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Nitrate availability modulates the temperature effect on N_2O and N_2 production from denitrification

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Abstract

Nitrous oxide (N_2O) can be both produced and subsequently reduced to dinitrogen gas (N_2) via canonical denitrification, making the balance between these steps a key control on the net flux of this potent climate gas. Through a meta-analysis, we showed that net N_2O and N_2 production from denitrification respond differently to temperature, exhibiting distinct temperature sensitivities. In addition, nitrate availability plays a critical role in regulating this balance, yet only few studies have examined the combined effects of temperature and nitrate availability in natural sediments. Using ^{15}N-isotope labelling and anoxic sediment incubations, we found that temperature effects on N_2O and N_2 production from denitrification were evident only under high nitrate levels (100 μM), while no significant temperature response occurred under low nitrate concentration (10 μM). At high nitrate availability, N_2 production increased at higher temperatures, whereas net N_2O production declined, leading to a lower production ratio of N_2O to N_2 at warmer temperatures. These findings suggest that in nitrogen-limited ecosystems, substrate availability plays a stronger role than temperature in regulating denitrification. More broadly, they provide insights into how nutrient loading and climate warming interact to shape nitrogen cycling and greenhouse gas emissions in aquatic ecosystems.

Introduction

N_2O has 273 times the warming potential of carbon dioxide (CO_2) (Masson-Delmotte et al., 2021), is currently the primary driver of stratospheric ozone depletion (Ravishankara et al., 2009) and, most importantly, its atmospheric concentration has already risen by 25% since the 1850s (Meinshausen et al., 2011). Freshwater systems, especially small lakes and ponds, are increasingly recognised as significant sources of N_2O, with their emissions increased by 126% over the same period (Li et al., 2024).

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Microbial denitrification which proceeds via the pathway $NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$ (Knowles, 1982), is a key process for both the production and reduction of N_2O in freshwaters (Beaulieu et al., 2014; Beaulieu et al., 2011; Maavara et al., 2019), as well as in marine (Codispoti et al., 2001; Ji et al., 2018) and terrestrial ecosystems (Yu et al., 2023). As denitrification involves both the formation and consumption of N_2O, its net emission to the atmosphere depends on the relative rates between these two steps. The activity of each step is regulated by environmental factors such as nitrate availability, dissolved oxygen concentration, and temperature (Codispoti et al., 2001; Ji et al., 2018; Kuypers et al., 2018).

Temperature is a key regulator of total N_2O and N_2 production via denitrification, with both generally increasing as temperature rises (Bailey and Beauchamp, 1973; Holtan-Hartwig et al., 2002; Keeney et al., 1979; Seitzinger et al., 1984; Silvennoinen et al., 2008). However, the effect of temperature on net N_2O production – defined as total N_2O production minus its consumption - varies widely among studies. Some studies report a positive relationship between net N_2O production and temperature, suggesting that N_2O production exceeds its reduction to N_2 at higher temperatures (Dobbie and Smith, 2001; Duan et al., 2019; McKenney et al., 1984; Myrstener et al., 2016; Smith et al., 1998). Conversely, others observed a negative relationship, where N_2 production increases more than N_2O production as temperature rise, resulting in lower net N_2O production (Silvennoinen et al., 2008). In addition, some studies find no significant relationship between temperature and net N_2O production (Bailey, 1976; Del Prado et al., 2006; Lai and Denton, 2018). This variability underscores the complexity of temperature effects on denitrification and highlights the need for further investigation into how temperature interacts with other environmental drivers to regulate N_2O dynamics and the balance in N_2O:N_2.

The availability of nitrate (NO_3^-) is another critical factor regulating the production of N_2O and N_2 via denitrification (Baulch et al., 2011; Beaulieu et al., 2011). However, many studies investigating the temperature dependence of N_2O and N_2 production have focused on nitrate-rich environments (Silvennoinen et al., 2008) or have experimentally added NO_3^- concentrations far in excess of ambient concentrations (Jørgensen, 1989; Lai and Denton, 2018; Myrstener et al., 2016; Rysgaard et al., 2004). In some cases, sediments were subjected to prolonged exposure to high NO_3^- enrichment for over three months, as in estuarine mesocosm studies, which significantly increased the temperature sensitivity of N_2 production (Nowicki, 1994).

To date, studies examining the combined effects of nitrate availability and temperature on denitrification remain scarce (Lai and Denton, 2018; Myrstener et al., 2016). As a result, it is still unclear how the temperature sensitivity of N_2O and N_2 production varies across different nitrate concentrations. Most studies that have characterised the temperature sensitivities of both gases have focused on soils at typically high experimental NO_3^- concentrations, whereas

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few have addressed aquatic sediments that are major sites of denitrification and nitrogen loss as N_2O or N_2 (Silvennoinen et al., 2008). Of the limited research in aquatic sediments, only two studies have characterised the temperature dependency of net N_2O production from denitrification (Myrstener et al., 2016; Silvennoinen et al., 2008), and none has done so using direct measurements of ^{15}N-labelled N_2O. This is a critical gap, as measuring bulk N_2O concentrations alone makes it difficult to disentangle the multiple microbial processes contributing to both N_2O production and reduction (Zhu et al., 2025).

In this study, we first conducted a meta-analysis of existing data to predict the temperature sensitivities of N_2O and N_2 production, as well as their ratio, in N-rich soils and aquatic sediments. To assess how temperature and nitrate availability regulate N_2O and N_2 production in understudied N-limited aquatic sediments, we experimentally quantified the temperature dependencies of ^{15}N-labelled N_2O and N_2 production from denitrification under varying ^{15}N-nitrate concentrations. We addressed two key questions – (1) How does the net production of N_2O and N_2, and their ratio, respond to temperature variation in N-limited aquatic ecosystem? and (2) Does nitrate availability influence the temperature sensitivity of N_2O and N_2 production in such system?

