). Note that **b** was plotted only with a subset of data when N_2O saturation was less than 200% (log10 = 2.3), while no significant effect of temperature was found when including the 10 data points with N_2O saturation higher than 200%. n = 291 measurements, 10 environment types from 22 studies. **c**, N_2O saturation seems to be consistent over a range of pH. n = 108 measurements, 8 environment types from 11 studies. The dashed line denotes 100% atmospheric equilibrium for N_2O. The solid lines in **b** represent the generalized additive mixed effects model (GAMM) which includes temperature and the interaction between temperature and ecosystem type in the smooth term, along with a random intercept to account for variation across the different studies. Note the log10 scale on the y-axes of all plots.

1.7.5 Relationship between temperature and sources and sinks of N_2O

As mentioned in section 1.5, temperature is a key regulator of N_2 fixation (Fig. 2) and could also be important for regulating N_2O fixation (*see* section 1.6). However, due to the different temperature dependencies of multiple processes that are related to the production or reduction of N_2O, the overall effect of temperature on the dynamic of N_2O is complicated.

N_2O emissions often increase with higher water temperatures in fresh (Soued et al., 2016; Stow et al., 2005; Zhao and Zhang, 2021) and marine waters (Barnes and Upstill-Goddard, 2011). From a dataset compiled from 58 reservoirs across the world, N_2O emission was also found to increase with higher air temperature (Deemer et al., 2016). These positive correlations between N_2O emission and temperature are probably due to that N_2O production from nitrification and denitrification increasing at higher temperatures (Berounsky and Nixon, 1990; He et al., 2012; Keeney et al., 1979). Conversely, N_2O production could also decrease at higher temperatures, e.g., in soils (Melin and Nômmik, 1983) and estuary sediments (Ogilvie et al., 1997), which could be due to the lower N_2O to N_2 ratio at higher temperatures as a result of higher activation energy of N_2O reduction than N_2O production from denitrification, e.g., 0.64 eV compared to 0.29 eV in soils (Holtan-Hartwig et al., 2002).

For studies that have reported N_2O sinks, e.g., in agricultural waters, net N_2O emission increased at higher water temperatures (Xia et al., 2013), with most N_2O sinks found during the coldest days (Xie et al., 2022). Boreal lakes, ponds, and rivers can act as N_2O sinks, with stronger N_2O sinks under colder temperatures (Soued et al., 2016). Similarly, in boreal peatlands, in Canada, N_2O emission increased at higher temperatures, with most N_2O sinks occurring below 13°C (Schiller and Hastie, 1994). In soils, some N_2O sinks were also found under colder temperatures (Donoso et al., 1993; Ryden, 1983), although a data compilation of N_2O sinks from multiple studies indicates that the effect of temperature on N_2O reduction is not so clear (Chapuis-Lardy et al., 2007). Nevertheless, these studies generally lack a clear

explanation for N_2O undersaturation found in the cold, and to my best knowledge, no study has explored the effect of temperature on N_2O fixation.

Here, through a meta-analysis, I have shown a significant relationship between temperature and N_2O saturation in freshwaters, while it was rather insignificant in the marine waters (Fig. 5**b**). Many cases of N_2O undersaturation were found near either 5°C or 20°C, which could be related to different processes reducing N_2O at low and high temperatures. As the reduction of N_2O to N_2 generally increases at higher temperatures, denitrification could explain N_2O undersaturation found at higher temperatures, whereas N_2O sinks found at lower temperatures could be due to a different N_2O-reducing process, such as N_2O fixation.

1.7.6 Other regulating factors on the sources and sinks of N_2O

Overall, the effect of pH on N_2O saturation was not evident across different studies (Fig. 4**f**). This agrees with the non-significant effect of pH on N_2O emission found in agricultural watersheds (Xia et al., 2013). In addition, other factors, such as salinity (Barnes and Owens, 1999), water stratification (Webb et al., 2019), and dissolved or particulate organic carbon (Baulch et al., 2011; Harrison and Matson, 2003) could also affect the sources and sinks of N_2O, but these factors are generally not considered as major stressors and studies on these are relatively scarce.

If N_2O fixation is also mediated by a nitrogenase enzyme, then it might be affected by similar environmental factors that are important for N_2 fixation. For example, since nitrogenase is inhibited by O_2, N_2 fixation is an O_2-sensitive process. Diazotrophs developed different ways to avoid this effect: some separate N_2 fixation and photosynthesis by fixing N_2 in the dark; while others fix N_2 with the presence of light either by confining N_2 fixation in specialized anaerobic cells (i.e., heterocysts) or by performing unique cell division (separate the N_2 fixing cells from the photosynthetic cells) in the colony as demonstrated by the non-heterocyst

forming *Trichodesmium* spp. (Berman-Frank et al., 2001; Zehr, 2011). As such, different phylotypes of diazotroph might prefer distinct light conditions and exhibit different diel-patterns on nitrogenase gene expression (Chen et al., 1998; Church et al., 2005). Further, N_2 fixation might also be affected the community diversity of diazotrophs. As reported by (Hsu and Buckley, 2009), the rate of N_2 fixation positively correlated with the higher community diversity of diazotrophs in soils. Thus, light and community diversity of diazotrophs could also be important for N_2O fixation. Nevertheless, further studies are needed to reduce the uncertainties on the regulatory factors of N_2O dynamics.

1.8 Aims and questions of this thesis

To date, great uncertainties remain around estimations for the budget of N_2O in aquatic ecosystems. Undersaturation in N_2O has been reported in various fresh and marine waters, yet many remain unaccounted for. N_2O fixation had been reported in the Eastern South Pacific (Cornejo et al., 2015; Farías et al., 2013) and soybean nodules (Mozen and Burris, 1954), which could explain these unaccounted for N_2O sinks in natural waters. However, to the best of my knowledge, the existence of N_2O fixation has not been explored in any freshwater ecosystem.

In Chapter 1, I have shown that N_2O sinks are more likely to exist in N-limited waters (Fig. 4**a**-**c**, Table 5, $p < 0.01$). Temperature could be a key stressor for N_2O fixation, as N_2O fixation would be more energetically favorable than N_2 fixation, especially in the cold (Section 1.5, Eq. 2 and Eq. 3). With potentially both the last step in denitrification ($N_2O \rightarrow N_2$) and N_2O fixation ($N_2O \rightarrow NH_4^+$) providing sinks for N_2O, it is ecologically important to distinguish between these two parts of total N_2O reduction. Further, any genuine direct N_2O fixation ($N_2O \rightarrow NH_4^+$) needs to be distinguished from indirect N_2O fixation i.e., that after the initial reduction of N_2O to N_2 ($N_2O \rightarrow N_2 \rightarrow NH_4^+$). Despite the few studies (Cornejo et al., 2015; Farías et al., 2013; Mozen and Burris, 1954) documenting N_2O fixation so far, no one has

characterised N_2O fixation in relation to canonical N_2 fixation through the dual-use of $^{15}N_2O$ and $^{15}N_2$ in natural communities. Moreover, with potentially both the last step in denitrification ($N_2O \rightarrow N_2$) and N_2O fixation ($N_2O \rightarrow NH_4^+$) providing sinks for N_2O, it is ecologically important to distinguish between these two parts of total N_2O reduction. Further, any genuine direct N_2O fixation ($N_2O \rightarrow NH_4^+$) needs to be distinguished from indirect N_2O fixation i.e., that after the initial reduction of N_2O to N_2 ($N_2O \rightarrow N_2 \rightarrow NH_4^+$).

Therefore, during my PhD, I have characterised the existence of N_2O fixation in freshwaters, the possible fates of N_2O reduction, and characterised the effect of temperature on N_2O reduction. In this thesis, I address the following questions:

- Does N_2O-dependent N fixation exist in freshwater communities?
- Is N_2O fixation important, e.g., can N_2O fixation provide a significant sink for N_2O in natural waters?
- What are the fates of the reduced N_2O? Can we distinguish different pathways for N_2O reduction, i.e., the dissimilatory reduction of N_2O to N_2 from canonical denitrification and assimilatory N_2O fixation?
- What is the effect of temperature on N_2O fixation compared to N_2 fixation? Would N_2O be preferred over N_2 in the cold?

To answer these questions, I performed a series of experiments, mainly with biomass from 20 experimental ponds in East Stoke, Dorset, UK (2°10'W, 50°30'N). These ponds (each with 1 m^3 water volume, 0.5 m depth) were set up in 2005, to experimentally study the whole-ecosystem effects of climate warming (Barneche et al., 2021; Yvon-Durocher et al., 2017; Zhu et al., 2020). Here, however, I exploited the fact that the ponds are also N-limited (Barneche et al., 2021), being fed only by rainwater, i.e., with supplies of N only coming from N fixation or N deposition from the atmosphere, to characterise any potential N_2O fixation in a controlled, experimental system. Despite being artificial, the ponds have well-established freshwater

ecosystems (Barneche et al., 2021; Yvon-Durocher et al., 2015; Yvon-Durocher et al., 2017; Zhu et al., 2020) with diverse cyanobacteria communities (Yvon-Durocher et al., 2015; Yvon-Durocher et al., 2010), among which some Nostocales (Hayes et al., 2019; Lesser, 2008) and Oscillatoriales (Stal and Krumbein, 1987) are known to fix N_2. The N-limited condition and the presence of diverse N-fixers indicate that these ponds have a high potential for N fixation and could potentially act as N_2O sinks.

In summary, I measured dissolved N_2O and N_2 to demonstrate the existence of both N_2O and N_2 sinks in the freshwater ponds. With incubations of biomass from the ponds using ^{15}N stable isotope techniques, I quantified the rates of N_2O and N_2 fixation and characterised the temperature dependence of the two N fixation processes. Further, to gain a more complete view of N_2O dynamics in freshwaters, I explored the production of N_2O from either denitrification or nitrification and characterised the potential temperature dependence of these two processes. My thesis advances our understanding of the different processes of N cycle in natural waters, and the dynamics of the atmospherically potent gas N_2O, especially under the current global warming scenario.

1.9 Structural outline of the thesis

Chapter 1

In the first chapter, I introduced the biological pathways for the production and consumption of N_2O. Most N_2O sinks found in oxygenated waters remain unaccounted for, as the canonical N_2O reduction to N_2, which occur in anoxic conditions, could not explain these N_2O sinks. Although a novel pathway for N_2O reduction – N_2O fixation was reported in the surface ocean and soybean nodules, the regulation of it is unknown. Therefore, I compiled data from various studies that reported N_2O sinks in natural waters and tried to identify the regulatory factors of N_2O sinks. From the meta-analysis, I showed that N_2O sinks are mostly found in N-limited

waters, with a positive correlation between N_2O saturation and fixed-N species (NO_3^-, NO_2^- + NO_3^-, NH_4^+). In addition, temperature is important in regulating the production and consumption of N_2O and could be a key factor for differentiating N_2O and N_2 fixation.

Chapter 2

From the meta-analysis in chapter 1, N_2O sinks seem to occur more often in N-limited ecosystems (Fig. 4**a**-**c**, Table 5, $p < 0.01$), and we have some well-established, N-limited experimental ponds set up already. Therefore, I measured dissolved N_2O and N_2 in the ponds to evaluate whether the ponds could act as sinks for either of the two gases and explored the possible seasonal patterns. Based on the wind speed around the pond and the saturation of N_2O and N_2, I estimated the fluxes of the gases and the contribution of either gas to the primary production in the ponds.

As the ponds are generally sinks for N_2O, to see whether these sinks are driven by N_2O fixation, I incubated biomass fractions from the ponds using ^{15}N-isotope techniques to trace whether N_2O can be fixed. Further, based on the distinct seasonal patterns of dissolved N_2O and N_2 which were probably driven by changes in temperature, I explored the temperature sensitivity of both N_2O and N_2 fixation. From the incubations, I found that both N_2O and N_2 can be assimilated by the biomass, yet they have different temperature sensitivities, which reflects the seasonal patterns of N_2O and N_2 saturation measured in the ponds.

In addition, it is important to confirm whether N_2O assimilation is from direct N_2O fixation, or indirect N_2O fixation i.e. after the reduction of N_2O to N_2 followed by N_2 fixation. Here I demonstrate that N_2O fixation is mainly direct, based on the ^{15}N-labelling of the respective N_2O and N_2 substrates and the measured rates of $^{15}N_2O$ and $^{15}N_2$ assimilation.

Chapter 3

In chapter 2, I show that N_2O can be assimilated into biomass and the reduction of N_2O to N_2 is a minor part in total N_2O reduction. As the reduction of N_2O to N_2 and N_2O fixation could both contribute to N_2O reduction, I quantified the proportion of the two processes and showed that N_2O reduction by the biomass mainly came from N_2O fixation rather than denitrification. Further, total reduction of N_2O potentially consists of multiple end-products from different pathways. Apart from the reduction of N_2O to N_2 and the assimilation of N_2O into the biomass, N_2O can be fixed as NH_4^+ and further oxidised to NO_x^-. These fixed, inorganic N-species can be lost to the water where they are available to the wider ecosystem.

Chapter 4

After characterizing the effect of temperature on N_2O fixation, I further explored the effect of temperature on the production of N_2O via denitrification and nitrification. As N_2O can be both produced or further reduced to N_2 via denitrification, the net product ratio of N_2O to N_2 from denitrification is important for the N_2O budget.

The N_2O produced from nitrification or denitrification is either being recycled within the ecosystem by N_2O fixation, or being reduced to N_2 then emitted from the ecosystem to the atmosphere. From the results of Chapter 2, the ratio of N_2O to N_2 fixation is higher in the cold (Fig. 8). Therefore, the higher product ratio of N_2O to N_2 from denitrification in the cold (Chapter 4, Fig. 7) indicates that the N in N_2O could be recycled by N_2O fixation and conserved in the cold, rather than being reduced to N_2 and "lost" by emission to the atmosphere. Overall, combining the patterns of N_2O production and N_2O reduction provide a more comprehensive picture of N_2O dynamics – compared to N_2, the accumulation and fixation of N_2O are both more pronounced in the cold, suggesting a link between N_2O production and reduction in natural cold waters.

Chapter 5

In the final chapter, I summarise the results from previous chapters, discuss the main findings from my PhD work, and draw some conclusions. I then discuss the significance of my research and possible directions for future research to further advance our understanding of N_2O dynamics in natural ecosystems.

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Chapter 2 Direct biological fixation provides a freshwater sink for N_2O

Abstract

Undersaturation in N_2O reported in natural waters has been routinely attributed to the reduction of N_2O to N_2 in the last step in denitrification under anoxic conditions. From the meta-analysis of published data in Chapter 1, I showed that N_2O undersaturation has also been found in many oxic waters, with most of these remaining unaccounted for. Although a sink for N_2O through biological fixation has been observed in the Pacific, the regulation of N_2O- compared to canonical N_2-fixation is unknown. Here I show that both N_2O and N_2 can be fixed by freshwater communities but with distinct seasonalities and temperature dependencies. N_2O fixation appears less sensitive to temperature than N_2 fixation, driving a strong sink for N_2O in winter. Moreover, by quantifying both N_2O and N_2 fixation I show that, rather than N_2O being first reduced to N_2 through denitrification, N_2O fixation is direct and could explain the widely reported N_2O sinks in natural waters.

2.1 Introduction

As shown in Chapter 1, although many accounts of N_2O undersaturation has been found in well-oxygenated, shallow freshwaters (Baulch et al., 2011; Diem et al., 2012; Guérin et al., 2008; Hendzel et al., 2005; Lemon and Lemon, 1981; Liu et al., 2011b; Soued et al., 2016; Webb et al., 2019; Whitfield et al., 2011) and surface-ocean-waters (Bange et al., 1998; Butler et al., 1989; Cornejo et al., 2015; Farías et al., 2013; Priscu et al., 1990; Rees et al., 1997; Verdugo et al., 2016; Walter et al., 2006; Zhan et al., 2015) (*see* Table 1, Chapter 1), the reasons for these N_2O undersaturation are poorly understood.

N_2O fixation (e.g. $N_2O \rightarrow NH_4^+$) provides an alternative pathway for N_2O reduction to the last step in denitrification ($N_2O \rightarrow N_2$), which makes it important to explore whether N_2O fixation could be responsible for some of the N_2O undersaturation found in natural waters. However, despite the few studies (Cornejo et al., 2015; Farías et al., 2013; Mozen and Burris, 1954) documenting N_2O fixation so far, to the best of my knowledge, no one has characterised N_2O fixation in relation to canonical N_2 fixation through the dual-use of $^{15}N_2O$ and $^{15}N_2$ in natural communities.

N_2O fixation (e.g. $N_2O \rightarrow NH_4^+$) may be related to N_2 fixation (e.g. $N_2 \rightarrow NH_4^+$) as it is a competitive inhibitor for N_2 fixation and could also be used as a substrate by nitrogenase (Jensen and Burris, 1986; Repaske and Wilson, 1952; Rivera-Ortiz and Burris, 1975; Wilson and Roberts, 1954). There could be enzymes other than nitrogenase responsible for N_2O fixation. However, as no known freshwater candidates for N_2O fixation to date and the few recognised N_2O fixers are diazotrophs (Cornejo et al., 2015; Farías et al., 2013; Mozen and Burris, 1954), it is possible that the recognised N_2 fixing community also fix N_2O. Whether N_2O fixation is mediated by the total nitrogenase (*nifH*) community simply in relation to the relative availability of N_2O to N_2, or it is preferentially mediated by a subset of the *nifH* community is unknown.

As shown by Eq.2 and Eq.3 in Chapter 1, if they are both performed by nitrogenase with ferredoxin as the electron carrier, N_2O fixation would be more energetically feasible than N_2 fixation (Alberty, 2005). Moreover, the dissociation energy of the N bond in N_2O ($N{\equiv}N{-}O$) is only half of that for N_2 (Shestakov and Shilov, 2001). N_2 fixation is known to have a high activation energy due to its strong $N{\equiv}N$ bond (Allen et al., 2005; Welter et al., 2015), which makes fixing N_2 in the cold energetically unfavourable. Therefore, compared to fixing N_2, fixing N_2O could be ecologically beneficial to microbes in the cold. Due to the different

thermodynamics of N_2 and N_2O fixation, it is important to characterise how N_2O and N_2 fixation would respond to changing temperature.

In addition, with both the last step in denitrification ($N_2O \rightarrow N_2$) and N_2O fixation ($N_2O \rightarrow NH_4^+$) providing sinks for N_2O it is ecologically important to distinguish between these two parts of total N_2O reduction. Further, any genuine direct N_2O fixation ($N_2O \rightarrow NH_4^+$) needs to be distinguished from indirect N_2O fixation i.e., that which occurs after the initial reduction of N_2O to N_2 ($N_2O \rightarrow N_2 \rightarrow NH_4^+$).

As N-rich environments typically act as sources of N_2O (Baulch et al., 2011; Walter et al., 2006) that would likely inhibit N_2O fixation, N-limited environments could be more likely to act as N_2O sinks and would be ideal to characterise any N_2O fixation. Indeed, many accounts of N_2O undersaturation have been reported in N-limited environments (Diem et al., 2012; Farías et al., 2013; Lemon and Lemon, 1981; Soued et al., 2016; Verdugo et al., 2016).

In 2005, 20 experimental ponds (each with 1 m^3 water volume, 0.5 m depth) in East Stoke, Dorset, UK were set up to experimentally study the whole-ecosystem effects of climate warming (Barneche et al., 2021; Yvon-Durocher et al., 2015; Yvon-Durocher et al., 2017; Yvon-Durocher et al., 2010; Yvon-Durocher et al., 2011a; Yvon-Durocher et al., 2011b; Zhu et al., 2020). Half of the ponds have been warmed by 3-5 °C above ambient temperatures since September 2006, with the long-term warming already showing significant effects on the total carbon cycle, methane emissions, trophic transfer efficiency, and metabolic balance of ecosystems (Barneche et al., 2021; Yvon-Durocher et al., 2017; Yvon-Durocher et al., 2010; Zhu et al., 2020).

Here, however, I exploited the fact that the experimental ponds are also N-limited (Barneche et al., 2021), being fed only by rain water, to characterise any potential N_2O fixation in a controlled, experimental system. Despite being artificial, the ponds have well established freshwater ecosystems (Barneche et al., 2021; Yvon-Durocher et al., 2015; Yvon-Durocher et

al., 2017; Zhu et al., 2020) with diverse communities that potentially fix N_2 (Yvon-Durocher et al., 2015), such as some Anabaenas, Azotobacter (Mozen and Burris, 1954), Nostocales (Hayes et al., 2019) and Oscillatoriales (Stal and Krumbein, 1987).

The key questions in this chapter are:

- Would the N-limited experimental ponds act as sinks for N_2O?
- Could N_2O fixation be responsible for N_2O sinks found in natural waters?
- Would N_2O and N_2 fixation respond differently to temperature? For example, with the potential ecological advantage of N_2O fixation in the cold, would there be a higher proportion of N_2O fixation relative to N_2 fixation at lower temperatures?

In this chapter, I show that the N-limited experimental ponds are sinks for both N_2O or N_2 by measuring the dissolved concentrations of both N_2O and N_2 in all ponds for 11 months from November 2019 to April 2022. With these results I further hypothesized that the pond communities fix both N_2O and N_2 to support primary production. To test these, I used incubations with pond biomass and $^{15}N_2$ and $^{15}N_2O$ stable isotope techniques to quantify their fixation activity, distinguish direct from indirect N_2O fixation and characterise the temperature dependence of both N-fixing processes.

2.2 Methods

2.2.1 Nutrient analysis

On each sampling date, temperature and O_2 concentration were measured in the experimental ponds using HQD portable meters (Hach), before any possible disturbance caused by sampling for gases, water, or biomass.

Water samples for nutrient analysis were collected from each pond using a syringe and filtered through clean 0.45 μM Syringe PES filters (ø = 25mm, Gilson) into a 15 ml sterilized

Falcon tube. The syringes and filters were pre-washed with deionized water (18.2 MΩ.cm, Elga) in the laboratory, and rinsed with sample water on site before use. Samples were immediately frozen at -20 °C upon returning to the laboratory. Before analysis, samples were thawed at 4°C overnight. Nitrite (NO_2^-), nitrate (NO_3^-), ammonium (NH_4^+), and soluble reactive phosphorous (SRP) were measured simultaneously on a continuous flow wet-chemistry autoanalyzer (San^{++}, SKALAR Analytical B.V.) with standard colorimetric techniques (Kirkwood, 1996). A calibration curve was prepared by diluting a mixture of NH_4Cl, $NaNO_2$, KNO_3, and KH_2PO_4 standard solutions (certified reference materials, traceable to NIST) in deionized water. The detection limits were 0.05 μM and 0.1 μM for NO_2^- and NO_x^- (NO_2^- + NO_3^-), respectively, with NO_3^- measured after reduction to NO_2^- through a cadmium-copper column. The detection limit was 0.2 μM for NH_4^+, and 0.05 μM for SRP. TIN (Total inorganic nitrogen) was calculated as the sum of NO_2^-, NO_3^- and NH_4^+. The ratios of N to P were determined from TIN and SRP concentrations, where values of SRP and TIN below the detection limit were omitted from any calculations.

2.2.2 Dissolved N_2 and N_2O

For dissolved N_2 and N_2O analysis, water samples were taken at mid-depth (~20 cm from the surface) of each pond. Samples were taken carefully using a 60 mL syringe with silicone tubing without introducing any bubbles. For each pond, five gas-tight vials (12 mL Exetainer, Labco) were over-filled with water at least three times, 50 μL of 50% (w/v) $ZnCl_2$ added as a bactericide using a long needle (Lansdown et al., 2016) and the vials closed and mixed by hand. Atmospheric air was also sampled at the same time as water collection. Extra pond waters were taken and preserved with $ZnCl_2$ for preparing references for N_2 saturation later.

Upon return to the laboratory, the extra pond waters were equilibrated with the atmosphere for over 2 d in a temperature-controlled room (22 °C) with temperature monitored

continuously to 0.01 °C using a data logger (HOBO Pendant, Onset). After full equilibration, references were prepared using the same method as field samples. Laboratory air was also sampled at the same time as references collection. Before gas analysis, a 2 ml helium (99.999% purity) headspace was created in all vials. All water samples were then equilibrated on an orbital shaker (SSL1, Stuart) in the temperature-controlled room for over 24 h. During gas-sample processing, all vials were weighed to determine the exact volume of headspace and water in each for calculating gas saturations, below.

For N_2O analysis, 100 μL of headspace were extracted by an autosampler and injected into a gas chromatograph fitted with a μECD (Agilent Technology UK Ltd., South Queensferry, UK) using conditions described previously (Nicholls et al., 2007) and air samples measured at the same time as water samples. Calibrations were performed against known concentrations of N_2O from NOAA standard (traceable to the SI unit "amount of substance fraction") at 359.73 ppb or 120 ppb, 1.04 ppm, and 96 ppm from BOC, UK cross-calibrated to the NOAA standard.

The concentration of dissolved N_2O in the vial ($C_{measured}$, μmol L^{-1}) was calculated as:

$$C_{measured} = \left(C_{hs} \times V_{hs} + C_{aq} \times V_{aq}\right)/V_{aq} \quad (1)$$

Where C_{hs} and C_{aq} are the concentrations of N_2O (μmol L^{-1}) in the headspace and aqueous phase in the vial. V_{hs} and V_{aq} are the volume of headspace and aqueous phase in the vial, respectively. C_{hs} was calculated from the mole fraction of headspace (X_{hs}, ppm or μmol mol^{-1}, acquired from GC measurement) and molar volume of N_2O at laboratory temperature (V_m, L mol^{-1}):

$$C_{hs} = X_{hs}/V_m \quad (2)$$

Whereas C_{aq} was calculated from X_{hs} and the equilibrium constant of N_2O at laboratory temperature (K_{lab}, mol L^{-1} atm^{-1}) based on (Weiss and Price, 1980):

$$C_{aq} = X_{hs} \times K_{lab} \quad (3)$$

The expected N_2O concentration ($C_{expected}$) is the concentration of dissolved N_2O when pond water is fully equilibrated with the atmosphere and was calculated based on the mole fraction of N_2O in the field air (X_{field}, acquired from GC measurement) and the equilibrium constant of N_2O at the field temperature (K_{field}):

$$C_{expected} = X_{field} \times K_{field} \quad (4)$$

N_2O saturation was then calculated as the ratio of measured to expected dissolved N_2O concentration:

$$N_2O \text{ Saturation } (\%) = C_{measured}/C_{expected} \times 100 \quad (5)$$

For the analysis of dissolved N_2 in the ponds, I used the N_2:Ar method (Eyre et al., 2002). Argon has a similar temperature-dependence of solubility to N_2, and was used as a conservative tracer (Hamme and Emerson, 2004). 100 μL sample headspace was injected into an elemental analyzer (Flash EA 1112 series, Thermo Finnigan), where O_2 in the samples was removed by the hot-copper reduction column. N_2 and Ar were then analyzed by the interfaced continuous flow isotope ratio mass spectrometer (CF-IRMS, Delta V Plus, Thermo Finnigan). Throughout each run, air samples were analyzed to correct for machine drift. The expected concentrations of N_2 or Ar in the headspace (C_{hs}) of the samples were calculated using the solubility of N_2 and Ar for samples of field air under both field (K_{field}) and laboratory (K_{lab}) temperature (Weiss, 1970):

$$C_{hs} \times V_{hs} + C_{hs} \times K_{lab} \times V_{aq} = C_{field} \times K_{field} \times V_{aq} \quad (6)$$

Similarly, the expected concentrations of N_2 and Ar in the headspace of the references were calculated using the solubility of N_2 and Ar for samples of laboratory air under laboratory temperature. The saturation of N_2 in the samples was then derived by comparing the measured to expected ratio of N_2 to Ar in the samples to that in the references (Loeks-Johnson and Cotner, 2020):

$$N_2 \text{ Saturation } (\%) = \left(\frac{N_2/Ar \text{ measured}}{N_2/Ar \text{ expected}}\right)_{Sample} / \left(\frac{N_2/Ar \text{ measured}}{N_2/Ar \text{ expected}}\right)_{reference} \times 100 \quad (7)$$

Where the measured ratio of N_2 to Ar was derived by comparing the peak area of N_2 and Ar from the CF-IRMS. The precision of the ratio of N_2 to Ar for triplicate references and air standards was 0.1% and 0.05% (coefficient of variation), respectively. In addition, I also tested the difference in the calculation of N_2 saturation when using different references. The ratio of deionized water to pond water as a reference was on average 99.7% and 99.82% on two different sampling months (n = 20 and n = 8, respectively). The effect of preservative was more evident - not using $ZnCl_2$ for the deionized water reference could result in a 5.15% over-estimation in the calculated N_2 saturation in the samples (n = 4).

It should be noted that the nutrient and gas saturation data collected for February, 2020, were omitted from further analysis, due to the exceptionally heavy rainfall before the sampling date. In February 2020, England had 209 mm of rain, which is the highest for February since 1862. The nearby (2.5 km) town of Wareham had ~ 200% of the average rainfall for February between 1981-2010 (Source: Met Office National Climate Information Centre). This possibly contributed to some changes in nutrient concentrations (e.g., diluted NO_2^- and NO_3^-) and re-equilibrated atmospheric gases in the ponds at the time of sampling.

2.2.3 Incubations to characterise the temperature dependence of N_2 and N_2O fixation

In typical $^{15}N_2$ fixation studies, the fixation of $^{15}N_2$ is confirmed by the enrichment of ^{15}N in incubated biomass samples. Therefore, I characterised the fixation of N_2 or N_2O by incubating biomass from the ponds with $^{15}N_2$ or $^{15}N_2O$ tracers. Temperature was manipulated in the incubations to characterise the respective temperature dependencies of N_2 and N_2O fixation.

