







COMMUNICATIONS earth & environment

ARTICLE


<https://doi.org/10.1038/s43247-021-00104-y>

OPEN

Biological nitrous oxide consumption in oxygenated waters of the high latitude Atlantic Ocean

Andrew P. Rees ¹, Ian J. Brown ¹, Amal Jayakumar ², Gennadi Lessin ¹, Paul J. Somerfield¹ & Bess B. Ward ²

Nitrous oxide (N₂O) is important to the global radiative budget of the atmosphere and contributes to the depletion of stratospheric ozone. Globally the ocean represents a large net flux of N₂O to the atmosphere but the direction of this flux varies regionally. Our understanding of N₂O production and consumption processes in the ocean remains incomplete. Traditional understanding tells us that anaerobic denitrification, the reduction of NO₃⁻ to N₂ with N₂O as an intermediate step, is the sole biological means of reducing N₂O, a process known to occur in anoxic environments only. Here we present experimental evidence of N₂O removal under fully oxygenated conditions, coupled with observations of bacterial communities with novel, atypical gene sequences for N₂O reduction. The focus of this work was on the high latitude Atlantic Ocean where we show bacterial consumption sufficient to account for oceanic N₂O depletion and the occurrence of regional sinks for atmospheric N₂O.

¹Plymouth Marine Laboratory, Prospect Place, Plymouth, UK. ²Department of Geosciences, Guyot Hall, Princeton University, Princeton, NJ, USA.
email: apre@pml.ac.uk

N₂O is now the 3rd most important greenhouse gas¹ and number one stratospheric ozone depleting compound². Constraining the magnitude of production and consumption processes is therefore essential to understand how the biotic environment contributes to atmospheric concentrations³. Sixty percent of the increasing atmospheric concentration has a natural source, approximately 30% of which is produced in coastal and oceanic waters⁴. Canonical understanding of the nitrogen cycle tells us that the only biological process that consumes N₂O is the final stage of denitrification⁵ where N₂O is reduced to N₂ under anoxic conditions by the enzyme nitrous oxide reductase (N₂OR), encoded by the *nosZ* gene⁶.

Whilst the global ocean is considered on balance to represent a strong source of N₂O to the atmosphere⁴, this source is not uniformly distributed. Regions of the northern and southern polar waters are often undersaturated and provide a sink for atmospheric N₂O^{7–10}. Indeed from the limited number of observations made, it appears that undersaturation is the prevailing condition for the ice-free surface waters of the Arctic^{11–13}. Zhan et al.^{10,13} argued that the extant undersaturation must be driven solely by physical processes, whilst Verdugo et al.¹⁴ proposed potential biological mechanisms.

Experimental evidence indicates the potential for dissolved N₂O to be biologically consumed, even when oxygen is replete. The bacteria *Paracoccus pantotrophus* (previously *Thiosphaera pantotropha*) and *Pseudomonas stutzeri* have been shown under laboratory conditions to utilise both nitrate and oxygen as terminal electron acceptors in respiration during a process termed aerobic denitrification^{15,16}. Aerobic denitrifiers have now been observed in diverse environments which include freshwater¹⁷ and saline¹⁸ wastewater treatments, in soils¹⁹ and in coastal marine sediments²⁰. Whilst there are no reported observations of N₂O consumption in oxygenated ocean waters, several authors have indicated the presence of microbial communities with the potential to reduce N₂O^{21–26}. In the Arabian Sea, Wyman et al.²⁴ found a close affiliation between a group of alphaproteobacteria expressing *nosZ* and *Trichodesmium*, a colonial, nitrogen fixing cyanobacterium. Coates and Wyman²¹ extended the geographical coverage of these observations to include the tropical and subtropical Red Sea, Atlantic and Indian Oceans, and hypothesised denitrifying activity associated with anoxic microsites within the cyanobacterial colony. Raes et al.²³ reported *nosZ* genes associated with *Rhodobacteraceae* in oxic waters of the south-eastern Indian Ocean whilst Farias et al. reported the potential for cultured and natural diazotrophs to consume N₂O, though under conditions far in excess of ambient concentrations²².

The *nosZ* gene occurs as two equally abundant but distinct clades, one of which had previously been unaccounted for with respect to the reduction of N₂O²⁷. The well characterised conventional denitrifiers of the alpha-, beta- and gamma- proteobacteria are grouped as Clade I, whereas Clade II organisms possess the novel, atypical *nosZ* gene sequences that are found in organisms previously not associated with denitrification, including Firmicutes, CFB (Cytophaga-Flavobacteria-Bacteroides) supergroup and Verrucomicrobia. Sun et al.²⁶ showed that Clade II genes were particularly abundant in oxygen minimum zones (OMZs), even in surface waters, and suggested that they might be associated with aerobic N₂O reduction. Both Clades I and II of the *nosZ* gene were detected in several geographical regions associated with OMZ of the Eastern Tropical Pacific and the Arabian Sea, and were also observed in oxygenated surface waters of the Arctic and Southern Oceans²⁸. Neither N₂O production rates nor the distribution of N₂O consuming microbes has been investigated in the high latitude waters represented by this study.

