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

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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 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. Highest N2O production from ammonium oxidation (AO) of 0.16 ± 0.003 nmol L-1 d-1 occurred in the upper oxycline at O2 concentrations of 10 - 30 µmol L-1 which coincided with highest archaeal amoA transcripts/genes. 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.
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 oxygen (O₂) gradients (oxygencline), 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 O₂ may decline to functionally anoxic conditions (O₂ <10 nmol kg⁻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⁻¹ O₂) which are strong net N₂O sources (Codispoti 2010; Babbin et al. 2015). Latest estimates of global, marine N₂O fluxes (Buitenhuis et al. 2018, Ji et al. 2018) agree well with the 3.8 Tg N y⁻¹ (1.8 – 9.4 Tg N y⁻¹) reported by the IPCC (2013), but have large variability in the resolution on the regional scale, particularly along coasts where N₂O 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, Schmidtko et al. 2017). This might lead to further intensification of marine N₂O emissions, which will constitute a positive feedback on global warming (Battaglia and Joos, 2018). However, decreasing N₂O 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 N₂O production and consumption in global ocean models is crucial for realistic future predictions, and therefore better understanding of their controlling mechanisms is needed.

N₂O can be produced by both nitrification and denitrification. Nitrification is a two-step process, comprising the oxidation of ammonia (NH₃) to nitrite (NO₂⁻) (ammonia oxidation, AO) and nitrite to nitrate (NO₃⁻) (nitrite 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 

amoA
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 N₂O as a by-product (Anderson 1964; Vajrala et al. 2013; Stein 2019) and AOA contribute significantly to N₂O production in the ocean (Santoro et al. 2011; Löschler et al. 2012). While hydroxylamine (NH₂OH) 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 N₂O in AOA remains controversial. Stieglmeier et al. (2014) concluded that NO is derived from NO₂⁻ reduction to form N₂O, while Carini et al. (2018) hypothesized that NO is derived from NH₂OH oxidation, which can then form N₂O. A hybrid N₂O production mechanism in AOA has been suggested, where NO from NO₂⁻ reacts with NH₂OH from NH₄⁺, which is thought to be abiotic, i.e., non-enzymatic (Koslovski et al. 2016). Abiotic N₂O production from intermediates like NH₂OH, NO or NO₂⁻ 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:O production in ODZs is still lacking.

When O\textsubscript{2} concentrations fall below 20 \(\mu\text{mol L}^{-1}\), nitrifiers produce N\textsubscript{2}O from NO\textsubscript{3}\textsuperscript{-}, a process referred to as nitrifier - denitrification (Frame & Casciotti 2010), which has been observed in cultures of AOB (Frame & Casciotti 2010) and AOA (Santoro et al. 2011). During nitrifier-denitrification, two NO\textsubscript{3}\textsuperscript{-} molecules form one N\textsubscript{2}O, which thus differentiates this process from hybrid N\textsubscript{2}O production. Overall, the yield of N\textsubscript{2}O per NO\textsubscript{3}\textsuperscript{-} increases with decreasing O\textsubscript{2} concentrations, which favors higher N\textsubscript{2}O production by nitrification in hypoxic waters (Cohen & Gordon 1978; Yoshida 1988; Goreau et al. 1980; Löscher et al. 2012, Ji et al. 2015a, 2018a).

The anaerobic oxidation of ammonia by nitrite (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 nitrate to elemental N\textsubscript{2} via nitrite, 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 under saturation 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}\textsuperscript{-} or NO\textsubscript{2}\textsuperscript{-} concentrations (Ji et al. 2018), 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 al. 2007, Babbin et al. 2015, Ji et al. 2015a, Yang et al. 2017). They show the importance of denitrification in N\textsubscript{2}O production in the ETNP from model outputs (Babbin et al. 2015) and in the ETSP from tracer incubation experiments (Dalsgaard et al. 2012, Ji et al. 2015a), based on natural abundance isotopes in N\textsubscript{2}O (Casciotti et al. 2018) or from water mass analysis of apparent N\textsubscript{2}O production (\(\Delta\text{N2O}\)) and O\textsubscript{2} utilization (AOU) (Carrasco et al. 2017).\textsuperscript{5,6}N\textsubscript{2}O production from the addition of \(^{15}\text{N}\)-labeled NH\textsubscript{4}\textsuperscript{+}, NO\textsubscript{2}\textsuperscript{-} and/or NO\textsubscript{3}\textsuperscript{-} revealed nitrification as a source of N\textsubscript{2}O within the oxic-anoxic interface, but overall denitrification dominated N\textsubscript{2}O production with higher rates at the interface and in anoxic waters (Ji et al. 2015a, 2018a). Denitrification is driven by organic matter exported from the photic zone and fuels blooms of denitrifiers leading to high N\textsubscript{2} production (Dalsgaard et al. 2012, Jayakumar et al. 2009, Babbin et al. 2014). Denitrification to N\textsubscript{2} 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\textsubscript{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\textsubscript{2}O fluxes.