Biomass collection

Two different biomass types were collected from the ponds for incubations (Fig. 1). Floating assemblages on the surface of the ponds e.g., assemblages containing *Oedogonium* spp. and microorganisms attached to the filaments (Fig. 1**b**), were sampled into 50 ml sterilized Falcon tubes. Pilot studies by former Master student Betty Boyse, found that floating assemblages containing *Oedogonium* spp. is capable of reducing N_2O, and they are commonly found in the experimental ponds. Benthic assemblages, for example, green or yellow mats or assemblages formed on the bottom of the pond (Fig. 1**c**) were taken separately with extra care to avoid sampling the sandy sediments beneath. Collected samples of floating and benthic assemblages in pond water were put in a temperature-controlled room (15 °C) overnight before the incubations to acclimatise the culture to nitrogen starvation.

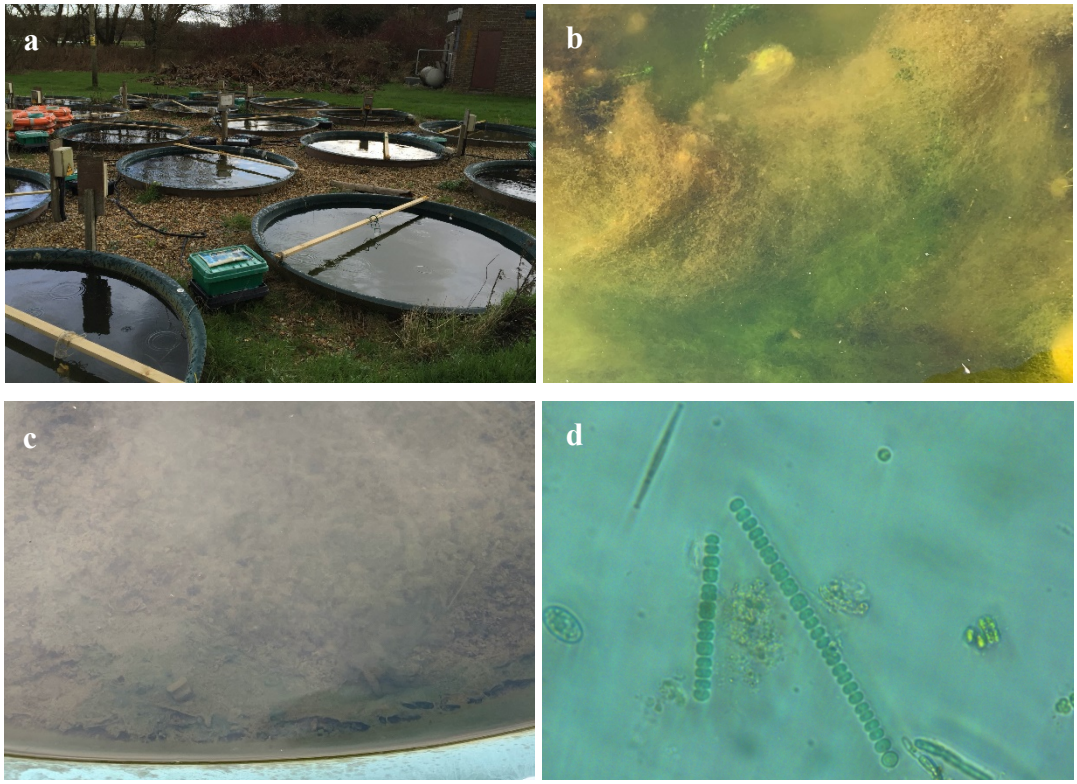

Fig. 1 | View of the experimental ponds and biomass types used in the incubations. a, The artificial, experimental ponds in East Stoke, Dorset, UK, established in September 2005 (Yvon-

Durocher et al., 2010). **b**, Close-up showing floating assemblages, dominated by *Oedogonium* sp., on the surface of the ponds. **c**, Close-up showing organic benthic material covering the bottom of a pond. **d**, Microscopy image showing filaments of *Anabaena sp*. – a N_2-fixing cyanobacterium, identified in the floating assemblages in the ponds. Copyright: **a** – **c,** Yueyue Si, **d**, Danielle Marchant.

Artificial freshwater medium

Despite the ponds being very low in dissolved inorganic nitrogen (Barneche et al., 2021), I standardized the nutrient concentrations to minimize the dissolved inorganic nitrogen present in the incubations. I used an artificial pond water medium by dissolving high-grade chemicals in deionized water (18.2 MΩ.cm, Elga) comprising: $CaCl_2$ (0.5 mM), KCl (1 mM), $MgSO_4$ (0.25 mM), $KHCO_3$ (0.7 mM) and $NaHCO_3$ (0.5 mM). P was added to 0.08 μM of $NaPO_4$, based on measured SRP concentrations in the ponds. Dissolved inorganic nitrogen was excluded from the medium, as the addition of combined N might have suppressed any N fixation activity (Knapp, 2012).

Preparation of stock solutions for $^{15}N_2$ and $^{15}N_2O$ substrate additions

Although the $^{15}N_2$ bubble addition method has been used widely for estimating N_2 fixation rates, N_2 fixation rates could be greatly underestimated due to the presence of un-equilibrated $^{15}N_2$ gas in the bottles, especially during short-term incubations (Mohr et al., 2010). The equilibration of N_2 between gaseous and aqueous phases could take more than 24 hours (Mohr et al., 2010), which exceeds the typical incubation time in most N_2 fixation studies. The uneven distribution of gaseous substrate in the incubation bottle means that the N-fixers could be exposed to lower concentrations of N substrate, while higher concentrations remain in the micro-environment around the gas bubbles. Therefore, to obtain more accurate measurements

of N fixation rates, I made an aqueous $^{15}N_2$ stock by pre-equilibrating the medium with $^{15}N_2$ gas.

$^{15}N_2$ gas (98% atom % ^{15}N, Sigma-Aldrich) was injected into an evacuated gas sampling bag (PTFE/silicone septum, FEP film) and 200 ml of artificial medium was injected into a separate evacuated 0.5 L gas sampling bag. $^{15}N_2$ stock was then prepared by transferring 40 ml of $^{15}N_2$ gas from the gas bag into the medium bag with a gastight syringe. Stock solutions were inverted, allowing the water to seal the outlet, to prevent gas loss and possible contamination from the atmosphere, followed by shaking for over 24 h to reach full dissolution. This dissolution method ensured that the ^{15}N tracers were distributed uniformly upon addition, hence avoiding substrate heterogeneity during the incubations. $^{15}N_2O$ stock solutions were prepared by replacing 3 ml of water with $^{15}N_2O$ gas (98% atom % ^{15}N, Cambridge Isotope Laboratories, Inc.) in a 50 mL sealed serum bottle (DWK Life Sciences Wheaton™) pre-filled with deionized water.

As the solubility of N_2 is quite low, to maximize $^{15}N_2$ labelling I added a relatively large amount (~2 ml) of $^{15}N_2$ stock solution to a 12 ml exetainer (~16% v/v). To keep the dissolved nutrients and gases at background levels in the $^{15}N_2$ treatments as in the controls and $^{15}N_2O$ treatment, the artificial freshwater medium, instead of deionized water, was used for preparing $^{15}N_2$ stocks. In contrast to N_2, N_2O is highly soluble in water, with only ~100 μL of $^{15}N_2O$ stock being needed in each treatment (~0.8% v/v) to reach the comparable concentration of ^{15}N-N_2 addition (~10 μM). Before each experiment, $^{15}N_2O$ and $^{15}N_2$ stocks were freshly made and tested with pseudo-samples (processed in the same way as the real samples except with no biomass added) to ensure adequate $^{15}N_2$ and $^{15}N_2O$ addition to the medium. The stocks of $^{15}N_2$ and $^{15}N_2O$ could also be reused by refilling with each respective ^{15}N-gas.

Incubations of biomass with $^{15}N_2$ and $^{15}N_2O$ tracers

Floating or benthic biomass (~3 g wet weight) from the ponds was aliquoted into 12 mL gas-tight vials and those as controls or the $^{15}N_2O$ treatment were then filled with artificial pond water medium and closed. For the $^{15}N_2O$ treatment, 100 μL of medium was replaced by $^{15}N_2O$ stock solution with a gastight syringe, whereas the controls were left un-amended. For the $^{15}N_2$ treatment, each vial was filled with 10 mL of medium and 2 ml of $^{15}N_2$ stock without any bubbles, then closed immediately. As the solubility of gases is temperature-dependent, all the samples were incubated without any headspace to make sure that the ^{15}N substrate accessible to the biomass was the same under different temperatures.

T_0 (Time zero) samples were killed with 200 μL of formaldehyde (37 wt. %) immediately and stored at room temperature (22°C). Other samples were incubated in temperature-controlled orbital incubators (SI500, Stuart) with gentle shaking (50 cycles min^{-1}) and a 12 h light/12 h dark regime. After the incubation, T_f (Time final) samples were killed as above and brought to room temperature. A 2 ml helium headspace was then created in all the vials, followed by shaking over 24 h to ensure fully-equilibration.

2.2.4 Characterising the total reduction of $^{15}N_2O$

The concentration of ^{15}N-N_2O (^{15}N from both $^{45}N_2O$ and $^{46}N_2O$) in the samples was measured on a CF-IRMS (Delta V Plus, Thermo-Finnigan) with an automated trace gas pre-concentrator (PreCon, Thermo-Finnigan) (Nicholls et al., 2007). A sub-sample from the headspace of each sample was transferred to a 12 mL air-filled gas-tight vial. Due to the high labelling of $^{15}N_2O$ in the N_2O pool (on average, 97.7% of $^{46}N_2O$) from the $^{15}N_2O$ treatment, only a small fraction of sub-samples from the sample headspace (~10 μL) was needed to keep it within the measurable range of the $^{46}N_2O$ signal using the Precon. The concentration of $^{15}N_2O$ in the samples was calibrated against air, 0.12 ppm, 1.04 ppm, and 96 ppm N_2O standards. The

reduction of $^{15}N_2O$ was then calculated by subtracting $^{15}N_2O$ concentrations in T_f samples from their corresponding T_0 samples.

2.2.5 Characterising any dissimilatory reduction of $^{15}N_2O$ to $^{15}N_2$

The possible production of $^{15}N_2$ from $^{15}N_2O$ treatments was measured by the CF-IRMS (Delta V Plus, Thermo Finnigan), bypassing the copper reduction column to avoid the reduction of $^{15}N_2O$ to $^{15}N_2$ before the isotopic analysis (Trimmer and Nicholls, 2009). $^{15}N_2$ abundance was calculated as the ratio in the peak areas of m/z 29 ($^{15}N^{15}N$) and 30 ($^{15}N^{15}N$) to the total N_2 mole masses (including 28: $^{14}N^{14}N$, 29, and 30). The enrichment of $^{15}N_2$ abundance during the incubation was then calculated to check if $^{15}N_2$ was produced from $^{15}N_2O$, with possible isotopic fractionation corrected for by subtracting the enrichment in the corresponding controls. The detection limit for $^{15}N_2$ production is ~0.03 μM from repeated measurements of standard air.

2.2.6 Characterising the assimilation of $^{15}N_2O$ or $^{15}N_2$ into biomass

After all the gas measurements, samples were centrifuged, supernatants then filtered through clean 0.45 μM Syringe PES filters into a 15 ml sterilized Falcon tube. The remaining content was completely dried in the oven for calculating dry weight-specific rates later. The dried biomass was homogenized, with sub-samples then weighed on a microbalance (UMX2, Mettler Toledo) and packed into tin caps (6 x 4 mm, Elemental Microanalysis). Isotopic analysis of N in biomass samples was performed with a combined combustion and isotope ratio mass spectrometer (Integra2, Sercon, UK). Urea samples (~0.5 mg, Sigma Aldrich) were measured before each batch (~60 samples) to optimize parameters, while a protein standard (OAS/Isotope, Elemental Microanalysis) was inserted throughout each batch for isotope correction and N content quantification. The ^{15}N abundance (^{15}N atom %) of the samples was derived from the

delta-^{15}N value from the mass spectrometer. The ^{15}N enrichment in the treatments was then calculated by the difference in excess ^{15}N atom % relative to its corresponding controls (taken from the same pond and incubated under the same conditions) to correct for possible isotopic fractionation during the incubation, where the excess ^{15}N atom % is the difference in ^{15}N atom % between T_0 and T_f:

$$^{15}N \text{ enrichment} = (\text{excess } ^{15}N \text{ atom \%})_{Treatment} - (\text{excess } ^{15}N \text{ atom \%})_{Control} \quad (8)$$

Biomass rates of ^{15}N assimilation (nmol ^{15}N g^{-1} day^{-1}) into particulate organic nitrogen (PON) were calculated as:

$$^{15}N \text{ assimilation rate} = PON \times {}^{15}N \text{ enrichment}/(\Delta t \times dw) \quad (9)$$

Where PON denotes the particulate organic nitrogen content of the biomass. Δt is the incubation time, and dw denotes the dry weight of biomass (g). Furthermore, the total assimilation of N_2O or N_2 into the biomass was calculated from the rate of $^{15}N_2O$ assimilation divided by F_{N2O} (the fraction of ^{15}N in N_2O) and $^{15}N_2$ assimilation divided by F_{N2} (the fraction of ^{15}N in N_2). The average F_{N2O} in the $^{15}N_2O$ treatment was 0.978, while the average F_{N2} in the $^{15}N_2$ treatment was 0.018.

2.2.7 Estimating N_2 and N_2O fluxes across the water to air interface in the ponds

The wind speed around the ponds was mostly less than 3 m s^{-1} throughout the year (median 1.8 m s^{-1}), as recorded using a Datahog 2 logger connected to an on-site weather station (Skye Instruments). The gas transfer velocity is typically independent of such low wind speeds (Zappa et al., 2007) and would possibly be underestimated if it were calculated based on empirical gas exchange-wind speed relationships (Wanninkhof, 2014; Zappa et al., 2007). Here, I estimated the gas transfer velocity for N_2O (k_{N2O}, cm h^{-1}) using previously determined gas

transfer velocities for CH_4 (k_{CH4}, cm h^{-1}) in the ponds (Zhu et al., 2020) and the ratio of their respective Schmidt numbers (unitless):

$$k_{N2O} = k_{CH4} \times \left(\frac{Sc_{N2O}}{Sc_{CH4}}\right)^{-0.66} \quad (10)$$

Where Sc_{N2O} and Sc_{CH4} are the Schmidt numbers for N_2O and CH_4 at *in situ* water temperature, respectively, with the corresponding exponent (-0.66) applied for the smooth water surface of the ponds (Wanninkhof, 2014). N_2O flux across the water to air interface in the ponds (F_{N2O}, μmol m^{-2} d^{-1}) was then derived by:

$$F_{N2O} = k_{N2O} \times (Cw_{N2O} - Ca_{N2O}) \quad (11)$$

Where Cw_{N2O} and Ca_{N2O} are the measured and air-equilibrated concentrations of N_2O in the ponds, respectively. N_2 fluxes were derived in the same way as for N_2O using the appropriate Schmidt and equilibration numbers.

As a result, N_2O flux into the ponds was -1.33 μmol N_2O m^{-2} d^{-1}, on average, with a range of -3.65 to 0.02 μmol N_2O m^{-2} d^{-1}, including low emissions to the atmosphere in summer. While N_2 flux into the ponds was 3,934 μmol N_2 m^{-2} d^{-1}, on average.

2.2.8 Estimating *in situ* rates of N_2O reduction in the ponds required to balance the N_2O flux

The kinetic effect of N_2O concentration on the rate of N_2O reduction was characterised experimentally using incubations with floating biomass enriched with different concentrations of N_2O (9.2 nM – 20,000 nM, Fig. 9). I estimated rates of N_2O reduction (y, nmol g^{-1} DW h^{-1}) at *in situ* concentrations of N_2O in the ponds (x, nM) according to:

$$\ln(y) = 0.95 \times \ln(x) - 4.96 \quad (12)$$

Therefore, with an ambient concentration of N_2O of 10 nM at the annual average temperature of 15°C in the pond water (Fig. 2), N_2O reduction would be 0.06 nmol g^{-1} DW h^{-}

[1], on average, where DW denotes the dry weight of floating biomass. Although I measured the conversion factor of dry to wet weight of the biomass collected from the ponds, as floating biomass is non-homogenous both within a pond and between different ponds, e.g., percentage volume infested (PVI, %) of the filamentous algae ranged from 0.1 to 40 in the ponds, estimating N_2O reduction in the ponds with the weight of floating biomass would be difficult. As benthic biomass is distributed relatively-evenly in the ponds, and from my incubations, rates of total $^{15}N_2O$ reduction were consistent between the floating and benthic biomass ($p = 0.9$, *t*-test), instead I applied the kinetic function (12) to the benthic biomass.

2.2.9 Statistical analysis

Statistical analysis and plotting were performed in R (Team, 2021) using RStudio (Version 1.3.1093). I used generalized additive mixed effects models (GAMMs) (Zuur et al., 2009) to characterise the seasonal patterns in N_2 and N_2O saturation in the overlying pond water. Sampling month was treated as a fixed effect to characterise the seasonality of N_2 or N_2O saturation, with an interaction term for sampling month by gas (N_2 or N_2O) to explore any distinct seasonality between N_2 and N_2O saturation. Each replicate pond was treated as a random effect to account for any repeat measures in each pond. Models were ranked by the small sample-size corrected Akaike Information Criterion (AICc) using the 'MuMIn' package and the best fitting model determined by the lowest delta AICc and the highest AICc weight (Table 1).

As the data for rate of ^{15}N assimilation and total $^{15}N_2O$ reduction were strongly skewed, potentially due to normalizing the rate to a unit of dry biomass which may not account for the abundance of N_2 and N_2O consumers. Therefore, I presented 95% of the dataset (2.5% to 97.5% percentiles) and applied quantile regression models using the 'quantreg' package (Koenker, 2021) to fit the median rather than mean to the data for rate of ^{15}N assimilation to minimise

any bias from the clearly visible outliers. Simple first-order linear models were used to characterise the temperature sensitivity of $^{15}N_2O$ fixation.

2.3 Results

2.3.1 Temperature and nutrients in the ponds

The average daily temperature was 13.7°C and 17.2°C in the ambient and warmed ponds, respectively between January 2019 to December 2021, with an overall difference between the ambient and warmed ponds of 3.5 °C, on average (Fig. 2). However, I compiled the data for ambient and warmed ponds together for all the other analyses, as the effect of pond warming treatment was not significant on any of the key elements I investigated, such as the concentrations of each nutrient species, or the assimilation rate of N_2O and N_2.

The concentrations of dissolved inorganic nutrients (NO_2^-, NO_3^-, NH_4^+, and SRP) were low in the ponds, with NO_2^-, NO_3^- and NH_4^+ often at or below the limit of detection (Fig. 3). The concentration of total inorganic nitrogen (TIN as the sum of NO_2^-, NO_3^-, and NH_4^+) was 0.85 ± 0.03 μM across all sampling months (Fig. 1**d**). SRP concentrations were 0.14 ± 0.01 μM, on average, and, at 5 to 1, the median N to P ratio was markedly lower than Redfield (16 to 1), indicating primary production in the ponds to be N limited (Fig. 1**h**). Further, the concentrations of NO_2^-, NO_3^-, NH_4^+ and TIN all showed significant seasonality (Table 1), whereas the concentration of SRP was consistent across the sampling months.

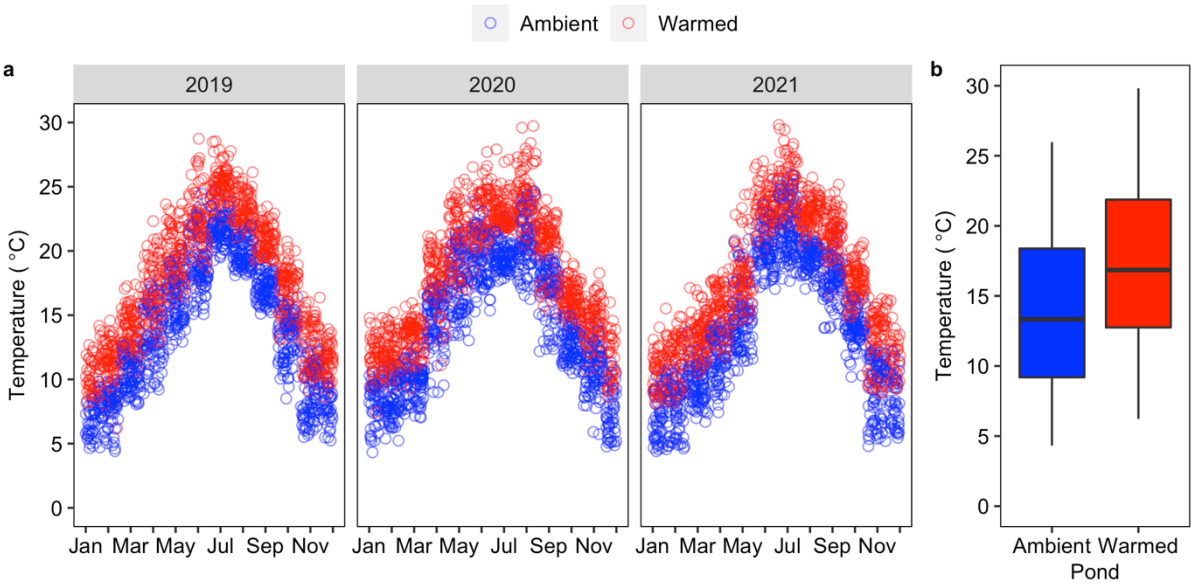

Fig. 2 | Daily average temperature in the pond water combined for the warmed and ambient ponds between January 2019 to December 2021. a, Seasonal changes in daily average temperature. The overall average temperature difference between the ambient and warmed ponds was 3.5 °C. n = 6546 (18 ponds, as temperature logger failed in 2 out of the 20 ponds). **b**, Box-whisker plots of daily average temperature. Each box in **b** shows the 25th and 75th percentiles, with the horizontal lines inside each box as the median, open circles denoting the outliers, and whisker extents correspond to 1.5 × the interquartile range.

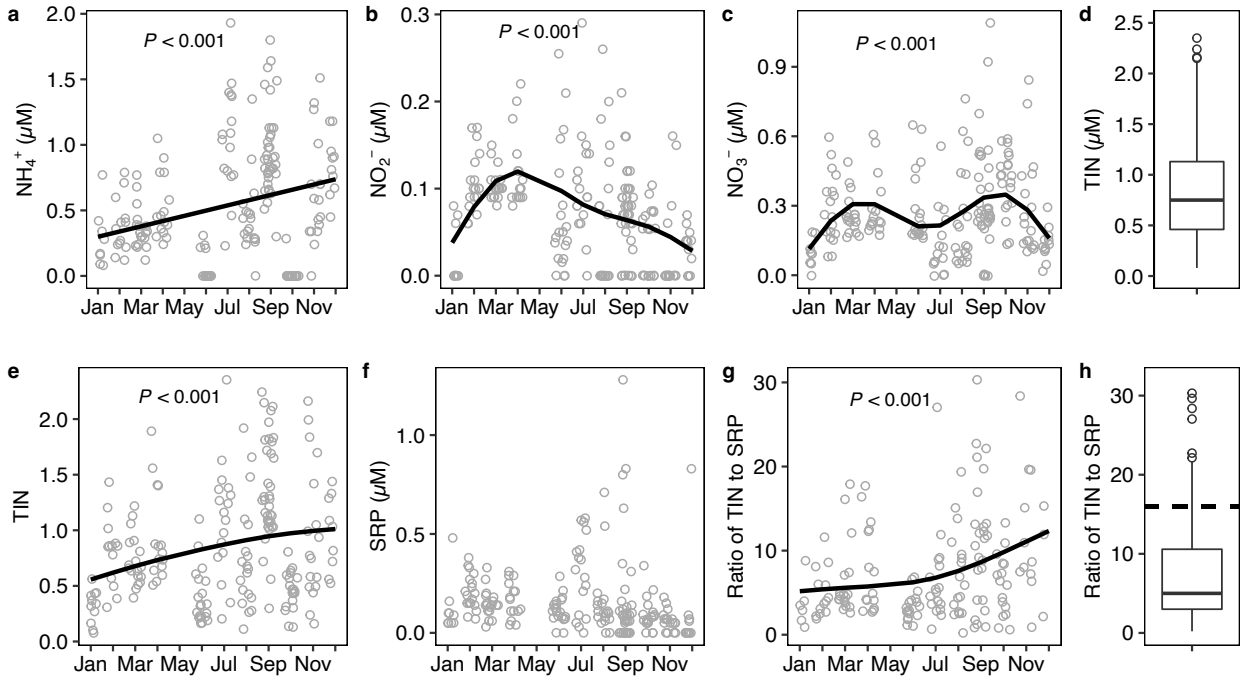

Fig. 3 | Concentrations of dissolved inorganic nutrients and the ratio of N to P across 11 different sampling months in the 20 experimental ponds. The concentrations of **a**, NH_4^+, **b**, NO_2^-, **c**, NO_3^-, **e**, TIN (total dissolved inorganic nitrogen), **f**, SRP (soluble reactive phosphorous), and **g**, the ratio of TIN to SRP in the ponds. The solid lines in **a** - **g** denote the fit from the best GAMM models (*see* Table 1). **d,** The concentration of total inorganic nitrogen (TIN) in the ponds. **h,** The ratio of TIN to soluble reactive phosphorous (SRP) in the ponds. Data were omitted where SRP was below the detection limit. Each box in **d** and **h** shows the 25th and 75th percentiles, with the horizontal lines inside each box as the median, and open circles denotes the outliers. The dashed line in **h** denotes the Redfield ratio of 16:1, with values below the dashed line indicating N-limitation in the ponds.

Table 1 | Multi-GAMM model selection for fitting the data of nutrient species, TIN, SRP, and the ratio of TIN to SRP across 11 different sampling months in the 20 experimental ponds (Fig. 3, a-c, e-g). Sampling month was treated as the fixed effects, i.e., smooth term s(), with a random intercept (1|Pond) to account for variation across the 20 experimental ponds.

Models were ranked by the corrected Akaike Information Criterion (AICc) and the best models (in **bold**) were selected as those with the lowest in AICc, suggesting the best goodness of fit. Models for each nutrient category (**M1 - M6**) were compared to their respective null models (N1 - N6, which only have an intercept denoted as 1) to evaluate whether including the smooth term 'Month' improved model fit. A lower AICc in the **full model** compared to the respective null model suggests seasonality for that inorganic nutrient species. The Chi-squared statistic (χ^2) and the corresponding *p*-value (*P*) were compared to the best model in each panel using the Log-likelihood ratio test (LogLik), degree of freedom (d.f.).

Model	d.f.	AICc	LogLik	χ^2	*P*
NH_4^+					
M1: NH_4^+~s(Month)	**5**	**241.3**	**-116.46**		
N1: NH_4^+~1	3	256.1	-124.97	17.01	<0.001
NO_2^-					
M2: NO_2^-~s(Month)	**5**	**-641.8**	**325.17**		
N2: NO_2^-~1	3	-607.4	306.74	36.85	<0.001
NO_3^-					
M3: NO_3^-~s(Month)	**5**	**-124.8**	**66.40**		
N3: NO_3^-~1	3	-106.5	56.33	20.14	<0.001
TIN					
M4: TIN~s(Month)	**5**	**310.6**	**-151.07**		
N4: TIN~1	3	322.4	-158.16	14.17	<0.001
SRP					
M5: SRP~ s(Month)	5	-163.8	86.06		
N5: SRP~1	3	-162.5	84.32	3.48	0.18
Ratio of TIN to SRP					
M6: TIN:SRP~s(Month)	**5**	**1102**	**-546.83**		
N6: TIN:SRP~1	3	1120	-556.97	20.28	<0.001

2.3.2 Contrasting seasonalities in undersaturation for N_2 and N_2O

As the ponds were N-limited (Fig. 3), primary production must be sustained by N fixation (and any unknown atmospheric N deposition), which could lead to the undersaturation of N_2 and N_2O in the ponds.

Indeed, concentrations of dissolved N_2O and N_2 were both significantly below atmospheric equilibration ($p < 0.001$, *t*-test, Fig. 4**a**) and the ponds are sinks for both atmospheric N_2O and N_2. Overall, N_2O was more under-saturated than N_2, with a mean value of 79.1% ± 1.1% (mean ± s.e., as below) of air saturation compared to 98.5% ± 0.2% for N_2. Furthermore, the seasonality in N_2O saturation was far more pronounced than for N_2 (Best fitting GAMMs, Table 2), with a strong minimum for N_2O (Fig. 4**b**; Table 2) in December and maximum saturation in summer. Conversely, N_2 saturation peaked in winter and was lower in spring and summer, although the seasonal amplitude in N_2 saturation was milder compared to N_2O (Fig. 4**c,** Table 2).

Interestingly, N_2O saturation increased with water temperature ($p < 0.001$, Fig. 4**d**), suggesting relatively higher net reduction of N_2O in the cold. Whereas N_2 saturation showed the opposite pattern, with relatively more net N_2 reduction at higher temperatures ($p < 0.001$, Fig. 4**e**) in spring and summer. Moreover, the saturation of dissolved O_2 in the ponds (at the same depth where the samples for N_2 and N_2O were collected) was generally around air-equilibration (104.8% ± 1.8%, median 99.6%), with N_2 saturation decreasing at higher O_2 saturations, while N_2O saturation increased with higher O_2 saturation (Fig. 5). Oxygen saturation was positively correlated with temperature (Fig. 5**c**), probably due to higher temperatures in spring and summer promoting primary production. Therefore, maximum N_2 undersaturation was probably related to higher primary production in spring and summer (Yvon-Durocher et al., 2017). The negative and positive correlations between N_2 or N_2O and O_2 respectively, indicated different controls for the reduction of N_2 and N_2O.