There are regions of the polar oceans which are undersaturated in N₂O relative to the atmosphere and therefore offer

a sink to atmospheric N₂O, this is recognised^{10,13} but not wholly explained. During two high latitude research expeditions in northern and southern waters of the Atlantic Ocean, including those presented in²⁸ we performed incubation experiments and collected samples which indicated that N₂O was consumed and that both Clade I and II denitrifiers were present in near surface waters. In the North Atlantic, measurements of dissolved N₂O confirmed the existence of an N₂O sink throughout the upper 100 m of the water column, with saturations in the upper 50 m of 90 ± 1% for areas north of 58.44° N. The consumption of N₂O was observed at the base of the upper mixed layer (40 to 85 m) where rates were comparable to rates of net production. In near surface waters, production of N₂O was not detected and the mean rates of N₂O consumption were greater than the sum of the N₂O supply from diapycnal transport of deep waters and surface exchange with the atmosphere. The determination of significant rates of N₂O consumption coincident with the detection of organisms possessing atypical gene sequences for N₂O reduction provides evidence of a biological mechanism that contributes to N₂O sink conditions in these high latitude waters.

Results

Research cruise JR271 onboard the RRS James Clark Ross during June 2012 made observations at 14 stations on a track between the northern North Sea, Greenland Sea, Fram Straits and the Barents Sea (Supplementary Fig. 1; Table 1). At five of these stations (E01 – E05), dissolved N₂O was assayed at time of collection and then at 48 and 96 h in seawater from the near surface (10–20 m), which had been incubated under in-situ conditions of light and temperature for four days. At four out of the five stations N₂O concentration decreased over the 96 h period at rates of between 0.17 and 0.31 nM d⁻¹ (linear regression of concentration over time: $r^2 = 0.97, 0.99, 0.72, 0.78$ for E01 to E04 respectively, Table 1). Logistical constraints meant that there was no sample replication within these experimental treatments and so data from the 5 stations were pooled and treated as replicates. To account for the heterogeneity of near-surface conditions, N₂O concentrations were normalised to the initial ambient concentration and presented as percentage of the time zero value (Fig. 1a). The linear regression of N₂O through time, averaged across the whole set of observations reveals a significant loss of N₂O (adjusted $r^2 = 0.439, p < 0.005$). At stations E01 to E04 the decrease equates to removal of 1.4–2.4% of the ambient N₂O concentration per day. There was no indication of N₂O removal at station E05 in the Barents Sea, here the mean N₂O concentration remained at 10.37 ± 0.04 nM over the four-day period. Removing this station from the linear regression of decreasing N₂O over time improved the fit to an adjusted r^2 of 0.721 ($p < 0.0005$).

At the same five stations, seawater was also collected and incubated, this time in triplicate, from the base of the seasonal upper mixed layer (40–60 m), depths that were generally coincident with, or close to, maxima in the N₂O concentration profile. All incubated samples showed an increase in N₂O concentration over the first 48 h with net rates of N₂O production (change in concentration over time) from 0.2–3.3 nMd⁻¹ followed by a decrease in concentration (net consumption) of 1.0–3.7 nMd⁻¹ over the subsequent 48 h (Fig. 1b, Table 1). Again using the pooled measurements from the 5 experiments as replicates, a one-way ANOVA showed that the mean N₂O concentrations at each time point were significantly different ($F_{2,12} = 10.2, p < 0.003$). After 96 h of incubation, N₂O had decreased in four (E01–E04) out of five incubations to a level below that of the initial concentration.

Table 1 Location of experiments and environmental variables encountered during JR271 (June 2012) and JR274 (January–February 2013).

Station	Latitude	Longitude	Depth (m)	Temp (°C)	NO ₃ ⁻ (μM)	NH ₄ ⁺ (nM)	O ₂ (μM)	N ₂ O concn. (nM)	N ₂ O production (nMd ⁻¹)	N ₂ O consumption (nMd ⁻¹)	Location
JR271											
E01	56.267°N	02.633°E	10	8.50	0.02	4.2	311	10.7	ND	0.18	North Sea
			50	6.70	0.65	44	269	11.5	2.97	3.13	
E02	60.594°N	18.857°W	20	10.44	5.2	163	300	9.7	ND	0.23	South of Iceland
			60	9.49	9.2	452	281	10.3	3.32	3.65	
E03	76.175°N	02.549°W	19	1.55	9.3	67	361	12.2	ND	0.16	Greenland Sea
			60	0.16	10.1	502	360	12.9	0.77	1.02	Ice-Edge
E04	78.353°N	03.664°W	10	-1.61	6.0	88	370	15.1	ND	0.31	Greenland Sea
			40	-1.76	7.8	69	345	14.9	0.17	1.16	In-Ice
E05	72.892°N	26.001°E	9	6.47	5.8	76	320	10.4	ND	ND	Barents Sea
			60	6.01	8.8	178	304 (†306)	10.4	1.81	1.79	
JR274											
B03	52.69°S	36.63°W	85	0.89	25.8	1196	320 (†323)	15.6	1.10	1.39	North of South Georgia
B04	58.08°S	25.93°W	46	-1.2	21.5	893	342 (†335)	15.8	0.58	0.70	East of S. Sandwich Islands

ND not detected, † O₂ concentration after 96 h of incubation, measurements at 0, 24, 48 and 96 h showed coefficient of variation of 1.1 and 1.4% for B03 and B04 respectively.