N\textsubscript{2}O concentration profiles around ODZs appear to be at steady state (Babbin et al. 2015), but are much more variable in regions of intense coastal upwelling where high N\textsubscript{2}O emissions can occur (Arévalo-Martínez et al. 2015). The contributions of and controls on the two N\textsubscript{2}O production pathways under different conditions of O\textsubscript{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\textsubscript{2}O production around ODZs in order to better constrain how future changes in O\textsubscript{2} concentration and carbon export will impact production, distribution and emissions of oceanic N\textsubscript{2}O. Our goal was to determine the impact of O\textsubscript{2} and particulate organic matter on N\textsubscript{2}O production rates using \(^{15}\text{N}\) tracer experiments in combination with 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
N₂O production rates from the different pathways. ¹⁵N-labelled NH₄⁺ and NO₂⁻ was used to trace the production of single- (¹⁵N:O) and double-labelled (¹⁵N:O) N₂O to investigate the importance of hybrid N₂O production during AO along an O₂ gradient.

2 Materials and Methods

2.1 Sampling sites, sample collection and incubation experiments

Seawater was collected from 9 stations in the upwelling area off the coast of Peru in June 2017 onboard R/V Meteor (Figure 1). Water samples were collected from 10 L Niskin bottles on a rosette with a conductivity-temperature-depth profiler (CTD, seabird electronics 9plus system). In-situ O₂ concentrations (detection limit 2 µmol L⁻¹ O₂), temperature, pressure and salinity were recorded during each CTD cast. NO₂⁻ and NO₃⁻ concentrations were measured on board by standard spectrophotometric methods (Hydes et al. 2010) using a QuAAtro autoanalyzer (SEAL Analytical GmbH, Germany). NH₄⁺ concentrations were determined fluorometrically using ortho- phthalaldehyde according to Holmes et al. (1999). 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 O₂ concentration along a natural and artificial O₂ gradient and experiment 3 targeted the impact of large particles (>50 µm) on N₂O production. Serum bottles were filled from the Niskin bottles with Tygon tubing after overflowing three times to minimize O₂ contamination. Bottles were sealed bubble free with grey butyl rubber septa (National Scientific) and crimped with aluminum seals immediately after filling. A 3 mL helium (He) headspace was created and samples from anoxic water depths were He purged for 15 min. Natural abundance 1000 ppm N₂O carrier gas (50 µL in He) was injected to trap the produced labeled N₂O and to ensure a sufficient mass for isotope analysis. ¹³N-NO₂⁻,¹⁵N-NO₂⁻, and ¹⁵N-NH₄⁺ tracer (¹⁵N/¹⁴N = 99 atom-%) were injected into five bottles each from the same depth to a final concentration of 0.5 µmol L⁻¹, except for the NO₂⁻ incubations where 2 µmol L⁻¹ final concentration were anticipated to obtain 10 % label of the NO₂⁻ pool. The fraction labeled of the substrate pools was 0.76 – 0.99 for NH₄⁺, 0.11 – 0.99 for NO₂⁻, 0.035 – 0.1 for NO₃⁻. In the ¹³N-NO₂⁻ treatment, ¹⁴N-NO₂⁻ was added to trap the label in the product pool for nitrate reduction rates and in the ¹⁵N-NH₄⁺ treatment, ¹⁴N-NO₂⁻ 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, headspace volume was adjusted depending on the amount of site water added and all samples were He purged. Site water from the incubation depth was shaken and exposed to air to reach full O₂ saturation. Then 0.2, 0.5, 2 and 5 mL O₂ saturated seawater was added into serum bottles and to reach final measured O₂ concentrations of 0 ± 0.18 µM, 0.4 ± 0.24 µM, 1.6 ± 0.12 µM, 5.2 ± 0.96 µM and 11.7 ± 1.09 µM in seawater. For the ¹³N-NO₂⁻ incubations two more O₂ treatments with 13.6 ± 1.4 µM and 21.5 ± 3.35 µM O₂ were carried out. 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). 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 3 different depths were collected with a Challenger stand-alone pump system (SAPS in situ pumps, Liu et al. 2005), autoclaved and He purged.
before 200µL of POC solution were added to each serum bottle. The final concentrations and C/N ratios varied between 0.18 – 1.37 µM C and 8.1 – 15.4, respectively (Table 2). The concentration and C/N ratio of PON and POC of the stock solutions were analyzed by mass spectrometry using GV 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 time for each experiment varied from 12 hours (at the shelf stations) to 24 hours (at the slope stations). Incubation was terminated by adding 0.1 mL 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. (2015). Briefly, serum bottles were flushed with He for 35 min (38 ml min⁻¹), N₂O was trapped by liquid nitrogen, H₂O removed with an ethanol trap, a Nafion® trap and a Mg(ClO₄)₂ trap and CO₂ removed with an Ascariid CO₂-Adsorbance column and afterwards mass 44, 45, 46 and isotope ratios 45/44, 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 ¹⁵N¹⁸O sixteen or ¹⁴N¹⁵O for ⁴⁵N₂O and the less abundant ¹⁵N¹⁵O sixteen for ⁴⁶N₂O. To evaluate the analyses of ¹⁵N-enriched N₂O samples, internal isotope standards for ¹⁵N₂O were prepared by mixing natural abundance KNO₂ 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 ¹⁵NO₂⁻ 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 (Mellvin 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. 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 ¹⁴NH₄⁺ and unlabeled NO₃⁻, it is assumed that AO produces ⁴⁰N₂O from two labeled NH₄⁺ (equation 1) and some ⁴⁰N₂O-labeled N₂O based on binominal distribution (equation 2). If more single labelled N₂O is produced than what is expected (equation 2 and 3) than a hybrid formation of one nitrogen atom from NH₄⁺ and one from NO₃⁻ (equation 4) is taking place as found in archaeal ammonia oxidizers (Kozlowski et al. 2016). In incubations with ¹⁵NO₃⁻, we assume that ⁴⁰N₂O comes from nitrifier-denitrification or denitrification, which cannot be distinguished (equation 1). Hence, any production of ⁴⁰N₂O not attributed to denitrification stems from hybrid N₂O formation by archaeal nitrifiers (equation 4). In incubations with ¹⁵NO₃⁻, denitrification produces ⁴⁰N₂O and was the only process considered and hence was calculated based on equation (1). Rates (R) are calculated as nmol N₂O L⁻¹ d⁻¹ (Trimmer et al. 2016):