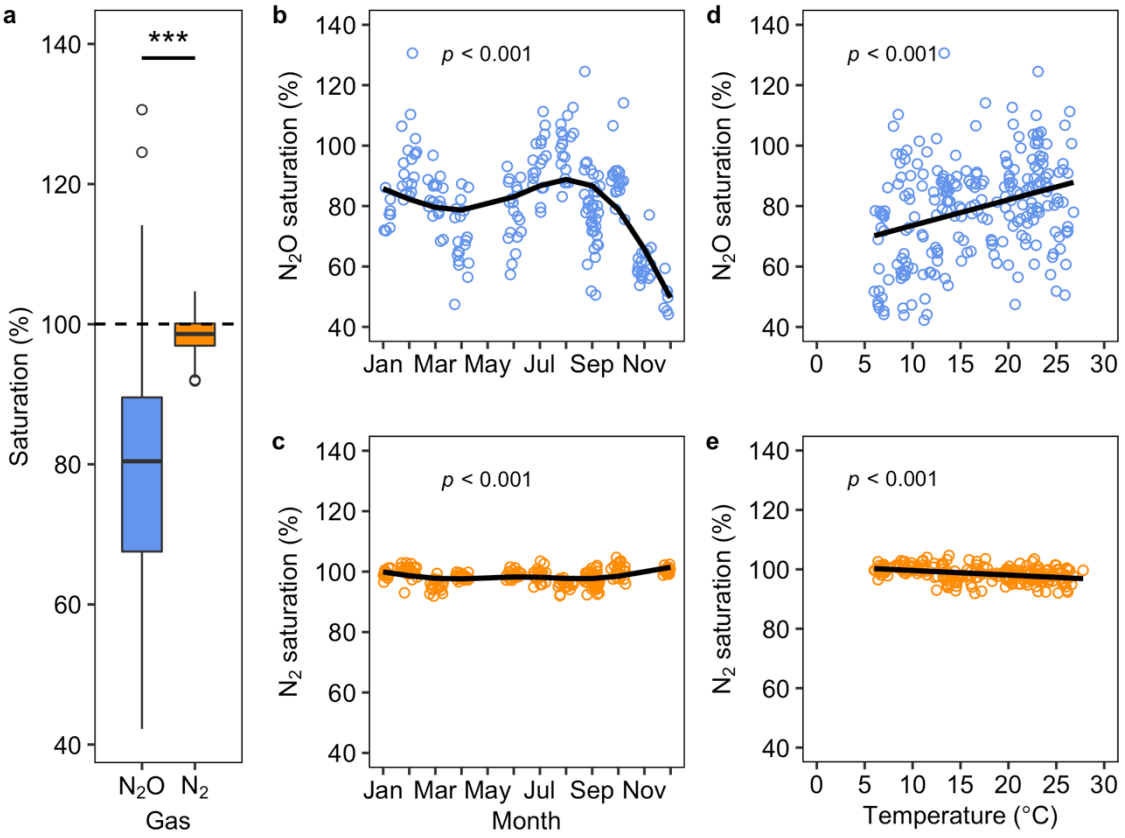

Fig. 4 | Seasonal and overall levels of N_2O and N_2 saturation in the ponds. Here I present the data on the same scale for N_2O and N_2 which partially masks the significant seasonality in N_2 ($p < 0.001$, M7 compared to the null model M8 for the smooth term fitted to 'Month', Table 1) and its significant negative correlation with temperature (Fig. 1**e**). **a,** Box-whisker plots showing the saturation of N_2O was lower than that for N_2 in the ponds (***: $p < 0.001$, M0 compared to M3, Table 2). The dashed line denotes 100% atmospheric equilibrium for the gases. **b** and **c** show the saturation in N_2O had a stronger seasonal pattern than N_2. The solid lines in **b** and **c** represent the best fitting GAMM models (Table 2). **d**, N_2O saturation increased at higher *in situ* water temperatures and, in contrast, **e**, N_2 saturation declined at higher temperatures. Each box in **a** shows the 25th to 75th percentiles, with the horizontal line inside the box giving the median, open circles denoting the outliers, and whiskers corresponding to 1.5 × the interquartile range. The lines in **d** and **e** are simple first order linear regressions. *n* =

230 and n = 215 for N_2O and N_2 saturation, respectively (11 months for 20 ponds, from November 2019 to April 2022).

Table 2 | Multi-GAMM selection for exploring seasonal patterns in N_2O and N_2 saturation over the year (Fig. 4, a-c). Here, I treated both Month (i.e., seasonal pattern) and either gas (N_2 or N_2O) as fixed effects and fitted different smooth terms s() i.e., shape or pattern, along with a random intercept (1|Pond) to account for variation among the 20 experimental ponds. The term 'by=Gas' within s() denotes a different shape for the smooth term describing saturation for either N_2 or N_2O, whereas '+Gas' denotes a different intercept (i.e., median saturation) for either gas. Models were ranked by the corrected Akaike Information Criterion (AICc) and the best fitting models (in **bold**) to the data were judged as those with the lowest AICc. Here the best fitting model (**M0**) showed that N_2 and N_2O had different seasonal saturation patterns, with different median of their saturation. I then further validated whether the seasonal patterns for N_2 or N_2O saturation were significant by modelling the data separately for N_2 and N_2O. Models M5 and M7 were compared to their respective null models (M6 and M8, which only have an intercept denoted by 1) to evaluate whether including the smooth term 'Month' improved model fit. The lower AICc in **M5** and **M7**, compared to their null models, suggested that both N_2 and N_2O saturation showed strong seasonality. n = 230 and n = 215 for N_2O and N_2, respectively (11 months for 20 ponds, with N_2O data for September measured in two different years). Models were compared to the best model in each panel using the Log-likelihood ratio test (LogLik, d.f., degrees of freedom) showing Chi-squared statistic (χ^2) and the corresponding p-value (p).

Model	d.f.	AICc	LogLik	χ^2	p
N_2 or N_2O Saturation					
M0: Sat~s(Month, by=Gas)+Gas	**8**	**3226.9**	**-1605.3**		
M1: Sat~s(Month)+Gas	6	3389.4	-1688.6	166.63	<0.001
M2: Sat~Gas	4	3480.0	-1735.9	261.28	<0.001
M3: Sat~s(Month, by=Gas)	7	3555.7	-1770.7	330.83	<0.001
M4: Sat~s(Month)	5	3636.9	-1813.4	416.16	<0.001
N_2O saturation					
M5: N_2O.sat1~s(Month)	**5**	**1824.8**	**-906.9**		
M6: N_2O.sat2~1	6	1946.8	-970.3	126.67	<0.001

N_2 saturation					
M7: N_2.sat1~s(Month)	**5**	**983.3**	**-486.5**		
M8: N_2.sat2~1	6	1019.6	-506.7	40.45	<0.001

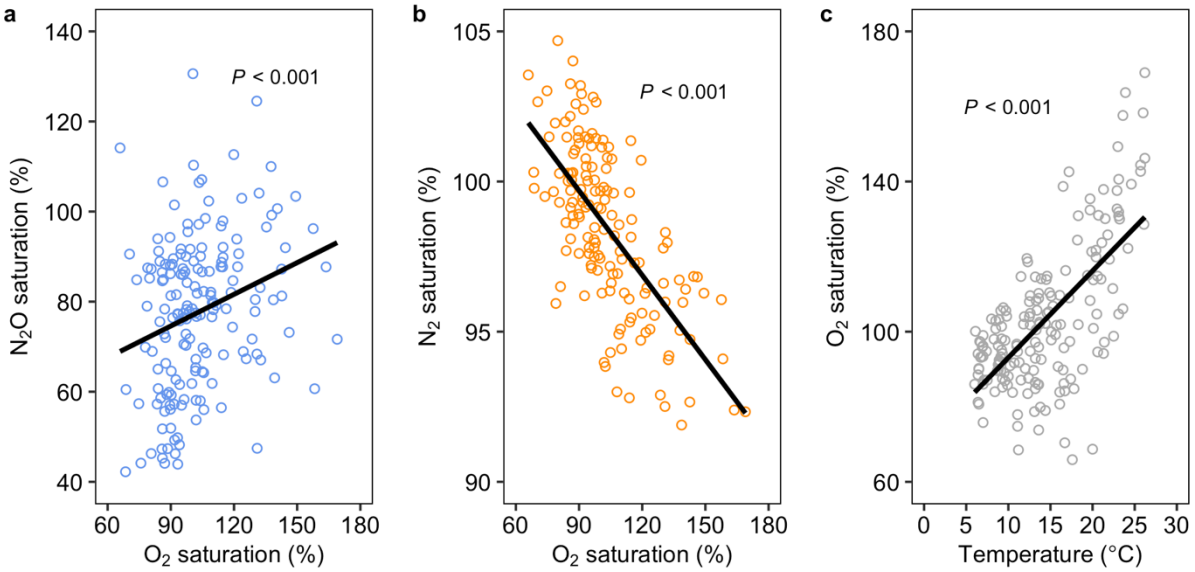

Fig. 5 | Correlation between saturation of dissolved O_2 and N_2, N_2O, or temperature in the pond water. a, Correlation between dissolved oxygen saturation and N_2O saturation in the ponds. **b**, Correlation between dissolved oxygen and N_2 saturation in the ponds. **c**, Correlation between dissolved oxygen saturation and temperature in the ponds indicating maximum primary production in spring and summer. The lines in **b** and **c** are simple first order linear regression models. n = 175, n = 156, and n = 180 in **a**, **b**, and **c**, respectively (n represents data for 9 months in 20 ponds).

2.3.3 N_2O and N_2 fixation by biomass in the ponds

In order to understand the undersaturation in both N_2O and N_2 in the ponds, I characterised any fixation for both gases by incubating biomass collected from the ponds (Fig. 4) with either $^{15}N_2O$ or $^{15}N_2$ (at a range of temperatures, see section 2.3.4). I found ^{15}N assimilated into biomass from either $^{15}N_2$ or $^{15}N_2O$ in the majority of the incubations (87%, 572 out of 658 incubations, Fig. 6**a**), with higher rates of ^{15}N assimilation with $^{15}N_2$ than for $^{15}N_2O$ with both floating and benthic biomass (p < 0.001, t-test, Fig. 7). On average, 11.5 ± 0.9 and 5.3 ± 0.3

nmol g^{-1} d^{-1} (mean ± s.e.) of ^{15}N were assimilated into biomass with either $^{15}N_2$ or $^{15}N_2O$, respectively (Fig. 7). The rate of $^{15}N_2$ assimilation was higher in the floating than the benthic biomass ($p < 0.001$, *t*-test), while $^{15}N_2O$ assimilation was consistent between the two biomass types ($p = 0.21$, *t*-test).

To distinguish any genuine direct N_2O fixation ($N_2O \rightarrow NH_4^+$) from any indirect fixation i.e., that after an initial reduction of N_2O to N_2 ([$N_2O \rightarrow N_2$] $N_2 \rightarrow NH_4^+$) through denitrification, I first checked for any production of $^{15}N_2$ in the incubations with $^{15}N_2O$ (Fig. 6**b**). Overall, $^{15}N_2$ production was a minor part of total $^{15}N_2O$ reduction and I was unable to detect any $^{15}N_2$ in 51% of the incubations enriched with $^{15}N_2O$ (median of 0%, Fig. 6**b**). Some denitrification is expected, especially for the sediments biomass that has the recognised capacity to consume oxygen (Zhu et al., 2020). The 12h/12h light/dark incubation-cycle generated oxygen minima overnight that likely facilitated the reduction of N_2O to N_2 via denitrification (for the experiement where O_2 was monitored, the median O_2 saturation decreased to 35% after 12 hour of dark incubation, $n = 12$).

I then compared the rates of ^{15}N assimilation with either $^{15}N_2$ or $^{15}N_2O$ against a theoretical upper threshold for indirect assimilation of $^{15}N_2O$ after reduction to $^{15}N_2$ (Table 3 and Fig. 7). Any $^{15}N_2$ from $^{15}N_2O$ would be assimilated in proportion to the ^{15}N-labelling of the total N_2 pool (FN_2, Table 3), dominated by the large ambient background of $^{14}N_2$ (~487 μM, Table 3). As a result, any indirect assimilation of ^{15}N from $^{15}N_2O$ should have been ~14-fold lower than that measured in the incubations where I added $^{15}N_2$ directly e.g. 0.8 nmol N g^{-1} d^{-1} *vs.* 11.5 nmol N g^{-1} d^{-1} (Table 3). In contrast, I measured far higher rates of assimilation of 5.3 nmol N g^{-1} d^{-1} with $^{15}N_2O$, compared to the upper threshold of 0.8 nmol N g^{-1} d^{-1}, on average (Fig. 7). Such disproportionately high assimilation of ^{15}N from $^{15}N_2O$, coupled to the overall low production of $^{15}N_2$ from $^{15}N_2O$, argues for ^{15}N mainly being assimilated directly from $^{15}N_2O$ into biomass from the freshwater ponds.

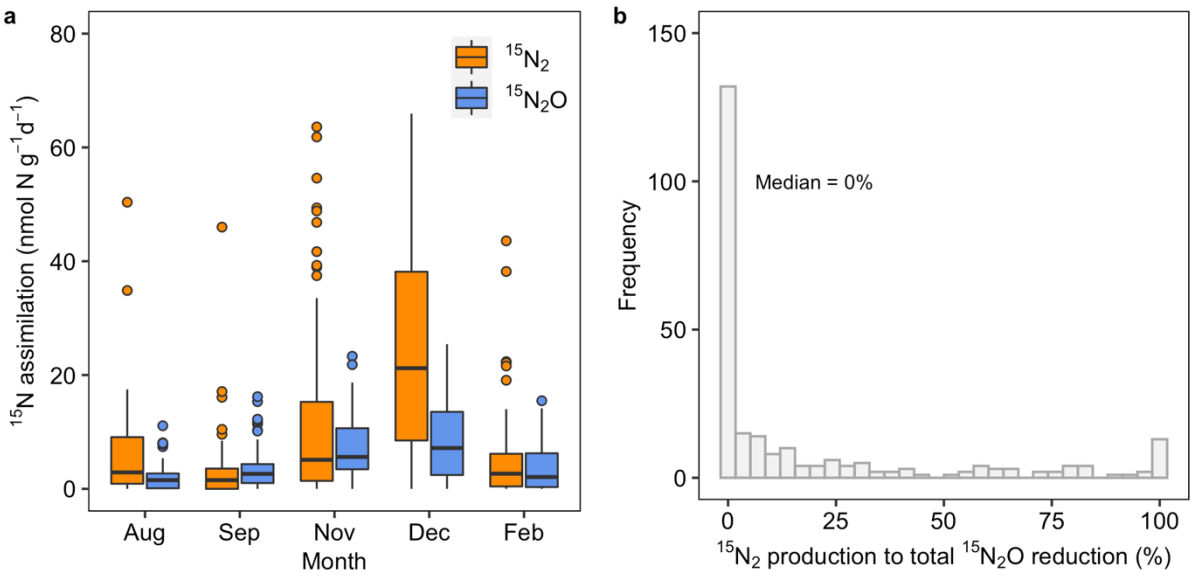

Fig. 6 | Direct assimilation of ^{15}N into biomass after 24-hour incubation from either $^{15}N_2$ or $^{15}N_2O$. ^{15}N from either $^{15}N_2$ or $^{15}N_2O$ was consistently assimilated into biomass from the ponds in different months. To exclude extreme outliers the data plotted are 95% of the dataset (2.5% to 97.5% percentiles). **a**, Each box shows the 25th to 75th percentiles, with the horizontal line inside each box giving the median, open circles denoting the outliers, and whiskers extending to 1.5 times the interquartile range. **b**, The total $^{15}N_2$ production as a percentage of the total $^{15}N_2O$ reduction for all the $^{15}N_2O$-amended biomass incubations. Overall, $^{15}N_2$ production was not detected in half (51%) of the incubations enriched with $^{15}N_2O$, giving a median of 0% i.e., no $^{15}N_2$ production detected. *n* = 252 incubation bottles. Data collected in 5 calendar months over a 13-month period, 2 biomass types for 8 to 10 ponds per sampling date.

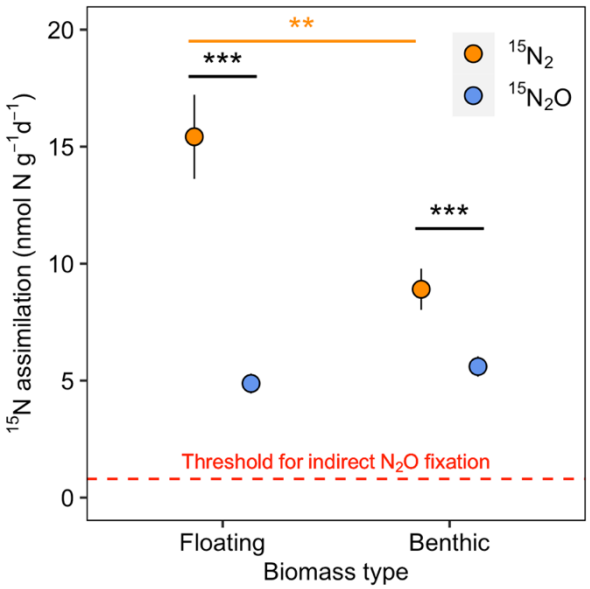

Fig. 7 | Rates of $^{15}N_2$ assimilation were higher than for $^{15}N_2O$ assimilation in both biomass types after 24-hour incubation. The red dashed line marks the upper threshold for indirect ^{15}N assimilation after reduction of $^{15}N_2O$ to $^{15}N_2$ (*see* Table 1). Statistical differences compared between treatments or biomass types for either treatment (*$p < 0.05$; ** $p < 0.01$; *** $p < 0.001$). $n = 303$ and $n = 322$ for $^{15}N_2$ and $^{15}N_2O$ treatments, respectively. Data collected in 5 calendar months over a 13-month period, 2 biomass types for 8 to 10 ponds per sampling date.

Table 3 | Rationalising N_2O assimilation as direct N_2O fixation. The ambient background concentrations for $^{14}N_2$ and $^{14}N_2O$ in both the $^{15}N_2$ and $^{15}N_2O$ treatments were ~487 µM and 0.01 µM, respectively. I then added $^{15}N_2$ and $^{15}N_2O$ at 9 µM and 10 µM, respectively. F_{N2} and F_{N2O} denote the initial ^{15}N labelling of the $^{15}N_2$ and $^{15}N_2O$ pools i.e. $^{15}N/[^{14}N + {}^{15}N]$, respectively. Here, when I added $^{15}N_2$ directly, F_{N2} was ~0.018. In contrast, if $^{15}N_2O$ assimilation was indirect and assuming all reduced N_2O is converted to N_2, at most 0.63 µM $^{15}N_2$ would have been produced and F_{N2}^* would have been <=0.0013 i.e., 14-fold lower. Accordingly, the absolute upper threshold (in red) for indirect $^{15}N_2O$ fixation would have been 0.8 nmol N g^{-1} d^{-1}, which is far lower than the rate I measured for $^{15}N_2O$ fixation (in blue, 5.3 nmol N g^{-1} d^{-1}, on average, Fig. 7). Therefore, the measured $^{15}N_2O$ assimilation activity was most likely due primarily to direct $^{15}N_2O$ fixation. n.a. = not applicable.

Treatment	**Process**	**F_{N2}** or F_{N2}*	**F_{N2O}**	**^{15}N assimilation** (nmol N g^{-1} d^{-1})
$^{15}N_2$	N_2 fixation	~0.018= [9/(9+487)]	n.a.	11.5
$^{15}N_2O$	*Indirect N_2O fixation	<= 0.0013 [0.63/(0.63+487)]	n.a.	**<= 0.8**
$^{15}N_2O$	Direct N_2O fixation	n.a.	0.98	**5.3**

Predicted maximum ^{15}N-labelling of the N_2 pool F_{N2} resulting from the initial reduction of $^{15}N_2O$ to $^{15}N_2$.

2.3.4 The temperature dependence of N_2O fixation

As seasonal changes in temperature drove contrasting patterns in N_2O and N_2 saturation, I experimentally characterised the effect of temperature on N_2O and N_2 reduction by incubating biomass from the ponds at temperatures from 6°C to 25°C.

Assimilation of ^{15}N from $^{15}N_2$ increased at higher temperatures (Fig. 8, $p < 0.05$), with an estimated Q_{10} of 1.38. In contrast, assimilation of ^{15}N from $^{15}N_2O$ was consistent across all temperatures with no discernible temperature sensitivity. As the assimilation data were highly skewed, a simple average linear model was inappropriate, and instead I applied median regression models to minimize bias from outliers. The large variance in Fig. 8 may in part be due to simply normalizing the ^{15}N assimilation data to a unit of dry biomass in each incubation, whereas the communities responsible for N_2 or N_2O assimilation could be heterogeneous in the biomass samples and across different months of the year.

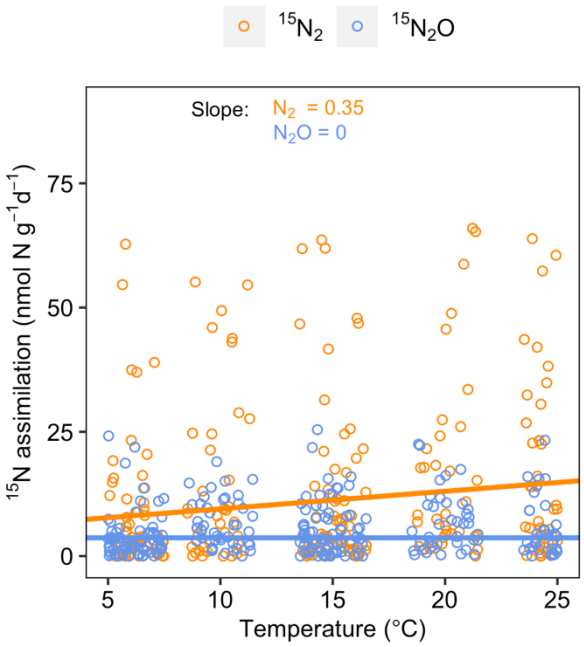

Fig. 8 | The respective temperature sensitivities of ^{15}N assimilation from $^{15}N_2$ and $^{15}N_2O$ treatments. Temperature sensitivities of ^{15}N assimilation from $^{15}N_2$ and $^{15}N_2O$ treatments were different ($p < 0.05$). $^{15}N_2$ assimilation increased at higher temperatures, whereas $^{15}N_2O$ assimilation was not sensitive to changes in temperature. As the data were highly skewed, I used median regression models instead of simple first-order linear regression models to minimise bias from outliers. The median regression was performed using the whole dataset, while data presented 95% of the dataset (2.5% to 97.5% percentiles), n = 303 and n = 322 incubation bottles for $^{15}N_2$ and $^{15}N_2O$ treatments, respectively (5 months and 2 biomass types for each treatment). As the temperature dependencies of ^{15}N assimilation were consistent between floating and benthic incubations, I pooled the data from the two biomass types together here.

2.3.5 Linking N_2 and N_2O fluxes to gross primary production

To put my estimates of N fixation into an ecological context, I compared estimates of the N_2 flux with former estimates of gross primary production (GPP) in the ponds (Yvon-Durocher et

al., 2017). Overall, the higher N_2 flux in the warmed compared to ambient ponds corresponded with the higher GPP estimates in the warmed ponds, determined previously (Table 4). The average net N_2 flux into the ambient and warmed ponds was 3,934 μmol N_2 m^{-2} d^{-1}, which, assuming Redfield ratios of 106:16 for C:N, could sustain GPP of 52,126 μmol C m^{-2} d^{-1} and which is comparable to GPP measured previously of 51,488 to 70,792 μmol C m^{-2} d^{-1} (Yvon-Durocher et al., 2017).

Moreover, the seasonal dynamics in N_2 flux in my study also matched that of GPP reported previously (Yvon-Durocher et al., 2017), with both peaking in summer (Fig.4**b**). In contrast, while the flux of N_2O is comparatively minor (~0.03 %) in terms of supporting GPP, it is great enough to maintain a strong sink for N_2O.

Table 4 | Average net N_2 and N_2O flux in the ambient and warmed ponds.

Pond	N_2 flux (μmol m^{-2} d^{-1})	N_2O flux (μmol m^{-2} d^{-1})	C flux* (μmol m^{-2} d^{-1})	GPP 2007# (μmol C m^{-2} d^{-1})	GPP 2012# (μmol C m^{-2} d^{-1})
Ambient	-3860	-0.72	-51153	47283	50662
Warmed	-4843	-0.76	-64179	55639	89521

*: Estimated carbon flux in the ponds from N fluxes (N_2 flux plus N_2O flux) assuming a C-N-ratio of 106:16 based on the Redfield ratio; #: Gross primary production (GPP) of the ponds in 2007 and 2012 (Yvon-Durocher et al., 2017).

2.3.6 Estimating *in situ* rates of N_2O reduction in the ponds

In the incubations, I added $^{15}N_2O$ to the incubations at concentrations many times higher than atmospheric equilibration (10 μM *vs.* 0.01 μM) and the rates of $^{15}N_2O$ assimilation are likely upper-potentials. In addition, the kinetic effect of N_2O concentration on total N_2O reduction was characterised from 9.2 nM (atmospheric equilibration) to 20,000 nM (Fig. 9), which

enabled me to estimate N_2O reduction by biomass at *in situ* concentrations in the ponds. I then scaled these *in situ* N_2O reduction estimates by the amount of benthic biomass in the ponds and compared them to the estimates of N_2O flux into the ponds calculated using the measurements of N_2O saturation (Fig. 4).

Firstly, based on the conversion factor of wet weight to dry weight (WW/DW = 12.17), N_2O reduction per unit wet weight would be 0.06 nmol g^{-1} DW h^{-1}/12.17 = 0.005 nmol g^{-1} WW h^{-1}. Then, based on the estimated wet-bulk density of benthic biomass (~1.01 g cm^{-3}) (Avnimelech et al., 2001), I converted the N_2O reduction rate to per unit volume of biomass as: 0.005 nmol g^{-1} WW h^{-1} × 1.01 g cm^{-3} = 0.005 nmol cm^{-3} h^{-1}.

Finally, using the depth of oxic benthic biomass (~0.006 m) (Zhu et al., 2020) in the ponds, I estimated *in situ* rates of N_2O reduction in the ponds per unit area of biomass as: 0.005 nmol cm^{-3} h^{-1} × 1000000 × 0.006 m = 31.12 nmol m^{-2} h^{-1}, i.e., 0.75 µmol m^{-2} d^{-1}, which is equivalent to 56% of the N_2O flux into the ponds of -1.33 µmol N_2O m^{-2} d^{-1}, on average (range of -3.65 to 0.02 µmol N_2O m^{-2} d^{-1}, including low emissions to the atmosphere in summer). The remaining ~44% of the N_2O flux is probably driven by microbes associated with the floating biomass (Fig. 2c) and water column (Yvon-Durocher et al., 2015) and I am confident that the laboratory biomass incubations can rationalise the undersaturation in N_2O I measured in the ponds.

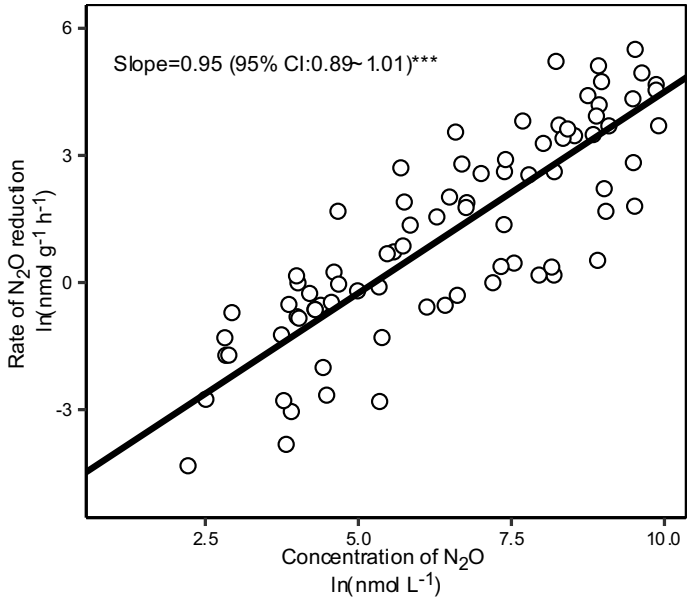

Fig. 9 | Kinetic effect of N_2O concentration on the rate of N_2O reduction after 24-hour incubation. The rate of N_2O reduction increased as a function of N_2O concentration (9.2 nM – 20,000 nM) in independent incubations of floating biomass (n = 81, floating biomass collected from 12 ponds). The overall kinetic effect of N_2O concentration on the rate of N_2O reduction was characterised by mixed-effect models, with variation across ponds accounted for by including each pond as a random effect on the intercept. The significance of slope was tested using log-likelihood-ratio comparing full to simpler, reduced models (***: p<0.001).

2.4 Discussion

In ecosystems with limited availability of fixed nitrogen (e.g. inorganic NO_2^-, NO_3^-, NH_4^+), primary production is dependent on N-fixation from the atmosphere – typically recognised to be N_2 gas. The undersaturation in N_2O reported here means that the reduction of N_2O was greater than its rate of delivery either from exchange with the atmosphere or biological sources in the ponds, which shows that these N-limited ponds were overall sinks for N_2O, including the direct fixation of the N from N_2O into biomass.

Here, N_2O fixation appears to have been direct. Others have argued for direct N_2O fixation based on the premise that if N_2 production was not detected in the presence of N_2O then N_2O fixation was direct (Farías et al., 2013; Mozen and Burris, 1954). Apart from not detecting any $^{15}N_2$ production in most (51%) of my incubations (Fig. 2**b**), here I also characterized both $^{15}N_2$ and $^{15}N_2O$ fixation that provides alternative evidence for direct N_2O fixation. In addition, my estimation of *in situ* direct N_2O fixation helps to rationalise the undersaturation and resultant flux of N_2O into the ponds (*see* section 2.3.6).

As the scale of N_2O undersaturation in the ponds (Fig. 1**a**) is in line with many other studies that also reported undersaturation in N_2O in freshwaters (typically ~70% to 100%) (Diem et al., 2012; Hendzel et al., 2005; Liu et al., 2011b; Webb et al., 2019; Whitfield et al., 2011), this indicates that direct N_2O fixation could explain the unaccounted for N_2O undersaturation in many freshwaters (Baulch et al., 2011; Diem et al., 2012; Guérin et al., 2008; Hendzel et al., 2005; Lemon and Lemon, 1981; Liu et al., 2011b; Soued et al., 2016; Webb et al., 2019; Whitfield et al., 2011).