In order to interrogate the pattern of production and apparent consumption of N₂O, further incubations were performed at Stations BO3 and BO4 in the South Atlantic during research expedition JR274 onboard RRS James Clark Ross (Supplementary Fig. 1). Similar incubations performed in triplicate on water from the base of the mixed layer were performed with an increased sampling resolution over a 5 day period (Fig. 1c, Table 1). The same pattern of production (linear regression analysis of the combined dataset over the first 48 h; slope = +0.035 ± 0.007, adjusted $r^2 = 0.83$, $p = 0.007$) followed by consumption (slope = -0.041 ± 0.01, adjusted $r^2 = 0.741$, $p < 0.02$) as in northern waters was observed. Production and consumption rates were comparable to those in the north (Table 1) and consumption continued up to 120 h after the start of the incubation. At 120 h the observed concentration of N₂O at both stations was lower than both the start point and the atmospheric equilibration value. 2.0 and 0.9 nM of N₂O were removed from sites in the vicinity of South Georgia and the South Sandwich Islands, equivalent to 8.9% and 4.4% per day of ambient concentrations respectively.

The first step of nitrification, the aerobic oxidation of ammonium to nitrite, is the dominant source of N₂O in the ocean except for in surface water overlying oxygen minimum zones²⁹, but measurements of nitrification are limited in the polar regions. The observations of N₂O production presented in Table 1 for samples collected from the base of the upper mixed layer are higher than those reported previously elsewhere³⁰, which are typically less than 1.7% of nitrite production. These rates of N₂O production are not entirely unexpected if associated with high rates of nitrification, such as reported for the Southern Ocean³¹ and the North Sea³², and considering that N₂O yield in culture of up to 10% have been recorded³³. A N₂O yield of 1.7% would imply nitrification rates of 10–195 nmol L⁻¹d⁻¹ at these stations. The pattern of reversal observed between production and consumption (Fig. 1b, c) during incubations argues that production is limited by a finite substrate not identified here, but likely NH₄⁺: NH₃, and that the observed consumption maintains a tight balance between the two processes.

nosZ gene abundance and diversity. Diverse *nosZ* genes, both in DNA and cDNA and including both conventional Clade I and atypical Clade II sequences, were detected in every sample (Fig. 2). Thus a surprising range of microbes, many of which were actively transcribing the functional gene for N₂O reduction, were

detected in oxygenated surface waters of these high latitude regions. The species richness of cDNA Archetypes (an Archetype represents organisms possessing a *nosZ* gene sequence with ≥ 87% identity to the probe sequence) exceeded that of DNA Archetypes at all stations except EO1 and geographically cDNA and DNA species richness was significantly higher at the northern vs southern stations ($p < 0.023$) (diversity measures are reported in Supplementary Table 1). cDNA and DNA community compositions were very similar within stations, with the exception of Station BO4. Multi-response permutation procedure analysis indicated that community composition was significantly different between the North Atlantic and Southern Ocean regions ($p < 0.002$). The community compositions represented by cDNA and DNA were not different ($p < 0.875$). Among the Clade I *nosZ* genes, Archetype NosZ20 (representing cultivated denitrifying strains *Pseudomonas stutzeri* and *P. fluorescens*) was among the most abundant archetypes in the northern samples, but was a minor component of those from the Southern Ocean. Clade II Archetypes, representing atypical *nosZ* genes with greatest identity to *Anaeromyxobacter* genes, were present in all samples but dominated the *nosZ* assemblage in the southern samples. Archetypes WnosZ16 and WnosZ21 had the highest relative abundance of all WnosZ archetypes in all samples.

The suite of environmental and experimental variables collected for all samples from all stations is presented in Table 1 and Supplementary Table 2 and the Pearson correlation coefficient for these in Supplementary Table 3. Highly correlated variables were omitted from further ordination analysis. The ambient N₂O concentration was negatively correlated with temperature ($p < 0.0001$) although both N₂O production and consumption rates were positively correlated with temperature ($p < 0.0001$). Further study is required, not only to investigate the extent and distribution of these processes on geographic and seasonal scales, but also within the context of our warming environment. N₂O concentration is largely governed by temperature dependent solubility, and as water temperatures increase there is potential for greater consumption (and production) which in concert with decreased solubility indicate an increase in the polar ocean N₂O sink. Despite the geographical differences in species richness and community composition noted above, N₂O production and consumption were also correlated with *nosZ* copy number in both DNA and cDNA ($p < 0.0003$). Normalising the consumption rate to cDNA count (Fig. 3a) reveals a positive