\[
(1) \quad R_{\text{external}} = \text{slope} \times (f_o)^{−2}
\]

\[
(2) \quad R_{\text{expected}} = \text{slope} \times (1 − f_o) \times (f_o)^{−1}
\]
(3) \[ R_{\text{above}} = \text{slope}^{45}N_2O - p^{45}N_2O_{\text{expected}} \]

(4) \[ R_{\text{hybrid}} = (f_h)^{-1} \times (\text{slope}^{45}N_2O + 2 \times \text{slope}^{46}N_2O \times (1 - f_h)) \]

(5) \[ R_{\text{total}} = pN_2O_{\text{external}} + pN_2O_{\text{hybrid}} \]

where \( f_h \) is the fraction of \(^{15}\text{N} \) in the substrate pool (NH\(_4\)\(^+\), NO\(_3^-\) or NO\(_2^-\)) which is assumed to be constant over the incubation time. Hence, changing \( f_h \) due to any other concurrent N-consumption or production process during the incubation is neglected. For example, accounting for a decrease in \( f_h \) of the nitrate pool by active nitrite oxidation, the process with highest rates (Sun et al. 2017), had an effect of only \( \pm 0.2\% \) on the final rate estimate. Slope of \(^{46}\text{N}_2\text{O} \) and slope of \(^{45}\text{N}_2\text{O} \) represent the \(^{46}\text{N}_2\text{O} \) and \(^{45}\text{N}_2\text{O} \) production rates, which were tested for significance based on a linear regression (n=5, student t-test, 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 Sigma Plot 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\(^+\) oxidation to NO\(_2^-\) and NO\(_2^-\) reduction to NO\(_3^-\) were calculated based on the slope of the linear regression of \(^{15}\text{NO}_2^-\) enrichment over time (n = 5) (equation 6).