Methods

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

To derive the temperature sensitivities of net N_2O and N_2 production from denitrification, and their ratios, here we compiled a list of studies that reported these rates and ratios under different temperatures in both aquatic sediments and terrestrial soils (Table 1). We fitted the net rate of N_2O production, N_2 production, and the N_2O:N_2 ratio into linear mixed-effect models to evaluate their temperature sensitivities (Bates et al., 2014). We then estimated the apparent activation energy ($\bar{E}$) by fitting the natural log-transformed rate or ratio against the centred temperature term $\left(\frac{1}{kT_C} - \frac{1}{kT_i}\right)$ (Yvon-Durocher et al., 2014; Zhu et al., 2020):

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

k is the Boltzmann constant (8.62×10^{-5} eV K^{-1}, 1 eV = 96.485 kJ mol^{-1}), while 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). T_i is the absolute temperature from study i (i =1, 2, … ,16). Further, to account for variances across the different studies, we included random slope (a_i) and random intercept (b_i) terms in the mixed-effects models.

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Table 1. The studies used for the meta-analysis to characterise the temperature sensitivity of net N_2O or N_2 production attibuted to denitrification in aquatic sediments and terrestrial soils. In most cases, denitrification was inferred rather than conclusively separated from other potential sources of N_2O or N_2. Inferred: N_2O or N_2 attributed to denitrification without confirmatory evidence to exclude other sources. Distinguished: N_2O or N_2 production from denitrification confimed by isotope-pairing techniques, which separated different sources. Number of measurements (*n*). In total, the dataset consisted of 920 measurements from 23 studies, i.e., 251 and 669 measurements in aquatic and terrestrial ecosystems, respectively. n.a.: not applicable.

Ecosystem	Measurement	Denitrification	Substrate	Studies
Aquatic	N_2O	Inferred	NO_3^-: 57 µM	(Myrstener et al., 2016)
	N_2	Inferred	Unspecified DIN	(Nowicki, 1994)
	N_2	Inferred	Ambient	(Seitzinger et al., 1984)
	$^{15}N_2$	Distinguished	$^{15}NO_3^-$: 100 µM	(Brin et al., 2014; 2017)
	$^{15}N_2$	Distinguished	$^{15}NO_3^-$: 50 µM	(Rysgaard et al., 2004)
	$^{15}N_2$	Inferred	$^{15}NO_3^-$: 5.9 to 20.2	(Veraart et al., 2011)
	N_2O, $^{15}N_2$	Inferred	NO_3^-: 30 µM	(Silvennoinen et al., 2008)
Terrestrial	N_2O	Inferred	NO_3^-: 10204 µM	(Dobbie and Smith, 2001)
	N_2O	Inferred	NO_3^-: 8571 µM	(Smith et al., 1998)
	N_2O	Inferred	NO_3^-: 2536 µM	(Benoit et al., 2015)
	N_2O	Inferred	NO_3^-: 10093 µM	(Del Prado et al., 2006)
	N_2O	Inferred	Ambient NO_3^-: 286 µM	(McKenney et al., 1984)
	N_2O	Inferred	NO_3^-: > 85 times ambient	(Kurganova and Lopes de Gerenyu, 2010)
	$^{15}N_2O$	Inferred	$^{15}NO_3^-$: >18000 µM	(Duan et al., 2019)
	N_2	Inferred	NO_3^-: 4 µM	(Qin et al., 2014)
	N_2	Inferred	NO_3^-: 5497 µM	(Castaldi, 2000)
	N_2	Inferred	NO_3^-: 4063 µM	(Holtan-Hartwig et al., 2002)
	N_2O, N_2	Inferred	NO_3^-: 4286 µM	(Bailey, 1976)
	N_2O, N_2	Inferred	NO_3^-: 8700 µM	(Keeney et al., 1979)
	$^{15}N_2O$, $^{15}N_2$	Inferred	$^{15}NO_3^-$: >8000 µM	(Lai and Denton, 2018)
	$^{15}N_2O$, $^{15}N_2$	Distinguished	$^{15}NO_3^-$: 8333 µM	(Yu et al., 2023)
	N_2O/N_2	Inferred	Ambient NO_3^-: 65 - 387 µM	(Maag and Vinther, 1996)

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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, we carried out a trial experiment to determine the optimal substrate concentration and incubation time for the experiments with sediments collected from our well-established freshwater experimental ponds (Si et al., 2023).

Sediment cores were collected from the experimental ponds in December 2020, with each pond sampled at three different locations. Intact cores were transported to the laboratory in the dark on ice packs (4-hour trip) and then kept overnight at 4ºC. Before the experiment, the cores and pre-weighed vials (12 mL Exetainer, Labco) 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 water medium (Si et al., 2023) with OFN for 20 min. The top 2 cm of the cores for each pond were homogenized, then 2 mL of the sediment and 4 mL of the medium were added to each gas-tight vial to make a slurry. The slurry was added carefully without any sediment remaining inside the thread of the lid, as this may affect the sealing of the vial and lead to air contamination. The vials were then closed and pre-incubated in a temperature-controlled room (15ºC) for 16 hours to remove any residual porewater NO_x^- and oxygen (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 100 µL of formaldehyde (37 wt. %), with vials then equilibrated at room temperature (22ºC) until further analysis.

As the formaldehyde used to preserve the gas samples interferes with the colorimetric assay for NO_x^- and NH_4^+ analyses, we prepared parallel samples for separate measurement of gases and nutrients. Samples for the nutrient measurement were immediately centrifuged and supernatants frozen at -20 °C at each time point until further analysis. To obtain the concentrations of porewater nutrients and the ^{15}N-labelling of the NH_4^+ pool (FA), nutrient concentrations were measured by the automated wet-chemistry autoanalyzer (San^{++}, SKALAR Analytical B.V.) with standard colorimetric techniques (Kirkwood, 1996). Calibration was performed against certified reference materials, traceable to NIST. The limits of detection were 0.05 µM, 0.1 µM and 0.2 µM for nitrite (NO_2^-), nitrite plus nitrate (NO_x^-: NO_2^- + NO_3^-), and ammonium (NH_4^+), respectively.