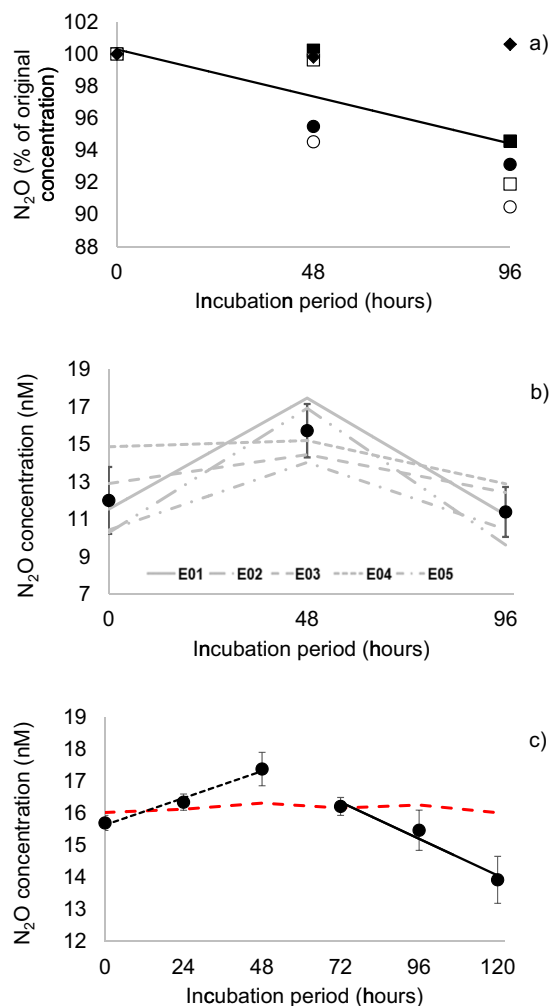


Fig. 1 N₂O in incubated samples. **a, b** From the North Atlantic during research cruise JR271 from near surface and base of the upper mixed layer respectively; **c** from cruise JR274 to the South Atlantic, samples collected from the base of the upper mixed layer at stations B03 and B04. In **a** due to the large environmental variability individual station data is presented as percentage of the original N₂O concentration. Stations were located at North Sea (E01 - filled black circle), South of Iceland (E02 - open black circle), Greenland Sea - ice edge (E03 - filled black square), Greenland Sea - in ice (E04 - open black square), Barents Sea (E05 - filled red diamond). In **b** mean N₂O concentrations at each time point, for each station are joined by the grey lines as indicated. In **b** and **c** data from all stations were combined, data points (filled black circle) represent mean concentrations ± 1 standard deviation. In **a** and **c** the linear regression line representing loss of N₂O over time is shown as the solid black line, in **c** production of N₂O over the first 48 h is represented by the dotted line, the red dashed line represents the mean expected value of N₂O concentration assuming complete equilibration with the atmosphere.

exponential relationship with temperature for Clade II archetypes which is not evident for the Clade I community.

In the redundancy analysis (Fig. 3b) combining community composition and the six most important environmental variables, the first two axes explained 49% and 37% of the variability, respectively. The two southern stations clustered closely and were distinct from those from the north. NosZ65 (*Marinobacter*-like), and NosZ -20, -22 and -42 (all derived from saltmarsh sediments) signals were positively correlated with qPCR_cDNA and were prominent in the North Sea station EO1 samples, which

can explain why station EO1 was the only one that exhibited a strong correlation with gene copy number in cDNA (qPCR-cDNA). The other northern stations at which NosZ20 was a major signal in both the DNA and cDNA clustered in the upper left quadrant and were not highly correlated with any of the environmental variables.

Discussion

At both of the polar stations (E04 and B04) presented in²⁸ there was a notable lack of the *nirS* gene, which is responsible for the reduction of NO₂⁻ during the denitrification process, though the two dominant Clade II *nosZ* archetypes were abundant. This suggests a decoupling of the denitrification pathway and provides the potential for an efficient N₂O scavenging mechanism in the absence of complete denitrification. The dominance of the WnosZ Archetypes at both of the southern stations, as well as their high relative abundance in all northern samples for both DNA and cDNA, suggests an important role for the Clade II microbes. This form of *nosZ* is preferentially associated with microbes that do not possess a full denitrification pathway, i.e., they do not possess the upstream enzymes to respire the soluble N oxides, and have been implicated in N₂O consumption in soils^{27,34}. It seems unlikely that the uncultivated microbes from agricultural soil and the cultivated *Anaeromyxobacter* strains, from which Archetypes WnosZ16 and WnosZ21, respectively, are derived, are present in the ocean. Rather, we suggest that N₂O consuming marine microbes with highly similar enzymes are performing a similar function in the ocean.

The prevalence of the Clade II *nosZ* genes and the observed consumption of N₂O at all geographical positions sampled argues very strongly that this is an important process, which had previously been unrecognised, in these high latitude waters. At the base of the upper mixed layer, the removal of N₂O by the WnosZ archetypes moderates the flux of N₂O from the deeper ocean to the near surface. In the near surface waters the rate of consumption is equivalent to the sum of fluxes from the atmosphere and deeper water (Fig. 4a) and thus provides a mechanism to contribute to the undersaturation of surface waters, which is prevalent in the polar regions^{7,11-13} and which was observed at all stations north of 58°N during this study (Fig. 4b).

