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Published in: Biogeosciences

DOI: 10.5194/bg-17-2263-2020

Publication date: 2020

Document version: Final published version

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Regulation of nitrous oxide production in low-oxygen waters off the coast of Peru

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Received: 3 December 2019 – Discussion started: 9 December 2019
Revised: 1 March 2020 – Accepted: 19 March 2020 – Published: 22 April 2020

Abstract. Oxygen-deficient zones (ODZs) are major sites of net natural nitrous oxide (N2O) production and emissions. In order to understand changes in the magnitude of N2O production in response to global change, knowledge on the individual contributions of the major microbial pathways (nitrification and denitrification) to N2O production and their regulation is needed. In the ODZ in the coastal area off Peru, the sensitivity of N2O production to oxygen and organic matter was investigated using 15N tracer experiments in combination with quantitative PCR (qPCR) and microarray analysis of total and active functional genes targeting archaeal amoA and nirS as marker genes for nitrification and denitrification, respectively. Denitrification was responsible for the highest N2O production with a mean of 8.7 nmol L−1 d−1 but up to 118 ± 27.8 nmol L−1 d−1 just below the oxic–anoxic interface. The highest N2O production from ammonium oxidation (AO) of 0.16 ± 0.003 mmol L−1 d−1 occurred in the upper oxycline at O2 concentrations of 10–30 µmol L−1 which coincided with the highest archaeal amoA transcripts/genes. Hybrid N2O formation (i.e., N2O with one N atom from NH4+ and the other from other substrates such as NO3−) was the dominant species, comprising 70%–85% of total produced N2O from NH4+. Regardless of the ammonium oxidation rate or O2 concentrations, oxygen responses of N2O production varied with substrate, but production and yields were generally highest below 10 µmol L−1 O2. Particulate organic matter additions increased N2O production by denitrification up to 5-fold, suggesting increased N2O production during times of high particulate organic matter export. High N2O yields of 2.1% from AO were measured, but the overall contribution by AO to N2O production was still an order of magnitude lower than that of denitrification. Hence, these findings show that denitrification is the most important N2O production process in low-oxygen conditions fueled by organic carbon supply, which implies a positive feedback of the total oceanic N2O sources in response to increasing oceanic deoxygenation.

1 Introduction

Nitrous oxide (N2O) is a potent greenhouse gas (IPCC, 2013) and precursor for nitric oxide (NO) radicals, which can catalyze the destruction of ozone in the stratosphere (Crutzen, 1970; Johnston, 1971) and is now the single most important ozone-depleting emission (Ravishankara et al., 2009). The ocean is a significant N2O source, accounting for up to one-third of all natural emissions (IPCC, 2013), and this source may increase substantially as a result of eutrophication, warming and ocean acidification (see, e.g., Capone and Hutchins, 2013; Breider et al., 2019). Major sites of oceanic N2O emissions are regions with steep oxy-
gen (O\textsubscript{2}) gradients (oxycline), which are usually associated with coastal upwelling regions with high primary production at the surface. There, high microbial respiratory activity during organic matter decomposition leads to the formation of anoxic waters also called oxygen-deficient zones (ODZs), in which O\textsubscript{2} may decline to functionally anoxic conditions (O\textsubscript{2} < 10 nmol kg\textsuperscript{-1}, Tiano et al., 2014). The most intense ODZs are found in the eastern tropical North Pacific (ETNP), the eastern tropical South Pacific (ETSP) and the northwestern Indian Ocean (Arabian Sea). The anoxic waters are surrounded by large volumes of hypoxic waters (below 20 µmol L\textsuperscript{-1} O\textsubscript{2}), which are strong net N\textsubscript{2}O sources (Codispoti, 2010; Babbin et al., 2015). Latest estimates of global, marine N\textsubscript{2}O fluxes (Buitenhuis et al., 2018; Ji et al., 2018a) agree well with the 3.8 Tg N yr\textsuperscript{-1} (1.8–9.4 Tg N yr\textsuperscript{-1}) reported by the IPCC (2013) but have large variability in the resolution on the regional scale, particularly along coasts where N\textsubscript{2}O cycling is more dynamic. The expansion of ODZs is predicted in global change scenarios and has already been documented in recent decades (Stramma et al., 2008; Schmitzko et al., 2017). This might lead to further intensification of marine N\textsubscript{2}O emissions, which will constitute a positive feedback on global warming (Battaglia and Joos, 2018). However, decreasing N\textsubscript{2}O emissions have also been predicted based on reduced nitrification rates due to reduced primary and export production (Martinez-Rey et al., 2015; Landolfi et al., 2017) and ocean acidification (Beman et al., 2011; Breider et al., 2019). The parametrization of N\textsubscript{2}O production and consumption in global ocean models is crucial for realistic future predictions, and therefore better understanding of their controlling mechanisms is needed.

N\textsubscript{2}O can be produced by both nitrification and denitrification. Nitrification is a two-step process, comprising the oxidation of ammonia (NH\textsubscript{3}) to nitrite (NO\textsubscript{2}) (ammonia oxidation, AO) and NO\textsubscript{2} to nitrate (NO\textsubscript{3}) (NO\textsubscript{2} oxidation). The relative contributions to AO by autotrophic ammonia-oxidizing archaea (AOA) and ammonia-oxidizing bacteria (AOB) have been inferred, based on the abundance of the archaeal and bacterial amo\textsubscript{A} genes, which encode subunit A of the key enzyme ammonia monoxygenase (e.g., Francis et al., 2005; Mincer et al., 2007; Santoro et al., 2010; Wuchter et al., 2006). These studies consistently revealed the dominance of archaeal over bacterial ammonia oxidizers, particularly in marine settings (Francis et al., 2005; Wuchter et al., 2006; Newell et al., 2011). In oxic conditions, AO by AOB and AOA forms N\textsubscript{2}O as a by-product (Anderson, 1964; Vajrala et al., 2013; Stein, 2019), and AOA contribute significantly to N\textsubscript{2}O production in the ocean (Santoro et al., 2011; Löscher et al., 2012). While hydroxylamine (NH\textsubscript{2}OH) was long thought to be the only obligate intermediate in AO, NO has recently been identified as an obligate intermediate for AOB (Caranto and Lancaster, 2017) and presumably AOA (Carini et al., 2018). Both intermediates are present in and around ODZs and correlated with nitrification activity (Lutterbeck et al., 2018; Korth et al., 2019). Specific details about the precursor of NO to form N\textsubscript{2}O in AOA remain controversial. Stiegelmeier et al. (2014) concluded that NO is derived from NO\textsubscript{2} reduction to form N\textsubscript{2}O, while Carini et al. (2018) hypothesized that NO is derived from NH\textsubscript{2}OH oxidation, which can then form N\textsubscript{2}O. A hybrid N\textsubscript{2}O production mechanism in AOA has been suggested, where NO from NO\textsubscript{2} reacts with NH\textsubscript{2}OH from NH\textsubscript{3}\textsubscript{+}, which is thought to be abiotic, i.e., nonenzymatic (Kozlowski et al., 2016). Abiotic N\textsubscript{2}O production, also known as chemodenitrification, from intermediates like NH\textsubscript{2}OH, NO or NO\textsubscript{2} can occur under acidic conditions (Frame et al., 2017), or in the presence of reduced metals like Fe or Mn and catalyzing surfaces (Zhu-Barker et al., 2015), but the evidence of abiotic N\textsubscript{2}O production/chemodenitrification in ODZs is still lacking.

When O\textsubscript{2} concentrations fall below 20 µmol L\textsuperscript{-1}, nitrifiers produce N\textsubscript{2}O from NO\textsubscript{2}, a process referred to as nitrifier denitrification (Frame and Casciotti, 2010), which has been observed in cultures of AOB (Frame and Casciotti, 2010) and AOA (Santoro and Casciotti, 2011). During nitrifier denitrification (and denitrification), two NO\textsubscript{2} molecules form one N\textsubscript{2}O, which thus differentiates this process from hybrid N\textsubscript{2}O production. It has also been suggested that high concentration of organic particles creates high-N0\textsubscript{2} and low-0\textsubscript{2} microenvironments enhancing nitrifier denitrification (Charpentier et al., 2007). Overall, the yield of N\textsubscript{2}O per NO\textsubscript{2} generated from AO is lower in AOA than AOB (Hink et al., 2017a, b), but it should be noted that the degree to which N\textsubscript{2}O yield increases with decreasing O\textsubscript{2} concentrations varies with cell density in cultures and among field sites (Cohen and Gordon, 1978; Yoshida, 1988; Goreau et al., 1980; Frame and Casciotti, 2010; Santoro et al., 2011; Löscher et al., 2012; Ji et al., 2015b, 2018a).

The anaerobic oxidation of ammonia by NO\textsubscript{2} (anammox) to form N\textsubscript{2} is strictly anaerobic and important in the removal of fixed N from the system, but it is not known to contribute to N\textsubscript{2}O production (Kartal et al., 2007; van der Star et al., 2008; Hu et al., 2019). In suboxic and O\textsubscript{2}-free environments, oxidized nitrogen is respired by bacterial denitrification, which is the stepwise reduction of NO\textsubscript{3} to elemental N\textsubscript{2} via NO\textsubscript{2}, NO and N\textsubscript{2}O. N\textsubscript{2}O as an intermediate can be consumed or produced, but at the core of the ODZ N\textsubscript{2}O consumption through denitrification is enhanced, leading to an undersaturation in this zone (Bange, 2008; Kock et al., 2016). Reducing enzymes are highly regulated by O\textsubscript{2} concentrations, and of the enzymes in the denitrification sequence, N\textsubscript{2}O reductase is the most sensitive to O\textsubscript{2} (Zumft, 1997), which can lead to the accumulation of N\textsubscript{2}O along the upper and lower ODZ boundaries (Kock et al., 2016). N\textsubscript{2}O accumulation during denitrification is mostly linked to O\textsubscript{2} inhibiting the N\textsubscript{2}O reductase, but other factors such as sulfide accumulation (Dalsgaard et al., 2014), pH (Blum et al., 2018), high NO\textsubscript{3} or NO\textsubscript{2} concentrations (Ji et al., 2018b), or copper limitation (Granger and Ward, 2003) may also be relevant. Recent studies contrast the view of nitrification vs. denitrification as the main N\textsubscript{2}O source in ODZs (Nicholls et
They show the importance of denitrification in N$_2$O production in the ETPN from model outputs (Babbin et al., 2015) and in the ETSP from tracer incubation experiments (Dalsgaard et al., 2012; Ji et al., 2015b), based on natural abundance isotopes in N$_2$O (Casciotti et al., 2018) or from water mass analysis of apparent N$_2$O production ($\Delta$N$_2$O) and apparent O$_2$ utilization (AOU) (Carrasco et al., 2017). 45,46N$_2$O production from the addition of 15N-labeled NH$_4^+$, NO$_2^-$ and/or NO$_3^-$ revealed nitrification as a source of N$_2$O within the oxic–anoxic interface, but overall denitrification dominated N$_2$O production with higher rates at the interface and in anoxic waters (Ji et al., 2015b, 2018a). Denitrification is driven by organic matter exported from the photic zone and fuels blooms of denitrifiers, leading to high N$_2$ production (Dalsgaard et al., 2012; Jayakumar et al., 2009; Babbin et al., 2014). Denitrification to N$_2$O is enhanced by organic matter additions, and the degree of stimulation varies with quality and quantity of organic matter (Babbin et al., 2014). Because N$_2$O is an intermediate in denitrification, we hypothesize that its production should also be stimulated by organic matter, possibly leading to episodic and variable N$_2$O fluxes.