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For concentration of ^{15}N-N_2O, sub-samples from the headspace of each vial were transferred to an air-filled 12 mL gas-tight vial and measured on a continuous flow isotope ratio mass spectrometer (CF-IRMS, Delta V Plus, Thermo Finnigan) with an automated trace gas pre-concentrator (Precon, Thermo Finnigan). Calibration was performed with air, 0.12 ppm, 1.04 ppm, and a series of diluted 96 ppm N_2O standards (BOC Limited), with a linear increase in the peak area over a range of 0.08 nmol to 5.85 nmol N_2O in the vial. Concentrations of $^{15}N_2$ were measured on the CF-IRMS in 100 μL of sample headspace. Drift in the signal of mass 30 was corrected by inserting air standards for every 10 samples (Si et al., 2023).

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 (Si et al., 2023). The production of ^{15}N-N_2O ($^{45}N_2O + 2 \times {}^{46}N_2O$) and ^{15}N-N_2 ($^{29}N_2 + 2 \times {}^{30}N_2$) was calculated from the excess gas production in the $^{15}NO_3^-$ treatments compared to that in the 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 determine 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	$^{45}N_2O$, $^{46}N_2O$, $^{29}N_2$, $^{30}N_2$

Characterising the temperature sensitivities of N_2O and N_2 production from denitrification

After we determined the optimal incubation conditions based on the trial experiment, we collected further sediment cores from eight experimental 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 even after 16 hours of pre-incubation (Supplementary Fig. 1). Therefore, the vials were pre-incubated in a temperature-controlled room (15°C) for a longer period (24 hours) to further remove any residual porewater NO_x^-. After the pre-incubation, vials were amended with different doses of $^{15}NO_3^-$ to a final concentration of 0 (controls), 10, or 100 μM (Table 3). Each concentration set was incubated for 3 hours, which is the optimal time determined from the trial experiment

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(Supplementary Fig. 2). Other details for sediment collection, sample preparations and measurements remained the same as in the trial experiments.

Characterising the potential of anammox and nitrification

As both denitrification and anammox ($NH_4^+ + NO_2^- \rightarrow N_2$) produce N_2 (Dalsgaard et al., 2003; Kuypers et al., 2003; Trimmer et al., 2013), distinguishing between these two processes is critical. This can be achieved by analysing the production of $^{29}N_2$ ($^{14}N^{15}N$) and $^{30}N_2$ ($^{15}N^{15}N$) from different combinations of ^{15}N-labeled substrates (Brin et al., 2014; Dalsgaard et al., 2003; Kuypers et al., 2003; Trimmer et al., 2013). According to isotope-pairing principles, the presence of anammox is indicated by the production of excess $^{29}N_2$ production in a treatment with $^{14}NO_3^-$ and $^{15}NH_4^+$, relative to a treatment with $^{15}NH_4^+$ alone - since anammox couples one nitrogen atom from $^{14}NO_3^-$ and one from $^{15}NH_4^+$ to form N_2 (Dalsgaard et al., 2003; Trimmer et al., 2013). To test for the potential of the anammox reaction, two additional sets of incubations were performed using the same sediment samples as in the denitrification experiments: a $^{15}NH_4^+$ treatment, and a $^{15}NH_4^+$ plus $^{14}NO_3^-$ treatment (Table 3). Each treatment included two concentrations of $^{15}NH_4^+$ (final concentration of 10 or 60 μM, prepared from 98% ^{15}N-NH_4^+, Sigma Aldrich) to test for anammox activity under both low and high substrate availability. In the combined treatment, $^{14}NO_3^-$ was added at a final concentration of 100 μM, consistent with the high $^{15}NO_3^-$ concentration used in the denitrification experiment above. After incubations, $^{29}N_2$ concentrations were measured in each treatment to assess anammox activity.

In addition, we also tested the potential of N_2O production from nitrification ($NH_4^+ \rightarrow NH_2OH \rightarrow N_2O$). Sediment cores were collected from eight ponds in February 2022, with the same sampling techniques as used in the denitrification experiments. In the laboratory, the top 2 cm of sediments for each pond were homogenised, then 2 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. Vials were amended with $^{15}NH_4^+$ (final concentration 22 μM or 44 μM) with or without allylthiourea (ATU) (100 μL from 2.8 mM stock, final concentration ~80 μM), where ATU was used to block the oxidation of NH_4^+ (Ginestet et al., 1998). 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 (Supplementary Table 1). Un-amended vials were prepared as controls to account for any activity of nitrification from the background NH_4^+ (Table 3). Independent vials were used for each pond and treatment and incubated for 0, 3, 8, 18, or 24 hours. All vials were incubated at 15°C, which was the annual average temperature in the ambient ponds (Si et al., 2023). The microbial activities in samples were terminated by formaldehyde at each time point, with N_2O measured with the same methods as used in the denitrification experiments.

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Table 3. Experiments designed to characterise the temperature sensitivities of N_2O and N_2 production via denitrification, and also test for any anammox potential, and N_2O production from nitrification. As both denitrification and anammox produce N_2, isotopic labelling with different ^{15}N-substrates was used to distinguish between the two processes. Anammox activity can be confirmed if excess $^{29}N_2$ is produced in the $^{15}NH_4^+$+$^{14}NO_3^-$ treatment relative to $^{15}NH_4^+$-only treatment. All treatments were applied to independent sediment samples collected from eight ponds.

Treatment	**$^{15}NO_3^-$ (μM)**	**$^{15}NH_4^+$ (μM)**	**Temperature (°C)**	**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	$^{29}N_2$
Nitrification				
Control	n.a.	0	15	$^{45}N_2O$, $^{46}N_2O$
$^{15}NH_4^+$	n.a.	22 or 44	15	$^{45}N_2O$, $^{46}N_2O$
$^{15}NH_4^+$+ATU	n.a.	44	15	$^{45}N_2O$, $^{46}N_2O$

Statistical analysis

To derive the temperature sensitivities of net N_2O and N_2 production, and the N_2O:N_2 ratio from denitrification in the N-limited ponds, we estimated the apparent activation energy by fitting the natural log-transformed rate of net production of N_2O or N_2 against the centred temperature term $\left(\frac{1}{kT_C}-\frac{1}{kT_i}\right)$ using equation (1), except that i (i =1, 2, … ,8) now denotes the different ponds and T_C denotes the average incubation temperature (15°C).