The melting of sea-ice has been shown to contribute to undersaturation of N₂O in surface waters^{7,11} whilst the convection of low N₂O water from the deep ocean coupled with air-sea exchange is used by Zhan et al.¹³ to account for what they describe as a permanent sink in the Nordic Seas. Neither of these mechanisms is necessarily exclusive to our observations, indeed all three are likely to contribute to N₂O undersaturation. This current study presents coincident observations, at both poles of the Atlantic Ocean, of Clade II archetype WnosZ abundance and robust measures of apparent N₂O consumption. This is a novel observation of the diverse presence and activity of an atypical gene sequence which enables an N₂O removal mechanism not previously observed in oxygenated seawater. Although our measurements were restricted to summertime observations in both hemispheres it is clear that N₂O consumption is occurring in oxygenated surface waters and is contributing to the presence of N₂O understaturation in high latitude waters. However, more rigorous investigations are required over greater scales of time and space before incorporating this process into budgetary exercises at regional or global scales.

Methods

Seawater samples were collected from Niskin bottles deployed on a titanium frame at the near surface (10–20 m) and at the base of the surface mixed-layer (40 to 85 m) directly into ~4.5 L polycarbonate incubation bottles (NalgeneTM) (Table 1,

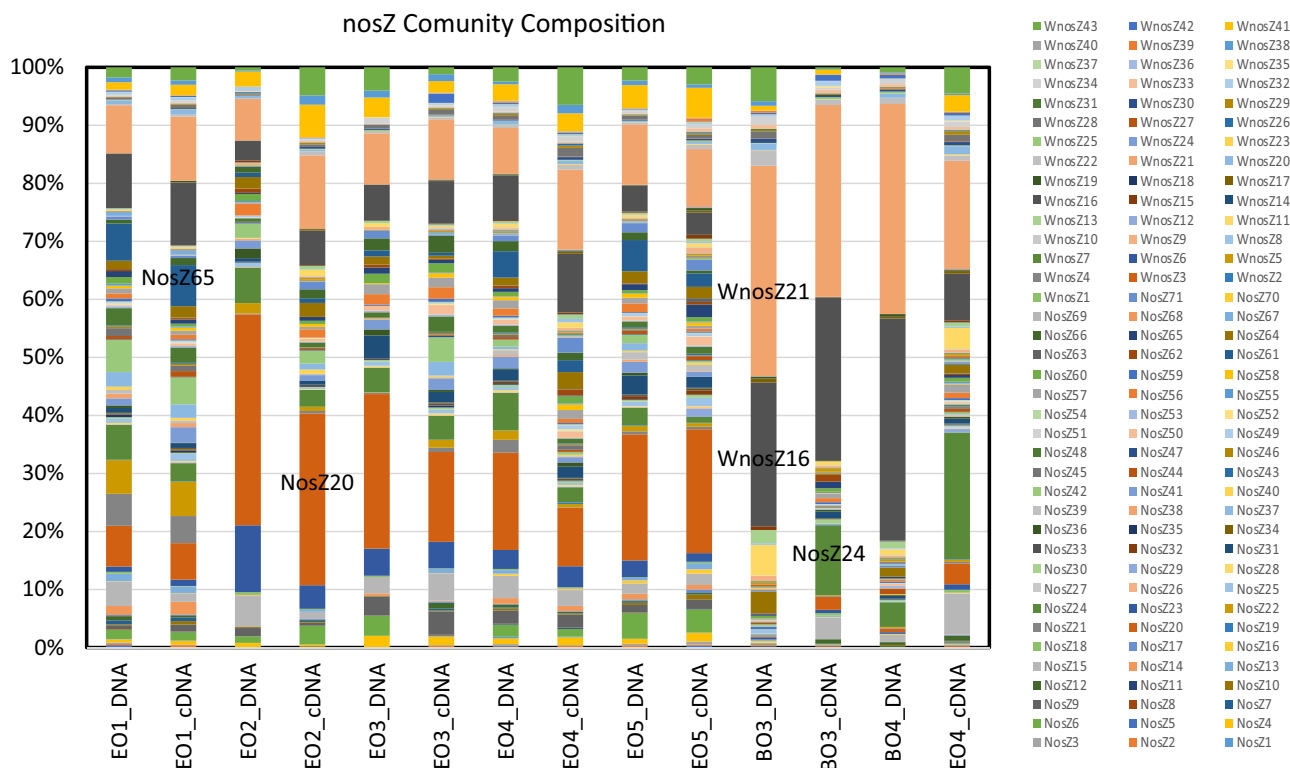


Fig. 2 Stacked bar plot of nosZ genes. Showing the community composition identified by microarray analysis during research cruises JR271 and JR274.