N$_2$O concentration profiles around ODZs appear to be at a steady state (Babbin et al., 2015) but are much more variable in regions of intense coastal upwelling where high N$_2$O emissions can occur (Arévalo-Martínez et al., 2015). The contributions and controls of the two N$_2$O production pathways under different conditions of O$_2$ and organic matter supply are not well understood and may contribute to this variability. Hence, the goal of this study is to understand the factors regulating N$_2$O production around ODZs in order to better constrain how future changes in O$_2$ concentration and carbon export will impact production, distribution and emissions of oceanic N$_2$O. Our goal was to determine the impact of O$_2$ and particulate organic matter (POM) on N$_2$O production rates using 15N tracer experiments in combination with quantitative PCR (qPCR) and functional gene microarray analysis of the marker genes, nirS for denitrification and amoA for AO by archaea, to assess how the abundance and structure of the community impacts N$_2$O production rates from the different pathways. 15N-labeled NH$_4^+$ and NO$_2^-$ were used to trace the production of single-labeled (45N$_2$O) and double-labeled (46N$_2$O) N$_2$O to investigate the importance of hybrid N$_2$O production during AO along an O$_2$ gradient.

2 Materials and methods

2.1 Sampling sites, sample collection and incubation experiments

Seawater was collected from nine stations in the upwelling area off the coast of Peru in June 2017 on board R/V Meteor (Fig. 1). Water samples were collected from 10L Niskin bottles on a rosette with a conductivity–temperature–depth profiler (CTD, Sea-Bird Electronics 9plus system). In situ O$_2$ concentrations (detection limit 2 µmol L$^{-1}$ O$_2$), temperature, pressure and salinity were recorded during each CTD cast. NO$_2^-$ and NO$_3^-$ concentrations were measured on board by standard spectrophotometric methods (Hydes et al., 2010) using a QuAAtro autoanalyzer (SEAL Analytical GmbH, Germany). NH$_4^+$ concentrations were determined fluorometrically using ortho-phthaldialdehyde according to Holmes et al. (1999). For N$_2$O, bubble-free triplicate samples were immediately sealed with butyl stoppers and aluminum crimps and fixed with 50 µL of saturated mercuric chloride (HgCl$_2$). A 10 mL He headspace was created, and after an equilibration period of at least 2 h the headspace sample was measured with a gas chromatograph equipped with an electron capture detector (GC/ECD) according to Kock et al. (2016). The detection limit for N$_2$O concentration is 2 nM ± 0.7 nM. At all experimental depths nucleic acid samples were collected by filtering up to 5 L of seawater onto 0.2 µm pore size Sterivex-GP capsule filters (Millipore, Inc., Bedford, MA, USA). Immediately after, collection filters were flash frozen in liquid nitrogen and kept at –80°C until extraction.

Three different experiments were carried out at coastal stations, continental slope and offshore stations. Experiments 1 and 2 aimed to investigate the influence of O$_2$ concentration along a natural and artificial O$_2$ gradient, and experiment 3 targeted the impact of large particles (> 50 µm) on N$_2$O production. Serum bottles were filled from the Niskin bottles with Tygon tubing after overflowing three times to minimize O$_2$ contamination. Bottles were sealed bubble-free with grey butyl rubber septa (National Scientific) and crimped with aluminum seals immediately after filling. The grey butyl rubber septa were boiled in Milli-Q for 30 min to degas and kept in

![Image](https://example.com/image.png)
a He atmosphere until usage. A 3 mL helium (He) headspace was created, and samples from anoxic (O₂ < below detection) water depths were He purged for 15 min. He purging removed dissolved oxygen contamination, which is likely introduced during sampling, and the headspace prevents possible oxygen leakage from the rubber seals (De Brabantere et al., 2012). Natural abundance 2000 ppb N₂O carrier gas (1000 µL in He) was injected to trap the produced labeled N₂O in He to ensure a sufficient mass for isotopic analysis. For all experiments, 15N-N₂O_3, 15N-N₂O_3 and 15N-NH₄⁺ tracer (15N/(14N + 15N) = 99 at. %) were injected into five bottles each from the same depth to a final concentration of 0.5 µmol L⁻¹, except for the NO₃⁻ incubations where 2 µmol L⁻¹ final concentration were anticipated to obtain 10% label of the NO₃⁻ pool. The fraction labeled of the substrate pools was 0.76–0.99 for NH₄⁺, 0.11–0.99 for NO₃⁻ and 0.055–0.11 for NO₅⁻. In the 15N-N₂O_3 treatment, 14N-N₂O_3 was added to trap the label in the product pool for NO₃⁻ reduction rates, and in the 15N-NH₄⁺ treatment, 14N-N₂O_3 was added to a final concentration of 0.5 µmol L⁻¹ to trap the label in the product pool for AO rates.

For the O₂ manipulation experiments, all serum bottles were He purged, and after the addition of different amounts of air saturated sit water a final headspace volume of 3 mL was achieved. Site water from the incubation depth was shaken and exposed to air to reach full O₂ saturation. Then 0, 0.2, 0.5, 2 and 5 mL of O₂-saturated seawater was added into serum bottles and to reach final measured O₂ concentration of 0±0.18, 0.4±0.24, 1.6±0.12, 5.2±0.96 and 11.7±1.09 µM in seawater. For the 15N-N₂O_3 incubations two more O₂ treatments with 21.5 ± 2.8 and 30.2 ± 3.35 µM O₂ were carried out to extend the range of a previous study in which N₂O production from 15NO₃⁻ did not decrease in the presence of up to 7 µM O₂ (Ji et al., 2018b). The O₂ concentration was monitored with an O₂ sensor spot in one serum bottle per treatment using an O₂ probe and meter (FireSting, PyroScience, Aachen, Germany; Fig. S1 in the Supplement). The sensor spots are highly sensitive in the nanomolar range and prepared according to Larsen et al. (2016).

For the organic matter additions, concentrated particles > 50 µm from three different depths were collected with a Challenger stand-alone pump system (SAPS in situ pumps, Liu et al., 2005), autoclaved and He purged. A total of 200 µL of POC (particulate organic carbon) solution was added to each serum bottle before 15N-N₂O_3 or 14N-N₂O_3 tracer injection. The final particle concentrations and C/N ratios varied between 0.18 and 1.37 µM C and between 8.1 and 15.4, respectively (Table 2). The concentration and C/N ratio of PON (particulate organic nitrogen) and POC of the stock solutions were analyzed by mass spectrometry using a GC Isoprime mass spectrometer.

A set of five bottles was incubated per time course. One bottle was sacrificed at t₀, two bottles at t₁ and two at t₂ to determine a single rate. Total incubation times were adjusted to prevent bottle effects, which become significant after 20 h based on respiration rate measurements (Tiano et al., 2014). Hence, experiments lasted from 12 h (at the shelf stations) to 24 h (at the slope stations). Incubation was terminated by adding 0.1 mL of saturated mercuric chloride (HgCl₂). All samples were stored at room temperature in the dark and shipped back to the lab.

### 2.2 Isotope measurement and rate determination

The total N₂O in each incubation bottle was extracted with a purge–trap system according to Ji et al. (2015b). Briefly, serum bottles were flushed with He for 35 min (38 mL min⁻¹); N₂O was trapped by liquid nitrogen; H₂O was removed with an ethanol trap, a Nafion® trap and a Mg(ClO₄)₂ trap; CO₂ was removed with an Ascarite CO₂ adsorbance column; and afterwards mass 44, 45, and 46 and isotope ratios 45/44 and 46/44 were detected with a GC–IRMS system (Delta V Plus, Thermo). Every two to three samples, a 20 mL glass vial with a known amount of N₂O gas was measured to calibrate for the N₂O concentration (linear correlation between N₂O peak size and concentration, r² = 0.99). The isotopic composition of the reference N₂O was δ15N = 1.75±0.10‰ and δ18O = 1.9±0.19‰ present in 15N₁₄N₁₆O or 14N₁₅N₁₆O for 45N₂O and the less abundant 15N₁₅N₁₀O for 46N₂O. To evaluate the analyses of 15N-enriched N₂O samples, internal isotope standards for 15N₂O were prepared by mixing natural abundance KNO₃ of known δ15N values with 99% Na¹⁵NO₃ (Cambridge Isotope Laboratories) and converted to N₂O using the denitrifier method (Sigman et al., 2001; Weigand et al., 2016). Measured and expected values were compared based on a binominal distribution of 15N and 14N within the N₂O pool (Frame et al., 2017).

After N₂O analysis, samples incubated with 15NH₄⁺ and 15NO₃⁻ were analyzed for 15NO₃⁻ to determine rates of NH₄⁺ oxidation and NO₃⁻ reduction, respectively. The individual sample size, adjusted to contain 20 nmol of N₂O, was transferred into 20 mL glass vials and He purged for 10 min. NO₃⁻ was converted to N₂O using sodium azide in acetic acid (McIlvin and Altabet, 2005), and the nitrogen isotope ratio was measured on a Delta V Plus (Thermo).