Statistical analysis and plotting were performed in R version 4.5.0 (Team, 2021) using RStudio. For meta-analysis, as the studies used different normalisation methods, we standardised the data by subtracting the study-specific intercept from the rate of N_2O or N_2 production for each study (Yvon-Durocher et al., 2014; Zhu et al., 2020). For our incubations with pond sediments, we standardised the data by subtracting the pond-specific intercept from the net production rate of N_2O or N_2 for each pond. 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 and the activation energies ($\bar{E}$), in the unit of eV, derived from the slope of the best-fitting models.

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Results

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

From the meta-data analysis of previous studies, net N_2O production from denitrification increased at higher temperatures, with an apparent activation energy (Ea) of 0.41 eV (95% CI of 0.29 to 0.54 eV, Fig. 1**a**). The temperature response of net N_2O production was consistent across aquatic sediments and terrestrial soils (Supplementary Table 2, likelihood ratio test comparing M0 and M2 for net N_2O, $p > 0.05$), although data from aquatic sediments remain limited. Similarly, N_2 production from denitrification was higher at warmer temperatures, with an activation energy at 0.6 eV (95% CI of 0.45 to 0.75 eV, Fig. 1; and Supplementary Table 2 comparing best-fitting model M0 and null model, $p < 0.001$). The temperature sensitivity of N_2 production was also consistent between aquatic and terrestrial ecosystems (Supplementary Table 2, likelihood ratio test comparing M0 and M2 for N_2, $p > 0.05$).

From studies reporting parallel measurements of net N_2O and N_2 production, we derived the temperature sensitivity of the N_2O:N_2 ratio, which showed a negative relationship with temperature (Fig. 2). We also predicted the temperature sensitivity of the N_2O:N_2 ratio using the independent datasets of net N_2O and N_2 shown in Fig. 1. The activation energy of the N_2O:N_2 ratio derived from the two approaches did not differ statistically ($\chi^2 = 1.46$, $p = 0.23$; linear hypothesis test for fixed effects using the 'car' package in R). These results showed that while both net N_2O and N_2 production from denitrification increased with temperature, their differing temperature sensitivities led to a negative relationship between the N_2O:N_2 ratio and temperature.

However, while these previous studies commonly inferred denitrification as the source of the measured N_2O or N_2 production, most did not explicitly distinguish it from other microbial pathways contributing to N_2O or N_2 production (Table 1). As a result, the reported temperature sensitivities may reflect a composite signal from multiple processes, rather than denitrification alone.

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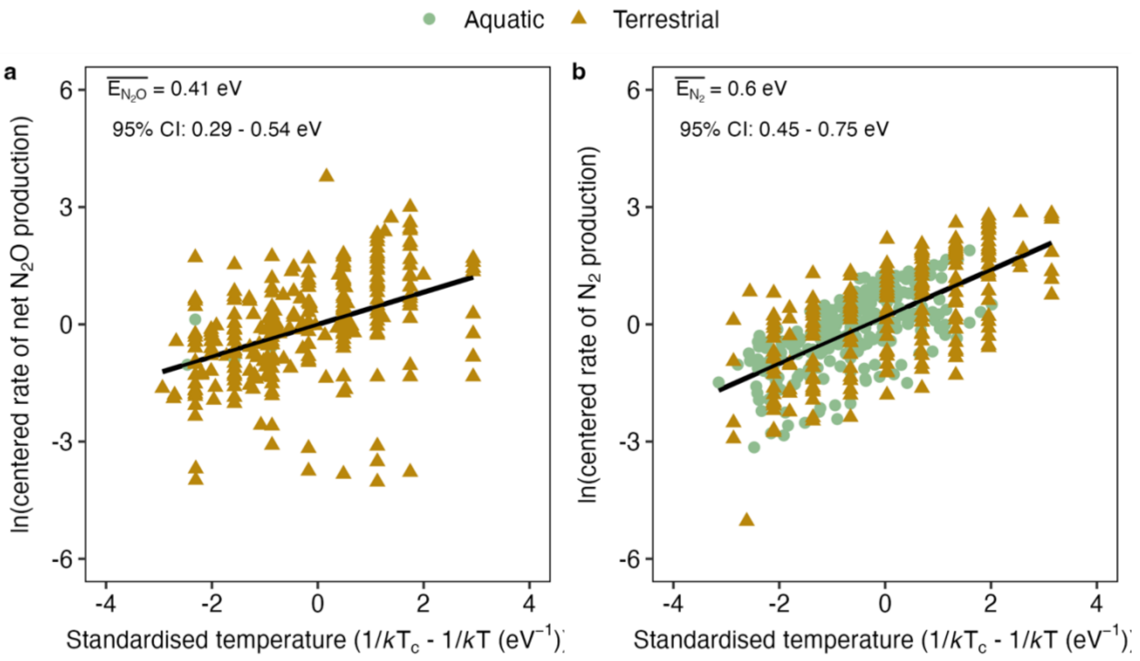

Fig. 1 | Meta-analysis fitting published rates of net N_2O and N_2 production inferred from denitrification as a function of temperature with both aquatic sediments and terrestrial **soils.** The rate of net N_2O and N_2 production increased at higher temperatures in both aquatic sediments and soils. We visualised the data using the “Visreg" package in R (Breheny and Burchett, 2017) plotting the data as the partial residuals (brown and green circles) from the best fitting models after the random effects were accounted for and with the overall estimate for temperature sensitivity given as black lines (Supplementary Table 2) . 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. For net N_2O production, n = 8 and 265 measurements in aquatic and terrestrial, respectively, whereas for N_2 productions, n = 239 and 208 measurements in aquatic and terrestrial, respectively. Because normalisation methods differed among studies, we standardised the data by centring each study’s N_2O or N_2 production rates on its specific intercept.