\[ R = f_h^{-1} \times \text{slope}^{15}\text{NO}_2^- \]

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

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

\[ \text{Yield}_{\text{NH}_4} = \frac{N^- - N\_N\_O\_2^- (\frac{dN}{d\tau})}{N^- - N\_N\_O\_2^- (\frac{dN}{d\tau})} \times 100\% \]

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

\[ \text{Yield}_{\text{NO}_3} = \frac{N^- - N\_N\_O\_2^- (\frac{dN}{d\tau})}{N^- - N\_N\_O\_2^- (\frac{dN}{d\tau}) + N^- - N\_N\_O\_2^- (\frac{dN}{d\tau})} \times 100\% \]

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

2.3 Molecular Analysis – qPCR, Microarrays

DNA and RNA were extracted using the DNA/RNA ALLPrep 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 Quant-iT OliGreen ssDNA Quantification Kit (life technologies) was used for cDNA quantification.

The abundances of total and active nir\(S\) and archaeal amo\(A\) communities were determined by quantitative PCR (qPCR) with assays based on SYBR Green staining according to methods described previously (Jayakumar et al. 2013, Peng et al. 2013). Primers nir\(S\)1F and nir\(S\)3R (Braker et al. 1998) were used to amplify a 260-bp conserved region within the nir\(S\) gene. Primers Arch-amo\(A\)F and Arch-amo\(A\)R (Francis et al. 2005) were used to quantify archaeal amo\(A\) abundance. A standard curve containing 6 serial dilutions of a plasmid with either an archaeal amo\(A\) fragment or a nir\(S\) 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, ddUaa was incorporated into DNA and cDNA targets during linear amplification with random octomers 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 oligo 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). 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 cDNA 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/) at NCBI (National Center for Biotechnology Information) under GEO Accession No XXXX.

2.4. Data analysis

Spearman Rank correlation was performed from all N\textsubscript{2}O production rates, AO and nitrate reduction rates, environmental variables, nirS and archael amoA gene and transcript abundance as well as 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 & Legendre 2012) was used to visualize differences in community composition dependent upon environmental conditions using the software PAST (Hammer et al. 2001). Before CCA analysis, 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.lefse 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 (http://huttenhower.sph.harvard.edu/lefse/) to test for discriminatory archetypes between O\textsubscript{2} 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 O$_2$ 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 hot spot for N$_2$O emissions (Arévalo-Martínez et al. 2015) with 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 N$_2$O concentration profiles (Kock et al. 2016) and N$_2$O emissions (Arévalo-Martínez et al. 2015). The Peru Coastal Water (PCW, temperature $<$19.5°C, salinity 34.9 - 35.1) and the equatorial subsurface waters (ESSW, temperature 8-12°C, salinity 34.7 – 34.9) (Pietri et al. 2013) were the dominant water masses off the Peruvian coast sampled for N$_2$O production rate measurements (Table 1). At the southern-most transect at 15.5° – 16°S a meso-scale anticyclonic mode water eddy (McGillicuddy et al. 2007), which was about to detach from the coast, was detected from deepening/shoaling of the main/seasonal pycnoclines (Bange et al. 2018, Figure S1). Generally, the stations were characterized by a thick anoxic layer (254 m – 427 m) reaching to the seafloor at two shelf stations (894, 883). NO$_2$ concentration accumulated only up to 2 µmol L$^{-1}$ in the secondary nitrite maximum (SNM) at the northern transect (stations 882, 883), but up to 7.19 µmol L$^{-1}$ along the southern transect (Figure 2, station 907, 912). N$_2$O concentration profiles showed a high variability with respect to depth and O$_2$ concentrations (Figure 2). The southern transect (station 907,912) showed the lowest N$_2$O concentrations (5 nmol L$^{-1}$) in the center of the anoxic zones. At the same time, station 912 in the center of the eddy showed highest N$_2$O concentration with 78.9 nmol L$^{-1}$ at [O$_2$] below detection limit in the upper part of the anoxic zone. Above the ODZ, the maximum N$_2$O peak ranged from 57.9 – 78.9 nmol L$^{-1}$ and was found at an O$_2$ concentration range from below detection (883, 894, 892, 912) up to 67 µmol L$^{-1}$ (907). Three stations (892, 894 and 904) showed high surface N$_2$O concentrations of 64 nmol L$^{-1}$.