I can see similar seasonal trends to what I report here in previous studies that have also reported undersaturation in N_2O. For example, boreal lakes, ponds, and rivers can also act as N_2O sinks, with the strongest sinks being found at the coldest temperatures (Soued et al., 2016). In Boreal peatlands, N_2O was undersaturated at most sites in spring, increased to the maximum oversaturation in summer, then decreased to near air equilibration in autumn (Schiller and Hastie, 1994). From the same study, soils acted as net N_2O sources at higher temperatures, while most N_2O sinks occurred below 13°C (Schiller and Hastie, 1994). In the surface waters of the Baltic Sea, N_2O was mostly undersaturated in winter, in December, but was oversaturated in summer and autumn, June and September, respectively (Bange et al., 1998). However, these studies lacked a clear explanation for N_2O undersaturation and its temperature dependence, which my work now offers.

To the best of my knowledge, my findings are the only evidence to demonstrate different temperature dependencies for N_2 and N_2O fixation. This difference in N_2 versus N_2O is supported not only by the opposing seasonal patterns in N_2 and N_2O saturation in the ponds, but also by the experimentally determined different temperature sensitivities for the assimilation of N_2 and N_2O by biomass in the incubations. Moreover, the results from the incubations support the seasonal patterns in N_2 and N_2O saturation in the ponds – with higher rates of N_2O reduction in incubations in winter than in summer matching the stronger N_2O undersaturation in the ponds in winter, and the elevated temperature effect on N_2 assimilation agrees with that for N_2 saturation in the ponds.

The contrasting temperature sensitivies of N_2O and N_2 fixation is probably associated with the energetic advantage of using N_2O instead of N_2 as a N-substrate for N-fixation (Shestakov and Shilov, 2001). In chapter 1, I compiled data for studies measuring N_2 fixation in both aquatic and terrestrial ecosystems (Fig. 2 and the references cited therein) which clearly shows N_2 fixation activity to increase at higher temperatures. On the other hand, as dissociating the N bond in N_2O only requires about half of the energy compared to N_2 (Howard and Rees, 1996), N_2O could be relatively easier to fix at colder temperatures and a higher proportion of total N fixation could be dependent on N_2O in the cold. For example, in the pond biomass the fraction of total N-fixation coupled to N_2O at 6°C was 26% higher than that at 25°C (Fig. 8). This thermodynamic advantage of N_2O fixation in the cold might explain why N_2O undersaturation in the ponds was strongest during the colder months and may also explain the undersaturation reported in cold, boreal environments and the Baltic Sea (Bange et al., 1998; Schiller and Hastie, 1994; Soued et al., 2016).

The fixation of N_2 is known to be down-regulated by the presence of inorganic N in both marine (Knapp, 2012) and freshwater (Marcarelli and Wurtsbaugh, 2007) communities, often with a threshold concentration of total inorganic nitrogen (TIN) around a few micromolar

(Knapp, 2012), which limits N_2 fixation to more pristine, N-limited ecosystems. The TIN in the ponds were maintained around 0.7 μM, which indicates a threshold TIN concentration that potentially limits N_2O fixation. This threshold probably also reflects the link between N_2O undersaturation (Fig. 4**a**) and the sub-micro-molar concentration of TIN throughout the year in the ponds (Fig. 3**d**). Indeed, N-rich ecosystems generally act as N_2O sources (Baulch et al., 2011; Walter et al., 2006), while N_2O sinks mediated through N_2O fixation are likely to be found in cold, pristine ecosystems (Diem et al., 2012; Priscu et al., 1990; Rees et al., 1997; Soued et al., 2016; Verdugo et al., 2016; Zhan et al., 2015).

2.5 Conclusions

In this chapter, I have shown that both N_2O and N_2 were undersaturated in the N-limited freshwater ponds, with distinct seasonalities and temperature dependencies. Further, with $^{15}N_2$ and $^{15}N_2O$ stable isotope techniques and biomass incubations, I showed that both N_2O and N_2 can be fixed by freshwater communities. Direct N_2O fixation can rationalise the undersaturation in N_2O in the ponds, which could also explain the various unaccounted for N_2O sinks – of similar magnitude – reported in natural, pristine waters (Diem et al., 2012; Hendzel et al., 2005; Priscu et al., 1990; Schiller and Hastie, 1994; Soued et al., 2016; Verdugo et al., 2016; Whitfield et al., 2011). As N_2O undersaturation is favoured in the cold, rising temperatures could erode these natural sinks for this potent climate-gas.

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Chapter 3 Characterising the multiple fates of N_2O reduction

Abstract

From my previous work, I have shown that N in N_2O can be assimilated directly into biomass, and the activity of N_2O fixation can explain the undersaturation in N_2O in the ponds. Moreover, the reduction of N_2O to N_2 is a minor component of total N_2O reduction, and the assimilation of N_2O into biomass cannot account for all of the reduced N_2O. Here, I characterise the multiple fates of total N_2O reduction and show that some of the remaining N_2O that had been reduced is present as dissolved inorganic nitrogen (DIN). N_2O could be fixed into NH_3, which could then be leaked into the water as NH_4^+ and further oxidised to NO_2^- and NO_3^-, with all DIN species being available to the wider community. Further, at lower temperatures, more N_2O is fixed and a higher proportion of that fixed N_2O was conserved as DIN or particulate organic N (PON) in the N-limited water, rather than being denitrified to N_2. In addition, the rate of N_2O fixation increased at higher relative availability of N_2O to N_2, which suggests that N_2O and N_2 could be fixed by some common communities.

3.1 Introduction

In chapter 2, I showed that $^{15}N_2O$ can be assimilated into floating and benthic biomass. I also checked for the possibility of dissimilatory reduction of N_2O to N_2 i.e., the final step in denitrification, to see whether or not ^{15}N assimilation comes from $^{15}N_2$ as the intermediate (i.e., $^{15}N_2O$ first being reduced to $^{15}N_2$ then being fixed). Overall, $^{15}N_2$ production was a minor component of total $^{15}N_2O$ reduction and N_2O fixation was primarily direct. In addition, the amount of ^{15}N assimilated into biomass could only account for 5.1% ± 0.9% (mean ± s.e.) of

total ^{15}N-N_2O reduction, which indicated that the production of inorganic N-species (NH_4^+, NO_2^- or NO_3^-) probably also contributed to the total reduction of $^{15}N_2O$.

Similar to N_2 fixation (e.g. $N_2 \rightarrow NH_4^+$), N_2O might also be fixed as NH_4^+ intracellularly (Eq.1 and Eq.2, Chapter 1), with some of the NH_4^+ then leaking into the water and further oxidised to NO_2^- or NO_3^- through nitrification. The few studies that have characterised N_2O fixation only measured the assimilation of $^{15}N_2O$ into the biomass (Cornejo et al., 2015; Farías et al., 2013; Mozen and Burris, 1954), and to the best of my knowledge, no one has traced the multiple fates of N_2O reduction by characterising different N forms.

Apart from temperature, substrate availability, i.e., the concentration of N_2O and N_2 could also be a key factor regulating the rate of total N fixation or the proportion of N_2O to N_2 fixation. With the incubation looking at the kinetic effect of N_2O fixation, I showed that N_2O fixation increased at higher availability of N_2O (Fig. 9, Chapter 2). Similarly, in the studies that characterised N_2O fixation in marine waters, higher $^{15}N_2O$ concentrations also promoted the assimilation of ^{15}N-N_2O into particulate organic nitrogen (PON) (Cornejo et al., 2015). In addition, if N_2O is fixed by the same enzymatic nitrogenase complex as for N_2 fixation, then the availability of N_2 could also affect N_2O fixation. For example, the much higher concentration of dissolved N_2 (~ 490 μM, ~5000 times of dissolved N_2O concentration) in the pond water – an more widely - might facilitate the N-fixers to consume N_2 instead of N_2O (~ 10 nM).

In this chapter, I aim to answer the following questions:

- Would fixed N_2O be presented as dissolved inorganic nitrogen, such as NH_4^+, NO_2^- or NO_3^-? And if so, how can I characterise each of the N species?
- Would the removal of the large N_2 background, i.e., a much higher relative availability of N_2O to N_2, promote N_2O fixation?

Here, I characterised any production of different DIN species from $^{15}N_2O$ in incubations with biomass collected from the experimental ponds and traced the alternative fates for N_2O fixation to N_2O assimilation into ^{15}N-PON biomass (Fig. 6, Chapter 2). With all of the possible fates of $^{15}N_2O$ reduction characterised, I demonstrated some of the potential pathways related to the reduction of N_2O. Further, I explored whether the relative availability of N_2O to N_2 have an effect on N_2O fixation, by characterising any difference in $^{15}N_2O$ assimilation after removing the large N_2 background in the incubations.

In addition, the data for the assimilation of $^{15}N_2O$ and $^{15}N_2$ into PON were highly skewed, potentially due to normalizing the rate to a unit of dry biomass which may not account for the abundance of N_2O and N_2 consumers. With no known freshwater candidates for N_2O fixation to date, and the few recognised N_2O-fixers in marine and soil ecosystems also fix N_2 (Farías et al., 2013; Mozen and Burris, 1954), it seems reasonable to hypothesise that N_2O fixation are also carried out by N_2-fixers. Further, despite its non-constitutive expression, and the potential bias in its amplification due to the specificity of different primers (Angel et al., 2018), the *nifH* gene is genetically highy conserved and act as a common biomarker for identifying N_2-fixation. If N_2O and N_2 are both fixed by the *nifH* communities, then the activities of the two processes could be correlated to the abundance of *nifH* gene presented in the incubations. Therefore, I quantified the abundance of the functional gene *nifH* in the biomass, to explore whether I could relate the abundance of *nifH* to the rate of N_2O or N_2 fixation.

3.2 Methods

Samples for the characterisation of total N_2O reduction and the fixation of $^{15}N_2O$ into $^{15}NO_x^-$ were from the same biomass incubations that were used for characterising N_2O assimilation and any reduction of N_2O to N_2 in Chapter 2. Therefore, I do not present the methods for

biomass incubation in this chapter, and details - such as the analysis of total $^{15}N_2O$ reduction, and analysis of bulk changes in dissolved inorganic nitrogen- can be found in Chapter 2.

3.2.1 Multiple fates of total reduction of $^{15}N_2O$

As total $^{15}N_2O$ reduction includes both assimilatory $^{15}N_2O$ fixation and dissimilatory $^{15}N_2O$ reduction to $^{15}N_2$ (Table 1), total $^{15}N_2O$ fixation can be calculated by subtracting $^{15}N_2$ production from total $^{15}N_2O$ reduction:

Total $^{15}N_2O$ fixation = Total $^{15}N_2O$ reduction $-$ $^{15}N_2$ production (1)

Where total $^{15}N_2O$ fixation includes $^{15}N_2O$ assimilated into biomass, as well as any fixed $^{15}N_2O$ present in the pond water medium as dissolved inorganic nitrogen (^{15}DIN, e.g., $^{15}NH_4^+$, $^{15}NO_2^-$, and $^{15}NO_3^-$):

Total $^{15}N_2O$ fixation = $^{15}N_2O$ assimilation + ^{15}DIN production (2)

Table 1 | Multiple fates of N_2O reduction.

Fate	Process	Product
Assimilatory N_2O reduction	N_2O fixation	NH_4^+
Assimilatory N_2O reduction	N_2O fixation	PON
Assimilatory N_2O reduction	N_2O fixation	NO_x^-
Dissimilatory N_2O reduction	Denitrification	N_2

3.2.2 Characterising the fixation of $^{15}N_2O$ into $^{15}NO_x^-$

I characterised any $^{15}N_2O$ presented as $^{15}NO_x^-$ (i.e., $^{15}NO_3^- + {}^{15}NO_2^-$) by converting $^{15}NO_3^-$ to $^{15}NO_2^-$ using spongy cadmium (McIlvin and Altabet, 2005), then reducing $^{15}NO_2^-$ to $^{15}N_2$ using sulfamic acid (Lansdown et al., 2016). $^{15}N_2$ was measured by CF-IRMS, bypassing the copper reduction column (Trimmer and Nicholls, 2009), before the isotopic analysis to avoid reducing any residual $^{15}N_2O$ in the water to $^{15}N_2$.

Standard calibration was performed using a mixture of $^{15}NO_3^-$ (98 atom % ^{15}N) and NO_3^- (natural abundance), to achieve a range in both the concentration and the ^{15}N-labelling of the NO_3^- pool. The final concentration of ^{15}N-NO_3^- in the standards ranged from 0.025 to 1 μM, to cover the range of possible $^{15}NO_x^-$ production from $^{15}N_2O$ reduction (0.63 μM, on average). The natural stable isotopic ($\delta^{15}N$) signatures of the N_2 produced was plotted against the concentration of ^{15}N-NO_3^-. Air-equilibrated deionised water (18.2 MΩ.cm, Elga) was prepared as per samples and inserted throughout the analysis to correct for any signal drift.

As the incubation was terminated by formaldehyde (37 wt. %), I also examined the effect of formaldehyde on the $^{15}NO_x^-$ assay by prepare two sets of identical standard curves with and without formaldehyde. The proportion of formaldehyde added to the standards is the same as that used in the biomass incubations.

Over a concentration range of $^{15}NO_3^-$ from 0 to 1.5 μM, formaldehyde did not affect the conversion efficiency of $^{15}NO_2^-$ to $^{15}N_2$ (p = 0.48, t-test), whereas it decreased the overall conversion efficiency of $^{15}NO_3^-$ to $^{15}N_2$ by 47.6%. As the interference from formaldehyde on the assay was consistent over the tested concentration range of $^{15}NO_3^-$, I prepared calibration curves by adding the same amount of formaldehyde to standards as the samples, then calibrated the production of $^{15}NO_x^-$ in the samples against the standards.

3.2.3 Characterising the fixation of $^{15}N_2O$ into $^{15}NH_4^+$

With samples from the biomass incubations for $^{15}N_2O$ fixation, I also tried to measure the presence of $^{15}N_2O$ fixed directly as $^{15}NH_4^+$ by converting $^{15}NH_4^+$ to $^{15}NO_2^-$ followed by conversion of $^{15}NO_2^-$ to $^{15}N_2O$ (Liu et al., 2014). Briefly, $^{15}NH_4^+$ was converted to $^{15}NO_2^-$ using hypobromite (Zhang et al., 2007), and the resulting $^{15}NO_2^-$ was further converted to $^{15}N_2O$ with hydroxylamine hydrochloride ($NH_2OH{\cdot}HCl$).

First, to test the conversion efficiency of NH_4^+ to N_2O, I prepared two sets of calibration curves for ammonium and nitrite conversion, which covered a broad concentration range (1 to 40 μM, to check the optimum conversion efficiency) and a lower concentration range (0.1 to 2 μM, corresponding to the potential range of $^{15}NH_4^+$ produced from $^{15}N_2O$ in the samples). After the conversion of NH_4^+ or NO_2^- to N_2O, the bulk concentration of N_2O was measured on GC/μECD (*see* Chapter 2 for details).

Further, to test whether the ^{15}N content in $^{15}N_2O$ can be measured accurately after the conversion, two separate calibration curves were prepared for $^{15}NH_4^+$ and $^{15}NO_2^-$. Briefly, I created a range of ^{15}N-labelling of the NH_4^+ and NO_2^- pool by adding different amounts of $^{15}NH_4^+$ (98 atom % ^{15}N) to 15 μM of NH_4^+ (natural abundance), and similarly, $^{15}NO_2^-$ (98 atom % ^{15}N) to 15 μM of NO_2^- (natural abundance). The reason for adding 15 μM of natural abundance of NH_4^+ and NO_2^- into the standards is to maintain a relatively constant conversion efficiency.

After conversion of $^{15}NH_4^+$ and $^{15}NO_2^-$ to $^{15}N_2O$, the sub-sample was taken from the headspace of each standard and then transferred to a 12mL gas-tight vial (Exetainer, Labco) pre-filled with air. The N_2O background in the 12mL Exetainer was 0.17 nmol, assuming a concentration of atmospheric N_2O at 335 ppb. The concentration of ^{15}N-N_2O (^{15}N from both $^{45}N_2O$ and $^{46}N_2O$) in the samples was measured on a CF-IRMS (Delta V Plus, Thermo-Finnigan) with an automated trace gas pre-concentrator (PreCon, Thermo-Finnigan) (Nicholls et al., 2007), details can be found in Chapter 2.

3.2.4 Characterizing the effect of N_2 availability on N_2O fixation

To characterize the effect of N_2 availability on N_2O fixation, I removed the background N_2 from the biomass incubations by degassing the bottles with an artificial gas mixture (400 ppm CO_2, 21% O_2 and Ar balance 200 bar to mimic air without N_2, BOC). To evaluate the degassing efficiency, 10 mL of deionised water were injected into 12mL Exetainers, the caps sealed and

degassed with Helium for 0, 0.33, 0.67, 1, 2, 3, 5, 10, 15, 20 minutes with a flow rate at either 60 or 80 cm^3 min^{-1}.

For the biomass incubations, I collected floating biomass from the ponds (~4 g of wet weight) and transferred them into gas-tight serum bottles (62 mL) along with 30 mL of filtered (PES syringe filters, 0.45μm pore size, ø = 25mm) pond water. ^{15}N-N_2O (98% atom % ^{15}N, Cambridge Isotope Laboratories, Inc.) was injected with a gastight syringe as substrate for N-fixation to track the assimilation of N_2O (final concentration ~10 μM in the medium), with the unamended vials are the controls. Prior to incubation, half of the bottles were degassed for 10 minutes with the artificial gas mixture to remove the large N_2 background, then all samples were incubated on an orbital shaker at 15 ℃, and on a 12h/12h light/dark cycle for 144 hours. After the incubations, samples were measured for the isotopic signature $\delta^{15}N$ (‰ *vs.* air) of particulate organic nitrogen (PON) to check for any difference in rate of $^{15}N_2O$ assimilation under different availability of N_2. Details for the biomass measurement are described in Chapter 2.

3.2.5 Quantitative PCR of *nifH* gene abundance in samples of biomass

To quantify the abundance of *nifH* in samples of the biomass used in the incubations, I stored ~2 g biomass from each pond at -20°C at the time of preparing the biomass incubations (described in Chapter 2). DNA from ~0.5 g of wet biomass was extracted using DNeasy PowerSoil kit (Qiagen) as per the manufacturer's instructions. The abundance of the *nifH* gene was determined using qPCR with IGK3/DVV primers (forward, 5'-GCIWTHTAYGGIAARGGIGGIATHGGIAA-3'; reverse, 5'-ATIGCRAAICCICCRCAIACIACRTC-3') (Angel et al., 2018). The IGK3/DVV primers could, however, also amplify non-*nifH* sequences, leading to an overestimation of the *nifH* communities in the samples (Angel et al., 2018; Si et al., 2023).

Amplifications were performed using CFX384 Touch Real-Time PCR (Bio-Rad) in a total volume of 10 µl, containing 5 µl of SensiFAST SYBR No-ROX mastermix (Meridian Bioscience), 0.8 µl of each primer (10µM), 0.8 µl of DNA template and 2.6 µl molecular biology quality water. Standard curves (10^2 to 10^7 copies per µl) were constructed by serial dilution of plasmid DNA containing the *nifH* gene insert, including the controls (no *nifH*). The qPCR program included 98 °C for 3 minutes and 40 cycles at 98 °C for 15 s, 58 °C for 60 s, 72 °C for 60 s. The specificity of the products was confirmed by melt curve analysis after the final extension. The PCR efficiency was 97%, with a slope of -3.39, and an R^2 of 0.98.

3.2.6 Statistical analysis

Statistical analysis and plotting were performed in R (Team, 2021) using RStudio (Version 1.3.1093). As the data for $^{15}N_2O$ reduction were highly skewed, I combined 95% of the dataset (2.5% to 97.5% percentiles) from summer and winter to minimize the impact of outliers. The combined dataset was used for statistical analysis and later presentation of the data. Simple first-order linear models were used to characterise the temperature sensitivity of total $^{15}N_2O$ reduction and $^{15}NO_x^-$ production.

3.3 Results

3.3.1 The temperature dependence of total N_2O reduction

I found significant $^{15}N_2O$ reduction in the majority (289 out of 372 incubations, 78%) of the incubations enriched with $^{15}N_2O$. The mean rate of total $^{15}N_2O$ reduction was 266 ± 42 nmol g^{-1} d^{-1}, with the highest rate of total $^{15}N_2O$ reduction occurring in December for both floating and benthic biomass. To compare total $^{15}N_2O$ reduction activity between the summer and

winter, data from November, February, and December were pooled together as winter months, while data for August and September were pooled together as summer months.

Rates of total $^{15}N_2O$ reduction were highest in winter at 507 nmol g^{-1} d^{-1}, compared to 237 nmol g^{-1} d^{-1} in summer, on average ($p < 0.001$, *t*-test) in both floating ($p < 0.001$, *t*-test) and benthic ($p < 0.05$, *t*-test) biomass (Fig. 1**a**). The patterns in total $^{15}N_2O$ reduction measured in the incubations agreed with the seasonal pattern of N_2O saturation in the ponds (Fig. 4, Chapter 2): overall, N_2O was consumed in both seasons and the pond communities were net sinks for N_2O, with higher N_2O reduction in the winter, corresponding to greater undersaturation in N_2O in the ponds in winter.

Rates of $^{15}N_2O$ reduction were higher with the benthic rather than floating biomass in summer ($p < 0.05$, *t*-test), while it was relatively consistent between the two biomass types in winter ($p = 0.08$, *t*-test) (Fig. 1**a**). In summer, $^{15}N_2O$ reduction increased at higher incubation temperatures with benthic biomass (Fig. 1**b**, $p < 0.05$), while it was consistent in the floating biomass ($p = 0.25$). In winter, rates of $^{15}N_2O$ reduction were relatively consistent across the different incubation temperatures with either biomass type (Fig. 1**c**). The overall higher temperature dependence of total $^{15}N_2O$ reduction in the benthic rather than the floating biomass could reflect different mechanisms driving total N_2O reduction: i.e., possibly different proportions of assimilatory N_2O reduction (i.e., N_2O fixation) and dissimilatory N_2O reduction (i.e., the reduction of N_2O to N_2) via denitrification in the floating and benthic biomass.

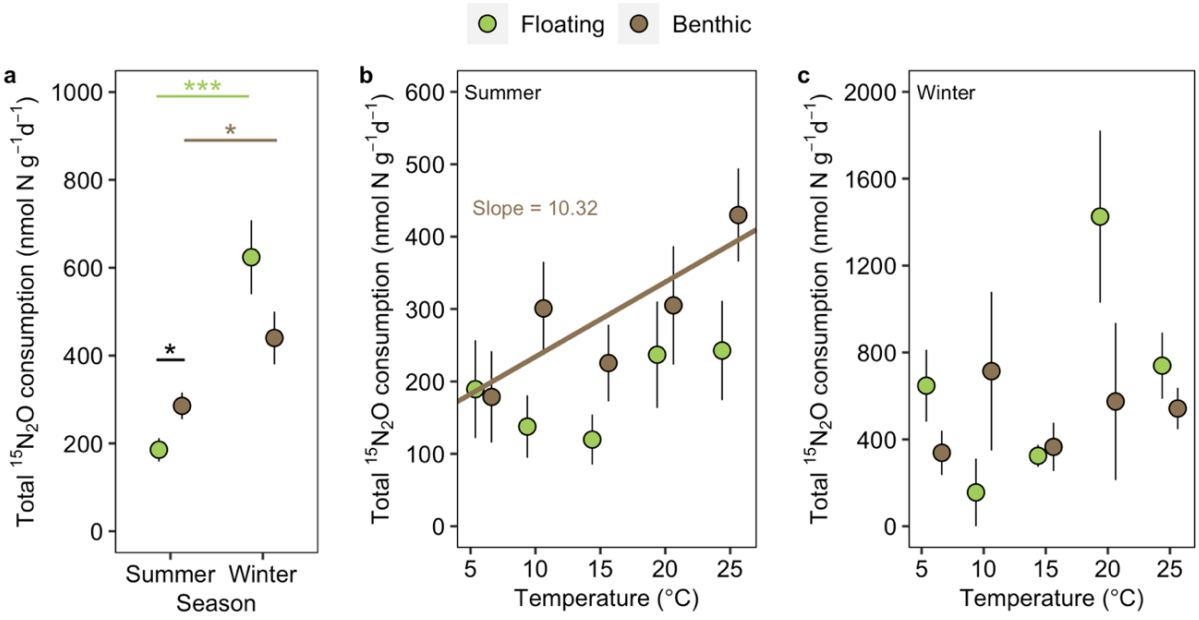

Fig. 1 | Total $^{15}N_2O$ reduction with either floating or benthic biomass the 12 mL incubations. a, Rates of total $^{15}N_2O$ reduction were highest in the winter than in the summer in both floating ($p < 0.001$) and benthic biomass ($p < 0.05$). In summer, $^{15}N_2O$ reduction was higher in the benthic than the floating biomass, while it was not statistically different between the two biomass types in the winter. Statistical significance of the means was compared between the seasons or between the biomass types in either season (*$p < 0.05$; ** $p < 0.01$; *** $p < 0.001$). **b** and **c,** The effect of temperature on $^{15}N_2O$ reduction in the summer and winter, respectively. $^{15}N_2O$ reduction by benthic biomass increased at higher temperatures in summer ($p < 0.05$, brown line), while it was relatively consistent across different temperatures with the floating biomass in summer and in either biomass type in winter. The brown line in **b** is a simple first-order linear regression model. Data plotted are means ± s.e. from 95% (2.5% to 97.5% percentiles) of the dataset for summer and winter. $n = 180$ and $n = 160$ in summer and winter, respectively (two months for summer, three months for winter). Note the difference in scale on the different y-axes.

3.3.2 Multiple fates for total $^{15}N_2O$ reduction

Apart from $^{15}N_2O$ being assimilated into biomass and the fraction reduced to $^{15}N_2$ (Chapter 2), some fixed ^{15}N could potentially "leak" into the pond-water medium as ^{15}N-dissolved inorganic nitrogen (DIN) – all of which comprise total $^{15}N_2O$ reduction. The total amount of inorganic ^{15}N in the medium and ^{15}N assimilated into biomass is termed total $^{15}N_2O$ fixation (assimilatory reduction of N_2O), which can be calculated by subtracting any $^{15}N_2$ production (dissimilatory reduction of N_2O) from the rate of total $^{15}N_2O$ reduction (equation 1).

Total N_2O reduction in both floating and benthic incubations mainly came from N_2O-dependent N fixation (Fig. 2**a**), rather than the reduction of N_2O to N_2 through denitrification. The ratio of $^{15}N_2O$ fixation to total $^{15}N_2O$ reduction was slightly higher in the floating than benthic biomass ($p < 0.01$, *t*-test), with a median of 100% and 97.6%, respectively (Fig. 2**a**).

Moreover, the proportion of N_2O fixation decreased at higher temperatures (Fig. 2**b**), which showed that the distribution of assimilatory and dissimilatory N_2O reduction was temperature-dependent. This suggests that N_2O is more likely to be conserved as fixed-N, rather than being reduced to N_2 at lower temperatures.

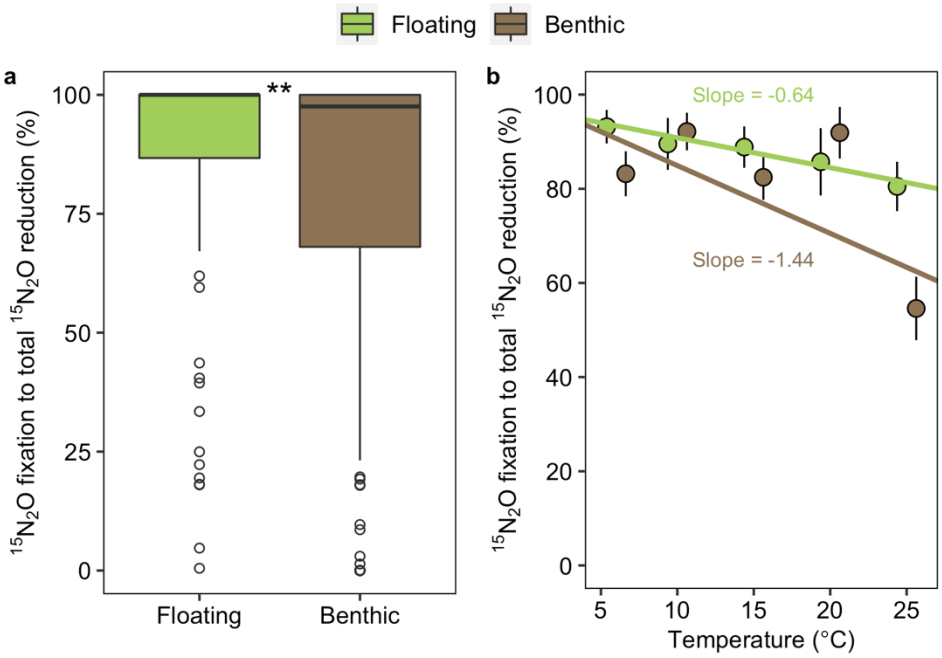

Fig. 2 | The proportion of $^{15}N_2O$ fixation to total $^{15}N_2O$ reduction in the 12 mL floating and benthic incubations. a, The majority of the total $^{15}N_2O$ reduced was fixed, rather than denitrified to N_2 in both floating and benthic incubations, with a higher proportion of $^{15}N_2O$ fixation in the floating compared to the benthic incubations. Each box shows the 25th to 75th percentiles, with the horizontal line inside each box giving the median, open circles denoting the outliers, and whiskers extending to 1.5 times the interquartile range. **b**, The proportion of $^{15}N_2O$ fixation to total $^{15}N_2O$ reduction decreased at higher temperatures in both floating and benthic incubations. Data in **b** are plotted as mean ± s.e., and the lines are simple first-order linear models. n = 102 and n = 136 for floating and benthic biomass incubations, respectively (5 months for 6 to 13 ponds).