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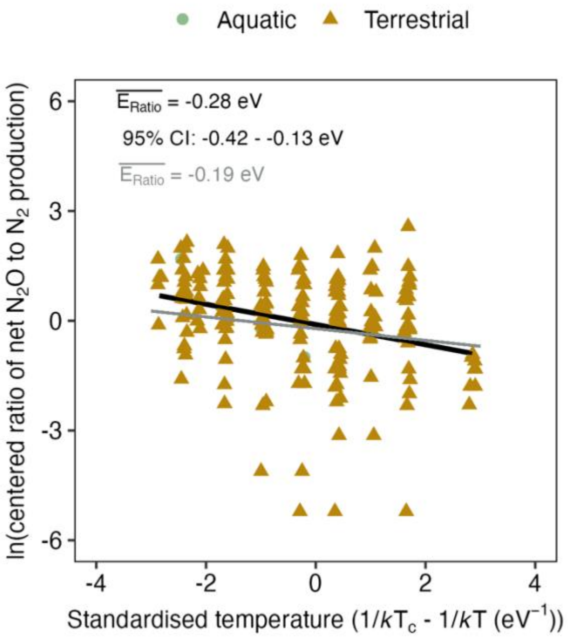

Fig. 2 | Ratio of net N_2O to N_2 production from denitrification as a function of temperature, based on incubations from both aquatic sediments and terrestrial soils. *n* = 4 and 196 measurements for aquatic sediments and terrestrial soils, respectively. The black line gives the activation energy derived from the slope of the best fitting linear mixed-effects model for actual parallel measurements of net N_2O and N_2 production (Supplementary Table 2, Model M0 for N_2O:N_2). The grey line shows the activation energy of the N_2O:N_2 ratio, predicted from the independent net N_2O and N_2 datasets shown in Fig. 1. The two estimates did not differ significantly ($\chi^2 = 1.46$, $p = 0.23$; linear hypothesis test for fixed effects using the 'car' package in R).

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

No excess production of $^{29}N_2$ was detected in either the $^{15}NH_4^+$ only treatments or those amended with $^{14}NO_3^-$ compared to the controls (Supplementary Fig. 3). Likewise, adding extra $^{14}NO_3^-$ to the $^{15}NH_4^+$ treatments did not increase $^{29}N_2$ concentrations ($p = 0.61$, Two-Sample *t*-test), confirming the absence of anammox. This agrees with previous studies showing no anammox activity in these pond sediments across three seasons (Warren, 2017). Nitrification was also negligible as a source of N_2O, detected only in one of eight ponds and at much lower rates than denitrification (Supplementary Fig. 4). These results indicate that denitrification was the dominant pathway responsible for both N_2O and N_2 production in the studied ponds.

In the trial experiments, ^{15}N-N_2O production peaked and was subsequently reduced during all incubations (Supplementary Fig. 2**a**). At $^{15}NO_3^-$ concentrations below 100 μM, ^{15}N-

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N_2O reached its peak within ~0.5 h of incubation and declined to zero before 24 h in most incubations. In contrast, with 100 µM $^{15}NO_3^-$, the ^{15}N-N_2O peak occurred later (at ~3 h) and reached higher production than in the lower-concentration treatments. Meanwhile, ^{15}N-N_2 accumulated continuously over 24 h in incubations with $^{15}NO_3^-$ additions higher than 10 µM, but at 10 µM of $^{15}NO_3^-$ it plateaued after ~12 h (Supplementary Fig. 2**b**).

The availability of nitrate influenced both the magnitude and the ration of N2O to N2?. Production rates of both ^{15}N-N_2O and N_2 increased with increasing $^{15}NO_3^-$ concentrations (Fig. 3**a**, 3**b**). Specifically, ^{15}N-N_2O production showed a near-linear increase across the full concentration range (0 - 100 µM), reaching a maximum of 223.27 nmol g^{-1} h^{-1} at 100 µM $^{15}NO_3^-$ (Fig. 3**a**). ^{15}N-N_2 production also increased with nitrate, but the response was gentler (Fig. 3**b**). The ratio of ^{15}N-N_2O to ^{15}N-N_2 production increased up to 50 µM) and then plateaued (Fig. 3**c**).

In addition, 43% of the $^{15}NO_3^-$ added was reduced to $^{15}N_2$ after 24 h of incubation, on average (Supplementary Fig. 5), which was consistent across different $^{15}NO_3^-$ concentrations (*p* = 0.52, df = 14, Kruskal-Wallis test). This indicates that the fraction of the NO_3^- reduced to the end-product N_2 was not affected by NO_3^- availability over the tested range of 10 to 100 µM.