3.2 Depth Distribution of N$_2$O production rates and total and active nirS and amoA abundance

N$_2$O production varied with depth and substrate (Figure 3, Table S3). In the oxycline, highest AO (34 ± 0.1 nmol L$^{-1}$ d$^{-1}$ and 35 ± 9.2 nmol L$^{-1}$ d$^{-1}$) coincided with highest N$_2$O production from AO (0.141 ± 0.003 nmol L$^{-1}$ d$^{-1}$ and 0.159 ± 0.003 nmol L$^{-1}$ d$^{-1}$) at both stations of the northern transect, stations 883 and 882, respectively (Figure 3(I)a, b). NH$_4^+$ oxidation and its N$_2$O production decreased to zero in the ODZ. The rates of the reductive source pathways for N$_2$O increased with depth. N$_2$O production from NO$_2^-$ and NO$_3^-$ displayed similar patterns with highest production at or below the oxic -anoxic interface (Figure 3(II)). N$_2$O production from NO$_2^-$ showed highest rates of 3.06 ± 1.17 nmol L$^{-1}$ d$^{-1}$ (912) and 2.37 ± 0.54 nmol L$^{-1}$ d$^{-1}$ (906) further south (Figure 3(II) m, q) compared to lower rates at northern stations, where the maximum rate was 0.71 ± 0.38 nmol L$^{-1}$ d$^{-1}$ (Figure 3(II) c, 883). A similar trend was found for N$_2$O production from NO$_3^-$: lower maximum rates at northern stations with 2.7 ± 0.4 nmol L$^{-1}$ d$^{-1}$ (882) and 5.7 ± 2.8 nmol L$^{-1}$ d$^{-1}$ (883, Figure 3(II) b) and highest rates in southern transects
with 7.2 ± 1.64 nmol L⁻¹ d⁻¹ ((Figure 3(II) l, 904) in transect 3 and up to 118.0 ± 27.8 nmol L⁻¹ d⁻¹ (Figure 3(II) p, 912) in transect 4. Generally, N₂O production rates from NO⁻² and NO₂⁻ were 10 to 100-fold higher than from AO.

qPCR analysis detected lowest gene and transcript numbers of archaeal amoA and nirS in the surface mixed layer (Figure 3(I) k, l, 3(II)r, s). Highest archaeal amoA gene and transcript abundance was in the oxycline (1 – 40 μmol L⁻¹ O₂) with 24,500 ± 340 copies mL⁻¹ and 626 ± 29 copies mL⁻¹ at station 883 (Figure 3(I)c, d). amoA gene and transcript number decreased in the ODZ to 1000 – 6500 gene copies mL⁻¹ and 20 - 250 transcript copies mL⁻¹. The profiles of nirS gene and transcript abundance were similar to each other (Figure 3(II) d, e) with highest abundance in the ODZ up to 1 x 10⁶ copies mL⁻¹ and 2.9 x 10⁵ copies mL⁻¹, respectively. Denitrifier nirS genes and transcripts peaked in the anoxic layer and were significantly correlated with N₂O production from NO⁻² but not from NO⁻¹. Archaeal amoA gene and transcript abundances were significantly correlated with AO and, N₂O production from AO (Figure S3). N₂O concentrations did not correlate with any of the measured variables (Figure S3).

3.3 Influence of O₂ concentration on N₂O production

N₂O production along the in situ O₂ gradient for the substrates NO⁻¹ and NO₂⁻ decreased exponentially with increasing O₂ concentrations (Figure 4b, c) while for NH₄⁺, the N₂O production was highest at highest sampled O₂ concentration (Figure 4a). At in situ O₂ levels above 8.4 μmol L⁻¹ N₂O production decreased by 100% and 98% from NO⁻¹ and NO₂⁻ respectively (Figure 4b, c).

In the manipulated O₂ treatments from the oxic - anoxic interface (S11, S19) a unimodal response of N₂O production from NH₄⁺ and NO₂⁻ to O₂ is apparent (Figure 4d, e). Increasing and decreasing O₂ concentrations inhibited N₂O production from NH₄⁺ and NO₂⁻ with the highest N₂O production rate between 1.4 - 6 μmol O₂ L⁻¹. However, this response was only significant in sample S11 (Figure 4d, e). There was no significant response to O₂ concentration of N₂O production from NO⁻¹. O₂ did not inhibit N₂O production from NO⁻¹ up to 23 μmol L⁻¹ (Figure 4f).