For each serum bottle, total N₂O concentration (moles) and 45N₂O/44N₂O and 46N₂O/44N₂O ratios were converted to moles of 44N₂O, 45N₂O and 46N₂O. N₂O production rates were calculated from the slope of the increase in mass 44, 45 and 46 over time (Fig. S2). To quantify the pathways for N₂O production, rates were calculated based on the equations for N₂ production for denitrification and anammox (Thamdrup and Dalsgaard, 2002). In incubations with 15NH₄⁺ and unlabeled NO₃⁻, it is assumed that AO produces 46N₂O from two labeled NH₄⁺ (Eq. 1) and some 45N₂O-labeled N₂O based on binominal distribution (Eq. 2). If more single-labeled N₂O is produced than expected (Eqs. 2 and 3), a hybrid formation of one nitrogen atom from NH₄⁺ and one from NO₃⁻ (Eq. 4) is assumed to be taking place as found in archaeal ammno-
nia oxidizers (Kozlowski et al., 2016). In incubations with $^{15}$NO$_2^-$, we assume that $^{46}$N$_2$O comes from nitrifier denitrification or denitrification, which cannot be distinguished (Eq. 1). Hence, any production of $^{45}$N$_2$O not attributed to denitrification stems from hybrid N$_2$O formation by archaeal nitrifiers (Eq. 4). In incubations with $^{15}$NO$_3^-$, denitrification produces $^{46}$N$_2$O, and it was the only process considered and hence was calculated based on Eq. (1). Rates ($R$) are calculated as nmol N$_2$O L$^{-1}$ d$^{-1}$ (Trimmer et al., 2016):

$$R_{\text{external}} = \text{slope}^{46}\text{N}_2\text{O} \times (f_N)^{-2},$$  
(1)

$$R_{\text{expected}} = \text{slope}^{46}\text{N}_2\text{O} \times 2 \times (1 - f_N) \times (f_N)^{-1},$$  
(2)

$$R_{\text{above}} = \text{slope}^{45}\text{N}_2\text{O} - p^{45}\text{N}_2\text{O}_{\text{expected}},$$  
(3)

$$R_{\text{hybrid}} = (f_N)^{-1} \times \left( \frac{\text{slope}^{45}\text{N}_2\text{O}}{+ 2 \times \text{slope}^{46}\text{N}_2\text{O} \times (1 - f_N^{-1})} \right),$$  
(4)

$$R_{\text{total}} = p^{46}\text{N}_2\text{O}_{\text{external}} + p^{46}\text{N}_2\text{O}_{\text{hybrid}},$$  
(5)

where $f_N$ is the fraction of $^{15}$N in the substrate pool (NH$_4^+$, NO$_3^-$ or NO$_2^-$), which is assumed to be constant over the incubation time. Hence, changing $f_N$ due to any other concurrent N-consumption or production process during the incubation is neglected. Nevertheless, the assumption of constant $f_N$ has implications that may affect the results. There is a potential for overestimating hybrid N$_2$O production in $^{15}$NO$_2^-$ incubations by 5% in samples with high NO$_3^-$ reduction rates. But in incubations from anoxic depths with high NO$_3^-$ reduction rates, no hybrid N$_2$O production was found at all. For example, accounting for a decrease in $f_N$ of the NO$_3^-$ pool by active NO$_3^-$ oxidation, the process with the highest rates (Sun et al., 2017) had an effect of only ±0.2% on the final rate estimate. The presence of dissimilatory nitrate reduction to ammonium (DNRA) complicates $^{15}$N-labeling incubations because it can change $f_N$ in all three tracer experiments. In $^{15}$NO$_3^-$ incubations, active DNRA produces $^{15}$NO$_2^-$ and $^{15}$NH$_3^+$ from $^{15}$NO$_3^-$, which can contribute to $^{46}$N$_2$O production by AO. Even if a maximum DNRA rate (20 nM d$^{-1}$, Lam et al., 2009) is assumed to produce 0.02 nM $^{15}$NH$_3^+$ during the 24 h incubations and all of it is oxidized (max. N$_2$O production from AO 0.16 nM d$^{-1}$, this study), its contribution to $^{46}$N$_2$O production is likely minor and within the standard error of the high N$_2$O production rates from NO$_3$. Hence an overestimation of the N$_2$O production rates is unlikely. The same applies in incubations with $^{15}$N-NO$_2^-$ when DNRA produces $^{15}$NH$_3^+$; additional $^{46}$N$_2$O can be produced with a hybrid mechanism by AO. In $^{15}$NO$_2^-$ incubations with high starting $f_N$ (> 0.7) the production of $^{14}$NO$_3^-$ by NO$_3$ reduction (which decreases $f_N$) leads to an underestimated by up to 9%, whereas in incubations with a low $f_N$ (< 0.3) the effect is less (up to 3% underestimation of N$_2$O production rates). In $^{15}$NH$_3^+$ incubations ($f_N > 0.9$), the maximum DNRA rate would lead to an underestimation of 3.5%. The slope of $^{46}$N$_2$O and slope of $^{45}$N$_2$O represent the $^{46}$N$_2$O and $^{45}$N$_2$O production rates, which were tested for significance based on a linear regression ($n = 5$, Student’s $t$ test, $R^2 > 0.80$, $p < 0.05$). Linear regressions that were not significantly different from zero were reported as 0. The error for each N$_2$O production rate was calculated as the standard error of the slope. Detection limits were 0.002 nmol L$^{-1}$ d$^{-1}$ for N$_2$O production from AO and 0.1 nmol L$^{-1}$ d$^{-1}$ for N$_2$O production from denitrification based on the average measured standard error for rates (Dalsgaard et al., 2012). The curve-fitting tool of SigmaPlot was used for the O$_2$ sensitivity experiments. A one-way ANOVA was performed on the N$_2$O production rates to determine if rates were significantly different between POM treatments.

The rates ($R$) of NH$_4^+$ oxidation to NO$_2^-$ and NO$_3^-$ reduction to NO$_3^-$ were calculated based on the slope of the linear regression of $^{15}$NO$_3^-$ enrichment over time ($n = 5$) (Eq. 6).

$$R = f_N^{-1} \times \text{slope}^{15}\text{NO}_3^-, \quad (6)$$

where $f_N$ is the fraction of $^{15}$N in the substrate pool (NH$_4^+$ or NO$_3^-$).

Yield (%) of N$_2$O production during NH$_4^+$ oxidation was defined as the ratio of the production rates (Eq. 7).

$$\text{Yield}_{\text{NH}_4} = \frac{N - \text{N}_2\text{O} (\text{nM} \text{ d}^{-1})}{N - \text{NO}_2^- (\text{nM} \text{ d}^{-1})} \times 100\% \quad (7)$$

Yields of N$_2$O production during denitrification were calculated based on the fact that NO$_2$ is not a side product during NO$_3^-$ reduction to NO$_3^-$ but rather the next intermediate during denitrification (Eq. 8).

$$\text{Yield}_{\text{NO}_3} = \frac{N - \text{N}_2\text{O} (\text{nM} \text{ d}^{-1})}{N - \text{NO}_2^- (\text{nM} \text{ d}^{-1}) + N - \text{N}_2\text{O} (\text{nM} \text{ d}^{-1})} \times 100\% \quad (8)$$

All rates, yields and errors are reported in Table S3 in the Supplement.

2.3 Molecular analysis – qPCR and microarrays

DNA and RNA were extracted using the AllPrep DNA/RNA Mini Kit (Qiagen) followed by immediate cDNA synthesis from purified and DNA-cleaned RNA using a SuperScript III First-Strand Synthesis System (Invitrogen). The PicoGreen dsDNA quantification kit (Invitrogen) was used for DNA quantification, and the Quant-iT OliGreen ssDNA quantification kit (Invitrogen) was used for cDNA quantification.

The abundances of total and active nirS and archaeal amoA communities were determined by qPCR with assays based on SYBR Green staining according to methods described previously (Jayakumar et al., 2013; Peng et al., 2013). Primers nirS1F and nirS3R (Braker et al., 1998) were used to amplify a 260 bp conserved region within the nirS gene. The nirS
primers are not specific for epsilon-proteobacteria (Murdock and Juniper, 2017), but in previous metagenomes from the ETSP epsilon-proteobacteria below 3%–4% of the reads or not found, except in very sulfidic, coastal stations (Stewart et al., 2011; Wright et al., 2012; Ganesh et al., 2014; Schunck et al., 2013; Kalvelage et al., 2015). Primers Arch-amoA and Arch-amOR (Francis et al., 2005) were used to quantify archaeal amoA abundance. A standard curve containing six serial dilutions of a plasmid with either an archaeal amoA fragment or a nirS fragment was used on respective assay plates. Assays were performed in a Stratagene Mx3000P qPCR cycler (Agilent Technologies) in triplicates of 20–25 ng DNA or cDNA, along with a no-primer control and a no-template control. Cycle thresholds (Ct values) were determined automatically and used to calculate the number of nirS or archaeal amoA copies in each reaction, which was then normalized to copies per milliliter of seawater (assuming 100% recovery). The detection limit was around 15 copies mL⁻¹ based on the Ct values of the no-template control.

Microarray experiments were carried out to describe the community composition of the total and active nirS and archaeal amoA groups using the DNA and cDNA qPCR products. Pooled qPCR triplicates were purified and cleaned using the QIAquick PCR Purification Kit (Qiagen). Microarray targets were prepared according to Ward and Bouskill (2011). Briefly, dUaa was incorporated into DNA and cDNA targets during amplification with random octamers and a Klenow polymerase using the BioPrime kit (Invitrogen) and then labeled with Cy3, purified and quantified. Each probe is a 90-mer oligonucleotide consisting of a 70-mer archetype sequence combined with a 20-mer reference oligonucleotide as a control region bound to the glass slide. Each archetype probe represents a group of related sequences with 87±3% sequence identity of the 70-mer sequence. Microarray targets were hybridized in duplicates on a microarray slide, washed and scanned using a laser scanner 4200 (Agilent Technologies), and analyzed with GenePix Pro 6.0. The resulting fluorescence ratio (FR) of each archaeal amoA or nirS probe was divided by the FR of the maximum archaeal amoA or nirS FR on the same microarray to calculate the normalized FR (nFR). The nFR represents the relative abundance of each archetype and was used for further analyses.

Two different arrays were used: BCO16, which contains 99 archaeal amoA archetype probes representing ∼8000 archaeal amoA sequences (Biller et al., 2012); and BCO15, which contains 167 nirS archetype probes representing ∼2000 sequences (collected from NCBI in 2009). A total of 74 assays were performed with 21 nirS cDNA targets, 21 nirS DNA targets, 16 amoA cDNA targets and 16 amoA DNA samples. The original microarray data from BCO15 and BCO16 are available via GEO (Gene Expression Omnibus; http://www.ncbi.nlm.nih.gov/geo/, last access: 16 April 2020) at NCBI (National Center for Biotechnology Information) under GEO accession no. GSE142806.