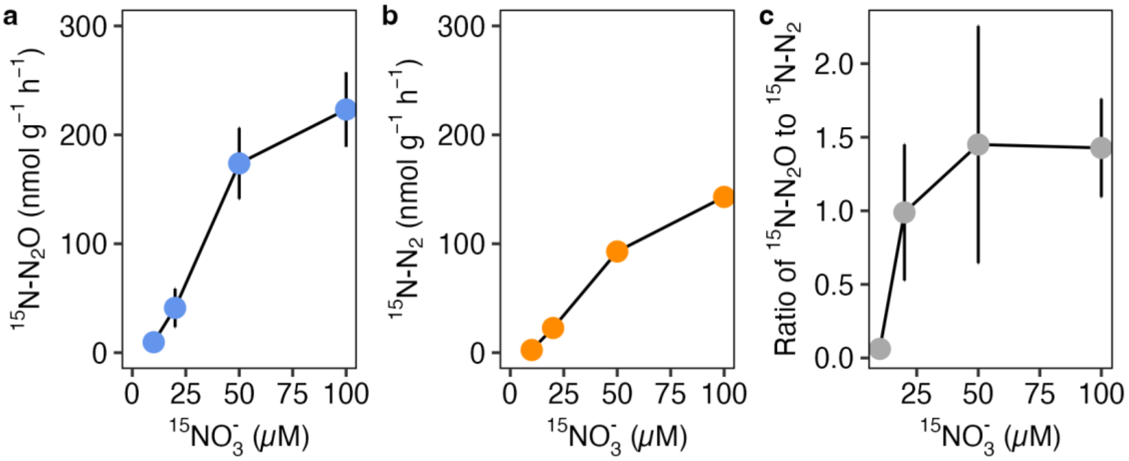

Fig. 3 | Rate of $^{15}N_2O$ and $^{15}N_2$ production and their ratios, from independent sediment incubations amended with varying concentrations of $^{15}NO_3^-$. a, Rate of $^{15}N_2O$ production calculated from the 3-h incubation. **b**, Rate of $^{15}N_2$ production calculated from the 24-hour incubation. Both gases showed increased production with higher $^{15}NO_3^-$ availability, with the rate of increase plateaued at 100 µM of $^{15}NO_3^-$. **c**, Ratio of $^{15}N_2O$ to $^{15}N_2$ production across $^{15}NO_3^-$ concentrations. Dots in each plot represent means, with error bars indicating standard error (*n* = 6 ponds per concentration of $^{15}NO_3^-$ at each time point).

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Temperature sensitivities of N_2O and N_2 net production from denitrification

Based on results from the trial experiments, sediments were incubated with 100 μM of $^{15}NO_3^-$ for approximately 3 h to characterise the temperature sensitivity of both the production of N_2O and N_2. In addition, as the concentration of NO_3^- in the ponds was low (< 2 μM, Supplementary Table 3), additional samples were incubated with a lower concentration of $^{15}NO_3^-$ (10 μM) to characterise the effect of lower substrate availability on the denitrification gas products.

After the 3-hour incubation, net production of both $^{15}N_2O$ and $^{15}N_2$ was detectable in the large majority of incubations (95%, 152 out of 160 incubations) with either 10 μM or 100 μM of $^{15}NO_3^-$ added. Production of N_2O and N_2 responded differently to temperature depending on the availability (concentration) of NO_3^-. At 10 μM $^{15}NO_3^-$ net production of both $^{15}N_2O$ and $^{15}N_2$ did not increase significantly at higher incubation temperatures (Fig. 4**a**, 4**c**, **M10.a** compared to M10.b for both N_2O and N_2, Supplementary Table 4). In contrast, with 100 μM $^{15}NO_3^-$, net production of both $^{15}N_2O$ and $^{15}N_2$ was sensitive to increasing temperature ($p <$ 0.001, **M100.a** compared to M100.b for both N_2O and N_2, Supplementary Table 4), but with opposite temperature sensitivities. That is, net N2O production decreased at -0.26 eV while N2 production increased at 0.43 eV (Fig. 4**b**, 4**d**). Between temperatures of 5ºC to 25ºC, $^{15}N_2O$ decreased, while $^{15}N_2$ production increased at higher temperatures.

Furthermore, at 10 μM $^{15}NO_3^-$, the $^{15}N_2O$:$^{15}N_2$ ratio was consistent across temperatures (Fig. 5**a,** $p > 0.05$, **M10.a** compared to M10.b for the ratio, Supplementary Table 4), whereas at100 μM $^{15}NO_3^-$, $^{15}N_2O$:$^{15}N_2$ decreased exponentially from 5ºC to 20ºC (Fig. 5**b,** $p < 0.001$, **M100.a** compared to M100.b for the ratio, Supplementary Table 4) at -0.7 eV (95% CI: -0.93 to -0.47 eV, Fig. 5**b**) leading to a higher accumulation of N_2O relative to N_2 from denitrification at colder temperatures.

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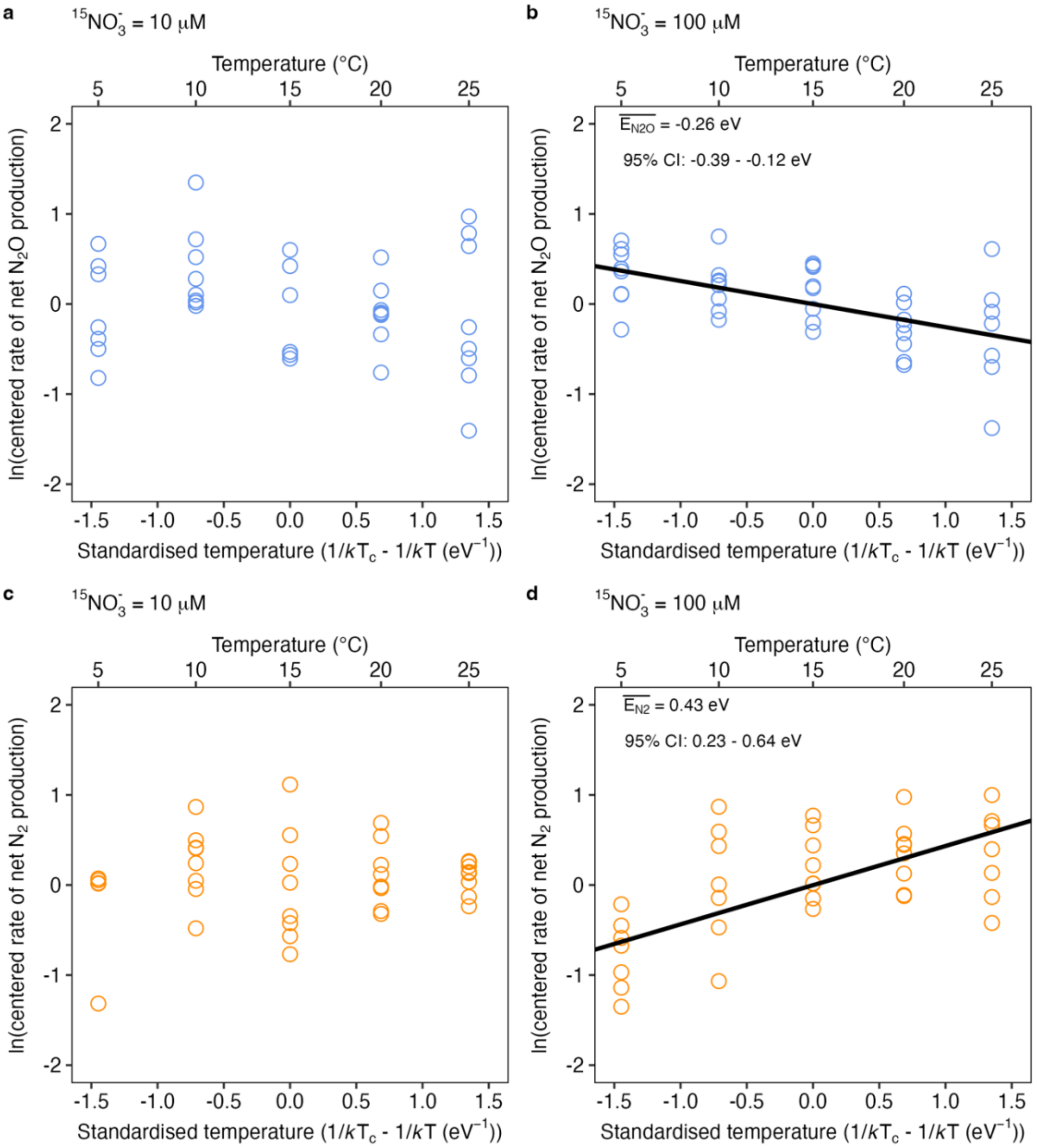