The proportion of hybrid N₂O produced during AO, i.e., the formation of N₂O from one ¹⁵N from the labelled NH₄⁺ and one ¹⁴N from a non - labelled N compound (excluding NH₄⁺) such as NO⁻³, NH₂OH or NO₃⁻, was consistently between 70 – 85 % across different O₂ concentrations for manipulated and natural O₂ concentrations (Figure 5a, c). Hybrid formation during N₂O production from NO₂⁻ varied between 0 and 95% along the natural O₂ gradient (Figure 5b). In manipulated O₂ treatments hybrid formation from NO₂⁻ did not change across different O₂ 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 120m of station 894 (Figure 5d).

Highest N₂O yields during AO (over 1%) occurred between 1.4 and 2 μmol O₂ L⁻¹, and decreased at both higher and lower O₂ concentrations (Figure 6a). However, only the increase in yield from nmol O₂ to 1.4 – 2 μmol L⁻¹ O₂ was significant (t-test, p<0.05) and the following decrease in yield was not (t-test, p>0.05). In the manipulated O₂ treatment of sample S19 (Figure 6c) the same significant pattern was observed, whereas in S11 highest yield was found at 12 μmol L⁻¹ O₂: N₂O yield during nitrate reduction to NO₂⁻ decreased to zero at 8.4 μmol L⁻¹ O₂ along the natural O₂ gradient (Figure 6b) while no significant response occurred in the manipulated O₂ treatments (Figure 6d). There, nitrate reduction was decreasing with increasing O₂ but N₂O production was steady with increasing O₂ leading to high yields between 38.8 ± 9 % - 91.2 ± 47 % at 23 μmol L⁻¹ O₂.
3.4 Effect of large particulate organic matter on N$_2$O production

The autoclaving of the concentrated POM solution liberated NH$_4^+$, reducing the N/C ratio of the particles compared to non-autoclaved particles (Table 2). The highest NH$_4^+$ accumulation is found in samples with the largest difference in N/C ratios (Table 2, 904-20m, 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 (Figure 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 (Figure 7b).

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. 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 (students t-test, $p > 0.05$), but were overall lower for RNA (3.2 ± 0.3) than DNA (3.8 ± 0.4) (Table S1). Principle 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 (Figure S2). 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 lese 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 (Figure 8a, b). Archetypes over 1% made up between 76% (DNA) - 83% (cDNA) of the amoA assemblage and only 61% (DNA) - 68% (cDNA) of the nirS assemblage. The 4 most abundant AOA archetypes AOA55, AOA3, AOA21 and AOA32 made up 20% - 65% of the total and active community (Figure 8a, b). DNA of archetypes AOA55 and AOA79, both related to uncultured AOA in soils, significantly correlated with $in situ$ NH$_4^+$ concentrations (Figure S3). 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 Nitrosopelagicus brevis (CN25), identified as the only archetype to be significantly correlated with N$_2$O production and yield from AO (Figure S3).

The total and active denitrifier communities were dominated by Nir7, derived from an uncultured clone from the ODZ in the ETSP (Lam et al. 2009), and Nir7 was significantly more enriched in the active community (Figure 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 (Figure 8c, d). Interestingly, these two samples were not divergent among the active nirS community (Figure 8c, d; Figure S2). DNA of Nir35, belonging to the Flavobacteriaceae derived from coastal waters of the Arabian Sea (Goréguès 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 5 abundant archetypes, were significantly enriched in oxic water masses (Table S2). nir166, belonging to Scalindua, and Nir23 were among the top 5 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 (Figure S4a, b). S18 (912, 5 m) is a surface sample with lowest NO\textsubscript{3} concentration (8 \( \mu \)mol L\(^{-1} \)), highest temperature and salinity of the data set and the DNA is positively related with \( O_2 \) 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 \( O_2 \) and NH\textsubscript{4}\textsuperscript{+} (Figure S3).

CCA clustered the denitrifier community DNA into one main group with a few exceptions (Figure S4 c). Two surface samples (S16, S18) clustered separate and were positively correlated with Nir4 and Nir14 and \( O_2 \).