2.4 Data analysis

Spearman Rank correlation was performed from all N₂O production rates, AO and NO₃⁻ reduction rates, environmental variables, nirS and archaeal amoA gene and transcript abundance, and the 20 most abundant archetypes of total and active nirS and amoA using R. Only significant values (p < 0.05) are shown. Archetype abundance (nFR) data were square root transformed, and beta-diversity was calculated with the Bray–Curtis coefficient. Alpha diversity of active and total nirS and amoA communities was estimated by calculating the Shannon diversity index using PRIMER6. Bray–Curtis dissimilarities were used to perform a Mantel test to determine significant differences between active and total communities of nirS and amoA using R (version 3.0.2, package vegan; Oksanen et al., 2019). Canonical correspondence analysis (CCA) (Legendre and Legendre, 2012) was used to visualize differences in community composition dependent upon environmental conditions using the software PAST (Hammer et al., 2001). Before CCA, a forward selection (Borcard et al., 1992) of the parameters that described the environmental and biological variables likely to explain the most significant part of the changes in the archetypes was performed.

The make.leafse command in mothur was used to create a linear discriminant analysis (LDA) effect size (LEfSe) (Segata et al., 2011) input file from the mothur shared file. This was followed by a LEfSe (https://huttenhower.sph.harvard.edu/galaxy, last access: 16 April 2020) to test for discriminatory archetypes between O₂ levels. With a normalized relative abundance matrix, LEfSe uses the Kruskal–Wallis rank sum test to detect features with significantly different abundances between assigned archetypes in the different O₂ levels and performs an LDA to estimate the effect size of each feature. A significant alpha of 0.05 and an effect size threshold of 2 were used for all marker genes discussed in this study.

3 Results

3.1 Hydrographic conditions

The upwelling system off Peru is a hotspot for N₂O emissions (Arévalo-Martínez et al., 2015), with the most intense upwelling in austral winter but maximum chlorophyll during December to March (Chavez and Messié, 2009; Messié and Chavez, 2015). The sampling campaign took place during austral fall in the absence of intense upwelling or maximum chlorophyll. The focus of this study was the region close to the coast, which has highly variable N₂O concentration profiles (Kock et al., 2016) and N₂O emissions (Arévalo-Martínez et al., 2015). The Peru coastal water (PCW, temperature < 19.5°C, salinity 34.9–35.1) and the equatorial subsurface waters (ESSW, temperature 8–12°C,
salinity 34.7–34.9) (Pietri et al., 2013) were the dominant water masses off the Peruvian coast sampled for N\textsubscript{2}O production rate measurements (Table 1). At the southernmost transect at 15.5–16.4° S a mesoscale anticyclonic mode water eddy (McGillicuddy et al., 2007), which was about to detach from the coast, was detected from deepening/shoaling of the main/seasional pycnoclines (Fig. S3). Generally, the stations were characterized by a thick anoxic layer (254–427 m) reaching to the seafloor at two shelf stations (894, 883). NO\textsubscript{2}O concentration accumulated only up to 2 µmol L\textsuperscript{-1} in the secondary NO\textsubscript{2}\textsuperscript{-1} maximum (SNM) at the northern transect (stations 882, 883) but up to 7.19 µmol L\textsuperscript{-1} along the southern transect (Fig. 2, station 907, 912). N\textsubscript{2}O concentration profiles showed a high variability with respect to depth and O\textsubscript{2} concentrations (Fig. 2). The southern transect (station 907, 912) showed the lowest N\textsubscript{2}O concentrations (5 nmol L\textsuperscript{-1}) in the center of the anoxic zones. At the same time, station 912 in the center of the eddy showed the highest N\textsubscript{2}O concentration with 78.9 nmol L\textsuperscript{-1} at [O\textsubscript{2}] below detection limit in the upper part of the anoxic zone. Above the ODZ, the maximum N\textsubscript{2}O peak ranged from 57.9 to 78.9 nmol L\textsuperscript{-1} and was found at an O\textsubscript{2} concentration range from below detection (883, 894, 892, 912) up to 67 µmol L\textsuperscript{-1} (907). Three stations (892, 894 and 904) showed high surface N\textsubscript{2}O concentrations of 64 nmol L\textsuperscript{-1}.

3.2 Depth distribution of N\textsubscript{2}O production rates and total and active nir\textsubscript{S} and amo\textsubscript{A} abundance

N\textsubscript{2}O production varied with depth and substrate (Fig. 3, Table S3). In the oxycline, the highest AO (34 ± 0.1 and 35 ± 9.2 nmol L\textsuperscript{-1} d\textsuperscript{-1}) coincided with the highest N\textsubscript{2}O production from AO (0.141 ± 0.003 and 0.159 ± 0.003 nmol L\textsuperscript{-1} d\textsuperscript{-1}) at both stations of the northern transect, stations 883 and 882, respectively (Fig. 3l.a, b). NH\textsubscript{4}\textsuperscript{+} oxidation and its N\textsubscript{2}O production decreased to zero in the ODZ. The rates of the reductive source pathways for N\textsubscript{2}O increased with depth. N\textsubscript{2}O production from NO\textsubscript{2}\textsuperscript{-1} and NO\textsubscript{3}\textsuperscript{-1} displayed similar patterns with the highest production at or below the oxic–anoxic interface (Fig. 3lI). N\textsubscript{2}O production from NO\textsubscript{2} showed the highest rates of 3.06 ± 1.17 nmol L\textsuperscript{-1} d\textsuperscript{-1} (912) and 2.37 ± 0.54 nmol L\textsuperscript{-1} d\textsuperscript{-1} (906) further south (Fig. 3lI, m, q) compared to lower rates at northern stations, where the maximum rate was 0.71 ± 0.38 nmol L\textsuperscript{-1} d\textsuperscript{-1} (Fig. 3lIc, 883). A similar trend was found for N\textsubscript{2}O production from NO\textsubscript{3}\textsuperscript{-1}: lower maximum rates at northern stations with 2.7 ± 0.4 nmol L\textsuperscript{-1} d\textsuperscript{-1} (882) and 5.7 ± 2.8 nmol L\textsuperscript{-1} d\textsuperscript{-1} (883, Fig. 3Iib) and the highest rates in southern transects with 7.2 ± 1.64 nmol L\textsuperscript{-1} d\textsuperscript{-1} (Fig. 3IIl, 904) in transect 3 and up to 118.0 ± 27.8 nmol L\textsuperscript{-1} d\textsuperscript{-1} (Fig. 3IIp, 912) in transect 4. Generally, N\textsubscript{2}O production rates from NO\textsubscript{2} and NO\textsubscript{3} were 10- to 100-fold higher than from AO.

qPCR analysis detected the lowest gene and transcript numbers of archaenal amo\textsubscript{A} and nir\textsubscript{S} in the surface mixed layer (Fig. 3Ik, l, IIr, s). The highest archaenal amo\textsubscript{A} gene and transcript abundance was in the oxycline (1–40 nmol L\textsuperscript{-1} O\textsubscript{2}) with 24 500 ± 340 and 626 ± 29 copies mL\textsuperscript{-1} at station 883 (Fig. 3Ic, d). amo\textsubscript{A} gene and transcript number decreased in the ODZ to 1000–6500 gene copies mL\textsuperscript{-1} and 20–250 transcript copies mL\textsuperscript{-1}.

The profiles of nir\textsubscript{S} gene and transcript abundance were similar to each other (Fig. 3II d, e), with the highest abundance in the ODZ up to 1 × 10\textsuperscript{6} and 2.9 × 10\textsuperscript{5} copies mL\textsuperscript{-1}, respectively. Denitrifier nir\textsubscript{S} genes and transcripts peaked in the anoxic layer and were significantly correlated with N\textsubscript{2}O production from NO\textsubscript{2} but not from NO\textsubscript{3}. Archaenal amo\textsubscript{A} gene and transcript abundances were significantly correlated with AO and N\textsubscript{2}O production from AO (Fig. S5). N\textsubscript{2}O concentrations did not correlate with any of the measured variables (Fig. S5).

3.3 Influence of O\textsubscript{2} concentration on N\textsubscript{2}O production

N\textsubscript{2}O production along the in situ O\textsubscript{2} gradient for the substrates NO\textsubscript{2} and NO\textsubscript{3} decreased exponentially with increasing O\textsubscript{2} concentrations (Fig. 4b, c), while for NH\textsubscript{4}\textsuperscript{+}, the N\textsubscript{2}O production was highest at the highest sampled O\textsubscript{2} concentration (Fig. 4a). At in situ O\textsubscript{2} levels above 8.4 µmol L\textsuperscript{-1}, N\textsubscript{2}O production decreased by 100 % and 98 % from NO\textsubscript{2} and NO\textsubscript{3}, respectively (Fig. 4b, c).

In the manipulated O\textsubscript{2} treatments from the oxic–anoxic interface (S11, S19) a unimodal response of N\textsubscript{2}O production from NH\textsubscript{4}\textsuperscript{+} and NO\textsubscript{3} to O\textsubscript{2} is apparent (Fig. 4d, e). Increasing and decreasing O\textsubscript{2} concentrations inhibited N\textsubscript{2}O production from NH\textsubscript{4}\textsuperscript{+} and NO\textsubscript{2}, with the highest N\textsubscript{2}O production rate between 1.4 and 6 µmol O\textsubscript{2} L\textsuperscript{-1}. However, this response was only significant in sample S11 (Fig. 4d, e). There was no significant response to O\textsubscript{2} concentration of N\textsubscript{2}O production from NO\textsubscript{3}. O\textsubscript{2} did not inhibit N\textsubscript{2}O production from NO\textsubscript{3} up to 23 µmol L\textsuperscript{-1} (Fig. 4f).

The proportion of hybrid N\textsubscript{2}O produced during AO, i.e., the formation of N\textsubscript{2}O from one \textsuperscript{15}NH\textsubscript{4} and one N compound (excluding NH\textsubscript{4}) such as NO\textsubscript{2}, NH\textsubscript{2}OH or NO, was consistently between 70 % and 85 % across different O\textsubscript{2} concentrations for manipulated and natural O\textsubscript{2} concentrations (Fig. 5a, c). Hybrid formation during N\textsubscript{2}O production from NO\textsubscript{3} varied between 0 % and 95 % along the natural O\textsubscript{2} gradient (Fig. 5b). In manipulated O\textsubscript{2} treatments hybrid formation from NO\textsubscript{3} did not change across different O\textsubscript{2} treatments but with respect to the original depth – 0 % in sample S11, which originated from 145 m of station 892, or 78 % in sample S19 from 120 m of station 894 (Fig. 5d).