Fig. 4 | Temperature sensitivities of the net production rate (nmol g^{-1}) of N_2O and N_2 from denitrification under nitrate-limited or nitrate-replete conditions. a, Net production rate of $^{15}N_2O$ was consistent between different temperatures with 10 µM $^{15}NO_3^-$ added. **b**, $^{15}N_2O$ 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. we 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 (Supplementary Table 4). The data shown are with the full range of incubation temperatures from 5ºC to 25ºC. *n* = 37,

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39, 38, and 38 incubations, respectively, for panels **a - d**, conducted using sediments from 8 different ponds for each $^{15}NO_3^-$ treatment.

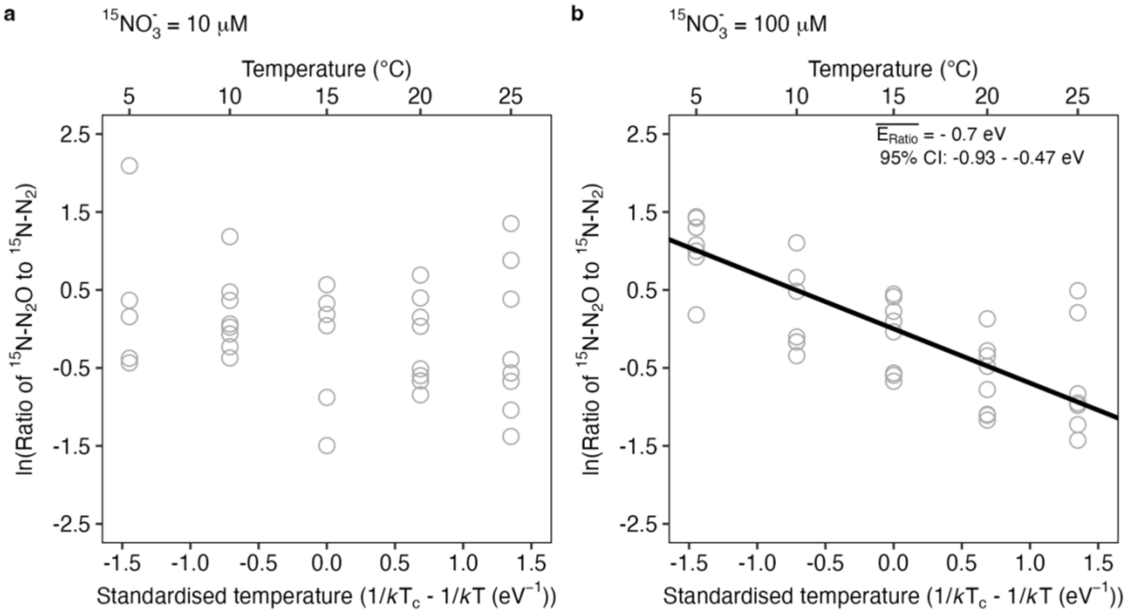

Fig. 5 | Temperature sensitivity of the ratio of $^{15}N_2O$ and $^{15}N_2$ net production from denitrification under nitrate-limited or nitrate-replete conditions. a, Ratio of net production rate of N_2O and N_2 was consistent between different temperatures with 10 μM $^{15}NO_3^-$ added. **b**, Ratio 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. We visualized the data using the "Visreg" package in R, with the solid line in **b** showing the best-fitting linear mixed-effect model (Supplementary Table 4). The data shown are with the full range of incubation temperatures from 5ºC to 25ºC. *n* = 35 and 38 incubations, respectively, for panels **a** and **b**, from 8 ponds for each $^{15}NO_3^-$ treatment.

Discussions

From the meta-data analysis of previous studies, both net N_2O and N_2 production from denitrification increased at higher temperatures, with apparent activation energies of 0.41 eV and 0.6 eV, respectively (Fig. 1). The temperature response of net N_2O and N_2 production appeared consistent across aquatic sediments and terrestrial soils, but available data from aquatic sediments remain sparse. In addition, the N_2O:N_2 ratio declined at higher temperatures, reflecting the differing temperature sensitivities of the two gases. However, most of these studies have been carried in N-rich environments or under high NO_3^- enrichment (Table 1),

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leaving N-limited ecosystems largely unexplored. Moreover, few studies have applied ^{15}N-labelling techniques, meaning that N_2O or N_2 production have often been attributed to denitrification without excluding contributions from other microbial pathways.