Tracing the fixation of $^{15}N_2O$ into $^{15}NO_x^-$

To further explore the possible fates of any N_2O being fixed, I characterised the production of $^{15}NO_x^-$ from $^{15}N_2O$ in incubations in December, 2020 (winter), when previously measured rates of total $^{15}N_2O$ reduction were at their highest.

The rate of $^{15}NO_x^-$ production was 280 ± 46 nmol g^{-1} d^{-1} (mean ± s.e.), which accounted for 11.7% (median) of total ^{15}N-N_2O reduction (Fig. 3). In addition, rates of $^{15}NO_x^-$ production were consistent across the range of incubation temperatures in both floating and benthic biomass (Fig. 3**b**). The consistency in $^{15}NO_x^-$ production at different temperatures agrees with the temperature response of $^{15}N_2O$ assimilation into PON (Fig. 8, Chapter 2), which, again, suggested that N_2O dependent fixation is not sensitive to temperature.

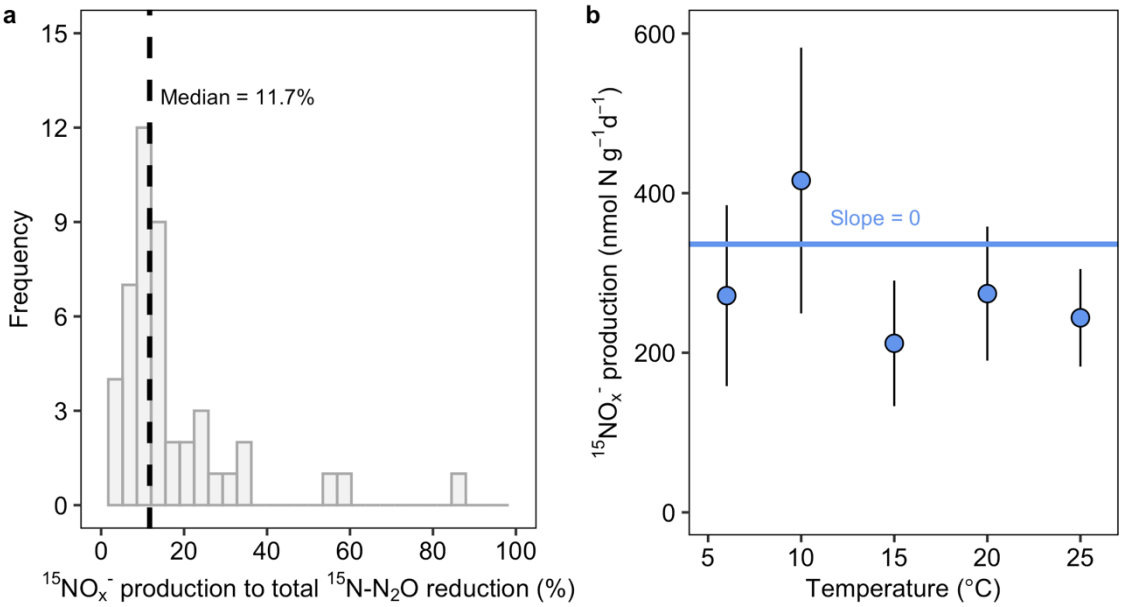

Fig. 3 | The production of $^{15}NO_x^-$ from $^{15}N_2O$ in the 12 mL biomass incubations. a, Distribution of the ratio of $^{15}NO_x^-$ production to total $^{15}N_2O$ reduction. The dashed lines denote the median value. **b**, Rate of $^{15}NO_x^-$ production from $^{15}N_2O$ was largely consistent over the range of incubation temperatures. Data are plotted as mean ± s.e., with the blue line denoting a simple first-order linear regression model. Data in both plots are from December, 2020, when the highest rate of total $^{15}N_2O$ reduction was measured. As the temperature dependencies of $^{15}NO_x^-$ production were consistent between floating and benthic incubations, I pooled the data from the two biomass types together here. n = 47 incubations for 5 temperatures and two biomass types.

Tracing the fixation of $^{15}N_2O$ into $^{15}NH_4^+$

To characterise any potential $^{15}NH_4^+$ produced from $^{15}N_2O$, I evaluated the method for converting $^{15}NH_4^+$ to $^{15}N_2O$ by preparing two sets of standards with high (1 to 40 μM) and low (0.1 to 2 μM) substrate concentrations. The higher concentration range was used to test the potential maximum conversion efficiency of the method, whereas the lower concentration range is the potential range for total $^{15}N_2O$ reduction from the biomass incubation.

As shown by the calibration curves, the conversion of both NH_4^+ and NO_2^- to N_2O increased linearly with higher substrate concentrations for both the high and low range of substrate concentration (Fig. 4). At the high concentration range (1 to 40 μM), the overall conversion efficiency of the two-step process of NH_4^+ to N_2O was 84%, and 88% for the direct conversion of NO_2^- to N_2O. At the lower substrate concentration of 0.1 to 2 μM, the conversion efficiency was considerably lower than the higher concentration range, at 41% and 72% for conversions starting from NH_4^+ and NO_2^-, respectively. Nevertheless, the constant conversion efficiency over this low range showed that using the conversion method to measure low concentrations of bulk NH_4^+ is appropriate.

Further, the isotopic signature ($\delta^{15}N$) in N_2O also increased linearly with the ^{15}N-labelling of the NH_4^+ and NO_2^- pool (Fig. 5), which showed that using the conversion of $^{15}NH_4^+$ to $^{15}N_2O$ to measure the low concentrations of $^{15}NH_4^+$ was also appropiriate.

Therefore, I then used this method to analyse the $^{15}NH_4^+$ content in the samples from the biomass incubations with $^{15}N_2O$ (*see* Chapter 2 for details of the biomass incubation). However, the high concentration of residual $^{15}N_2O$ in the samples interfered with the analysis of ^{15}N content in NH_4^+, which makes it impossible to accurately characterise any $^{15}N_2O$ production that has been fixed as $^{15}NH_4^+$ using this method. Further, as formaldehyde was used to preserve the samples, which interferes with the colorimetric assay of NH_4^+ measurement on the

automated wet-chemistry autoanalyzer, using the preserved samples to measure the bulk change in NH_4^+ was also inappropriate.

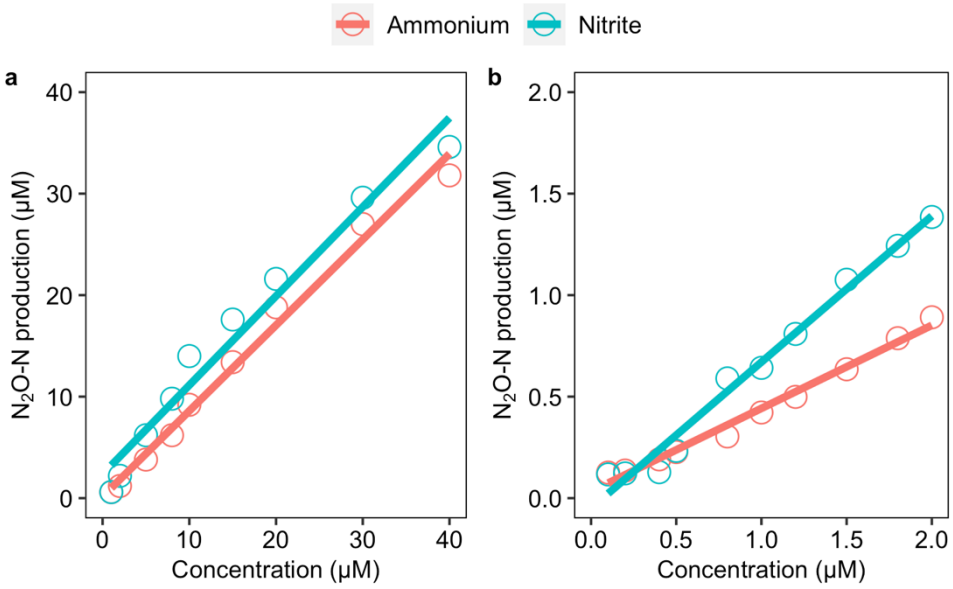

Fig. 4| Calibration curves for the conversion of ammonium (NH_4^+) and nitrite (NO_2^-) to N_2O. a, Conversion of NH_4^+ and NO_2^- at high concentrations (1 to 40 μM) and **b**, conversion of NH_4^+ and NO_2^- at low concentrations (0.1 to 2 μM). The lines are simple first-order linear models. The overall conversion efficiency of the two-step process of NH_4^+ to N_2O was 84%, and 88% for the direct conversion of NO_2^- to N_2O in **a**, and 41% and 72% in **b**.

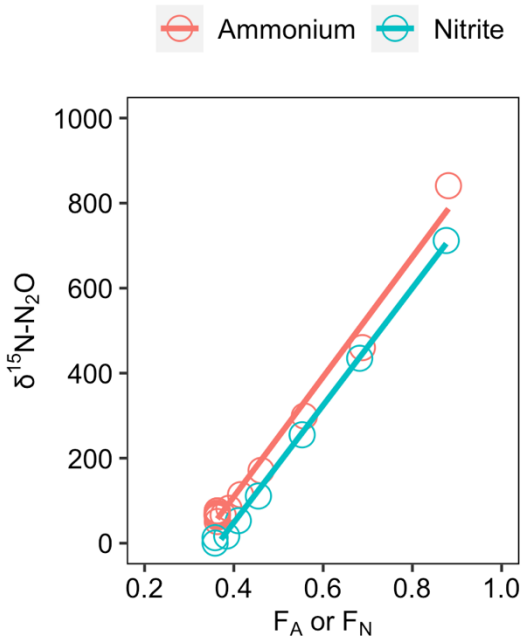

Fig. 5 | Calibration curves for the conversion of $^{15}NH_4^+$ and $^{15}NO_2^-$ to $^{15}N_2O$. F_A and F_N are the ^{15}N-labelling of the NH_4^+ and NO_2^- pool, respectively. The lines are simple first-order linear models.

Tracing the fixation of N_2O into DIN by measuring bulk changes in DIN

The methods I tried above showed that measuring the production of $^{15}NH_4^+$ in the preserved samples from the previous biomass incubations was inapproporiate due to the residual background in added $^{15}N_2O$. Alternatively, to explore whether fixed N_2O can be quantified as NH_4^+ in the incubation water I performed additional incubations with parallel samples for gas (preserved with formaldehyde) and nutrient measurements (no formaldehyde, *see* Methods in Chapter 2 for details). Half of the 12 mL vials were amended with N_2O, half unamended as controls and incubated at 15 °C, with details of the biomass incubation the same as that described in Chapter 2.

With these incubations, I also characterised any production in NO_2^- and NO_3^- to further confirm whether or not N_2O initially fixed intracellularly as NH_4^+ could leak into the medium

i.e., to be available to the wider ecosystem, apart from confirming $^{15}NO_x^-$ production from the previous incubations (*see* Fig. 4). Concentrations of NH_4^+ and NO_x^- in the controls and N_2O treatments were measured by the automated wet-chemistry autoanalyzer (San^{++}, SKALAR Analytical B.V.) with standard colorimetric techniques (Kirkwood, 1996), while changes in N_2O concentrations were measured with GC/μECD (Nicholls et al., 2007) (*see* Chapter 2 for details).

The concentration of total inorganic nitrogen (TIN) was, on average, 0.32 μM higher in incubations enriched with N_2O than the controls ($p < 0.01$, Fig. 6**a**). Although the concentration of NH_4^+ was often below the limit of detection for the colorimetric assay (~0.2 μM), the stronger signal for NH_4^+ with N_2O ($p < 0.05$, Fig. 6**b**) indicated some N_2O fixed as NH_4^+ could "leak" into the medium. In addition, significant production of NO_2^- and NO_3^- was also detected in the N_2O treatments compared to controls (Fig. 6**b**).

With the multiple fates of N_2O reduction characterised, I plotted a simple diagram showing the potential processes and possible N species that are linked to N_2O reduction (Fig. 7). Apart from being assimilated into the biomass (PON), fixed N_2O can be lost as NH_4^+ to the water and further oxidized to NO_x^- through nitrification and both, in turn, could be further assimilated into PON by the wider community.

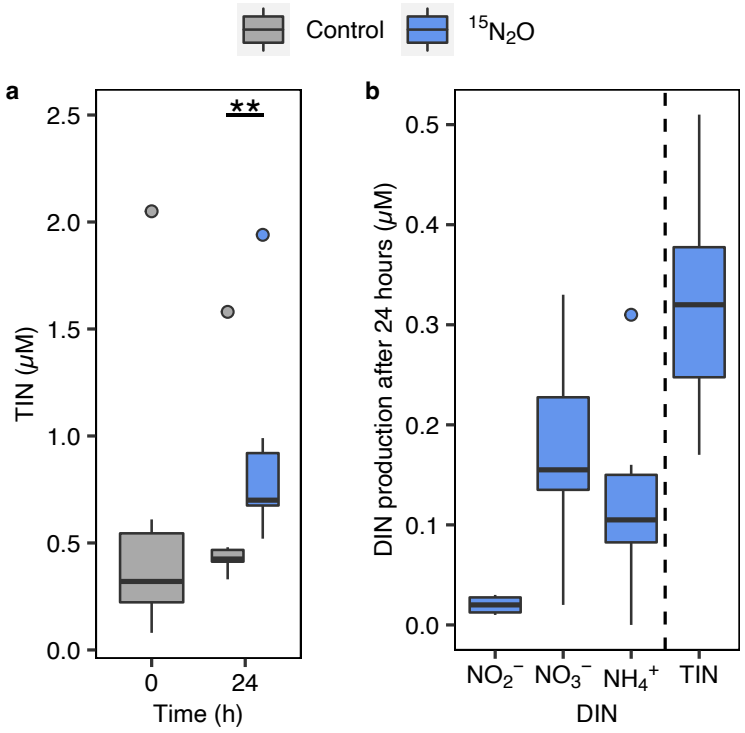

Fig. 6 | Production of different dissolved inorganic nitrogen species in the 12 mL biomass incubations with N_2O at 15 °C. a, Significant production of total inorganic nitrogen occurred in incubations with $^{15}N_2O$ relative to the controls. **b**, Production of each DIN species in $^{15}N_2O$ treatment. TIN: Total inorganic nitrogen. n = 6 (biomass from 6 independent ponds). Each box shows the 25th to 75th percentiles, with the horizontal line inside each box giving the median, open circles denoting the outliers, and whiskers extending to 1.5 times the interquartile range.

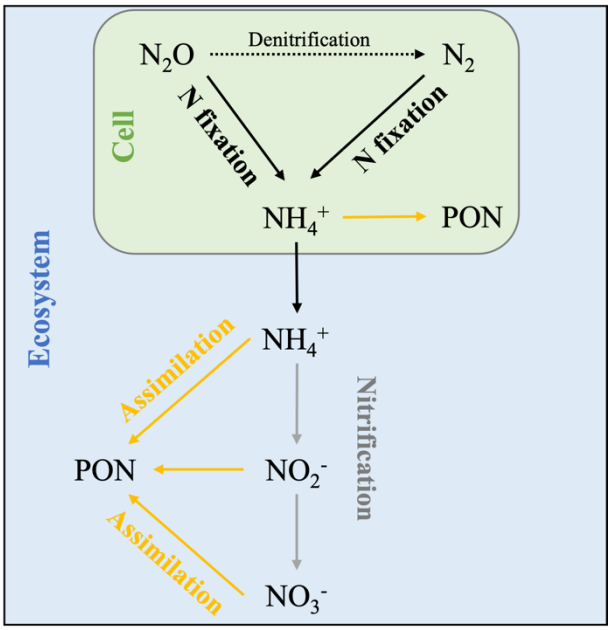

Fig. 7 | Simplified diagram showing possible pathways for N_2O reduction in relation to canonical N_2 fixation. N from N_2 or N_2O could be fixed as NH_4^+, which could be assimilated into biomass as PON. NH_4^+ could also "leak" into the water before being further oxidized to NO_x^-, with both then available to be assimilated to PON. Additionally, a minor proportion of N_2O could be reduced to N_2 through complete denitrification.

3.3.3 The effect of N_2 availability on N_2O fixation

The initial background concentration of dissolved N_2 in the incubation bottles was 491.3 µM, on average. After being degassed with helium at 80 cm^3 min^{-1} for 5 min, the concentration of dissolved N_2 decreased to 18 ± 2 µM, on average, corresponding to a removal efficiency of background N_2 of 96.33% (Fig. 8). In addition, extended degassing time (up to 20 min) did not lower the N_2 concentration any further.

Additional samples were also degassed at a lower flow rate 60 cm^3 min^{-1} for 5, 8, and 10 minutes to evaluate the effect of gas flow rate on the degassing efficiency. At a degas time of 10 min, no difference in the residual concentration of dissolved N_2 was found between flow rates at 60 cm^3 min^{-1} and 80 cm^3 min^{-1}.

Therefore, half of the biomass samples were degassed for 10 minutes with the artificial gas mixture before the incubations. After the degassing process, the ratio of N_2 and N_2O concentrations in the incubations decreased from 931 to 1 to 24 to 1, on average. With lower concentrations of N_2, the $\delta^{15}N$ (‰) of PON (Fig. 8) increased significantly (Two sample *t*-test, $p < 0.01$), which suggests a stronger activity of $^{15}N_2O$ assimilation into PON at a higher availability of N_2O relative to N_2.

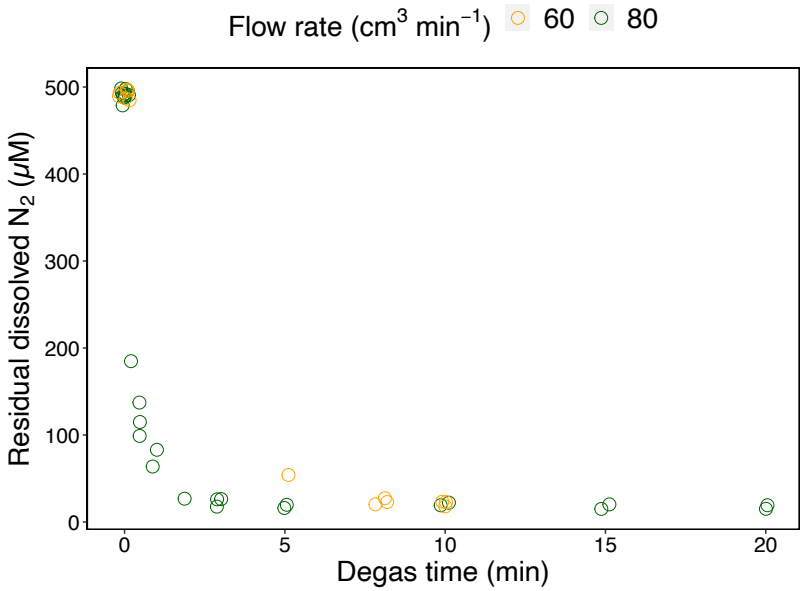

Fig. 8 | Residual dissolved N_2 in deionised water after degassing with Helium at two different flow rates.

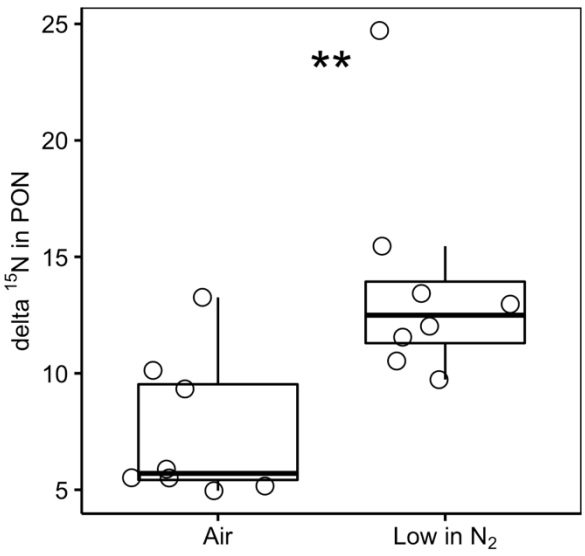

Fig. 9 | ^{15}N isotopic signature ($\delta^{15}N$, ‰) of PON in $^{15}N_2O$ treatments under different concentrations of background N_2 in the 62 mL incubations (with 32 mL headspace of artificial gas mixture). *n* = 8 in both air (ambient N_2) and low N_2 (most of N_2 in the biomass incubations removed by degassing the bottles with an artificial gas mixture for 10 min) treatments. Accordingly, the ratio of N_2 and N_2O concentrations were 931 to 1 and 24 to 1 for the 'Air' and 'Low in N_2' treatments, respectively.

3.3.4 The abundance of *nifH* in floating and benthic biomass

As N_2O fixation increases at the higher availability of N_2O relative to N_2, the fixation of both N_2O and N_2 are likely regulated by similar microbes, for example, the *nifH* community which typically fixes N_2. Therefore, here I quantified the copy numbers of the *nifH* gene in the biomass incubations, to see whether I could link the fixation of N_2O or N_2 to the abundance of *nifH*.

From the results, the assimilation of N_2O and N_2 into PON was not correlated with the abundance of *nifH* in the floating biomass (Fig. 10**a**, 10**c**). However, they both increased at higher *nifH* abundance in the benthic biomass ($p < 0.05$, Fig. 10**b**, 10**d**).

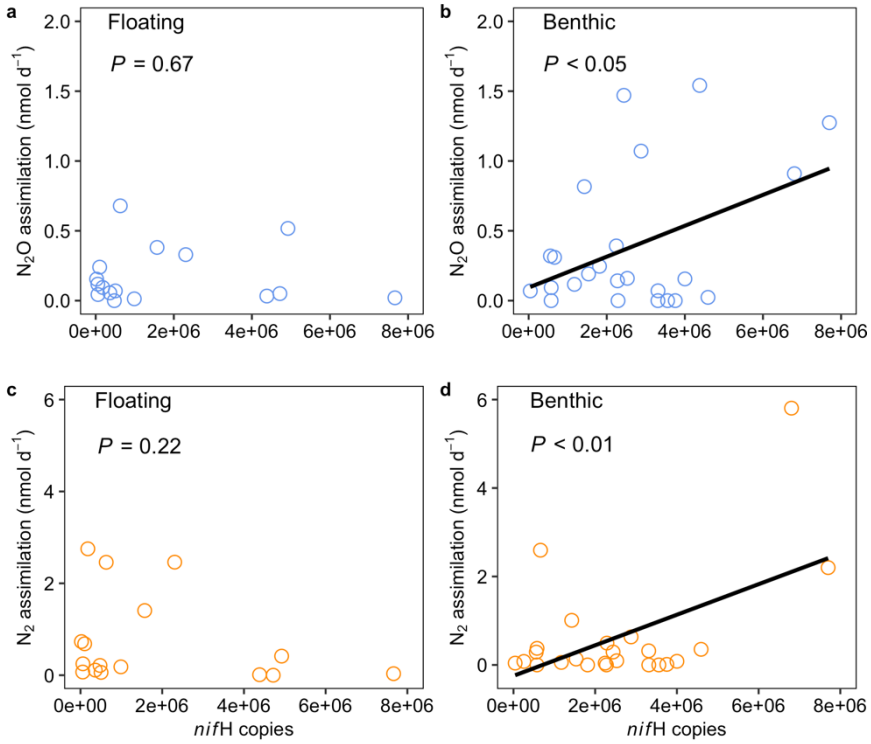

Fig. 10 | The correlation between the abundance of *nifH* and assimilation of N_2O and N_2 into particulate organic nitrogen (PON) in floating and benthic incubations. The lines in **b** and **d** are simple first-order linear regression models. n = 34 for floating and n = 49 for benthic incubations.

3.4 Discussions

In Chapter 2, I showed that undersaturation in N_2O was more pronounced in the ponds in the colder months compared to the warmer months. Here I found that the rates of total N_2O reduction were also higher in winter in the incubations with biomass collected from the ponds, which reflects the seasonal pattern of undersaturation in N_2O *in situ*. In addition, the proportion of N_2O fixation to total N_2O reduction was higher at the lower temperatures, which showed that in the cold, N_2O is being fixed, rather than being denitrified to N_2.

To characterise any alternative pathways for total N_2O reduction to the assimilation of N_2O into the biomass and the reduction of N_2O to N_2 (*see* Chapter 2), I measured the

accumulation of TIN in the N_2O treatments after the 24-hour incubations (Fig. 6). The enrichment in NH_4^+, NO_2^- and NO_3^- suggested that, apart from being assimilated intracellularly, N_2O fixed as NH_4^+ could "leak" into the medium and subsequently be oxidised to NO_2^- and NO_3^-. All these fixed-N species could then be assimilated by the wider community in the ecosystem.

In addition, theoretically, it is also thermodynamically feasible that N_2O could be oxidized directly to NO_3^- or NO_2^- through an undiscovered aerobic pathway (Kuypers et al., 2018):

$$N_2O + O_2 + H_2O = 2NO_2^- + 2H^+ \qquad (\Delta G^{0'} = -21 \text{ kJ/mol } N_2O) \qquad (3)$$

$$N_2O + 2O_2 + H_2O = 2NO_3^- + 2H^+ \qquad (\Delta G^{0'} = -89 \text{ kJ/mol } N_2O) \qquad (4)$$

The low energy yield of these oxidation pathways ($\Delta G^{0'}$ = –21 and –89 kJ mol^{-1} N_2O, respectively), however, could limit the competitiveness of the microorganisms for N_2O at ambient, nano-molar concentrations.

The measurable production of $^{15}N_2$ from $^{15}N_2O$ incubations suggested that both assimilatory (N_2O fixation) and dissimilatory N_2O reduction to N_2 (the terminal step in complete denitrification) were happening. As the reduction of N_2O to N_2 generally occurs in oxygen-limited or truly anoxic conditions (Babbin et al., 2015; Dalsgaard et al., 2012), the two processes could have occurred simultaneously in microhabitats characterised by different oxygen concentrations, or separately during the 12h/12h light/dark phases of the 24h incubations (Nielsen et al., 1990; Severin and Stal, 2008). Nevertheless, the minor contribution of N_2 production to N_2O reduction indicated that N_2O reduction was dominated by pathways other than dissimilatory N_2O reduction and N_2O fixation (e.g., into DIN and biomass) was the dominant pathway for N_2O reduction (Fig. 3).

N_2O fixation appears to be affected by the availability of both N_2O and N_2. Higher ^{15}N enrichment into the biomass from $^{15}N_2O$ occurred when most of background N_2 was removed

from the incubation (Fig. 9). Similarly, the kinetic experiment clearly showed that total N_2O reduction was limited by the availability of N_2O (Fig. 9, Chapter 2). These results show that the relative availability of N_2O to N_2 regulates N_2O fixation, with higher activity of N_2O fixation at higher proportions of N_2O relative to N_2. It is therefore likely that the fixation of N_2O and N_2 are competitive and potentially can be performed by similar microbes, e.g., *nifH* communities. Further, although the assimilation of N_2O and N_2 were not affected by the abundance of *nifH* in the floating biomass, they both increased at higher *nifH* abundance in the benthic biomass (Fig. 10).

As suggested by some earlier studies, N_2O is a competitive inhibitor for N_2 fixation and it could also be used by nitrogenase (Jensen and Burris, 1986; Repaske and Wilson, 1952; Rivera-Ortiz and Burris, 1975; Wilson and Roberts, 1954). All these results combined, suggest that N_2O and N_2 fixation are potentially regulated by some common communities, and together they support the primary production in N-limited waters. Although due to the much lower concentration of N_2O compared to N_2 in natural waters, the contribution of N_2O fixation to total ecosystem primary production will always be relatively minor compared to N_2 (Table 4, Chapter 2).

However, the distinct seasonal patterns I measured for N_2 and N_2O undersaturation (Fig. 4, Chapter 2), coupled with disproportionate rates of N_2O fixation (Fig. 6, Chapter 2) and the higher proportions of N_2O fixation at colder temperatures (Fig. 7**a**, Chapter 2) - all indicate that the community responsible for N_2O fixation seem to be different from N_2 fixation at colder temperatures. The contradictory results suggest that communities responsible for N_2O fixation may have different patterns in substrate preference. For example, some N_2O fixers are facultative and would fix more N_2O at a relatively higher availability of N_2O to N_2 (Fig. 9), while others are obligate N_2O fixers that always fix N_2O regardless of the large N_2 background (Fig. 6, Chapter 2).

3.5 Conclusions

Here I have shown that rates of total N_2O reduction are higher in winter compared to summer, which agrees with the stronger undersaturation in N_2O in the ponds in winter. The proportion of N_2O fixation to total N_2O reduction was also higher at lower temperatures. I also characterised different products in the biomass incubations to illustrate the multiple fates for total N_2O reduction. N_2O could be fixed into NH_4^+ intracellularly, which could then be released into the water, before being oxidised to NO_2^- and NO_3^- and becoming available to the wider communities. These results showed that at lower temperatures, not only more N_2O was being reduced, a higher proportion of the reduced N_2O was being conserved in the forms of dissolved inorganic N and particulate organic N, rather than being denitrified to N_2 in the N-limited ecosystem. In addition, the relative availability of N_2O to N_2 regulates N_2O fixation, with higher rates of N_2O fixation at higher availability of N_2O relative to N_2.

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Chapter 4 Temperature dependences of N_2O and N_2 production from denitrification

Abstract

As N_2O can be both produced and reduced to N_2 by canonical denitrification, the balance between these two steps is important in regulating the budget of this potent climate gas. With a metadata analysis of published rates of denitrification, I show that the total production of N_2O and N_2 from denitrification are both sensitive to increasing temperature, with an identical activation energy of 0.7 eV. Further, the availability of nitrate can also be crucial in regulating the balance of N_2O and N_2 from denitrification, yet only a few studies have characterised the combined effect of nitrate and temperature in natural waters. Here, using ^{15}N-isotope techniques and anoxic biomass incubations, I show that the production of N_2O and N_2 were only sensitive to changes in temperature with 100 μM of nitrate, whereas no temperature effect was found with 10 μM of nitrate. With the high nitrate, N_2 production increases at higher temperatures, while net N_2O production has an opposite temperature dependency. As a result, the net production ratio of N_2O to N_2 from denitrification increases at lower temperatures, which could provide more N_2O relative to N_2 for N fixation in the cold. In addition, N_2O production from nitrification was not detected from most of the experimental ponds, showing that denitrification was the dominant potential source for N_2O production.