The highest N\textsubscript{2}O yields during AO (over 1 %) occurred between 1.4 and 2 µmol O\textsubscript{2} L\textsuperscript{-1} and decreased at both higher and lower O\textsubscript{2} concentrations (Fig. 6a). However, only the increase in yield from nmol O\textsubscript{2} to 1.4–2 µmol L\textsuperscript{-1} O\textsubscript{2} was significant (t test, p < 0.05), and the following decrease in yield was not (t test, p > 0.05). In the manipulated O\textsubscript{2} treatment of sample S19 (Fig. 6c) the same significant pattern was observed, whereas in S11 the highest yield was found at
Table 1. Overview of characteristics of samples. bd – below detection limit of the Winkler method and Sea-Bird sensor (2 µmol L\(^{-1}\)); × – analysis includes qPCR and microarray with qPCR products; × – only qPCR, no microarray.

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<th>ID</th>
<th>Stat no.</th>
<th>Coordinates/ position</th>
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<td>14.78° W 78.04° N/</td>
<td>4128</td>
<td>140</td>
<td>interface</td>
<td>13.1</td>
<td>34.86</td>
<td>bd</td>
<td>17.3</td>
<td>3.9</td>
<td>0.0</td>
<td>POM addition (from 906)</td>
<td>NO$_3^-$</td>
<td>×</td>
<td>×</td>
</tr>
</tbody>
</table>

Figure 2. Depth profiles of $O_2$, nutrients and $N_2O$ in the upper 400 m for all stations. Panels (a)–(d) refer to the transect numbers 1–4.
Figure 3. (I) Profiles of AO (a, e, i), N₂O production rates from NH₄⁺ (b, f, j), archaeal amoA gene (c, g, k) and transcript copy numbers per milliliter (d, h, l). (II) Profiles of NO₃⁻ reduction rates (a, f, k, p), N₂O production rates from NO₃⁻ (b, g, l, q) and NO₂⁻ (c, h, m, r), and nirS gene (d, i, n, s) and transcript copy numbers per milliliter (e, j, m, t). In (I) and (II), the panel numbers 1–4 correspond to transect numbers. Negative values on the y axis represent shallower, oxic depths, and the positive values represent deeper, anoxic depth (0 = interface). Shaded area indicates the anoxic zone. Note the different scale for N₂O production rates.
Figure 4. O$_2$ dependence of N$_2$O production rates from NH$_4^+$ (a, d), NO$_3^-$ (b, e) and NO$_2^-$ (c, f). Upper panels (a–c) is N$_2$O production along a natural O$_2$ gradient from all stations. Panels (b) and (c) are additionally zoomed in to oxygen concentrations below 5 µmol L$^{-1}$. Lower panels (d–f) show N$_2$O production in manipulated O$_2$ experiments with water from the oxic–anoxic interface from slope station 892 (S11, 0 µmol L$^{-1}$ O$_2$, 145 m) and shelf station 894 (S19, 0 µmol L$^{-1}$, 120 m). Note the different scale for N$_2$O production rates from NH$_4^+$. Vertical error bars represent ± standard error ($n$ = 5 per time course). Horizontal error bars represent ± standard error of measured O$_2$ over the time of incubations ($n$ = 6).

12 µmol L$^{-1}$ O$_2$. N$_2$O yield during NO$_3^-$ reduction to NO$_2^-$ decreased to zero at 8.4 µmol L$^{-1}$ O$_2$ along the natural O$_2$ gradient (Fig. 6b), while no significant response occurred in the manipulated O$_2$ treatments (Fig. 6d). There, NO$_3^-$ reduction was decreasing with increasing O$_2$, but N$_2$O production was steady with increasing O$_2$, leading to high yields between 38.8 ± 9 % and 91.2 ± 47 % at 23 µmol L$^{-1}$ O$_2$.

3.4 Effect of large particulate organic matter on N$_2$O production

The autoclaving of the concentrated POM solution liberated NH$_4^+$ from the particles, reducing the N/C ratio of the particles compared to nonautoclaved particles (Table 2). The highest NH$_4^+$ accumulation is found in samples with the largest difference in N/C ratios between autoclaved and nonautoclaved particles (Table 2, 904–20, 898–100 m). Addition of 0.17–1.37 µmol C L$^{-1}$ of autoclaved particles > 50 µm (Table 2) produced a significant increase in N$_2$O production by up to 5.2- and 4.8-fold in 10 and 7 out of 19 additions for NO$_2^-$ and NO$_3^-$ respectively (Fig. 7a, b). There was no linear correlation of the origin (mixed layer depth, oxycline or anoxic zone), the quality (N/C ratio) or the quantity of the organic matter on the magnitude of the increase. Only samples S20 and S17 were not stimulated by particle addition, and N$_2$O production from denitrification did not significantly differ from the control (Fig. 7b).

Figure 5. O$_2$ dependency of hybrid N$_2$O formation from NH$_4^+$ (a, c) and NO$_3^-$ (b, d) along the natural O$_2$ gradient (a, b) and for the O$_2$ manipulations (c, d) from sample S11 (0 µmol L$^{-1}$ O$_2$, 145 m) and S19 (894, 0 µmol L$^{-1}$, 120 m).
Table 2. Quality (N/C), quantity (addition µmol L\(^{-1}\)) and origin (station and depth) of added, autoclaved and nonautoclaved particulate organic matter (POM) and increase in NH\(_4^+\) concentration after autoclaving.

<table>
<thead>
<tr>
<th>POM Feature</th>
<th>Station</th>
<th>Depth (m)</th>
<th>Addition (µmol L(^{-1}))</th>
<th>N/C of autoclaved POM</th>
<th>N/C of nonautoclaved POM</th>
<th>NH(_4^+) (µM) after autoclaving</th>
</tr>
</thead>
<tbody>
<tr>
<td>POM 1 mixed layer depth</td>
<td>898</td>
<td>60</td>
<td>0.55</td>
<td>0.10</td>
<td>0.15</td>
<td>0.7</td>
</tr>
<tr>
<td></td>
<td>904</td>
<td>20</td>
<td>0.17</td>
<td>0.09</td>
<td>0.17</td>
<td>1.56</td>
</tr>
<tr>
<td></td>
<td>906</td>
<td>50</td>
<td>0.48</td>
<td>0.07</td>
<td>0.11</td>
<td>0.57</td>
</tr>
<tr>
<td>POM 2 oxycline</td>
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<td>100</td>
<td>1.37</td>
<td>0.06</td>
<td>0.13</td>
<td>0.85</td>
</tr>
<tr>
<td></td>
<td>904</td>
<td>50</td>
<td>0.38</td>
<td>0.09</td>
<td>0.12</td>
<td>0.46</td>
</tr>
<tr>
<td></td>
<td>906</td>
<td>100</td>
<td>0.44</td>
<td>0.08</td>
<td>0.10</td>
<td>0.55</td>
</tr>
<tr>
<td>POM 3 anoxic zone</td>
<td>898</td>
<td>300</td>
<td>0.43</td>
<td>0.09</td>
<td>0.10</td>
<td>0.15</td>
</tr>
<tr>
<td></td>
<td>904</td>
<td>150</td>
<td>0.19</td>
<td>0.10</td>
<td>0.10</td>
<td>0.20</td>
</tr>
</tbody>
</table>

Figure 6. Yields (%) of N\(_2\)O production during NH\(_4^+\) oxidation (a, c) and during NO\(_3^-\) reduction (b, d) along the natural O\(_2\) gradient (a, b) and for the O\(_2\) manipulations (c, d) from sample S11 (892, 0 µmol L\(^{-1}\) O\(_2\), 145 m) and S19 (894, 0 µmol L\(^{-1}\), 120 m). Error bars present ± SD calculated as error propagation.

3.5 Diversity and community composition of total and active nirS and amoA assemblages and its correlation with environmental parameters

nFR values from functional gene microarrays were used to describe the nitrifier and denitrifier community composition of AOA and nirS assemblages, respectively. The nFR was averaged from duplicate microarrays, which replicated well \((R^2 = 0.89–0.99)\). Alpha-diversities of nirS and archaeal amoA were not statistically different for total and active communities (Student’s t test, \(p > 0.05\)) but were overall lower for RNA \(3.2 ± 0.3\) than DNA \(3.8 ± 0.4\) (Table S1). Principal coordinate analysis of Bray–Curtis similarity for each probe group on the microarray indicated that the community structure of archaeal amoA genes was significantly different from that of archaeal amoA transcripts, whereas community structure of nirS genes and transcripts did not differ significantly (Fig. S4). To identify which archetypes were important in explaining differences in community structure of key nitrification and denitrification genes, we identified archetypes that accounted for more than 1% of the total fluorescence for their probe set and that were significantly different with respect to ambient O\(_2\) using a LEfSe analysis (Table S2). Furthermore, we used CCA to test whether the community composition, or even single archetypes, could explain the N\(_2\)O production rates.

The nFR distribution showed greater variability in the active (cDNA) AOA community than in the total community (DNA) among depths, stations and O\(_2\) concentrations (Fig. 8a, b). Archetypes over 1% made up between 76% (DNA) and 83% (cDNA) of the amoA assemblage and only between 61% (DNA) and 68% (cDNA) of the nirS assemblage. The four most abundant AOA archetypes – AOA55, AOA3, AOA21 and AOA32 – made up 20%–65% of the total and active community (Fig. 8a, b). DNA of archetypes AOA55 and AOA79, both related to uncultured AOA in soils, significantly correlated with in situ NH\(_4^+\) concentrations (Fig. S5). DNA and cDNA from AOA3 and AOA83 were significantly enriched in oxic waters, and AOA7, closely related with crenarchaeote SCGC AAA288-M23 isolated from station ALOHA near Hawaii (Swan et al., 2011), was significantly enriched in anoxic and hypoxic waters for DNA and cDNA respectively (Table S2). All other archetypes did not vary with O\(_2\) levels. DNA of AOA 3, closely related to Candidatus Nitrosopelagicus brevis (CN25), identified as the only archetype to be significantly correlated with N\(_2\)O production and yield from AO (Fig. S5).

The total and active denitrifier communities were dominated by Nir7, derived from an uncultured clone from the
Figure 7. Bar plots of N₂O production after additions of autoclaved suspended and sinking particles > 50 µm (See Table 2). POM1: mixed layer depth; POM2: oxycline; POM3: ODZ. Error bars represent ± SE of linear regression. * indicates significant difference to control rate (p < 0.05).