Two anoxic samples from the eddy core (S17) and eddy edge (S15) clustered separate with S17 being driven by 3 nirS archetypes – Nir54, Nir10 and Nir167 and S15 by Nir23, Nir35 and Nir133 (Figure S4 c). Total and active nirS community composition did not differ as a function of \( O_2 \). Although, composition of active and total nirS communities were 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 \( O_2 \), temperature and salinity, whereas the anoxic eddy samples did not differ from the rest (Figure S4d). \( N_2O \) production from NO\textsubscript{2}– significantly correlated with nirS gene and transcript abundance but both reductive \( N_2O \) production pathways were not linked with a single dominant nirS archetype (Figure S3).

4. Discussion

Most samples originated from Peru Coastal Water (PCW) characterized by supersaturated \( N_2O \) concentrations (Kock et al. 2016, Bourbonnais et al. 2017). Only the deepest sample (S1, 882 - 350m) saw the presence of a different water mass, the equatorial subsurface waters. Thus, our findings about regulation of \( N_2O \) 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 (Biller et al. 2012, Hamdan et al. 2012) and a coupling of amo\textsubscript{A} 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 (Figure S4), for \( N_2O \) production rates correlations with physical and chemical parameters were not consistent. On one hand, oxidative \( N_2O \) production from NH\textsubscript{4}\textsuperscript{+} positively correlated with temperature, salinity, oxygen and negatively with depth and PO\textsubscript{4}\textsuperscript{3–} concentration, on the other hand, reductive \( N_2O \) production from NO\textsubscript{2}– positively correlated with NH\textsubscript{4}\textsuperscript{+} and NO\textsubscript{2}– concentrations, but negatively with NO\textsubscript{3}– concentrations (Figure S3). Both oxidative (AO) and reductive (NO\textsubscript{2}– and NO\textsubscript{3}– reduction) \( N \) cycling processes produced \( N_2O \) with differential effects of \( O_2 \) on them. Measured \( N_2O \) production rates were always highest from NO\textsubscript{2}–, followed by NO\textsubscript{3}– and NH\textsubscript{4}+, which is consistent with previous studies that showed denitrification as a dominant \( N_2O \) source in Peruvian coastal waters harboring an ODZ (Ji et al. 2015a, Casciotti et al. 2018). A low contribution of AO to \( N_2O \) production in low \( O_2 \) waters is in line with a previous study in this area estimating \( N_2O \) 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 \( N_2O \) production was between 0.5 – 6%, with one exception in the shallowest sample S5 with

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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 NO⁻ concentrations and strong N₂O depletion in the core of ODZ of the eddy result in reduced N₂O inside of this kind of eddies as they age and are advected westward (Cornejo D’Ottone et al. 2016, Arévalo-Martínez et al. 2016). Our study found similar patterns with largest SNM (5.23 µM NO⁻), lowest NO⁻ (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 120 nmol L⁻¹ d⁻¹ are the highest N₂O production rates from denitrification reported in the ETSP. Previously reported maximum rates ranged from 49 nmol L⁻¹ d⁻¹ (Bourbonnais et al. 2017) and 50 nmol L⁻¹ d⁻¹ (Farias et al. 2009) to 86 nmol L⁻¹ d⁻¹ (Dalsgaard et al. 2012). N₂ production was not measured, so it cannot be determined whether this high N₂O production represents a high N₂O/N₂ yield or if the N₂ production rates were also 10 times higher than outside of the eddy. Considering that at some depths only incomplete denitrification (also known as “stop- and go” denitrification) to N₂ is at work, it would not be surprising that N₂O production can reach the same order of magnitude as N₂ production from complete denitrification. Aged eddies also show lower N₂O 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 N₂O 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 N₂O concentrations and high N₂O production rates have a great potential for high N₂O emissions compared to aged eddies or waters surrounding eddies.