4.1 Introduction

N_2O can be either produced or further reduced to N_2 during microbial denitrification ($NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$) (Dalsgaard et al., 2012; Naqvi et al., 2010). With denitrification

both producing and consuming N_2O, the difference in the rate of the last two steps determines the net production or consumption of N_2O during denitrification.

Total N_2O and N_2 production from denitrification generally increase at higher temperatures (Bailey and Beauchamp, 1973; Boulêtreau et al., 2012; Holtan-Hartwig et al., 2002; Keeney et al., 1979; Palacin‐Lizarbe et al., 2018; Seitzinger et al., 1984; Silvennoinen et al., 2008), with an optimum range typically observed at 20 to 35ºC (Benoit et al., 2015; Braker et al., 2010; Brin et al., 2017; Lai et al., 2019; Wang et al., 2018). Occasionally, high optimum temperature for denitrification was reported, e.g., ~ 45ºC for total N_2O production in anaerobic agricultural soils (Fischer and Whalen, 2005). However, the effect of temperature on net N_2O production i.e. total N_2O minus N_2 production varies among different studies. Both positive (Keeney et al., 1979; McKenney et al., 1984; Myrstener et al., 2016) and negative (Adouani et al., 2015; Bailey, 1976; Ogilvie et al., 1997; Silvennoinen et al., 2008) relationships between net N_2O production from denitrification and temperature have been reported, whereas no significant relationship was found in others (Bailey and Beauchamp, 1973; Del Prado et al., 2006).

Furthermore, N_2O and N_2 produced from denitrification could be recycled for other processes, such as N fixation, with the ratio in the net production of N_2O to N_2 indicating the relative availability of N_2O to N_2. Despite a few studies reporting a non-significant effect of temperature on the product ratio of N_2O to N_2 from denitrification (Focht, 1974; Rudaz et al., 1999), lower ratios of N_2O to N_2 at higher temperatures have been found in both soils (Avalakki et al., 1995; Bailey, 1976; Bailey and Beauchamp, 1973; Keeney et al., 1979; Maag and Vinther, 1996; Melin and Nômmik, 1983) and river sediments (Silvennoinen et al., 2008).

These results suggest a different temperature sensitivity between N_2O production and N_2O reduction (i.e., N_2 production) from denitrification, e.g., a lower activation energy of N_2O production than N_2 production from denitrification would result in a lower product ratio of N_2O

to N_2 at warmer temperatures. The multiple microbial processes of denitrification may respond differently temperature changes (Saleh-Lakha et al., 2009; Zhang et al., 2015) with shifted community structures under warming (Xing et al., 2021), likely altering the proportion of gas products. In incubations with Arctic soils, abundance of *nosZ*, which is responsible for the reduction of N_2O to N_2, was higher at 10ºC than 4ºC, whereas abundance of *norB,* the gene for the reduction of NO to N_2O, decreased at the higher temperature (Jung et al., 2011). Other studies in soils showed that the higher N_2O emission at lower temperatures was either due to inhibited activity of N_2O reductase at very low temperatures, e.g., around 0ºC (Holtan-Hartwig et al., 2002; Öquist et al., 2007), or that the reduction of N_2O to N_2 was promoted at higher temperatures due to enhanced O_2-limited condition (Lai et al., 2019; Smith, 1997; Veraart et al., 2011).

As acetylene (C_2H_2) inhibits the reduction of N_2O to N_2, total production of N_2O from denitrification has been routinely characterised by N_2O production in the presence of C_2H_2 (Abdalla et al., 2009; Bonnett et al., 2013; Braker et al., 2010; Castaldi, 2000; Cui et al., 2016; De Klein and Van Logtestijn, 1996; Fischer and Whalen, 2005; Holtan-Hartwig et al., 2002; Jørgensen, 1989; Myrstener et al., 2016; Saleh-Lakha et al., 2009; Wertz et al., 2013; Westermann and Ahring, 1987), while N_2 production can be calculated by the difference between N_2O production with and without C_2H_2 (Castaldi, 2000; Holtan-Hartwig et al., 2002). However, it is known that the C_2H_2 method has a few disadvantages. For example, its incomplete inhibition of denitrification may underestimate N_2 production from denitrification (Qin et al., 2014). C_2H_2 can also inhibit nitrification, which could decrease the amount of NO_3^- produced from nitrification and therefore underestimate the production of gases from coupled nitrification and denitrification (Seitzinger et al., 1993). Alternatively, some studies have measured N_2 production directly, either by measuring the flux of N_2 (Nowicki, 1994; Qin et al., 2014; Seitzinger et al., 1984) or the production of ^{15}N-N_2 from $^{15}NO_x^-$ (Brin et al., 2014; 2017;

Rysgaard et al., 2004; Silvennoinen et al., 2008; Veraart et al., 2011). Nevertheless, to the best of my knowledge, only one study in soil measured ^{15}N-N_2O production from denitrification under different temperatures (Duan et al., 2019), and no study has characterised the temperature dependency of denitrification by measuring the ^{15}N content in both N_2 and N_2O.

Apart from temperature, the availability of NO_3^- can also be crucial in regulating the production of N_2 and N_2O from denitrification. As low availability of NO_3^- can suppress the rate of denitrification (Myrstener et al., 2016), many studies that have characterised the temperature dependence of denitrification either performed the experiments in N-rich natural environments or added replete NO_3^- (Jørgensen, 1989; Myrstener et al., 2016; Rysgaard et al., 2004; Silvennoinen et al., 2008; Veraart et al., 2011), to achieve a steady increase in the production of N_2O or N_2. A 3-month exposure to a high concentration of NO_3^- increased the temperature sensitivity of N_2 production in sediments collected from estuarine mesocosms (Nowicki, 1994). Another study showed a stronger temperature dependency of total N_2O production after the agricultural soils had been pre-incubated for 3 weeks with non-limiting NO_3^- (Braker et al., 2010). In addition, a study in N-limited boreal lakes showed that total N_2O production from denitrification increased at either higher concentrations of NO_3^- or higher temperatures (Myrstener et al., 2016). However, this study only characterised the temperature response with a relatively high addition of NO_3^- at 57 μM. To date, studies on the combined effect of nitrate and temperature on denitrification are very scarce, and it is not clear how the temperature response of N_2 and N_2O production would change under different concentrations of nitrate in natural waters.

Moreover, although the many studies listed above have characterised the temperature sensitivities of both N_2O and N_2 production from denitrification, most focused on soils, whereas similar studies are very limited in aquatic ecosystems (Silvennoinen et al., 2008). Therefore, here I try to narrow down these knowledge gaps by characterising the temperature

dependencies of net production of both N_2O and N_2 from denitrification using ^{15}N-tracers and our well-established freshwater experimental ponds (*see* Methods, Chapter 2). Furthermore, as N_2O produced from denitrification and nitrification could be a potential source for N_2O fixation, combining my findings from these processes, I will show how temperature regulates both the production and reduction of N_2O in aquatic ecosystems.

The key questions in this chapter are:

- How would the net production of N_2O and N_2 from denitrification change under different temperatures in freshwater? And
- How would the product ratio of N_2O to N_2 from denitrification change under different temperatures?
- Would the availability of NO_3^- affect the temperature sensitivities of N_2O and N_2 production from denitrification in the N-limited ecosystem?

From the results of my N_2O consumption experiments, the reduction of N_2O to N_2 occurred in some incubations and was more pronounced at higher temperatures (data not shown, Chapter 2). As the reduction of N_2O to N_2 typically happens under anoxic conditions (Dalsgaard et al., 2012; Knowles, 1982), this process might have occurred in the anoxic micro-environment or the 12-hour dark period of the incubations. Nevertheless, to characterise the temperature sensitivity of the reduction of N_2O to N_2 from denitrification, anoxic, rather than oxic incubations, are more appropriate as the potential of denitrification would be maximized (Silvennoinen et al., 2008).

Further, as denitrification and anammox ($NH_4^+ + NO_2^- \rightarrow N_2$) can both produce N_2 (Dalsgaard et al., 2003; Kuypers et al., 2003; Trimmer et al., 2013), it is important to differentiate the production of N_2 from the two processes. This is usually performed by applying different combinations of ^{15}N-substrates and characterising the production of $^{29}N_2$ ($^{14}N^{15}N$) and $^{30}N_2$ ($^{15}N^{15}N$) (Dalsgaard et al., 2003; Kuypers et al., 2003; Trimmer et al., 2013).

Based on one to one isotope-pairing , if anammox was present, excess $^{29}N_2$ would be produced from $^{14}NO_3^-$ plus $^{15}NH_4^+$ treatment relative to $^{15}NH_4^+$ only treatment: i.e. one N from $^{14}NO_3^-$ and one N from $^{15}NH_4^+$ from anammox (Dalsgaard et al., 2003; Trimmer et al., 2013).

Apart from denitrification and anammox, I also characterised the production of N_2O from nitrification. As N_2O can also be produced from nitrification via hydroxylamine oxidation ($NH_4^+ \rightarrow NH_2OH \rightarrow N_2O$), characterising both denitrification and nitrification would help us to better understand the production and reduction of N_2O and how temperature affects these processes in aquatic ecosystems.

4.2 Methods

4.2.1 Meta-analysis: Temperature sensitivities of N_2O and N_2 production from denitrification

To derive the temperature sensitivities of N_2O and N_2 production from denitrification, here I compiled a list of studies that reported denitrification rate under different temperatures in both aquatic and terrestrial ecosystems (Table 1). Different studies in the dataset measured different gaseous products for denitrification, including the total production of N_2O, N_2, and net production of N_2O (Table 1). I have separated the three different processes and fitted each into the linear mixed-effect models to evaluate the temperature sensitivity of each process.

I estimated the apparent AE of total N_2O, N_2, and net N_2O production ($\bar{E}$) by fitting the natural log-transformed rate against the centered temperature term $\left(\frac{1}{kT_C} - \frac{1}{kT_i}\right)$ (Yvon-Durocher et al., 2014; Zhu et al., 2020):

$$\ln F(T_i) = (\bar{E} + a_i)\left(\frac{1}{kT_c} - \frac{1}{kT_i}\right) + \overline{\ln F(T_c)} + b_i \quad (1)$$

where k is the Boltzmann constant (8.62×10^{-5} eV K^{-1}, 1 eV = 96.485 kJ mol^{-1}), T_c was calculated by the sum of maximum and minimum of the inverted absolute temperature (in

Kelvin) of the dataset, then divided by 2 ($T_c = (\max + \min)/2$), and T_i the absolute temperature from study i (i =1, 2, … ,16). Further, to account for variances across the different studies, I included random slope (a_i) and random intercept (b_i) terms in the mixed-effects models.

Statistical analysis and plotting were performed in R (Team, 2021) using RStudio (Version 1.3.1093). As the studies used different normalization methods, I standardised the data by subtracting the study-specific intercept from the rate of denitrification for each study (Yvon-Durocher et al., 2014; Zhu et al., 2020). Moreover, as the production of N_2O and N_2 often started to curve above the optimal temperature (Braker et al., 2010; Fischer and Whalen, 2005; Veraart et al., 2011), data were truncated to keep the log-transformed rate within the linear range where appropriate. Data were fitted using linear mixed-effect models (Bates et al., 2014), and then models were ranked by the small sample-size corrected Akaike Information Criterion (AICc) using the 'MuMIn' package. The best-fitting model was determined by the lowest AICc score (*see* Table 6) and, AE, in the unit of eV, was derived from the slope of the best-fitting model (Fig. 1).

Table 1. A summary of the studies used for the meta-analysis to explore the effect of temperature on N_2O or N_2 production from denitrification in aquatic and terrestrial ecosystems. In total, the dataset for the meta-analysis consists of 583 measurements from 31 studies, i.e., 327 and 256 measurements in aquatic and terrestrial ecosystems, respectively. n.a.: not applicable.

Ecosystem	Measurement	Method	Substrate	Studies
Aquatic	Total N_2O	C_2H_2 inhibition	NO_3^-	(Jørgensen, 1989; Myrstener et al., 2016; Saleh-Lakha et al., 2009; Westermann and Ahring, 1987)

	N_2	bulk N_2	n.a.	(Nowicki, 1994; Seitzinger et al., 1984)
	N_2	$^{15}N_2$	$^{15}NO_3^-$	(Brin et al., 2014; 2017; Rysgaard et al., 2004; Silvennoinen et al., 2008; Veraart et al., 2011)
	Net N_2O	bulk N_2O	NO_3^-	(Adouani et al., 2015; Silvennoinen et al., 2008; Wang et al., 2018)
Terrestrial	Total N_2O	C_2H_2 inhibition	NO_3^-	(Abdalla et al., 2009; Bonnett et al., 2013; Braker et al., 2010; Castaldi, 2000; Cui et al., 2016; De Klein and Van Logtestijn, 1996; Fischer and Whalen, 2005; Holtan-Hartwig et al., 2002; Wertz et al., 2013)
	Total N_2O	$^{15}N_2O$	$^{15}NO_3^-$	(Duan et al., 2019)
	N_2	bulk N_2	NO_3^-	(Qin et al., 2014; Wang et al., 2018)
	N_2	C_2H_2 inhibition	NO_3^-	(Castaldi, 2000; Holtan-Hartwig et al., 2002)
	Net N_2O	bulk N_2O	NO_3^-	(Benoit et al., 2015; Del Prado et al., 2006; Dobbie and Smith, 2001; Kurganova and Lopes de Gerenyu, 2010; McKenney et al., 1984; Smith et al., 1998)

4.2.2 Optimisation of incubation conditions for characterising N_2O and N_2 production from denitrification

Before characterising the temperature sensitivity of the gas products from denitrification, I did a trial experiment to determine the optimal ^{15}N-substrate concentration and incubation length for the experiments.

Sediment cores were collected from the ponds in December 2020, with each pond sampled at three different locations. Intact cores were kept at 4ºC on return to the laboratory. Before the experiment, the cores and pre-weighed 12 mL vials were put into an anaerobic glove box (5 ppm of residual O_2, Belle Technology) which was constantly flushed with oxygen-free N_2 (OFN) gas recycled through oxygen-scrubbing, catalytic cartridges. Anoxic medium was made by flushing N-free artificial pond medium (*see* Chapter 2 for details) with OFN for 20 min. The top 2 cm of the cores for each pond were homogenized, then 2mL of the sediment and 4mL of the medium were added to each vial to make a slurry. The slurry was added carefully without any sediment remaining inside the thread of the lid, as this would affect the sealing of the vial and lead to possible air contamination. The vials were then pre-incubated in a temperature-controlled room (15ºC) for 16 h to remove any residual porewater NO_x^- and/or O_2 (Trimmer et al., 2013).

After the pre-incubation, vials were amended with 50 µL of different stocks of $^{15}NO_3^-$ (98% of ^{15}N, Sigma Aldrich) to a final concentration range 10, 20, 50, or 100 µM, with un-amended vials as controls. Each concentration set was incubated for 0.5, 3, 6, 12, and 24 h (Table 2). Independent vials were used for each pond, substrate concentration, and time point. At each time point, the microbial activities in the vials were terminated by injecting 200 µL of formaldehyde (37 wt. %), with vials then equilibrated at room temperature (22ºC) until further analysis.

N_2O concentrations were measured on a gas chromatograph fitted with a micro-electron capture detector (GC/μECD, Agilent Technologies UK Ltd., South Queensferry, UK), see Chapter 2 for details. For concentration of ^{15}N-N_2O, sub-samples from the headspace of each vial were then transferred to an air-filled gas-tight vial (12 mL Exetainer, Labco, UK) and measured on a CF-IRMS (Delta V Plus, Thermo-Finnigan) with an automated trace gas pre-concentrator (Precon, Thermo Finnigan). The calibration curve was performed with air, 1.04 ppm, and a series of diluted 96 ppm N_2O standards, with a linear increase in the peak area over a range of 0.08 nmol to 5.85 nmol N_2O in the vial. Additionally, $^{15}N_2$ concentrations were measured on a CF-IRMS in 100 μL headspace from each sample (*see* Methods, Chapter 2).

The net production of N_2O and N_2 in each vial was derived from the headspace concentration and the solubility of gases under the equilibrium temperature based on (Weiss and Price, 1980) for N_2O and (Weiss, 1970) for N_2 (*see* Methods, Chapter 2). The production of ^{15}N-N_2O ($^{45}N_2O$ + $^{46}N_2O$) and ^{15}N-N_2 ($^{29}N_2$ + $^{30}N_2$) was calculated from the excess gas production in the $^{15}NO_3^-$ treatments compared to that in controls. After all the gas measurements, vials were centrifuged, supernatants removed and completely dried in the oven to obtain the dry weight for calculating weight-specific rates.

Table 2. Experiments designed to explore the optimal substrate and incubation length to characterise N_2O and N_2 production from denitrification.

Treatment	$^{15}NO_3^-$ (μM)	Time (h)	Targeted product(s)
Control	0	0, 0.5, 3, 6, 12, 24	$^{45}N_2O$, $^{46}N_2O$, $^{29}N_2$, $^{30}N_2$
$^{15}NO_3^-$	10, 20, 50, 100	0, 0.5, 3, 6, 12, 24	$^{45}N_2O$, $^{46}N_2O$, $^{29}N_2$, $^{30}N_2$

4.2.3 Characterising the temperature sensitivities of N_2O and N_2 production from denitrification

After optimising the incubation conditions from the trial experiment, I collected further sediment cores from 8 experimental ponds (4 ambient and 4 warmed ponds) in September 2021, to explore the temperature sensitivities of N_2O and N_2 production from denitrification.

As shown by the trial experiment, residual NO_3^- remained in the incubation after 16 hours of pre-incubation (Fig. 5). Therefore, the vials were pre-incubated in a temperature-controlled room (15ºC) for a longer period (24 h) to further remove residual porewater NO_x^- and/or O_2. After the pre-incubation, vials were amended with different doses of $^{15}NO_3^-$ (98% of ^{15}N, Sigma Aldrich) to a final concentration of 0 (controls), 10, or 100 μM (Table 3). Each concentration set was incubated for 3 h, which is the optimal time determined from the trial experiment (Fig. 3). Other details for sediment collection, sample preparations and measurements were the same as described in the trial experiments above.

In addition, I did a parallel study to detect if anammox was present in the pond sediments and to show whether denitrification is the only N_2 production process. Briefly, another two sets of incubations were performed with the same sediment samples collected as used in the denitrification experiments: $^{15}NH_4^+$ treatment, and $^{15}NH_4^+$ plus $^{14}NO_3^-$ treatment (Table 3) and two different concentrations of $^{15}NH_4^+$ (10 or 60 μM) to check for the presence of anammox activity under both low and high substrate availability. The concentration of $^{14}NO_3^-$ added to the $^{15}NH_4^+$+$^{14}NO_3^-$ was 100 μM, same as the high concentration of $^{15}NO_3^-$ used in the denitrification experiment. After the incubations, $^{29}N_2$ concentrations in each of the treatments were compared to that in the controls to calculate the production of $^{29}N_2$.

Table 3. Experiments designed to characterise the temperature sensitivities of N_2O and N_2 production from denitrification, and to check the presence of anammox. As

denitrification and anammox can both produce N_2, to differentiate the two processes, the presence of anammox activity was checked by using different sets of ^{15}N-substrates. The presence of anammox activity would be confirmed if excess $^{29}N_2$ is produced from $^{15}NH_4^+ + ^{14}NO_3^-$ treatment relative to $^{15}NH_4^+$ only treatments. Each treatment was applied to the sediments collected from 4 ambient and 4 warmed ponds.

Treatment	**$^{15}NO_3^-$ (µM)**	**$^{15}NH_4^+$ (µM)**	**Temperature (ºC)**	**Targeted product(s)**
Denitrification				
Control	0	n.a.	5, 10, 15, 20, 25	$^{45}N_2O$, $^{46}N_2O$, $^{29}N_2$, $^{30}N_2$
$^{15}NO_3^-$	10 or 100	n.a.	5, 10, 15, 20, 25	$^{45}N_2O$, $^{46}N_2O$, $^{29}N_2$, $^{30}N_2$
Anammox				
$^{15}NH_4^+$	n.a.	10 or 60	15	$^{29}N_2$
$^{15}NH_4^+ + ^{14}NO_3^-$	n.a.	10 or 60	15	$^{29}N_2$

4.2.4 Characterising the production of N_2O from nitrification

Apart from denitrification, I also characterised any production of N_2O from nitrification ($NH_4^+ \rightarrow NH_2OH \rightarrow N_2O$). Sediment cores were collected from 8 experimental ponds (4 ambient and 4 warmed ponds) in February 2022, with the same sampling techniques as used in the denitrification experiments (*see* section 4.2.2).

In the laboratory, the top 2 cm of sediments for each pond were homogenized, then 2 mL or 3 mL of the sediment and 2.7 mL of artificial pond medium were added to each 12 mL vial to make an oxic slurry. The slurry was added to the vials carefully without any sediment remaining inside the thread of the lid, as this would affect the sealing of the vial and lead to possible air contamination.

Vials were amended with $^{15}NH_4^+$ (98% of ^{15}N, Sigma Aldrich, final concentration 22 µM or 44 µM) with or without allylthiourea (ATU) (100 µL from 2.8mM stock, final concentration ~80 µM), where ATU was used to block the oxidation of NH_4^+ (Ginestet et al., 1998). Un-amended vials were also prepared as controls to account for any activity of nitrification from

the background NH_4^+ (Table 5). Independent vials were used for each pond and treatment and incubated for 0, 3, 8, 18, or 24 h. All vials were incubated at 15ºC (annual average temperature in the ambient ponds, Fig. 2, Chapter 2)

Further, as formaldehyde was used to preserve the gas samples, which interferes with the colorimetric assay of NO_x^- and NH_4^+ measurement on the automated wet-chemistry autoanalyzer, I prepared parallel samples for measuring gas and nutrients. The microbial activities in samples for the gas measurement were terminated by injecting 200μL of formaldehyde (37 wt. %) at each time point, with vials then equilibrated at room temperature (22ºC) until further analysis. Whereas samples for the DIN measurement were immediately centrifuged and supernatants frozen at -20°C at each time point.

Methods for measuring and calculating the production of N_2O and N_2 are the same as used in the denitrification experiments (*see* section 4.2.2). To obtain the concentrations of porewater nutrients and the ^{15}N-labelling of the NH_4^+ pool (FA), DIN concentrations in the nutrient samples were measured by the automated wet-chemistry autoanalyzer (San^{++}, SKALAR Analytical B.V.) with standard colorimetric techniques (Kirkwood, 1996).

Table 5. Experimental design to characterise the production of N_2O from nitrification. Each treatment was applied to the sediments collected from 4 ambient and 4 warmed ponds.

Treatment	$^{15}NH_4^+$ (μM)	Temperature (ºC)	Targeted product(s)
Control	0	15	$^{45}N_2O$, $^{46}N_2O$
$^{15}NH_4^+$	22 or 44	15	$^{45}N_2O$, $^{46}N_2O$
$^{15}NH_4^+$+ATU	44	15	$^{45}N_2O$, $^{46}N_2O$

4.2.5 Statistical analysis

Statistical analysis and plotting were performed in R (Team, 2021) using RStudio (Version 1.3.1093). The percentage of ^{15}N-N_2 produced to $^{15}NO_3^-$ added in the incubations was

calculated by $(\ ^{45}N_2+2\times{}^{46}N_2)/\ ^{15}NO_3^-\times100$, where the difference in the percentage between incubations with different $^{15}NO_3^-$ additions was tested by the Kruskal-Wallis test.

To derive the temperature sensitivities of net N_2O and N_2 production from denitrification, I estimated the apparent AEs by fitting the natural log-transformed rate of net production of N_2O or N_2 against the centered temperature term $\left(\frac{1}{kT_C}-\frac{1}{kT_i}\right)$ using equation (1), except that i (i =1, 2, … ,8) denotes the different ponds and T_C denotes the average incubation temperature (15°C). I standardised the data by subtracting the pond-specific intercept from the net production rate of N_2O or N_2 for each pond (Yvon-Durocher et al., 2014; Zhu et al., 2020). Further, the apparent AE of the ratio of N_2O to N_2 net production was also characterised using the same methods. Data were fitted into linear mixed-effect models (Bates et al., 2014), which were then ranked by the small sample-size corrected Akaike Information Criterion (AICc) using the 'MuMIn' package. The best-fitting model was determined by the lowest AICc score (*see* Table 8) and, AE, in the unit of eV, was derived from the slope of the best-fitting model (Fig. 6, Fig. 7).

4.3 Results

4.3.1 Meta-analysis: Temperature sensitivities of N_2O and N_2 production from denitrification

From the meta-analysis, the total production of both N_2O and N_2 from denitrification are sensitive to increasing temperature (Table 6, likelihood ratio test comparing best-fitting model M0 and null model M3: $\boldsymbol{\chi^2}$ = 24.1, $\boldsymbol{p}$ < 0.001), with a similar activation energy at 0.7 eV and 0.69 eV, respectively (Fig. 1). In addition, the temperature sensitivities of N_2O and N_2 production were both consistent between aquatic and terrestrial ecosystems ($\boldsymbol{p}$ > 0.05, Table 6, likelihood ratio test comparing M0 and M2 for N_2O, and M1 and M2 for N_2). These results

show that total N_2O and N_2 production in the denitrification process share a common response to higher temperatures.

The temperature dependency of net N_2O production from denitrification, on the other hand, shows a rather complex pattern. The temperature response of net N_2O production is statistically different between terrestrial and aquatic ecosystems ($p < 0.05$). Net N_2O production significantly increases at higher temperatures in the terrestrial ecosystems, with an activation energy of 0.82 eV (95% confidence intervals of 0.61 to 1 eV, Fig. 2). Whereas it was not sensitive to changes in temperature in aquatic ecosystems from the few studies that characterised net N_2O production (Adouani et al., 2015; Myrstener et al., 2016; Silvennoinen et al., 2008; Wang et al., 2018).

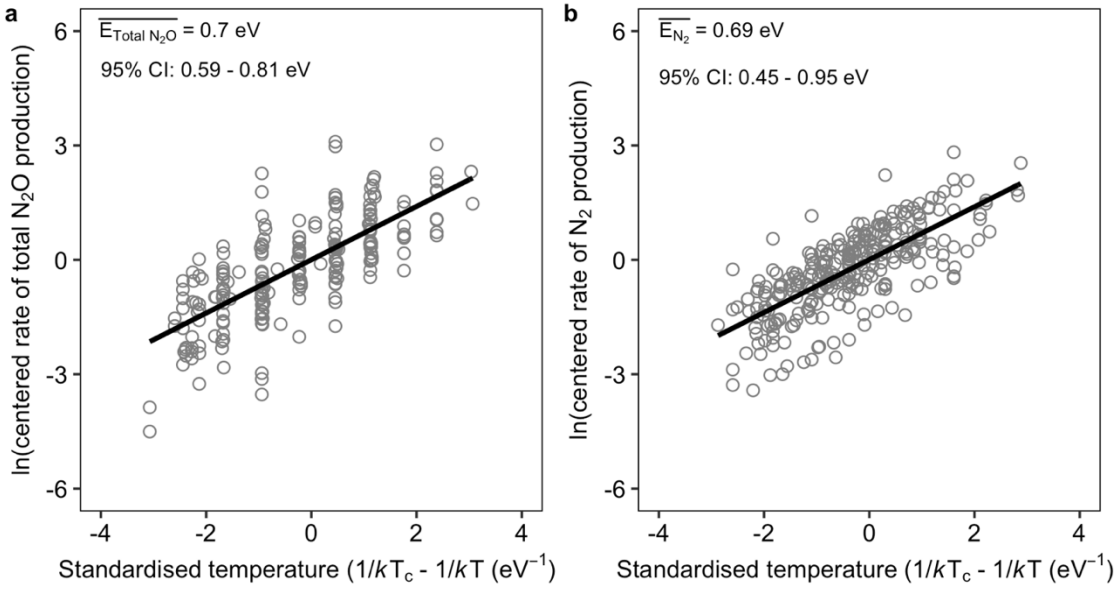

Fig. 1 | Meta-analysis fitting published rates of total N_2O and N_2 production from denitrification as a function of temperature in incubations with biomass from both aquatic and terrestrial communities. The rate of both total N_2O and N_2 production clearly increases at higher temperatures, which is consistent for both aquatic and terrestrial communities. I visualized the data using the "Visreg" package in R (Breheny and Burchett, 2017) showing the best-fitting linear mixed-effect models (black lines, Table 6) and partial

residuals (grey circles). The inverted absolute temperature was centered as Tc = (max+min)/2, while the rate of N_2O or N_2 production was natural log (ln) transformed and then centered by subtracting each study-specific intercept. *n* = 210 and 277 measurements for total N_2O and N_2 productions, respectively.

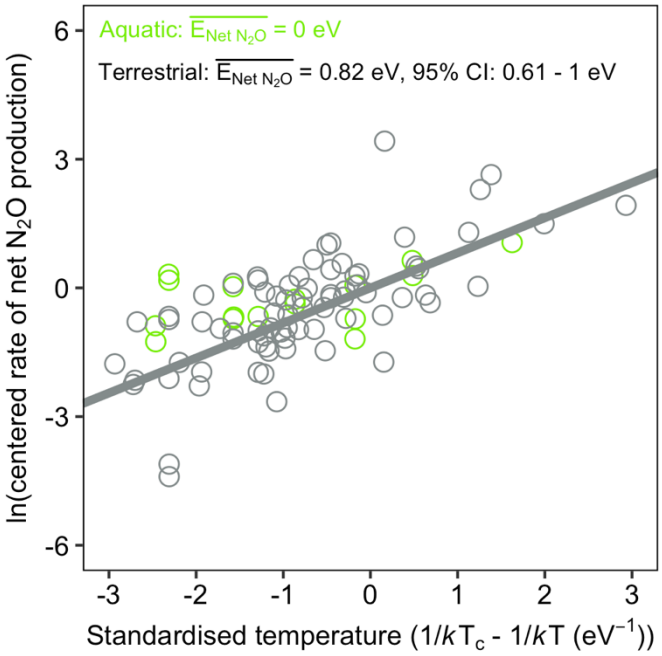

Fig. 2 | Meta-analysis fitting published rates of net N_2O production from denitrification as a function of temperature in incubations with biomass from both aquatic and terrestrial communities. The rate of net N_2O production increases at higher temperatures in terrestrial communities, while it was consistent at different temperatures in aquatic communities. Data were visualised using the "Visreg" package in R showing the best-fitting linear mixed-effect model (grey line) and partial residuals (grey and green circles). The inverted absolute temperature was centered as Tc = (max+min)/2, while the rate of net N_2O production was natural log (ln) transformed and then centered by subtracting each study-specific intercept. *n* = 16 and 76 measurements in aquatic and terrestrial ecosystems, respectively.