Figure 8. Stacked bar plot of community composition of AOA amoA archetypes (a, b) and nirS archetypes (c, d). Only archetypes over 1% contribution are shown. (a, c) Total community composition (DNA). (b, d) Active community composition (cDNA).
ODZ in the ETSP (Lam et al., 2009), and Nir7 was significantly more enriched in the active community (Fig. 8c, d). DNA from ODZ depths of the eddy, S15 (907, 130 m) and S17 (912, 90 m), diverged most obviously from the rest and from each other (Fig. 8c, d). Interestingly, these two samples were not divergent among the active nirS community (Figs. 8c and d, S2, and S4). DNA of Nir35, belonging to the Flavobacteriaceae derived from coastal waters of the Arabian Sea (Gorégués et al., 2004), was most abundant (12.3 %) at the eddy edge (S15) as opposed to the eddy center (S17) where nir167, representing anammox sequences from Peru, was most abundant (12.0 %). Interestingly, Nir4 and Nir14, among the top five abundant archetypes, were significantly enriched in oxic water masses (Table S2). The nFR signal of nir166, belonging to Candidatus Scalindua, and Nir23 were among the top five abundant archetypes and significantly enriched in anoxic depths.

CCA is a direct gradient analysis, where the gradient in environmental variables is known a priori and the archetypes are considered to be a response to this gradient. Composition from total and active AOA community did not differ between stations, and all samples cluster close together (Fig. S6a, b). S18 (912, 5 m) is a surface sample with the lowest NO3 concentration (8 µmol L−1) and the highest temperature and salinity of the data set, and the DNA is positively related with O2 and driven by AOA55, AOA32 and AOA79. RNA of S17 (912, 90 m) clusters with AOA70. AOA55 was abundant, and its distribution is driven by O2 and NH4+ (Fig. S5).

CCA clustered the denitrifier community DNA into one main group with a few exceptions (Fig. S6c). Two surface samples (S16, S18) clustered separately and were positively correlated with Nir4 and Nir14 and O2. Two anoxic samples from the eddy core (S17) and eddy edge (S15) clustered separately, with S17 being driven by three nirS archetypes – Nir54, Nir10 and Nir167 – and S15 by Nir23, Nir35 and Nir133 (Fig. S6c). Total and active nirS community composition did not differ as a function of O2. Although the composition of active and total nirS communities was not significantly different, the active community clustered slightly differently. For nirS RNA, surface and oxycline samples (S16 and S10) grouped together and were correlated positively with O2, temperature and salinity, whereas the anoxic eddy samples did not differ from the rest (Fig. S6d). N2O production from NO2 significantly correlated with nirS gene and transcript abundance, but both reductive N2O production pathways were not linked with a single dominant nirS archetype (Fig. S5).

4 Discussion

Most samples originated from Peru coastal water (PCW) characterized by supersaturated N2O concentrations (Kock et al., 2016; Bourbonnais et al., 2017). Only the deepest sample (S1, 882–350 m) saw the presence of a different water mass, the equatorial subsurface waters. Thus, our findings about regulation of N2O production at different stations probably apply to the region as a whole. Several studies indicate that water mass hydrography plays an important role in shaping microbial community diversity (Billing et al., 2012; Hamdan et al., 2012), and a coupling of amoA alpha diversity to physical conditions such as salinity, temperature and depth has been shown in coastal waters off Chile (Bertagnolli and Ulloa, 2017). While salinity, temperature and depth were prominent factors in shaping the community compositions of nitrifiers and denitrifiers (Fig. S6), for N2O production rates correlations with physical and chemical parameters were not consistent. On one hand, oxidative N2O production from NH4+ positively correlated with temperature, salinity, and oxygen and negatively with depth and PO4− concentration. On the other hand, reductive N2O production from NO2− positively correlated with NH4+ and NO3− concentrations but negatively with NO3− concentrations (Fig. S5), suggesting, when NO3− is abundant, denitrifiers are less likely to use NO2− for N2O production during denitrification. Both oxidative (AO) and reductive (NO− and NO3− reduction) N cycling processes produced N2O with differential effects of O2 on them. Measured N2O production rates were always highest from NO3−, followed by NO2− and NH4+, which is consistent with previous studies that showed denitrification as a dominant N2O source in Peruvian coastal waters harboring an ODZ (Ji et al., 2015b; Casciotti et al., 2018). A low contribution of AO to N2O production in low-O2 waters is in line with a previous study in this area estimating N2O production based on isotopomer measurements combined with a 3-D-reaction-advection-diffusion box model (Bourbonnais et al., 2017). The low percentage that AO contributed to total N2O production was between 0.5 % and 6 %, with one exception in the shallowest sample S5 with 30 µmol L−1 O2, where AO contributed 86 % to total N2O production. We found strong positive effects of decreasing O2 concentration and increasing particulate matter concentrations on N2O production in the upper oxycline.

The occurrence of an anticyclonic mode water eddy at 16°S (transect 4, stations 912, 907) at the time of sampling was not unusual, as such eddies have been reported at a similar position (Stramma et al., 2013). High N loss, a large SNM with low NO3− concentrations and strong N2O depletion in the core of ODZ of the eddy result in reduced N2O inside of this kind of eddy as the eddies age and are advected westward (Cornejo D’Ottone et al., 2016; Arévalo-Martínez et al., 2016). Our study found similar patterns with the largest SNM (5.23 µM NO3−) and the lowest NO3− (14 µmol L−1) and N2O (4 nmol L−1) concentrations in the eddy center. For the first time N2O production rates were measured in an eddy, and the rates of up to 118 ± 27 nmol L−1 d−1 are the highest N2O production rates from denitrification reported in the ETSP. Previously reported maximum rates were up to 86 nmol L−1 d−1 (Dalsgaard et al., 2012) based on 15N tracer
incubations. Much smaller maximum rates, 49 nmol L$^{-1}$ d$^{-1}$ (Bourbonnais et al., 2017) and 50 nmol L$^{-1}$ d$^{-1}$ (Farías et al., 2009), were obtained using N$_2$O isotope and isotopomer approaches, which provide time and process integrated signals. Hence, the deviation of maximum rates can be explained by (1) the different approaches and (2) the sampling of the core of the eddy. N$_2$O production measurements (from anammox and denitrification) were not performed in this study but should be in future studies to account for potential artefacts by co-occurring NO$_3^-$ reduction processes. Here, it cannot be determined whether the eddy only stimulated N$_2$O production but not N$_2$O production from denitrification (i.e., increasing the N$_2$/O$_2$/N$_2$ yield) or whether the eddy also increased complete denitrification to N$_2$ by 10 times compared to stations outside of the eddy. Considering that at some depths only incomplete denitrification (also known as “stop-and-go denitrification”) to N$_2$O is at work, it would not be surprising that N$_2$O production can reach the same order of magnitude as N$_2$ production from complete denitrification. Aged eddies also show lower N$_2$O concentration maxima at the upper oxycline (Arévalo-Martínez et al., 2016), which was not the case in this study where a young eddy was just about to detach from the coast. In fact, the eddy stations show the highest N$_2$O peak in the upper oxycline within this data set. Eddies and their age imprint mesoscale patchiness and heterogeneity in biogeochemical cycling. It appears that young eddies close to the coast with high N$_2$O concentrations and high N$_2$O production rates have a great potential for high N$_2$O emissions compared to aged eddies or waters surrounding eddies.

4.1 Effect of O$_2$ on reductive and oxidative N$_2$O production

The relationship between O$_2$ concentrations and N$_2$O production by nitrification and denitrification is very complex in ODHs. While poorly constrained, the reported O$_2$ threshold level (1.7 µmol L$^{-1}$ O$_2$) for reductive N$_2$O production is lower (Dalsgaard et al., 2014) than the reported O$_2$ threshold level (8 µmol L$^{-1}$) for N$_2$O consumption in the ETSP (Cornejo and Farías, 2012). Nevertheless, the suboxic zone between 1 and 8 µmol L$^{-1}$ O$_2$ carries high N$_2$O concentrations indicating higher N$_2$O production than consumption. In this study, we focused on these suboxic water masses above the ODZ and determined bulk kinetics of O$_2$ sensitivity in batch experiments, which reflect the metabolism of the microbial community. The effect of O$_2$ on N$_2$O production differed between natural O$_2$ concentrations with varying communities and manipulated O$_2$ concentrations within a community. While N$_2$O production from NO$_2^-$ and NO$_3^-$ decreased exponentially along the natural O$_2$ gradient, it did not always decrease for the manipulated O$_2$ treatments. Unchanged N$_2$O production with higher O$_2$ levels in NO$_3^-$ treatments showed that at least a portion of the community can respond very differently to a sudden increase in O$_2$ than predicted from natural O$_2$ gradients with communities acclimated to a certain O$_2$ concentration. In the ETNP, this pattern has been observed before (Ji et al., 2018b), but the mechanism behind it is unknown. Different responses of N$_2$O production rates to O$_2$ between in situ assemblages and incubated samples were not unexpected because different rates at different depths were likely not only due to O$_2$ differences but also other factors such as different organic matter fluxes and different amounts and types of N$_2$O producers at different depths. In addition, sampling with Niskin bottles and purging can induce stress responses (Stewart et al., 2012) and shift the richness and structure of the microbial community from the in situ community (Torres-Beltrán et al., 2019). The removal of other gases like H$_2$S during purging is another potential artifact. However, it is unlikely as measurable H$_2$S concentrations have mostly been found at very shallow coastal stations (< 100 m deep) (Callbeck et al., 2018), which are not the environment of this study. On the contrary, high abundances (up to 12 %) of sulfur-oxidizing gamma proteobacteria, like SUP05, can be found in eddy-transported offshore waters where they actively contributed to autotrophic denitrification (Callbeck et al., 2018). In this study, we cannot differentiate between autotrophic or organotrophic denitrification, but a contribution of autotrophic denitrification in the eddy center is likely. Off the Chilean coast, active N$_2$O production by denitrification was found at up to 50 µmol L$^{-1}$ O$_2$ (Farías et al., 2009). These results reinforce prior studies showing that distinct steps of multi-step metabolic pathways, such as denitrification, can differ in O$_2$ sensitivity (Dalsgaard et al., 2014; Bristow et al., 2016a, b). In various bacterial strains and natural communities, the NO$_3^-$ reductase enzyme (Nar) which catalyzes the first step in denitrification, is reportedly the most O$_2$ sensitive (Dalsgaard et al., 2014; McKenney et al., 1994; Kalvelage et al., 2011). The fact that N$_2$O production is insensitive to manipulated O$_2$ in the NO$_3^-$ treatments and not in the NO$_3^-$ treatments is evidence that it is not due to inhibition of the reduction of N$_2$O to N$_2$ at higher O$_2$ because then both treatments would look similar. It further indicates that high N$_2$O production from NO$_3^-$ in high-oxygen treatments is not likely an effect of anoxic microniches. While anoxic microniches in batch incubations can never be fully ruled out, there is no reason why they should systematically change N$_2$O production in NO$_3^-$ from NO$_3^-$ incubations at the same oxygen treatment. We suggest a stimulation of incomplete denitrification, which leads to the accumulation of N$_2$O in the serum bottles rather than a stimulation of overall denitrification rates to N$_2$. While NO$_3^-$ reduction was inhibited by higher O$_2$ concentrations, N$_2$O production was not, leading to very high yields of N$_2$O production per NO$_2^-$ produced. We hypothesize that there is a direct channeling of reduced NO$_3^-$ to N$_2$O without exchange of an internal NO$_3^-$ pool with the surrounding NO$_3^-$. Long turnover times for NO$_2^-$ have been inferred from δ$^{18}$O of NO$_2^-$, which was fully
Supplementary Information