Supplementary Fig. 1). Bottles from the near surface were sealed and transferred into incubators at ambient temperature and simulated light conditions to match those of collection. N₂O concentration was determined on single bottles immediately and then at several time points up to 96 h later. Sample bottles collected from the base of the surface mixed-layer were sealed and incubated in the dark at ambient temperature within a purpose-built experimental laboratory container allowing precise temperature control. N₂O concentration was determined on triplicate bottles immediately and then at several time points up to 120 h later. Temperature within a dummy incubation bottle was monitored using a traceable thermometer, while two recording thermometers were used to monitor air temperature in the incubator. Oxygen was determined by Winkler titration at the beginning of the experiment and for selected stations E05, B03 and B04 after 48 and 96 h. Samples were also collected for N₂O analysis from CTD casts performed at 14 stations including the five experimental stations during JR271 only (Supplementary Fig. 1).

At six positions on a transect through the Atlantic Ocean between the UK and the Falkland Islands (Supplementary Fig. 1), tests were performed to confirm the validity of this approach and to test the integrity of the polycarbonate bottles to N₂O diffusion. At each station up to 24 bottles were filled with seawater, sealed and incubated in the dark at collection temperature. A second set of bottles were collected which were poisoned with the addition of 1 ml saturated HgCl₂. N₂O concentration was determined on collection, and thereafter triplicate analyses of N₂O were made at several time points over 6 days. N₂O concentration remained stable during each of these storage tests (Supplementary Fig. 2), the coefficient of variation varied between 1.3 and 5.2% (mean 3.9%, *n* = 16 time points × 3 analyses). F tests between N₂O in killed samples and initial concentrations, and between killed samples and the expected (atmospheric equilibrium) concentration confirmed 86% and 87% similarity in variance respectively.

N₂O analysis. Samples were collected using acid cleaned Tygon tubing directly from CTD Niskin bottles or by siphoning from 4.5 L incubation bottles into 1 L borosilicate flasks. Single samples were taken from CTD bottles and triplicates from the incubated sample. Samples were overfilled in order to expel air bubbles, poisoned with 200 μL of saturated HgCl₂ solution and temperature equilibrated at 25.0 ± 0.5 °C. In all cases samples were analysed within 8 h of collection. N₂O was determined by single-phase equilibration gas chromatography with electron capture detection similar to that described by Upstill-Goddard et al.³⁵. Each individual sample was calibrated against three certified (± 5%) reference standards of 287, 402 and 511 ppb (Air Products Ltd) which are traceable to NOAA WMO-N₂O-X2006A scale for N₂O mole fractions. Mean instrument precision from daily, triplicate analyses of the three calibration standards (*n* = 81) was 0.95%. Concentrations of N₂O in seawater were calculated from solubility tables³⁶ at equilibration temperature (~25 °C) and salinity. To capture variability within experimental treatments at the appropriate scale, mean values from each location-

time combination were used as replicates in standard statistical analyses (linear regression and analysis of variance), which were performed using the Data Analysis ToolPak add-in module in Excel for Microsoft 365.

Air-sea flux. The exchange of N₂O between the ocean and the atmosphere, the sea-air flux density (*F*_{N₂O}), was estimated from:

$$F_{N2O} = (K_w(S_c/600)^{0.5}) \cdot (C_w - C_a)$$

Where *K_w* is the gas exchange coefficient adjusted for *S_c* the Schmidt number for N₂O as described in ref.³¹. *C_w* is the measured seawater concentration and *C_a* is the equilibrium concentration of N₂O in seawater based on the measured atmospheric value.

Diapycnal flux. The flux of N₂O from below the pycnocline to the surface layer (*Q_N*) was estimated from³²:

$$Q_N = K_z \cdot \delta N / \delta z$$

where: $\delta N / \delta z$ is the vertical gradient of N₂O across the pycnocline, and *K_z* is the vertical turbulent diffusion coefficient, estimated from

$$K_z = 0.24 \cdot \epsilon / N^2$$

where ϵ is the turbulent kinetic energy dissipation, and *N* is the buoyancy (Brunt-Vaisala) frequency

$$N^2 = -g / \rho \cdot \Delta \rho / \Delta z$$

where *g* is gravitational acceleration, ρ is the density and $\Delta \rho / \Delta z$ is the vertical density gradient across the pycnocline.

Nucleic acid manipulations and quantitative PCR analysis. Seawater samples (up to 8 L) were filtered onto 0.2 μm pore size Sterivex filters (Millipore, Billerica, MA) using a peristaltic pump, and filters were flash frozen in liquid nitrogen and stored at -80 °C. Total DNA and RNA were both extracted from the same Sterivex capsule filters using the ALLPrep DNA/RNA Mini Kit (Qiagen Sciences, Maryland, USA). Only one filter was extracted for each depth, i.e., no biological replicates. Reverse transcription from RNA to cDNA was performed using SuperScript R III First-Strand Synthesis System for RT-PCR (Invitrogen™ by Life Technologies™). Any remaining RNA was removed by RNase at the end of the synthesis. PCR and qPCR using SYBR Green and the Qiagen master mix (Qiagen Sciences, Maryland, USA) (as described for seawater samples³⁷) were used to amplify a 259 bp fragment of the nosZ gene using primers nosZ1F (WCSYTGTTTCMTCGACAGCCAG) and nosZ1R (ATGTCGATCARCTGVKCRITYTC)³⁸. Standardisation and verification of specificity for qPCR assays was performed as described in²⁸.