Table 6. Meta-analysis for published rates of total N_2O and N_2 from denitrification as a function of incubation temperature for biomass from both terrestrial and aquatic ecosystems (Fig. 1). Linear mixed-effects model selection included centered temperature (Tc) and the interaction between Tc and ecosystem type as fixed effects, with a random intercept (1|Study) and slope (0+Tc|Study) to account for variation across the different studies (Table 1). Here, the best-fitting model (**M0**) showed that the rate of denitrification increased at higher temperatures, and that the temperature sensitivity for denitrification was not different between aquatic and terrestrial ecosystems. Models were ranked by the small sample-size corrected Akaike Information Criterion (AICc) with the better models (in **bold**) having lower AIC values. M3 is the null model which only included an intercept, denoted by 1. lnN_2O and lnN_2 are the natural log (ln) transformed rate of total N_2O and N_2 production, respectively, and Ecosystem is ecosystem type (terrestrial or aquatic). Each model was compared to the best model using the Log-likelihood ratio test (LogLik, d.f. degrees of freedom) showing χ^2 (Chi-squared statistic) and *p* (the corresponding *p*-value).

Model	d.f.	AICc	LogLik	χ^2	*p*
Total N_2O production					
M0: lnN_2O~Tc	4	662.8	-327.3		
M1: lnN_2O~Tc+Ecosystem	5	664.6	-327.2	0.25	0.62
M2: lnN_2O~Tc*Ecosystem	6	666.6	-327.1	0.36	0.83
M3: lnN_2O~1	3	782.7	-388.3	122	**<0.001**
M4: lnN_2O~Ecosystem	4	784.6	-388.2	122	**<0.001**
N_2 production					
M0: lnN_2~Tc+Ecosystem	6	738.3	-363.0		
M1: lnN_2~Tc	5	739.6	-364.7	3.43	0.06
M2: lnN_2~Tc*Ecosystem	7	740.0	-362.8	0.37	0.54
M3: lnN_2~Ecosystem	5	753.4	-371.6	17.2	**<0.001**
M4: lnN_2~1	4	754.6	-373.2	20.5	**<0.001**

4.3.2 Optimal incubation conditions for characterising net production of N_2O and N_2 from denitrification

From the anammox experiments, no excess $^{29}N_2$ was detected in the treatments compared to the controls (Fig. 2). $^{29}N_2$ concentration did not increase when an additional 100 μM of $^{14}NO_3^-$

was added to the incubations with $^{15}NH_4^+$ (p = 0.61, Two-Sample t-test), which confirms that anammox was not present. This agrees with previous studies that show no anammox activity was detected in the sediments of these ponds over three different seasons (Warren, 2017). Therefore, denitrification was treated as the sole N_2 production process in these ponds for the later experiments.

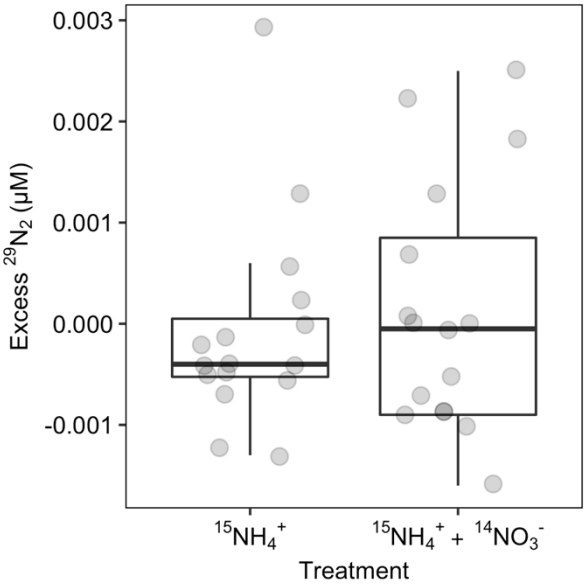

Fig. 2 | Excess $^{29}N_2$ in the treatments compared to controls from the experiments to confirm whether anammox activity is present in the experimental ponds. No additional $^{29}N_2$ was produced from $^{15}NH_4^+$ plus $^{14}NO_3^-$ treatments relative to the $^{15}NH_4^+$ only treatments, which indicated that anammox was not present in the ponds and that denitrification could be treated as the only N_2 producing process.

Before characterising the temperature sensitivities of N_2O and N_2 production from denitrification, I did the trial experiments to optimise the conditions by tracing the production of N_2 and N_2O over time with different concentrations of $^{15}NO_3^-$.

The production of ^{15}N-N_2O reached a peak, followed by its reduction during all incubations (Fig. 3**a**). With $^{15}NO_3^-$ less than 100 μM, ^{15}N-N_2O quickly peaked after ~0.5 h from

the start of the incubation, then decreased to 0 before 24 h in most incubations. Whereas, with 100 µM of $^{15}NO_3^-$ added, ^{15}N-N_2O peaked later at approximately 3 h, with higher production compared to that in the incubations with less $^{15}NO_3^-$ addition. In addition, ^{15}N-N_2 accumulated continuously during 24 h in most incubations with $^{15}NO_3^-$ additions higher than 10 µM, while it reached a plateau at ~12 h with 10 µM of $^{15}NO_3^-$ (Fig. 3**b**).

Further, 43% of $^{15}NO_3^-$ was reduced to $^{15}N_2$, on average, after 24 h of incubation (Fig. 4), which was consistent between different $^{15}NO_3^-$ concentrations (p = 0.48, df = 13, Kruskal-Wallis test). This indicates that the proportion of complete denitrification i.e., the percentage of the NO_3^- reduced to the end-product N_2, was not affected by the availability of NO_3^- over a concentration range of 10 to 100 µM.

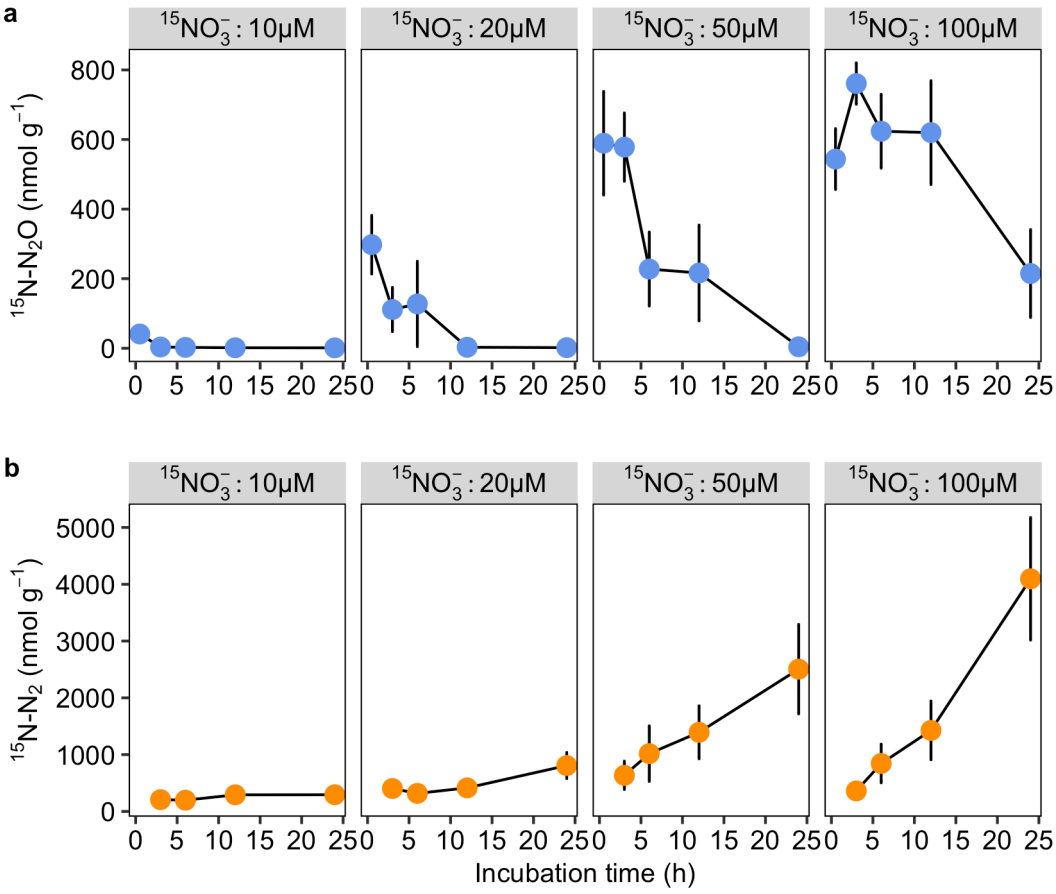

Fig. 3 | The accumulation of $^{15}N_2O$ and $^{15}N_2$ over time from independent sediment incubations amended with different concentrations of $^{15}NO_3^-$. a, $^{15}N_2O$ production quickly reached a peak and started to decline in incubations with $^{15}NO_3^-$ additions less than 100 μM, while it reached the peak later at ~3 hours with 100 μM of $^{15}NO_3^-$ added. **b**, $^{15}N_2$ continued to accumulate over the 24-hour incubation with $^{15}NO_3^-$ additions higher than 10 μM, while it reached the plateau at ~12 hours with 10 μM of $^{15}NO_3^-$ added. The dots in each plot are the means, with vertical bars showing the standard error (n = 6 ponds for each concentration of $^{15}NO_3^-$ at each time point).

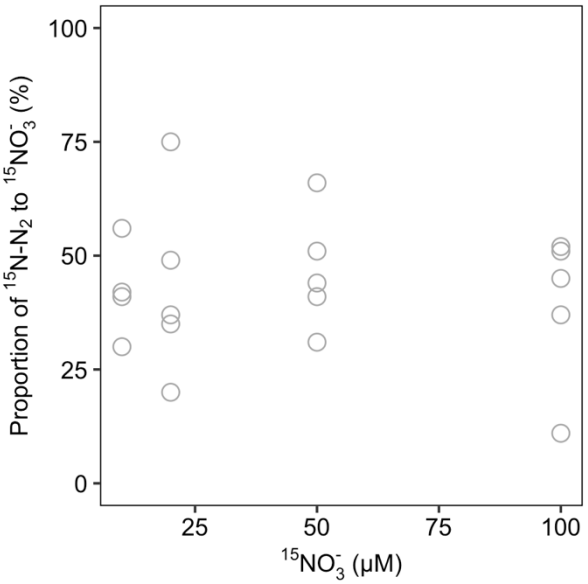

Fig. 4 | After 24 hours of incubation, the proportion of ^{15}N-N_2 production to ^{15}N-NO_3^- addition was consistent between different concentrations of $^{15}NO_3^-$.

As anammox was not present and denitrification was the only pathway for the dissimilatory reduction of NO_3^- to N_2O and N_2, the isotopic ratio of NO_3^- being denitrified (F_N) was calculated from that of N_2O production by $F_N = \frac{2 \times P^{46}N_2O}{P^{45}N_2O + 2 \times P^{46}N_2O}$, where $p^{45}N_2O$ and $p^{46}N_2O$ are the productions of $^{45}N_2O$ and $^{46}N_2O$ (Trimmer et al., 2006). If $^{15}NO_3^-$ is the sole

source of NO_x^- in the incubation, F_N should be constant across different doses of $^{15}NO_3^-$. However, F_N increased with the concentration of $^{15}NO_3^-$ added (Fig. 5), which showed by the lower ^{15}N-labelling of NO_3^- when less $^{15}NO_3^-$ was added. This indicates the presence of background $^{14}NO_x^-$ (NO_2^- plus NO_3^-) in the incubations that should have been constant in different treatments, which dilutes the ^{15}N-labelling of the NO_x^- pool with a stronger effect at lower $^{15}NO_3^-$ concentrations.

With less $^{15}NO_3^-$ added, the proportion of background ^{14}N-NO_x^- in the NO_x^- pool (^{14}N + ^{15}N) is higher, resulting in a higher chance of making $^{45}N_2O$ and $^{29}N_2$ (one N from $^{14}NO_x^-$ and one N from $^{15}NO_x^-$) rather than $^{46}N_2O$ and $^{30}N_2$ (both N from $^{15}NO_x^-$) during denitrification. This influence of background $^{14}NO_3^-$ on the nitrate pool being reduced would decrease with more $^{15}NO_3^-$ added and the isotopic ratios of the gas products would get closer to that expected for the ^{15}N-labelling of the added $^{15}NO_3^-$. As the artificial pond-water medium is N-free, the $^{14}NO_3^-$ was most likely introduced by the addition of the biomass despite that biomass being pre-incubated for 16h to remove residual nitrate. Thus, to eliminate the interference of background NO_x^-, a pre-incubation time longer than 16h would be needed for the next experiment.

Based on these results, to characterise the temperature sensitivities on the production of both N_2O and N_2, sediments should be incubated with ~100 μM of $^{15}NO_3^-$ for approximately 3 h. As the concentration of NO_3^- in the ponds was very low (typically < 1 μM, Table 7), additional samples should be treated with a low concentration of $^{15}NO_3^-$ to mimic the effect of low substrate availability on the gas productions (~10 μM, as the gas production might not be measurable below this concentration, Fig. 3).

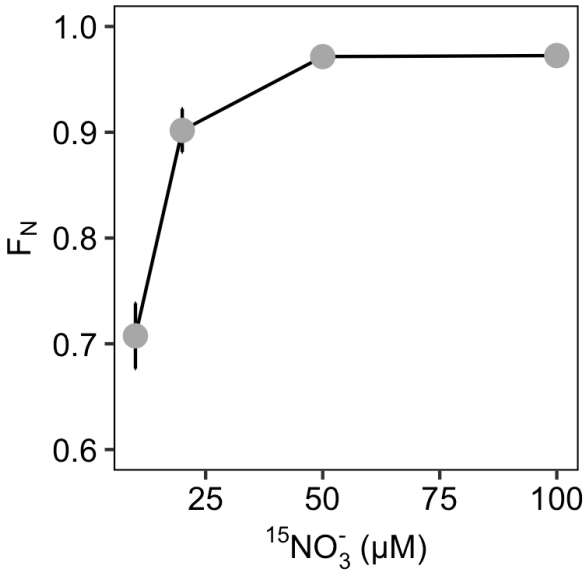

Fig. 5 | The ^{15}N-labelling of the NO_3^- pool being denitrified (F_N, calculated using the ^{15}N-labelling of N_2O produced in the incubations) in incubations with different concentrations of $^{15}NO_3^-$ added. F_N was lower in experiments with less $^{15}NO_3^-$ added, indicating the interference from background $^{14}NO_x^-$ that has a stronger effect on the ^{15}N-labelling at lower $^{15}NO_3^-$ concentrations. The dots are the means, with vertical bars showing the standard error (*n* = 6 ponds for each concentration of $^{15}NO_3^-$).

4.3.3 Temperature sensitivities of N_2O and N_2 net production from denitrification

After the 3-hour incubation, net production of both $^{15}N_2O$ and $^{15}N_2$ was detectable in the large majority of incubations (97.5%, 156 out of 160 incubations) with either 10 μM or 100 μM of $^{15}NO_3^-$ added. The proportion of $^{15}NO_3^-$ reduced to gaseous products (N_2O + N_2) was 34.3% and 11%, on average, with 10 μM and 100 μM of $^{15}NO_3^-$, respectively. In addition, the product ratio of $^{15}N_2O$ and $^{15}N_2$ was 0.01 and 3.63, on average, with 10 μM and 100 μM of $^{15}NO_3^-$, respectively. The relatively high denitrification proportion and exceedingly low product ratio of $^{15}N_2O$ and $^{15}N_2$, with 10 μM of $^{15}NO_3^-$, indicated that almost all of the $^{15}N_2O$ produced was

quickly reduced to $^{15}N_2$. In the meantime, with the higher availability of $^{15}NO_3^-$, at the end of the 3-hour incubation, $^{15}N_2O$ was still being produced and most of the produced $^{15}N_2O$ had not been reduced to $^{15}N_2$ yet.

Table 7. Concentrations of porewater nutrients (μM) in biomass incubations characterising the temperature sensitivities of N_2O and N_2 production from denitrification. s.e.: standard error (n = 8 ponds for each nutrient species).

Pond	NO_2^-	NO_3^-	NOx^-	NH_4^+	PO_4^{3-}
3	0.37	1.19	1.56	5.11	0.24
4	0.16	0.53	0.69	41.16	0.24
5	0.15	0.52	0.67	1.03	0.15
6	0.12	0.26	0.38	3.61	0.22
9	0.08	0.25	0.34	31.84	0.27
10	0.10	0.30	0.40	25.72	0.20
11	0.08	0.19	0.27	0.99	0.19
12	0.21	0.36	0.57	0.36	0.21
Mean	0.16	0.45	0.61	13.73	0.22
s.e.	0.03	0.11	0.15	5.83	0.01

The temperature sensitivities of the net production of both N_2O and N_2 from denitrification were not statistically different between the ambient and warmed ponds (p > 0.05). Therefore, here I focused on the effect of temperature on the production of N_2O and N_2 at different concentrations of NO_3^-.

With 10 μM $^{15}NO_3^-$ present, the net production rate of both $^{15}N_2O$ and $^{15}N_2$ was consistent between different temperatures (Fig. 6**a**, 6**c**, **M10.a** compared to M10.b for both N_2O and N_2, Table 8). With higher availability of $^{15}NO_3^-$ i.e., 100 μM, net production of both $^{15}N_2O$ and $^{15}N_2$ was sensitive to increasing temperature (p < 0.01, **M100.a** compared to M100.b for both N_2O and N_2, Table 8) with opposite temperature sensitivities (-0.27 eV and 0.51 eV for N_2O and N_2, respectively, Fig. 6**b**, 6**d**). The accumulation of $^{15}N_2$ started to curve at around 20ºC,

showing that the optimal temperature for complete denitrification is around 20ºC. At the temperature range of 5ºC to 20ºC, $^{15}N_2O$ decreased, while $^{15}N_2$ accumulated at higher temperatures, showing the reduction of N_2O to N_2 from denitrification.

Furthermore, with 100 µM of $^{15}NO_3^-$, the log-transformed ratio of $^{15}N_2O$ to $^{15}N_2$ decreased linearly over a temperature range of 5ºC to 20ºC ($p < 0.001$, **M100.a** compared to M100.b for the ratio, Table 8), with a highly negative activation energy of -0.76 eV (95% CI: -1.03 to -0.49 eV, Fig. 7**b**). This leads to a higher accumulation of N_2O relative to N_2 from denitrification in the colder temperatures.

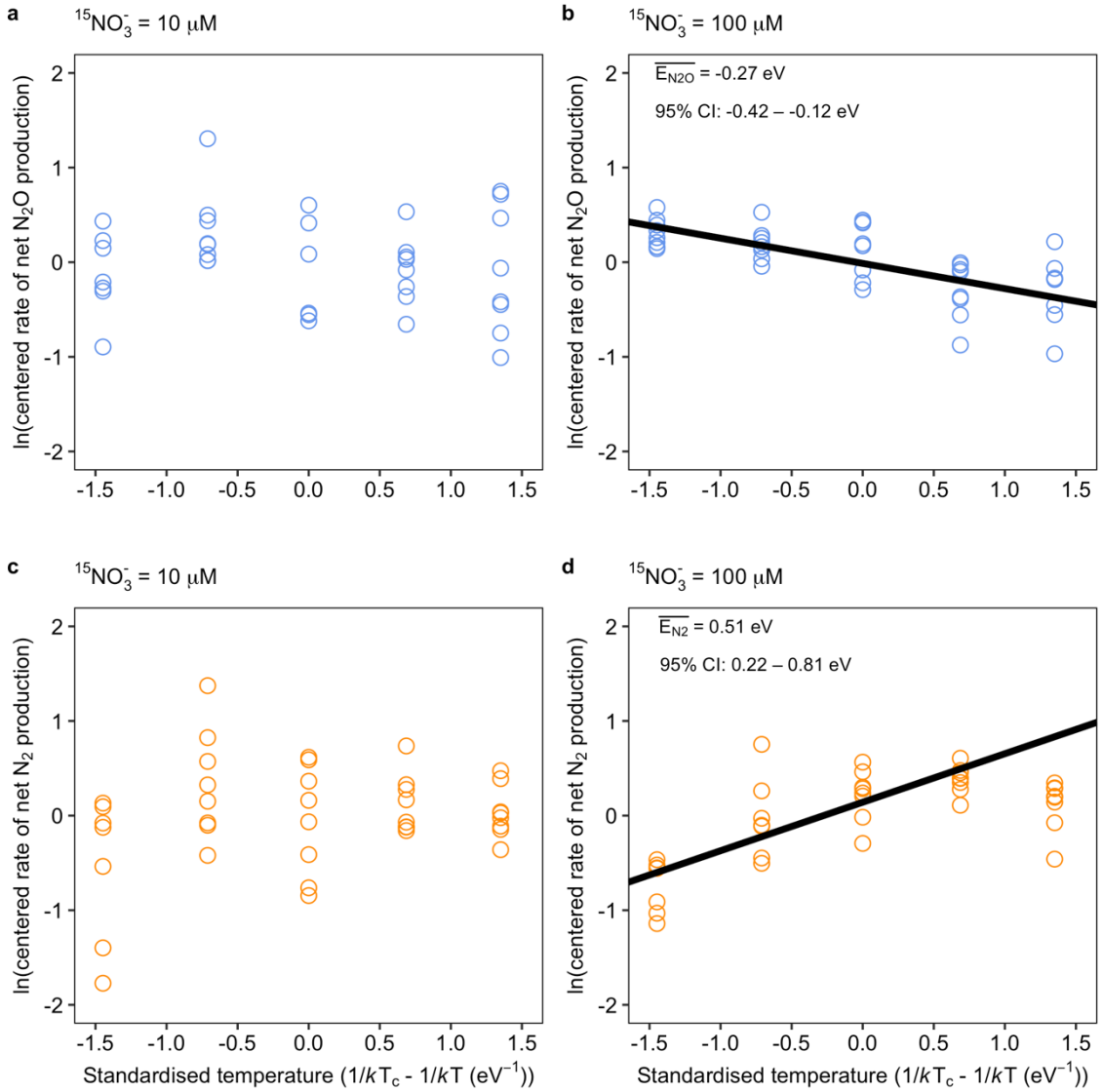

Fig. 6 | Temperature sensitivities of the net production rate (nmol g^{-1}) of N_2O and N_2 from denitrification. a, Net production rate of $^{15}N_2O$ was consistent between different temperatures with 10 μM $^{15}NO_3^-$ added. **b**, $^{15}N_2O$ decreased at higher temperatures with 100 μM of $^{15}NO_3^-$. **c**, Net production of $^{15}N_2$ was consistent between different temperatures with 10 μM $^{15}NO_3^-$ added. **d**, $^{15}N_2$ accumulated at higher temperatures with 100 μM of $^{15}NO_3^-$. The temperature was centered at the median temperature of all the data points, i.e. 15°C, while the net production rates of N_2O and N_2 were natural log (ln) transformed and then centered by subtracting the pond-specific intercepts. I visualized the data using the "Visreg" package in R (Breheny and Burchett, 2017), with the lines in **b** and **d** showing the best-fitting linear mixed-effect model (Table 8). The models were fitted with a subset of the dataset from 5ºC to 20ºC, as the net production of both N_2O and N_2 started to curve around 25ºC. The data shown are with the full range of incubation temperatures from 5ºC to 25ºC. n = 40 incubations from 8 ponds for each $^{15}NO_3^-$ treatment.

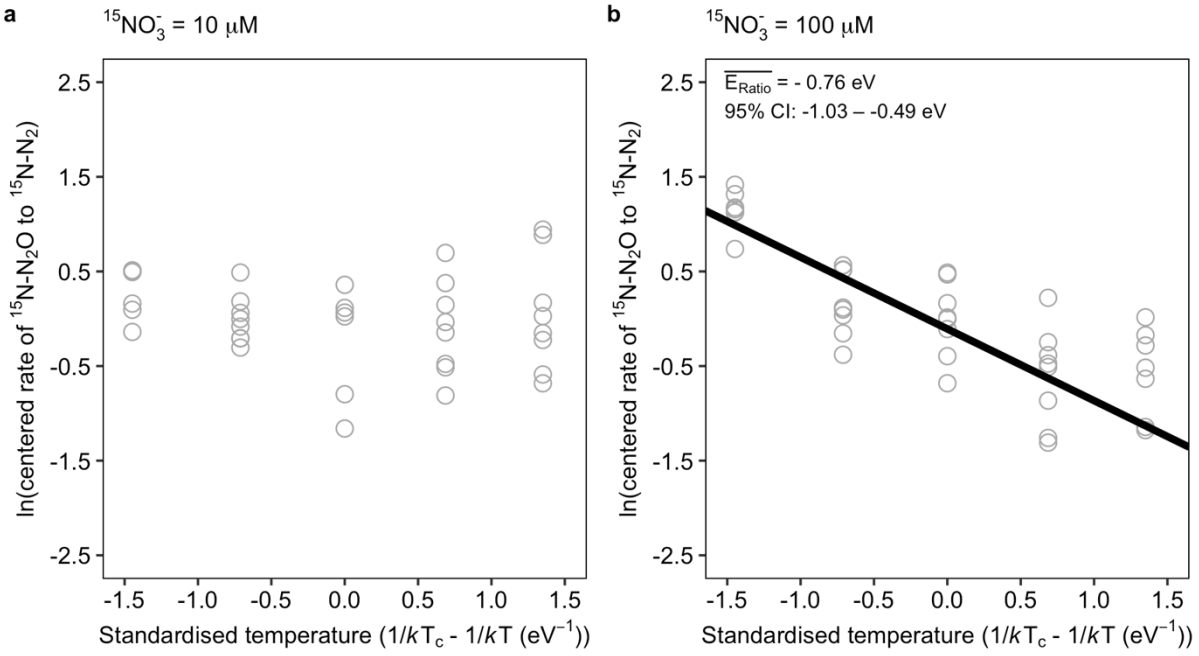

Fig. 7 | Temperature sensitivity of the ratio of $^{15}N_2O$ and $^{15}N_2$ net production from denitrification. a, Ratio of net production rate of N_2O and N_2 was consistent between different

temperatures with 10 μM $^{15}NO_3^-$ added. **b**, Ratio of net production rate of N_2O and N_2 decreased at higher temperatures with 100 μM of $^{15}NO_3^-$. The temperature was centered at the median temperature of all the data points, i.e. 15°C, while the ratios of N_2O and N_2 net production were natural log (ln) transformed and then centered by subtracting the pond-specific intercepts. I visualized the data using the "Visreg" package in R (Breheny and Burchett, 2017), with the solid line in **b** showing the best-fitting linear mixed-effect model (Table 8). The lines were fitted with a subset of the dataset at temperatures lower than 25ºC, as the ratio started to curve around 25ºC. n = 40 incubations from 8 ponds for each $^{15}NO_3^-$ treatment.

Table 8. Net production rate of N_2O and N_2 (Fig. 6) and their ratio (Fig. 7) as a function of incubation temperature at 10 μM or 100 μM of NO_3^- with biomass from the experimental ponds. Linear mixed-effects model selection included centered temperature (Tc) as fixed effects, with a random intercept and slope (1+Tc|Pond) to account for variation across the different ponds. Separate models were fitted for 10 μM and 100 μM of NO_3^-, which were denoted as M10 and M100, respectively. Models were ranked by the small sample-size corrected Akaike Information Criterion (AICc) with the better models (in **bold**) having lower AIC values. Null models only has an intercept, denoted by 1. $lnPN_2O$ and $lnPN_2$ is the natural log (ln) transformed rate of net production of N_2O and N_2, respectively, while lnRatio is the ratio of net production of N_2O and N_2. Each model was compared to the best model (in **bold**) using the Log-likelihood ratio test (LogLik, d.f. degrees of freedom) showing χ^2 (Chi-squared statistic) and p (the corresponding p-value).

Model	**d.f.**	**AICc**	**LogLik**	χ^2	p
N_2O net production					
M10.a: $lnPN_2O$~1	**4**	**103.1**	**-46.9**		
M10.b: $lnPN_2O$~Tc	5	105.2	-46.6	0.62	0.43
M100.a: $lnPN_2O$~Tc	**5**	**59.9**	**-23.8**		
M100.b: $lnPN_2O$~1	4	65.8	-28.1	8.74	<0.01
N_2 production					
M10.a: $lnPN_2O$~1	**4**	**90.5**	**-40.7**		
M10.b: $lnPN_2O$~Tc	5	90.6	-39.4	2.57	0.11

M100.a: lnPN$_2$O~Tc	**5**	**78.1**	**-32.8**		
M100.b: lnPN$_2$O~1	4	83.7	-37.0	8.41	<0.01
Ratio of N$_2$O to N$_2$					
M10.a: lnRatio~1	**4**	**101.6**	**-46.2**		
M10.b: lnRatio~Tc	5	102.3	-45.2	1.97	0.16
M100.a: lnRatio~Tc	**6**	**87.9**	**-36.2**		
M100.b: lnRatio~1	5	99.5	-43.5	14.6	<0.001

4.3.4 Net production of N_2O from nitrification

At the beginning of the incubation, the concentrations of NO_2^- and NO_3^- were 0.18 μM and 0.76 μM, respectively (Table 9). On average, 44.2 ± 1.5 μM (mean ± s.e., as below) of $^{15}NH_4^+$ and half of that were added to the high and low substrate treatments, respectively. With 44.2 μM of $^{15}NH_4^+$ added, the ^{15}N-labelling of NH_4^+ (F_A) in the treatment was 0.85 ± 0.07, on average (Table 9).