4.2 N\textsubscript{2}O yields and hybrid N\textsubscript{2}O formation from NH\textsubscript{4}\textsuperscript{+}

N\textsubscript{2}O yields of AO were 0.15\%–2.07\% (N\textsubscript{2}O-N mol NO\textsubscript{2}-N mol\textsuperscript{-1} = 1.5 \times 10^{-3} – 20.7 \times 10^{-3}), which are at the higher end of most marine AOA culture or field studies (Hink et al., 2017b; Qin et al., 2017; Santoro et al., 2011; Stieglmeier et al., 2014). A higher maximum yield of 3.14\% was reported off the coast of Peru only in 2015 (Ji et al., 2018a). While high N\textsubscript{2}O yields are usually found in low-O\textsubscript{2} waters (< 6 \mu mol L\textsuperscript{-1}), in this study AO also had high yields at higher oxygen concentrations: 0.9\% at 30 \mu mol L\textsuperscript{-1} O\textsubscript{2} compared to previous studies (0.06\% at > 50 \mu mol L\textsuperscript{-1}; Ji et al., 2018a). In near-coastal regions, higher N\textsubscript{2}O yield at higher O\textsubscript{2} concentrations expands the overall water volume where N\textsubscript{2}O production by AO contributes to high N\textsubscript{2}O concentration, which is more likely to be emitted to the atmosphere.

Insight into the production mechanism of N\textsubscript{2}O is gained from hybrid N\textsubscript{2}O formation based on differentiating between production of single-labeled (\textsuperscript{15}N\textsubscript{2}O) and double-labeled (\textsuperscript{15}\textsuperscript{15}N\textsubscript{2}O) N\textsubscript{2}O. If the production of \textsuperscript{15}\textsuperscript{15}N\textsubscript{2}O is higher than what is expected based on the binomial distribution, then an additional source of \textsuperscript{15}N can be assumed. In \textsuperscript{15}N\textsuperscript{4}H\textsuperscript{14}O\textsuperscript{2} incubations, as potential \textsuperscript{14}N substrates (besides NH\textsubscript{4}\textsuperscript{+}), NO\textsubscript{2}, NH\textsubscript{2}OH and HNO are most likely. Even though, in situ NH\textsubscript{4}\textsuperscript{+} is below detection in almost all water depths (f\textsubscript{N} < 0.9), there remains the potential for \textsuperscript{15}NH\textsubscript{4}\textsuperscript{+} pool dilution by remineralization and DNRA during the incubation. Studies have shown fast turnover for NH\textsubscript{4}\textsuperscript{+}, despite low NH\textsubscript{4}\textsuperscript{+} concentrations (e.g., Klawonn et al., 2019). Even if hybrid N\textsubscript{2}O production rates are overestimated, it remains the major N\textsubscript{2}O production mechanism from AO in this study. In future \textsuperscript{15}N-labeling studies, co-occurrence of NH\textsubscript{4}\textsuperscript{+} production by DNRA or degradation should be measured along with N\textsubscript{2}O production to account for pool dilution. Whether hybrid N\textsubscript{2}O formation is purely abiotic, a mix of biotic and abiotic or biotic reactions is debatable (Stieglmeier et al., 2014; Kozlowski et al., 2016; Carini et al., 2018; Lancaster et al., 2018; Stein, 2019). Hybrid N\textsubscript{2}O production from NO\textsubscript{2} was variable with depth and oxygen, which can be explained by the different proportions of nitrifier versus denitrifier NO\textsubscript{2}\textsuperscript{-} reduction to N\textsubscript{2}O. For example, in the interface sample S\textsubscript{19} (892, 144 m, 3.69 \mu mol L\textsuperscript{-1} NO\textsubscript{2}\textsuperscript{-}), N\textsubscript{2}O production from NO\textsubscript{2} (0.72 ± 0.19 mmol L\textsuperscript{-1} d\textsuperscript{-1}) was 20 times higher than from NH\textsubscript{4}\textsuperscript{+} (0.033±0.0004 mmol L\textsuperscript{-1} d\textsuperscript{-1}) and no hybrid N\textsubscript{2}O formation from NO\textsubscript{2} was found (Fig. 5d). There, the major N\textsubscript{2}O production mechanism seems to be by denitrification rather than nitrification, and even if there was a hybrid production we were not able to detect it within the given error ranges. Hybrid N\textsubscript{2}O production from NH\textsubscript{4}\textsuperscript{+} was independent of the rate at which N\textsubscript{2}O production took place and independent of the O\textsubscript{2} concentration and varied little (70\%–86\% of total N\textsubscript{2}O production) during AO. Therefore, a purely abiotic reaction outside and without the vicinity of the cell can be excluded because concentrations of potential substrates for abiotic N\textsubscript{2}O production like Fe(II), Mn, NO and NH\textsubscript{2}OH vary...
with depth and O$_2$ concentration (Zhu-Barker et al., 2015; Kondo and Moffet, 2015; Lutterbeck et al., 2018; Korth et al., 2019). Additionally, at four depths the potential for abiotic N$_2$O production in $^{15}$NO$_2$ addition experiments showed variations with depth and no significant impact of HgCl$_2$ fixation (Fig. S9). Hence, any $^{14}$N which is integrated into N$_2$O to produce a hybrid/single-labeled N$_2$O has to be passively or actively taken up by the cell first (Fig. 9). There, it reacts with an intermediate product ($^{15}$NO or $^{15}$NH$_2$OH) of AO inside the cell. With this set of experiments, it is not possible to disentangle if hybrid production is based on an enzymatic reaction or an abiotic reaction inside the cell. Caranto et al. (2017) showed that the main substrate of NH$_2$OH oxidation is NO, making NO an obligate intermediate of AO in AOB, and suggested the existence of an unknown enzyme that catalyzes NO oxidation to NO$_2^-$ (further details also in Stein, 2019). If NO is an obligate intermediate of AO in AOA (Lancaster et al., 2018), a constant rate of spontaneous abiotic or enzymatic N$_2$O production is very likely, which always depends on the amount of NO produced in the first place. This could explain why we consistently find $\sim$ 80% hybrid formation at high as well as at low AO rates. Further studies are needed to investigate the full mechanisms.

4.3 Effect of particulate organic matter on N$_2$O production

A positive stimulation of N$_2$O production from denitrification by particulate organic matter was found, indicating carbon limitation of denitrification in the ETSF. The experimental POM amendments simulated a low POC export flux and represented a flux that happens over 2–15 d, assuming an export flux of 3.8 mmol m$^{-2}$ d$^{-1}$ and that 8% of the total POC pool is $>50$ $\mu$m (Boyd et al., 1999; Martin et al., 1987; Haskell et al., 2015). We are aware that the POM collected by in situ pumps is a mix of suspended and sinking particles, and hence the flux should be considered a rough estimate. However, the particle size ($>50$ $\mu$m) used in the experiments is indicative of sinking particles. The stimulation of N$_2$ production from denitrification by particulate organic matter has been shown in ODZs before (Ward et al., 2008; Chang et al., 2014), with quantity and quality of organic matter influencing the degree of stimulation (Babbin et al., 2014). In this study, amendments of POM at different degradation stages resulted in variable magnitudes of N$_2$O production from NO$_2^-$ and NO$_3^-$ with no significant correlations between magnitude of the rates and amount, origin, or quality of POM added. The processing of the particles has reduced the original N/C ratios of POM from the mixed layer more than of the POM from the ODZ, resulting in similar N/C ratios of particles from different depths. This could be one possible explanation for a lack of correlation of N$_2$O production with origin of the POM. Furthermore, N$_2$ production was not quantified, and hence it is not possible to evaluate potential relationships between overall N loss and POM additions or whether the partitioning between N$_2$O and N$_2$ varied among treatments and depths. The N$_2$O/N$_2$ production ratio can vary from 0% to 100% (Dalsgaard et al., 2014; Bonaglia et al., 2016). A temporary accumulation of N$_2$O before further reduction to N$_2$ in the incubations can be ruled out as N$_2$O accumulated linearly over time. The only station where POM additions did not stimulate N$_2$O production was in the center of the young eddy (912-S17). There, the highest rates of N$_2$O production from NO$_2^-$ (118 nmol L$^{-1}$ d$^{-1}$) were found, indicating that denitrification was not carbon limited. This is consistent with previous studies on anticyclonic eddies, which have shown high N loss in the core of a young eddy that weakened with aging of the eddy (Stramma et al., 2013; Bourbonnais et al., 2015; Löscher et al., 2016). A direct link between the freshly produced POM fueling N loss on the one hand and decreased N loss with aging due to POM export out of the eddy on the other hand was proposed (Bourbonnais et al., 2015; Löscher et al., 2016). In this study, the young eddy is a hotspot for N$_2$O production.

Besides carbon availability as electron donor for denitrification, copper limitation and high NO$_3^-$ availability may play a role. Copper limitation has been argued to lead to N$_2$O accumulation by inhibiting the copper-dependent N$_2$O reductase (Granger and Ward, 2003; Bonaglia et al., 2016), but it was not a limiting factor for denitrification in the three major ODZs previously (Ward et al., 2008). Water sampling from Niskin bottles in our study was not trace metal clean and could be contaminated with copper from the sampling system, making a limitation of trace metals in our incubations unlikely. However, OM-fueled N$_2$O production may have become limited by the availability of copper during the incubation.