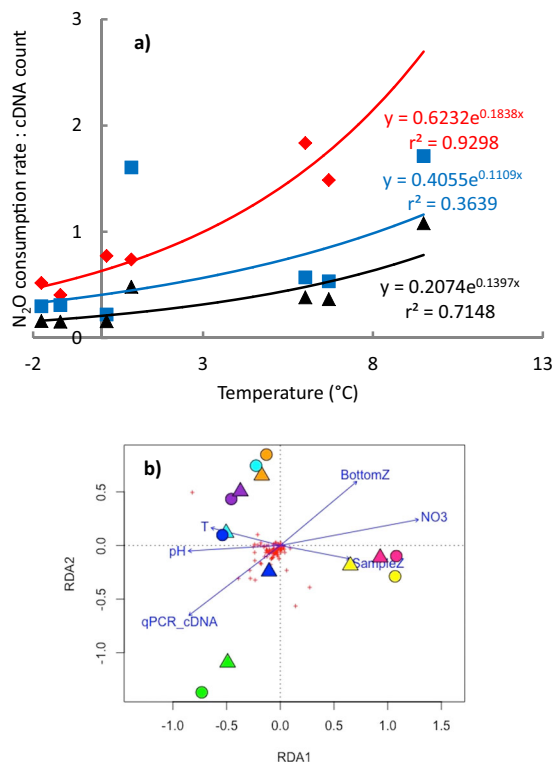


Fig. 3 Relationship between Clade I and Clade II nosZ archetypes and environmental variables for samples collected at the base of surface mixed layer in the North and South Atlantic. **a** N_2O consumption rate normalised to cDNA count (Red = Clade II, Blue = Clade I, Black = total), **b** RDA plot showing relationships between environmental variables (T = temperature, BottomZ = water column depth, SampleZ = depth sample collected) and NosZ/WnosZ community compositions based on Archetypes (red cross) from the microarray analysis. Circles = DNA, triangles = cDNA. Green = EO1, orange = EO2, cyan = EO3, blue = EO4, purple = EO5, pink = BO3, yellow = BO4.

Primers nosZ1F and nosZ1R targeted the Clade I genes; Clade II genes were not amplified separately. *Rhodospseudomonas palustris* (Clade I), was used as a positive control to optimise the reaction and to construct a plasmid for use as a standard in the qPCR assays. The amplified products were visualised after electrophoresis in 1.0% agarose gels stained with ethidium bromide. Assays were carried out within a single assay plate³⁹. Each assay included triplicates of the no template controls (NTC), no primer control (NPR), four or more serial dilutions for the standard curve, and triplicates of known quantity of the environmental DNA samples (20–25 ng). DNA was quantified using Pico Green fluorescence (MolecularProbes, Eugene, OR) calibrated with several dilutions of phage lambda standards. Quantitative PCR was performed using a Stratagene MX3000P (AgilentTechnologies, LaJolla, CA, USA). Automatic analysis settings were used to determine the threshold cycle (Ct) values. The copy numbers were calculated according to:

$$\text{Copy number} = (\text{ng} \times \text{number per mole}) / (\text{bp} \times \text{ng per g} \times \text{g per mole of bp})$$

and then converted to copy number ml^{-1} seawater filtered assuming 100% extraction efficiency. To maintain continuity and consistency among qPCR assays a subset of samples from the first assay was included in subsequent assays, as well as fresh dilution series for standard curves on every assay. Template DNA and plasmid DNA were quantified prior to every assay as above using Pico Green fluorescence to account for DNA loss that occurs upon repeated freeze-thaw cycles.

Microarray. The microarray (BC016) was developed following the archetype array approach described and employed previously (e.g.^{40,41}). An archetype is defined as all sequences that hybridise with an individual probe, here within 87% identify to the probe sequence. Each 90-mer oligonucleotide probe included a nosZ-specific 70-mer region and a 20-mer control region (5'-GTACTACTAGCCTAGGCTAG-3') bound to a glass slide. The design and spotting of the probes has been described previously⁴⁰. Microarray BC016 contained two probe sets (NosZ and WnosZ) for the nosZ gene²⁹. Clade I nosZ, most commonly found in marine and terrestrial heterotrophic denitrifying bacteria and associated with cultured strains representing