Total NH_4^+ decreased after 24 h of incubation ($p < 0.05$, Two Sample *t*-test), with an average reduction of 42.8 ± 2.6 μM, except for one pond with a very high concentration of porewater NH_4^+ (66.31 μM, Pond 9) where the reduction of NH_4^+ was only 4.8 μM. On the other hand, the concentration of NO_x^- did not increase significantly after the incubation (p = 0.57), indicating that processes other than nitrification could be present during the incubation, such as the assimilation of NH_4^+ into the biomass, and/or denitrification which could have consumed any NO_x^- that was produced from nitrification.

Table 9. Concentration of porewater nutrients and the ^{15}N-labelling of the NH_4^+ pool (F_A) with 44 μM of $^{15}NH_4^+$ were added at the start of the nitrification experiments. NA: not applicable. Mean: mean value of each category from all ponds. s.e.: standard error.

Pond	NO_2^-	NO_3^-	NH_4^+	PO_4^{3-}	F_A
2	0.31	0.77	3.78	0.57	0.89

5	NA	NA	NA	0.44	0.98
7	0.20	0.2	1.84	0.88	0.95
8	0.06	0.18	0.98	0.61	0.96
9	0.18	0.16	66.3	1.39	0.41
11	0.43	0.7	3.71	0.60	0.90
12	0.60	1.78	10.9	0.84	0.78
17	0.18	0.26	1.35	0.82	0.95
Mean	0.28	0.58	12.7	0.77	0.85
s.e.	0.07	0.22	9.03	0.10	0.07

Net production of N_2O was only detected from one out of eight ponds. For the one pond that showed significant N_2O production, i.e., pond 8, the maximum net production of N_2O occurred in the first 3 to 6 hours with $^{15}NH_4^+$ added (Fig. 8). The peak concentration in total N_2O was then followed by the reduction of N_2O, which indicates that denitrification and/or N_2O fixation was also present. However, the reduction of N_2O to N_2 through denitrification typically occurred in anoxic conditions and the incubations here are oxic, whereas the high availability of fixed N from the addition of $^{15}NH_4^+$ probably should have inhibited N_2O fixation.

Similarly, the production of ^{15}N-N_2O was not measurable i.e. no excess production of $^{45}N_2O$ and $^{46}N_2O$ was detected in the incubations, except for biomass from one pond (Pond 8, *see* Fig. 9). Pond 8 has the lowest concentration of porewater NO_2^-, NO_3^- and NH_4^+ (Table 9), which indicates that the production of N_2O could have been inhibited by the high porewater DIN from other ponds. Further, although detectable with biomass from pond 8, the net production of N_2O is still much lower than that from the denitrification experiments (median, 0.46 nmol compared to 37.22 nmol per vial at 15ºC), indicating that denitrification is the dominant process for N_2O production in the ponds. Therefore, due to undetectable net production of $^{15}N_2O$ in the majority of incubations, no further experiment was performed for characterising the temperature sensitivity of N_2O production from nitrification.

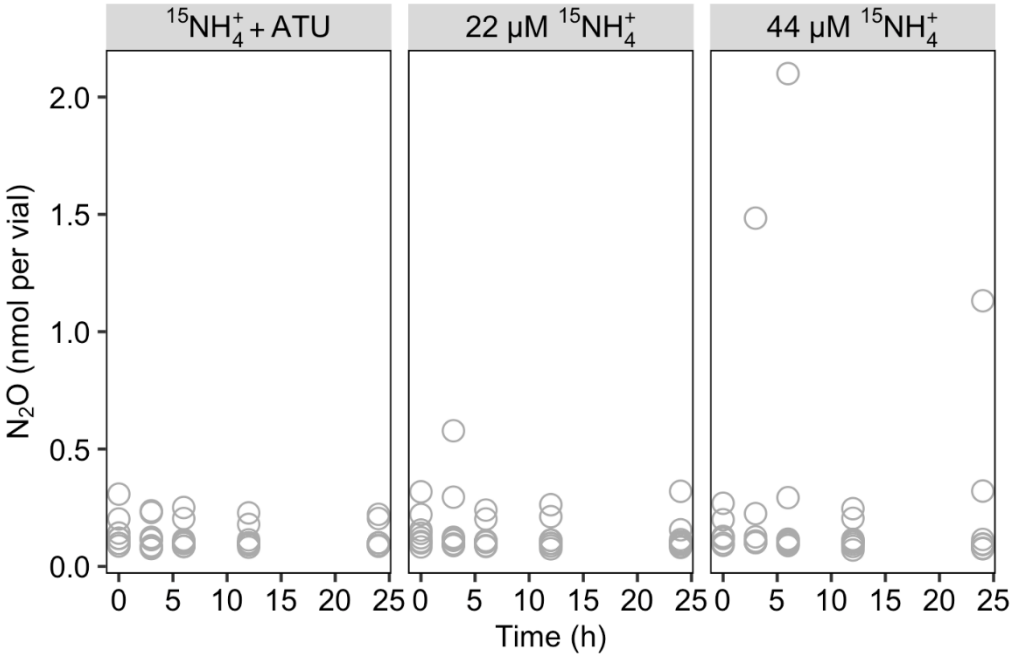

Fig. 8 | N_2O over time from the nitrification experiments with sediment collected from the ponds. Overall, total N_2O production was only detected in one (pond 8) out of eight ponds with the additional $^{15}NH_4^+$ (the points showing $N_2O > 0.5$ nmol per vial). For the sediment collected from pond 8, N_2O was produced rapidly, reaching a peak in the first 3h or 6 h with 22 or 44 µM of $^{15}NH_4^+$, respectively, while it was consistent over time when treated with ATU to block the first step in nitrification.

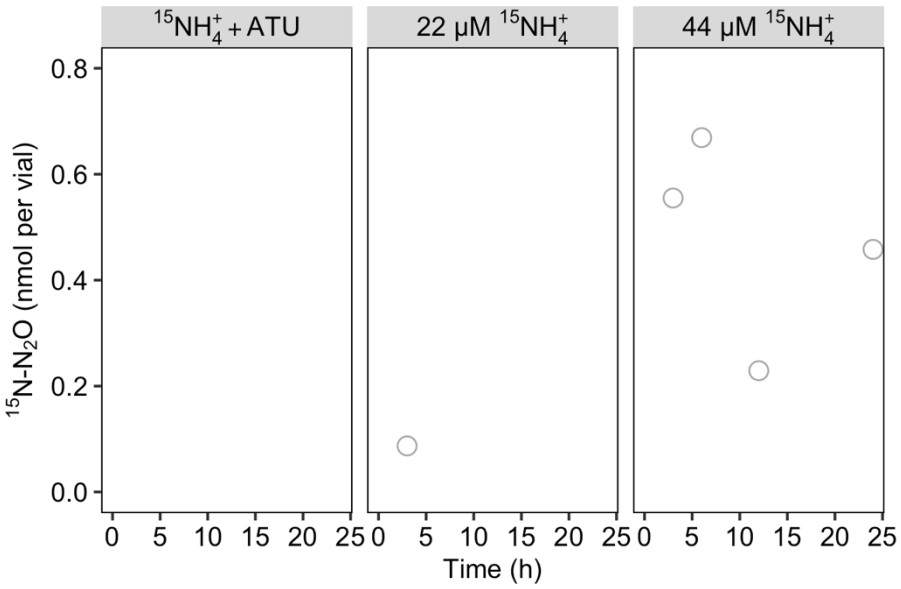

Fig. 9 | Net production of ^{15}N-N_2O from the nitrification experiments with sediment collected from Pond 8. Net production of ^{15}N-N_2O from pond 8 was detected with 44 μM of $^{15}NH_4^+$, while it was only detected at 3 h with 22 μM of $^{15}NH_4^+$ and remained undetected with ATU inhibition. Net production of ^{15}N-N_2O was not detected in any treatments with sediments from the other 7 ponds.

4.4 Discussions

Denitrification activity was present in the ponds and contributed to both the production and reduction of N_2O (Fig. 6), whereas the net production of N_2O from nitrification was only detected in one out of eight ponds studied (Fig. 9). Therefore, denitrification seems to be the primary potential process producing N_2O in the ponds. The organic matter content, e.g., organic nitrogen and carbon in the sediments of the experimental ponds are similar to natural lakes (Yvon-Durocher et al., 2017), where the decomposition of organic carbon may act as the source of electrons for the denitrifiers to reduce NO_3^-.

Moreover, while net production and reduction of N_2O were both detected across a temperature range of 5ºC to 25ºC with either 10 or 100 μM of $^{15}NO_3^-$, significant temperature effects only occurred with 100 μM of $^{15}NO_3^-$ (Fig. 6). This shows that the availability of NO_3^- affects the temperature dependency of N_2O and N_2 production. When substrate is limited, net production and reduction of N_2O did not respond to changes in temperature. The few studies that have characterised the effect of substrate on the temperature sensitivity of N_2O or N_2 production either focused on the effect of long-term adaption to NO_3^-, e.g., 3 weeks or 3 months (Braker et al., 2010; Nowicki, 1994), or did not separate the effect of NO_3^- and other stressor (Bonnett et al., 2013). The temperature sensitivity and rate of N_2 production were both much higher in the N-enriched mesocosms with estuarine sediments compared to the controls, with an activation energy of 1.1 eV compared to 0.4 eV (Nowicki, 1994). Another study suggested

that the strong temperature dependency of total N_2O production in agricultural soils could be linked to higher abundance of nitrate reducers after a 3-week adaption to nonlimiting NO_3^- conditions (Braker et al., 2010). Moreover, total denitrification (N_2O+N_2) and net N_2O production both increased at higher temperatures when the soils were enriched with water and NO_3^-, whereas they were consistent over different incubation temperatures with the unamended soils (Bonnett et al., 2013). However, this study did not separate the effect of water content and NO_3^-, making it difficult to evaluate the effect caused by NO_3^- alone.

N_2 production increased linearly from 5ºC to 20ºC, then started to decline, indicating that the optimal temperature for complete denitrification is around 20ºC (Fig. 6**d**). The effect of temperature on denitrification is often bell-shaped, with an optimal temperature, e.g., at approximately 20 to 35ºC (Benoit et al., 2015; Braker et al., 2010; Brin et al., 2017; Wang et al., 2018), above which the enzyme activity starts to decline. On the contrary, net N_2O production decreased from 5ºC to 20ºC, showing a lower accumulation of N_2O at higher temperatures. As a result, the ratio of net N_2O production to N_2 production was higher at lower temperatures, indicating a higher availability of N_2O relative to N_2 in the cold.

This opposite temperature dependency of net production of N_2O and N_2 has also been reported in river sediments, where N_2 production increased and N_2O production decreased under O_2-limited conditions (O_2 < 6.25 μM) (Silvennoinen et al., 2008). In addition, N_2O production was also higher under 10ºC than at 20ºC with nitrate-metabolising bacteria isolated from estuary sediments (Ogilvie et al., 1997). Similar trends were also found in soils - with N_2 production increasing while net N_2O production decreased from 10ºC to 30ºC (Bailey, 1976). These results indicate that while increasing temperatures would enhance the rates of complete denitrification, it does not appear to increase the net production of N_2O from denitrification.

From my results, N_2 production has a higher temperature sensitivity than net N_2O production in freshwater communities (Fig. 6), which leads to a lower production ratio of N_2O

to N_2 at higher temperatures. Others have also reported the negative effect of temperature on the ratio of N_2O to N_2 in soils (Avalakki et al., 1995; Bailey, 1976; Bailey and Beauchamp, 1973; Keeney et al., 1979; Maag and Vinther, 1996; Melin and Nômmik, 1983) and river sediments (Silvennoinen et al., 2008). Moreover, as predicted from a biogeochemical model, the ratio of N_2O to N_2 from denitrification will decrease with warming, leading to a 6% decrease in N_2O emission with a 1.8°C increase from forest soils across Europe (Kesik et al., 2006).

The increase in the ratio of N_2O to N_2 at lower temperatures could be caused by a higher temperature sensitivity of N_2O reductase relative to NO reductase, or an attenuated activity of N_2O reductase at lower temperatures (Melin and Nômmik, 1983; Wertz et al., 2013). For example, the abundance of the gene for NO reduction, *norB*, was lower at 10ºC than 4ºC in incubations with Arctic soils, whereas the abundance of other functional genes for denitrification, such as *nirK* and *nirS* (nitrite reduction), as well as *nosZ* (N_2O reduction) increased with warming (Jung et al., 2011). Some studies showed that the higher N_2O emissions at lower temperatures were mainly due to an inhibited activity of N_2O reductase at very low temperatures (e.g., ~0ºC), rather than different temperature sensitivities of N_2O production and N_2O reduction (Holtan-Hartwig et al., 2002; Öquist et al., 2007). In addition, others suggest that higher temperatures might enhance the O_2-limited condition in soils due to a stronger promotion of respiration relative to photosynthesis (Smith, 1997; Veraart et al., 2011), which could facilitate the reduction of N_2O to N_2.

Similar to the consistent temperature response of N_2O fixation between the ambient and warmed ponds (Chapter 2), I also did not find any statistical difference in the temperature sensitivities of the net production of both N_2O and N_2 between the ambient and warmed ponds ($p > 0.05$, **M0** compared to M2 for both N_2O and N_2, Table 8), even at higher $^{15}NO_3^-$ concentration. As the effect of short-term incubation temperature on denitrification is evident,

this means that the denitrifiers might have already acclimatised to the long-term 4ºC of warming (September 2006 to September 2021), or the long-term warming effect was masked by the short-term temperature manipulation. In any case, neither the temperature sensitivity (slope of the model) nor the capacity (intercept of the model at Tc) of denitrification differs between the ambient and warmed ponds, therefore it cannot be concluded that the long-term 4ºC of warming has induced any effects on the net production of N_2O or N_2 from denitrification.

Furthermore, apart from being emitted either directly or as N_2 from the waters, N_2O produced from denitrification could also be recycled and then used for N_2O fixation (Fig. 10). The negative relationship between the ratio of N_2O to N_2 and temperature from denitrification provide a higher relative availability of N_2O to N_2 at lower temperatures, which could enhance the proportion of N_2O fixation relative to N_2 fixation in the cold that would help to conserve N in N-limited ecosystems (Fig. 8, Chapter 2).

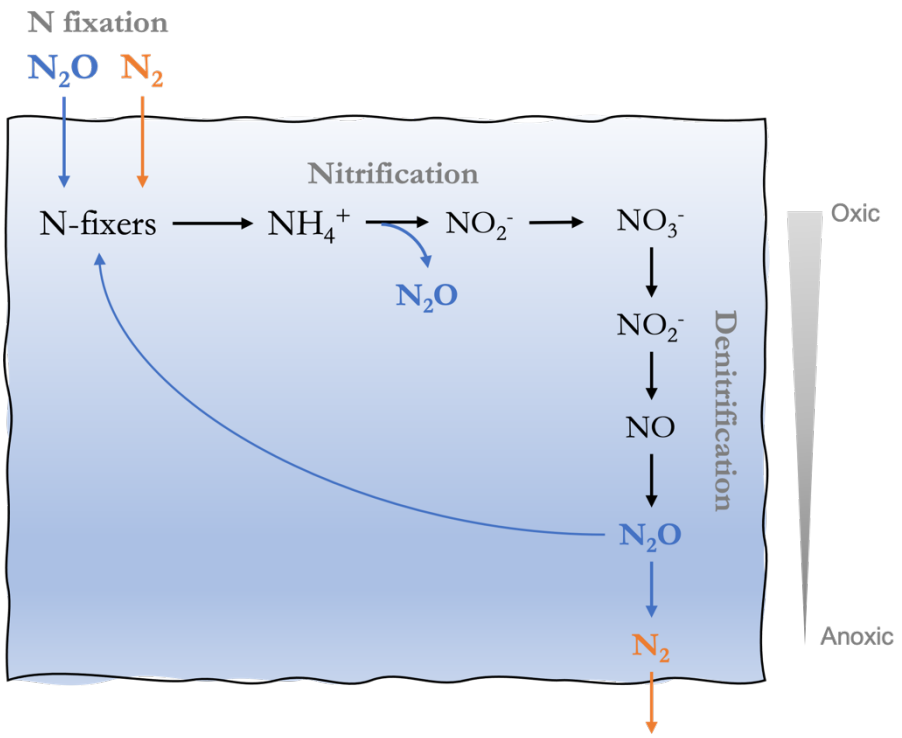

Fig. 10 | In cold, N-limited ecosystems, N_2O produced from denitrification could be recycled and then utilised for N_2O fixation. As net N_2O production and the product ratio of

N_2O to N_2 from denitrification are both higher at colder temperatures (*see* section 4.3.4), the availability of N_2O relative to N_2 should also be higher in the cold. As a result, a higher proportion of N fixation could come from N_2O fixation in the cold to support the N-limited ecosystem. This agrees with my findings from the N_2O fixation experiment - the ratio of N_2O fixation to N_2 fixation was also higher in the cold (Fig. 8, Chapter 3). These results indicate that in cold, N-limited ecosystems, N could be conserved through the intermediate production of N_2O.

4.5 Conclusions

In this chapter, I characterised the temperature dependencies of both N_2O production and N_2O reduction from freshwater ponds. This study would provide insights on how changes in temperature would affect N_2O emission from natural waters under the current global warming scenario. While increasing temperatures would enhance the rates of complete denitrification by reducing fixed-N into the gaseous product N_2, it might not increase the accumulation of the atmospherically potent gas N_2O. More importantly, warming might only affect the production of N_2O and N_2 when the substrate for denitrification is not limiting. In N-limited ecosystems, substrate availability might be more important in regulating the balance of N_2O to N_2 in the denitrification process than temperature. Furthermore, by considering the temperature effect on different N_2O production and reduction processes such as denitrification and N_2O fixation, I show that the two processes are potentially linked and could form a recycling loop for N_2O within the ecosystem.

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Chapter 5: Summary and suggestions for future work

5.1 Summary

5.1.1 Why do we need to explore N_2O fixation in natural waters?

Nitrous oxide (N_2O) is a potent climate gas, with ~265 times global warming potential of carbon dioxide (CO_2) (Stocker, 2014) and strong ozone-depleting properties (Ravishankara et al., 2009). The atmospheric concentration of N_2O has already risen by ~20% since 1750 and is expected to continue to increase in the following years (Meinshausen et al., 2011). Therefore, most research have been focused on N_2O sources, while studies on N_2O sinks are relatively scarce.

Oxygen is a key factor regulating the production and reduction of N_2O. For example, the reduction of N_2O was routinely attributed to its reduction to N_2 in the last step of microbial denitrification ($N_2O \rightarrow N_2$), which occurs under oxygen-limited or depeleted conditions (Codispoti, 2010; Naqvi et al., 2010). However, many accounts of undersaturation in N_2O have been found in oxic waters, e.g., the surface ocean and shallow freshwaters (Table 1, Chapter 1), which mostly remain unaccounted for.

There are some evidences for an alternative N_2O reduction pathway in oxic conditions, i.e. N_2O fixation, which could explain some of the unaccounted for N_2O sinks found in natural waters. Apart from early studies that showed $^{15}N_2O$ could be assimilated by soybean root nodules (Mozen and Burris, 1954), N_2O fixation has also been reported in pure cultures of the marine isolates *Trichodesmium* sp. and *Crocosphaera* sp. and surface marine waters (Cornejo et al., 2015; Farías et al., 2013). However, within the widespread accounts of N_2O undersaturation found in oxic waters, only a few studies mentioned the possibility of N_2O fixation (Cornejo et al., 2015; Farías et al., 2013; Verdugo et al., 2016) and it is not widely acknowledged.

Further, the ecological benefit of N_2O-, compared to canonical N_2-fixation is unknown. N_2 fixation, regulated by the enzymatic nitrogenase complex, is known to increase at higher temperatures with a high activation energy of approximately 1 eV (Fig. 2, Chapter 1), which makes fixing N_2 in the cold energetically costly. On the other hand, N_2O could be a competitive inhibitor for N_2 fixation and could also be used by nitrogenase (Jensen and Burris, 1986; Repaske and Wilson, 1952; Rivera-Ortiz and Burris, 1975; Wilson and Roberts, 1954), indicating that N_2O fixation (e.g. $N_2O \rightarrow NH_4^+$) could be related to N_2 fixation (e.g. $N_2 \rightarrow NH_4^+$). If N_2O is also fixed by a nitrogenase using ferredoxin as the electron carrier, fixing N_2O would be more energetically feasible than fixing N_2 (Eq. 2 and Eq. 3, Chapter 1). Further, as the dissociation energy of the N bond in N_2O is only half of that for N_2 (Shestakov and Shilov, 2001), being able to fix N_2O could confer an ecological advantage to microbes in the cold compared to fixing N_2.

The thermodynamic advantage of N_2O fixation compared to N_2 fixation leads us to wonder whether the temperature response of the two processes would be different. From my meta-analysis using published data from the literature, I show that temperature is also an important regulating factor on either sinks or sources of N_2O in freshwaters, with overall no significant effect found in marine waters. Many cases of N_2O undersaturation were found near either 5°C or 20°C, which could be related to different processes reducing N_2O at low and high temperatures. For example, as the reduction of N_2O to N_2 generally increases at higher temperatures, denitrification might have caused the N_2O undersaturation found at higher temperatures, whereas N_2O sinks found at lower temperatures could be due to a different N_2O-reducing process, such as N_2O fixation.

Moreover, undersaturation in N_2O is more likely to be found in N-limited waters, as higher concentrations of nitrate (NO_3^-) or ammonium (NH_4^+) often promote the production of

N_2O (Barnes and Upstill-Goddard, 2011; Garcia-Ruiz et al., 1998; Harrison and Matson, 2003; Richardson et al., 2004; Xia et al., 2013).

5.1.2 What have I found by characterising N_2O fixation?

With our well-established, N-limited freshwater ponds, I performed a series of experiments, to characterise any potential N_2O fixation in a controlled, experimental system. I measured dissolved N_2O and N_2 to demonstrate the existence of both N_2O and N_2 sinks in the freshwater ponds. With incubations of biomass from the ponds using ^{15}N stable isotope techniques, I quantified the rates of N_2O and N_2 fixation and characterised the temperature dependence of the two N fixation processes. Further, to gain a more complete view of N_2O dynamics in freshwaters, I explored the production of N_2O from either denitrification or nitrification and characterised the potential temperature dependence of these two processes.

As a recap, from my PhD work, I gained some potential answers to the following questions outlined in Chapter 1:

Does N_2O-dependent N fixation exist in freshwater communities?

By quantifying both N_2O and N_2 fixation I show that both gases can be fixed by freshwater communities. N_2O fixation appears to be direct, rather than N_2O being first reduced to N_2 through denitrification and then being fixed (Fig. 7, Table 3, Chapter 2).

Is N_2O fixation important, e.g., can N_2O fixation provide a significant sink for N_2O in natural waters?

By scaling the rates of total N_2O reduction from the laboratory biomass incubations (Section 2.3.6, Chapter 2), I show that total N_2O reduction can rationalise the significant undersaturation in N_2O I measured in the ponds (Fig. 4, Chapter 2). Further, rates of total N_2O reduction in the biomass incubations were higher in winter than in summer, reflecting the strong sink for N_2O

in the ponds in winter. As total N_2O reduction mainly came from N_2O-dependent N fixation (Fig. 2a, Chapter 3), rather than the reduction of N_2O to N_2 through denitrification, this suggests that N_2O fixation could provide a significant sink for N_2O in natural waters.

What are the fates of the reduced N_2O? Can we distinguish different pathways for N_2O reduction, i.e., the dissimilatory reduction of N_2O to N_2 from canonical denitrification and assimilatory N_2O fixation?

With the biomass incubations, I characterised the potential products from N_2O reduction. In summary, total N_2O reduction comprises two different categories: the dissimilatory reduction of N_2O to N_2 via canonical denitrification and assimilatory N_2O fixation (Table 1, Chapter 3). In addition, the assimilatory N_2O fixation can be further broken down into different pathways such as N_2O assimilation into the biomass as particulate organic nitrogen (PON), N_2O fixed as NH_3 that was then potentially 'leaked' into the water as NH_4^+, which could be further oxidised to NO_2^- and NO_3^- (Fig. 7, Chapter 3).

The contribution of dissimilatory reduction to total N_2O reduction can be quantified by comparing the production of $^{15}N_2$ to total $^{15}N_2O$ production, with the rest of total $^{15}N_2O$ production accounted for by assimilatory N_2O fixation. The two different pathways were also distinguished by characterising both $^{15}N_2O$ and $^{15}N_2$ fixation, with the disproportionate rate of the two suggesting that $^{15}N_2O$ fixation is direct (Fig. 7, Chapter 2). Overall, assimilatory N_2O fixation was the primary pathway for total N_2O reduction, whereas dissimilatory reduction of N_2O to N_2 was a minor part (Fig. 6, Chapter 2).

What is the effect of temperature on N_2O fixation compared to N_2 fixation? Would N_2O be preferred over N_2 in the cold?

N_2O fixation appears less sensitive to temperature than N_2 fixation. From the biomass incubations, N_2 fixation increased at higher incubation temperatures, which agrees with the typical pattern found in other natural waters, whereas N_2O fixation was not sensitive to changes in temperature (Fig. 8, Chapter 2). In addition, some of the fixed N_2O was oxidised to NO_x^-, and the production of NO_x^- was invariant at different temperatures (Fig. 3, Chapter 3), which again, suggests that N_2O fixation is not sensitive to temperature. As a result, the fraction of total N-fixation (N_2O fixation plus N_2 fixation) coupled to N_2O was higher in the cold, for example, 26% higher at 6°C than that at 25°C (Fig. 8, Chapter 2).

5.2 Suggestions for future work

With biological N_2O fixation identified and potentially contributing significantly to N_2O sinks in freshwaters, the existence and importance of N_2O fixation could be overlooked in the global nitrogen cycle. As many undersaturation in N_2O in natural waters remain unaccounted for, we need more studies to confirm whether N_2O fixation could also explain these N_2O sinks. Further, it could be vital to include N_2O fixation in the global N_2O budget to provide a better estimation.

With N-rich ecosystems typically act as N_2O sources, N_2O sinks mediated through N_2O fixation are likely to be found in cold, pristine ecosystems. More field campaigns would be needed to confirm whether cold, pristine regions can act as net sinks for N_2O. Further, as the relative proportion of N_2O fixation to N_2 fixation appears to be more pronounced at lower temperatures, N_2O fixation could be more important in supporting the primary production in these environments. Most importantly, with the current global warming scenario, rising temperatures could potentially cause the erosion of the N_2O sinks caused by N_2O fixation.

As studies on N_2O fixation are very limited, lots are unknown about the possible regulatory factors of this pathway. Although there is abundant evidence showing a positive correlation between N_2O emissions and the availability of dissolved inorganic nitrogen, studies

would be needed to characterise the inhibition threshold of DIN species on N_2O fixation and N_2O sinks. More studies *in situ* and laboratory settings would also be crucial to help us better understand other key stressors for N_2O fixation, such as temperature and the availability of oxygen.

To date, it is not clear which microorganisms are responsible for N_2O fixation in natural ecosystems. A few studies have reported N_2O fixation in surface marine waters (Cornejo et al., 2015; Farías et al., 2013) and soybean root nodules (Mozen and Burris, 1954), but only one study, on pure cultures of the marine cyanobacteria *Trichodesmium* sp. and *Crocosphaera* sp., has related the *nifH* gene to N_2O fixation(Farías et al., 2013).

My work shows that N_2O fixation can occur in the presence of an abundant concentration of N_2, as the $^{15}N_2O$ fixation in the incubations occurred against a natural N_2 background. This indicates that N_2O fixation could happen in natural ecosystems replete in N_2 and provides further insight into the communities responsible for N_2O fixation. For example, *nifH* communities could fix N_2 and N_2O randomly, with the ratio of N_2O to N_2 fixation being simply proportional to the relative availability of N_2O to N_2. However, the distinct seasonal patterns I measured for N_2 and N_2O undersaturation (Fig. 4, Chapter 2), coupled with disproportionate rates of N_2O fixation (Fig. 6, Chapter 2) and the higher proportions of N_2O fixation at colder temperatures (Fig. 7**a**, Chapter 2) - all indicate that the community responsible for N_2O fixation seem to be different from N_2 fixation at colder temperatures.

On the other hand, the higher $^{15}N_2O$ fixation under relatively higher availability of N_2O to N_2, and the similar pattern in the relationships between fixation of N_2O and N_2 and *nifH* abundance both indicate some similarity in the communities responsible for the two processes (Fig. 10, Chapter 3). Communities responsible for N_2O fixation may have different patterns in substrate preference. For example, some N_2O fixers are facultative and would fix more N_2O at a relatively higher availability of N_2O to N_2 (Fig. 9, Chapter 3), while others are obligate fixers

that always fix N_2O regardless of the large N_2 background (Fig. 6, Chapter 2). Nevertheless, further studies combining community analysis and the process measurement of N_2O fixation activity would be needed to identify the potential N_2O fixers and compare them to the typical N_2-fixing communities. The application of molecular microbial techniques, such as stable-isotope-probing (SIP) to label the DNA with ^{15}N (or, combinations of ^{13}C-CO_2 and ^{15}N-N_2O to possibly making heavier DNA), metagenomics, and 16S bacterial qPCR, combined with $^{15}N_2O$-enriched incubations of natural communities will be helpful in identifing potential N_2O-fixers. N_2O-enriched incubations may also select for microbes that grow on N_2O, which can help isolating potential N_2O-fixers and likely accelerating the confirmation of N_2O fixation by pure cultures. Other technical development, such as the design of new primer sets which are more specific for *nifH* sequences, could also be benificial.

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