Here, we addressed these limitations by applying ^{15}N-labelling techniques to directly isolate denitrification and quantify its temperature sensitivity in N-limited aquatic sediments, an ecosystem type that remains largely overlooked. Our results showed that while both net production and reduction of N_2O occurred across a temperature range of 5ºC to 25ºC under both 10 and 100 μM $^{15}NO_3^-$, significant temperature effects were only evident at the higher nitrate concentration (Fig. 4**b**, 4**d**). Under nitrate-limited conditions (10 μM $^{15}NO_3^-$), neither net N_2O production nor its reduction to N_2 responded significantly to temperature changes (Fig. 4**a**, 4**c**). Similarly, a previous study reported that both the rate and temperature sensitivity of N_2 production were substantially higher in nitrogen-enriched estuarine mesocosms compared with controls, with activation energies of 1.1 eV and 0.4 eV, respectively (Nowicki, 1994). Together, these results indicate that nitrate availability modulates the temperature sensitivity of N_2O and N_2 production from denitrification (Palacin-Lizarbe et al., 2018).

According to Arrhenius kinetics, enzyme-mediated rates generally increase with temperatures. However, when substrate availability is low, microbial activity can be constrained by substrate supply rather than enzyme kinetics, resulting in minimal temperature sensitivity. Beyond the denitrification patterns observed in this study, such invariant temperature responses under substrate limitation have also been reported for other microbial processes, including N_2O fixation (Si et al., 2023), ammonia oxidation (Horak et al., 2013; Zheng et al., 2020), and methane oxidation (Lofton et al., 2014; Szafranek-Nakonieczna et al., 2019). Under such conditions, heterotrophic denitrifiers could behave like autotrophs, which often operate under chronic substrate limitation and therefore exhibit little thermal responses.

In the high nitrate treatment (100 μM $^{15}NO_3^-$), N_2 production increased exponentially from 5 ºC to 25 ºC (Fig. 4**d**). On the contrary, net N_2O production decreased (Fig. 4**b**), resulting in lower N_2O accumulation at higher temperatures. This opposing temperature response of N_2O and N_2 production has also been reported in river sediments, where rising temperatures led to increased N_2 production and decreased N_2O production (Silvennoinen et al., 2008). Similar patterns have been found in soils - with N_2 production increasing while net N_2O production declined from 10 ºC to 30 ºC (Bailey, 1976). Elevated N_2O emissions have also been documented in soils under very low temperatures – for example, at -1 ºC compared to above 0 °C (Wertz et al., 2013), at 0 °C compared to 5 °C (Holtan-Hartwig et al., 2002), and at 4 °C compared to 20 °C (Melin and Nômmik, 1983). Together, these findings indicate that while increasing temperature enhances N_2 production via complete denitrification, it does not necessarily lead to increased net N_2O production.

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As N_2 production exhibited greater temperature sensitivity than net N_2O production in freshwater communities (Fig. 4), the ratio of N_2O to N_2 production declined at higher temperatures (Fig. 5). This is consistent with previous studies showing that while N_2 production from denitrification increases with temperature in both aquatic (Brin et al., 2014; 2017; Nowicki, 1994; Rysgaard et al., 2004; Seitzinger et al., 1984; Silvennoinen et al., 2008; Veraart et al., 2011) and terrestrial ecosystems (Bailey, 1976; Castaldi, 2000; Holtan-Hartwig et al., 2002; Keeney et al., 1979; Lai and Denton, 2018; Qin et al., 2014; Yu et al., 2023), lower product ratios of net N_2O to N_2 at higher temperatures were found in studies that have characterised the temperature sensitivity of both net N_2O to N_2 in soils (Bailey, 1976; Keeney et al., 1979; Lai and Denton, 2018; Maag and Vinther, 1996) and river sediments (Silvennoinen et al., 2008). Moreover, biogeochemical modelling supports this pattern: for example, the ratio of N_2O to N_2 from denitrification was predicted to decrease with warming, leading to a 6% decrease in N_2O emissions in response to a 1.8°C temperature increase in European forest soils (Kesik et al., 2006).

These findings suggest that N_2O production and its reduction to N_2 during denitrification exhibit differing temperature sensitivities. On possible reason is that N_2O production has a lower activation energy than its reduction to N_2, resulting in a declining N_2O:N_2 production ratio at increasing temperature. Supporting this, incubations of Arctic soils showed that the abundance of *nosZ* - the gene encoding N_2O reductase - was higher at 10°C than at 4°C, whereas the abundance of *norB*, which is involved in N_2O production, decreased with rising temperature (Jung et al., 2011). Additional studies in soils have suggested that increased N_2O emissions at low temperatures may result from the inhibited activity of N_2O reductase under cold conditions (e.g., around 0ºC) (Holtan-Hartwig et al., 2002; Öquist et al., 2007). Conversely, N_2O reduction to N_2 may be enhanced at higher temperatures, potentially due to reduced oxygen solubility or lower oxygen concentrations driven by elevated respiration relative to photosynthesis (Smith, 1997; Veraart et al., 2011).

Our results also showed that both net N_2O production and the N_2O: N_2 ratio decreased with increasing temperature (Fig. 4, Fig. 5), indicating greater relative accumulation of N_2O at colder temperatures. This additional N_2O pool could be available for other nitrogen cycling processes, including N_2O fixation, as suggested by our earlier work showing higher N_2O fixation:N_2 fixation ratios under cold conditions (Si et al., 2023). Such a shift may represent a nitrogen-conserving strategy in nitrogen-limited ecosystems, particularly when N_2 fixation is energetically less favourable, highlighting a potential link between denitrification and N_2O fixation in cold environments.

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Conclusions

Here, we characterised the combined effects of temperature and nitrate availability on net N_2O and N_2 production from denitrification in understudied N-limited aquatic sediments. Our results showed that the effects of warming on N_2O and N_2 production from denitrification are strongly modulated by nitrate availability. In N-limited ecosystems, substrate availability may outweigh temperature in determining the balance between N_2O to N_2 production. Under high nitrate concentrations, warming enhanced complete denitrification by reducing fixed nitrogen into N_2, without necessarily increasing emissions of the atmospherically potent gas N_2O. These findings highlight the need to consider both nutrient availability and temperature when assessing N_2O emissions and nitrogen balance in natural waters, as neglecting nutrient limitation may overestimate the impacts of warming.

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Author contributions

M.T and Y.S conceived the study. Y.S performed incubations, analysed the data and wrote the manuscript. Both authors contributed to revisions of the manuscript.

Competing Interests

The authors declare no competing interests.

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