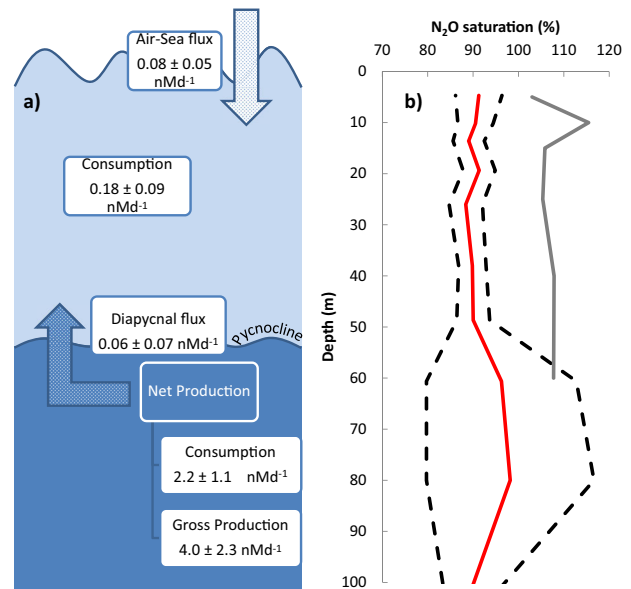


Fig. 4 Summary budget of N_2O fluxes and indication of N_2O undersaturation in northern Atlantic waters during research cruise JR271. **a** Mean (± 1 s.d.) rates of production, consumption & fluxes of N_2O observed for stations E01 to E05 inclusive. Gross production assumes that consumption processes are constant throughout, which the experimental design did not resolve. **b** Mean N_2O saturation (red line) ± 1 s.d. (dashed lines) for 13 stations north of 58.44°N during the same cruise. Grey line represents N_2O saturation at the North Sea station E01 (56.16°N).

alpha-, beta-, and gamma-Proteobacteria, was represented by 71 NosZ probes derived from whole genome sequences in public databases plus sequences obtained from clone libraries made using DNA extracts from the Great Sippewissett Marsh in Falmouth, MA, USA⁴². An additional 43 WnosZ probes were included to capture the atypical Clade II nosZ sequences^{27,34}. Cultivated members of the atypical WnosZ probe set include alpha-, beta- and delta-Proteobacteria, CFB supergroup, Firmicutes and Verrucomicrobia. The probe accession numbers and sequences are listed in Table S2 and the phylogenetic trees of the probe sequences are found in²⁸. Arrays were printed on glass slides⁴³ by Microarrays, Inc. (Huntsville, AL, USA). Targets were prepared from the amplified DNA produced in the qPCR assays above according to⁴¹. DNA concentration of the targets were measured on a Nanodrop spectrophotometer and the volume required for 200 ng of DNA was hybridised to duplicate arrays overnight in sealed tubes and then washed according to⁴¹. Arrays were scanned with an Agilent laser scanner 4300 (Agilent, Palo Alto, CA) and analysed using GenePix 6.0 software. Replicate features on the same array were averaged to calculate the Cy3/Cy5 ratio for each probe. Relative fluorescence ratio (RFR, the fraction that each probe fluorescence (Cy3/Cy5 ratio) contributes to the total fluorescence of the probe group) and normalised fluorescence ratio (FRn, the Cy3/Cy5 ratio of each probe normalised to the maximum Cy3/Cy5 detected on that array for the probe group) were used for plotting and statistical analysis. The vegan package in R (CRAN website; <http://www.R-project.org>)⁴⁴ was used for ordination and diversity analysis of the array data. FRn values were transformed (Arcsin(Square root)) in order to normalise the proportional data. Archetypes with FRn < 0.01 were considered absent. Environmental data were standardised around zero (decostand in vegan). The transformed data were used in redundancy analysis (RDA) using the vegan package in R⁴⁵.

Data availability

All N_2O data from this study are available from the British Oceanographic Data Centre according to <https://doi.org/10.5285/268dfd3b-dcc6-3f4a-e053-6c86abc0c2f9>⁴⁶. The original nosZ array data are available at Gene Expression Omnibus (<http://www.ncbi.nlm.nih.gov/projects/geo/>) at the National Center for Biotechnology Information under GEO Accession No. GSE121473. Further supporting datasets from both cruises (JR271 and JR274) can be found at: https://www.bodc.ac.uk/projects/data_management/uk/ukoa/data_inventories/cruise. All data used in Figs. 1–4 are available either from the locations above or are found within Table 1 and supplementary information.

Received: 3 June 2020; Accepted: 20 January 2021;

Published online: 18 February 2021

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Acknowledgements

We thank the captains and crew of the RRS James Clark Ross during research cruises JR271, JR274 and JR300. We are grateful to Eric Acterberg, Richard Sanders, Mark Moore and Mark Stinchcombe for nutrient and oxygen measurements and acknowledge the contributions made to this work by Vas Kitidis, Darren Clark and Phil Nightingale. This work was funded by the UK Natural Environment Research Council Grant, UKOA-Ocean Acidification impacts on sea surface biology, biogeochemistry & climate (NE/H017259/1) and further by NERC National Capability funding as part of the Climate Linked Atlantic Section Science (CLASS) programme (NE/R015953/1).

Author contributions

A.R., I.B., and B.W. designed the research; A.R. and I.B. measured and analysed the N₂O; A.J. and B.W. measured and analysed *nosZ*; Statistical analysis of the N₂O experiments was by PS; Interpretation of data and writing of manuscript was led by A.R. with contributions from B.W., G.L., P.S., I.B. and A.J.

Competing interests

The authors declare no competing interests.

Additional information

Supplementary information The online version contains supplementary material available at <https://doi.org/10.1038/s43247-021-00104-y>.

Correspondence and requests for materials should be addressed to A.P.R.

Peer review information Primary handling editor: Teresa Ortner.

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