High NO$_3^-$ availability increases N$_2$O production from denitrification in salt marshes (Ji et al., 2015a) and in soils (Weier et al., 1993), systems which are generally not carbon limited. Also, at the oxic–anoxic interface of the Chesapeake Bay, the ratio of NO$_2^-$ to NO$_3^-$ concentration was identified...
as a driver for high N$_2$O production from NO$_3^-$ (Ji et al., 2018b). This study also found higher N$_2$O production rates from NO$_3^-$ than NO$_2^-$, which linearly correlated with the ratio of NO$_3^-$/NO$_2^-$ concentrations (Fig. S8). Intracellularly produced NO$_2^-$ does not seem to exchange with the surrounding pool, but ambient NO$_3^-$ is directly converted to N$_2$O, a process identified as “NO$_3^-$ shunting” in N$_2$ production studies (de Brabandere et al., 2014; Chang et al., 2014). POM as electron donor is an important regulator for reductive N$_2$O production.

4.4 Effect of abundance of total and active community composition on N$_2$O production rates

The abundances of both amoA and nirS genes found in the ETSP are similar to those reported in earlier studies in the ETSP (Peng et al., 2013; Ji et al., 2015b; Jayakumar et al., 2013). The amoA gene abundances were similar to those reported for the coastal ETSP by Lam et al. (2009), but nirS abundances reported here were higher than the nirS abundances in that study, probably due to the use of different PCR primers. The community composition of AOA did not significantly differ along the O$_2$ gradient as shown previously (Peng et al., 2013), but a significant correlation between archaeal amoA transcript abundance and N$_2$O production was shown in this study. The combination of qPCR and microarray analysis offered a great advantage to relate the total abundances to the production rates and additionally link particular community components to biogeochemical activities. To determine whether a particular archetype drives the correlation of N$_2$O production by AO, a Bray–Curtis dissimilarity matrix revealed archetype AO3 related to Candidatus Nitrosopelagicus brevis (CN25) to be significantly correlated with the N$_2$O production by AO. This clade is abundant in the surface ocean and typically found in high abundances in the lower euphotic zone (Santoro et al., 2011, 2015). With the demonstration of high abundances of AO3 coincident with high nitrification rates and high N$_2$O production rates, we suggest that Candidatus Nitrosopelagicus brevis-related AO3 likely play an important role in N$_2$O production in near-surface waters in the eastern tropical South Pacific.

The lack of significant correlation between community composition or single members of the community and reductive N$_2$O production is consistent with the fact that nirS is not the enzyme directly synthesizing N$_2$O and nirS communities are sources as well as sinks for N$_2$O. Taxonomic analysis of the nirS gene and transcripts suggested that there is high taxonomic diversity among the denitrifiers, which is likely linked to a high variability of the total denitrification gene assembly (including nos, nor, nir). In particular the abundance and diversity of nitric oxide reductase (nor), the enzyme directly synthesizing N$_2$O, would be of interest, but it is present in nitrifiers and denitrifiers (Casciotti and Ward, 2005), and one goal of this study was to differentiate among N$_2$O produced by nitrifiers and denitrifiers. However, nirS gene and transcript abundance correlated with N$_2$O production from NO$_3^-$, making it a possible indicator for one part of reductive N$_2$O production. It is also worth noting that ammox-related nirS genes and transcripts (nirS 166, 167) contribute up to 12% of the total copy numbers, putting a wrinkle on nirS abundance as a marker gene for denitrifiers only. The subtraction of the ammox-related nirS genes from total copy numbers did not change the results from Bray–Curtis analysis. These data indicate that the extent to which gene or transcript abundance patterns or community composition of marker genes of processes can be used as proxies for process rate measurements is variable, likely due to complex factors, including the relative dominance of different community members, the modular nature of denitrification, differences in the level of metabolic regulation (transcriptional, translational and enzymatic) and the range of environmental conditions being observed.

4.5 Summary and conclusion

In this study we used a combined approach of $^{15}$N tracer techniques and molecular techniques in order to investigate the factors that control N$_2$O production within the upper oxycline of the ODZ in the ETSP. Our results suggest that denitrification is a major N$_2$O source along the oxic–anoxic interface of the upper oxycline. The highest N$_2$O production rates from NO$_3^-$ and NO$_2^-$ were found at or below the oxic–anoxic interface, whereas the highest N$_2$O production from AO was slightly shallower in the oxycline. Overall, the in situ O$_2$ threshold below 8 μmol L$^{-1}$ favored NO$_3^-$ and NO$_2^-$ reduction to N$_2$O and high N$_2$O yields from AO up to 2.2%. A different pattern was observed for the community response to increasing oxygen, with the highest N$_2$O production from NH$_4^+$ and NO$_2^-$ between 1.4 and 6 μmol L$^{-1}$ O$_2$ and high N$_2$O production from NO$_3^-$ even at O$_2$ concentrations up to 22 μmol L$^{-1}$. This study highlights the diversity of N$_2$O production regulation and the need to conduct further experiments where single community members can be better constrained. Our experiments provide the first insights into N$_2$O regulation by particulate organic matter in the ETSP with particles greatly enhancing N$_2$O production (up to 5-fold). Furthermore, the significant positive correlation between Candidatus Nitrosopelagicus brevis (CN25) and N$_2$O produced from AO could indicate its importance in N$_2$O production and points out the great value of combining biogeochemical rate measurements with molecular analysis to investigate multifaceted N$_2$O cycling. This study shows that short-term oxygen increase can lead to high N$_2$O production even from denitrification and extends the existing O$_2$ thresholds for high reductive N$_2$O production up to 22 μmol L$^{-1}$ O$_2$. Together with high N$_2$O yields from AO up to O$_2$ levels of 30 μmol L$^{-1}$, an expansion of low-oxygen waters around ODZs predicted for the future can significantly increase marine N$_2$O production.
Regardless of which processes are responsible for N$_2$O production in the ODZ, high N$_2$O production at the oxic–anoxic interface of the upper oxycline sustains high N$_2$O concentration peaks with a potential for intense N$_2$O emission to the atmosphere during upwelling events. An average total N$_2$O production rate of 3.1 nmol N$_2$O L$^{-1}$ d$^{-1}$ in a 50 m thick suboxic layer with 0–20 µmol L$^{-1}$ O$_2$ leads to an annual N$_2$O efflux of 0.5 Tg N yr$^{-1}$ in the Peruvian upwelling (2.22 × 10$^7$ km$^2$, Arévalo-Martínez et al., 2015), which is within the estimates based on surface N$_2$O concentration measurements from 2012 to 2013 (Arévalo-Martínez et al., 2015; Bourbonnais et al., 2017). The importance of the Peru upwelling system for global N$_2$O emissions (5%–22% of global marine N$_2$O emissions) is directly linked to the extreme N$_2$O accumulations in coastal waters. Coastal N$_2$O hotspots are well known (Bakker et al., 2014), and this study shows that they can be explained by considering denitrification as a major N$_2$O source. While this study does not help to resolve temporal variability, manipulation experiments give valuable insights into the short-term response of N$_2$O production to oxygen and particles. With the further parametrization of POM export as a driver for N$_2$O production from denitrification, models may be able to better predict N$_2$O emissions in highly productive coastal upwelling regions and to evaluate how fluxes might change with changing stratification and deoxygenation.

**Data availability.** The microarray data presented here are available via GEO (Gene Expression Omnibus; http://www.ncbi.nlm.nih.gov/geo; NCBI, 2020) at NCBI (National Center for Biotechnology Information) under GEO accession no GSE142806. The N$_2$O production rates were archived in the PANGAEA data archive (https://doi.pangaea.de/10.1594/PANGAEA.914948, Frey et al., 2020). The N$_2$O data are available from the Marine Methane and Nitrous Oxide (MEMENTO) database (https://memento.geomar.de/de/n2o, last access: 16 April 2020; Kock and Bange, 2015).

**Supplement.** The supplement related to this article is available online at: https://doi.org/10.5194/bg-17-2263-2020-supplement.

**Author contributions.** CF, HWB and BBW conceptualized the study. CF and MS performed the experiment. CF and ELP analyzed samples. RCX and EPA collected POM. DLAM sampled and measured N$_2$O concentrations. AJ performed qPCR. SO supported the mass spectrometer analysis. XS supported the experimental methods and assisted with data analysis. CF analyzed data and led the writing effort, with substantial contributions from all coauthors.

**Competing interests.** The authors declare that they have no conflict of interest.

Special issue statement. This article is part of the special issue “Ocean deoxygenation: drivers and consequences – past, present and future (BG/CP/OS inter-journal SI)”. It is a result of the International Conference on Ocean Deoxygenation, Kiel, Germany, 3–7 September 2018.

Acknowledgements. We thank the captain and crew of R/V Meteor. A great thanks goes to Annette Kock for helping to pack, ship, order material for the cruise. Moreover, we thank the Peruvian authorities for the permission to work in their territorial waters. We thank the editor S. Wajih A. Naqvi, Annie Bourbonnais and an anonymous reviewer for their thoughtful, constructive comments which greatly improved this paper.

**Financial support.** The work presented here was made possible by the DFG-funded Collaborative Research Center 754 (SFB754) phase III (http://www.sfb754.de, last access: 16 April 2020) and by a fellowship of the German Academic Exchange Service (DAAD) program Postdoctoral Researchers International Mobility Experience (PRIME, ID 57350888) awarded to Claudia Frey. Mingshuang Sun was supported by the China Scholarship Council (no. 201406330054). Elizabeth León-Palmero had a FPU PhD fellowship (014/02917) from the Spanish Ministry of Education and a PhD International Mobility scholarship from the Universidad de Granada. Carolin R. Löschler was funded by a EU H2020 Marie Curie Individual Fellowship (NITROX, grant no. 704272) and by the Villum Foundation (grant no. 16518). Eric P. Achterberg, Hermann W. Bange and Ruifang C. Xie were funded by the DFG-funded Collaborative Research Center 754 (SFB754) program. Bess B. Ward, Xin Sun and Amal Jayakumar were funded by the National Science Foundation (NSF, OCE-1657663). The article processing charges for this open-access publication were covered by a Research Centre of the Helmholtz Association.

**Review statement.** This paper was edited by S. Wajih A. Naqvi and reviewed by Annie Bourbonnais and one anonymous referee.

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Biogeosciences, 17, 2263–2287, 2020