4.1 Effect of O₂ on reductive and oxidative N₂O production

The relationship between O₂ concentrations and N₂O production by nitrification and denitrification is very complex in ODZs. While poorly constrained, the reported O₂: threshold level (1.7 µmol L⁻¹ O₂) for reductive N₂O production is lower (Dalsgaard et al. 2014) than the reported O₂: threshold level (8 µmol L⁻¹) for N₂O consumption in the ETSP (Cornejo and Farias 2012). Nevertheless, the suboxic zone between 1 – 8 µmol L⁻¹ O₂ carries high N₂O concentrations indicating higher N₂O production than consumption. In this study, we focused on this suboxic water masses above the ODZ and determined bulk kinetics of O₂ sensitivity in batch experiments, which reflect the metabolism of the microbial community. The effect of O₂ on N₂O production differed between natural O₂ concentrations with varying communities vs. manipulated O₂ concentrations within a community. While N₂O production from NO⁻ and NO⁻ decreased exponentially along the natural O₂ gradient, it did not always decrease for the manipulated O₂ treatments. Unchanged N₂O production with higher O₂: levels in NO⁻ treatments showed that at least a portion of the community can respond very differently to a sudden increase in O₂ than predicted from natural O₂ gradients with communities acclimated to a certain O₂ concentration. In the ETNP, this pattern has been observed before (Ji et al. 2018a) but the mechanism behind it is unknown. Off the Chilean coast, active N₂O production by denitrification was found at up to 50 µmol L⁻¹ O₂ (Farias et al. 2009). These results reinforce prior studies showing that distinct steps of multistep metabolic pathways, such as denitrification, can differ in O₂ sensitivity (Dalsgaard et al. 2014). In various bacterial strains and natural communities, the NO⁻ reductase enzyme (Nar) which catalyzes the first step in denitrification, is reportedly the most O₂ tolerant, followed by the more O₂ sensitive steps of NO₃⁻ reduction (Nir) and N₂O reduction (Körner und Zumft 1989, McKenney et
The fact, that we see this pattern only in the NO₃⁻ treatments and not in the NO₂⁻ treatments is evidence that it is not due to inhibition of the reduction of N₂O to N₂ at higher O₂. We suggest a stimulation of incomplete denitrification, which leads to the accumulation of N₂O in our serum bottles rather than a stimulation of overall denitrification rates to N₂. While nitrate reduction was inhibited by higher O₂ concentrations, N₂O production was not, leading to very high yields of N₂O production per nitrite produced. We hypothesize that there is a direct channeling of reduced NO₃⁻ to N₂O without exchange of an internal nitrite pool with the surrounding nitrite. If NO₃⁻ does not exchange, our rate estimates for NO₃⁻ reduction based on produced ¹⁵N-NO₃⁻ are underestimated resulting in high yields. A low NO₃⁻ exchange rate has been shown before (Ji et al. 2018b). Based on the assumption that all labelled N₂O from ¹⁵NO₃⁻ has gone through the NO₂⁻ pool, we include the NO₂⁻ pool into calculating f. In ¹⁵NO₃⁻ incubations the enrichment of the substrate pool was low (f = 0.05 – 0.1) and including NO₂⁻ resulted in an underestimation of no more than 5 % depending on the in situ NO₃⁻ concentration, and thus does not explain the high rates.

One N₂O producing process not considered in this study is fungal denitrification, but it deserves mentioning because in soils and coastal sediments it contributes substantially to N₂O production (Wankel et al. 2017, Shoun et al. 2012). With ¹⁵N-labelling experiments it is not possible to distinguish between bacterial and fungal denitrification. In ODZs, marine fungal communities show a wide diversity (Jebarah et al. 2012) and a high adaptive capability is suggested (Richards et al. 2012). Most fungal denitrifiers lack the capability to reduce N₂O to N₂, hence all nitrate reduction results in N₂O production (Richards et al. 2012). In a culture study, the fungus, Fusarium oxysporum, needed O₂ exposure before it started to denitrify (Zhou et al. 2001). To what extent marine fungi play a role in denitrification in open ocean ODZs and their O₂ sensitivity remains to be investigated.

N₂O production from NH₄⁺ did not decrease exponentially with increasing O₂ as shown previously for the ETSP (Qin et al. 2017, Ji et al. 2018a, Santoro et al. 2011). N₂O production rather increased with increasing in situ oxygen and had an optimum between 1.4 – 6 µmol O₂ L⁻¹ in manipulated O₂ treatments. A similar optimum curve was observed in cultures of the marine AOA Nitrosopumilus maritimus, where N₂O production reached maxima at O₂ concentrations between 2 - 10 µmol L⁻¹ (Hink et al. 2017). Furthermore, N₂O production by N. viennensis and N. maritimus was not affected by O₂ but instead by the rate of AO (Stieglmeier et al. 2014, Hink et al. 2017). To find out if this is the case in our study, we plotted AO rate against N₂O production from NH₄⁺ for natural and manipulated O₂ samples (Figure S5). The resulting significant linear fit (R² = 0.75, p<0.0001) implies that the rate of AO was the main driver for the intensity of N₂O production from NH₄⁺ and oxygen had a secondary effect.

Discrepancies in estimates of the O₂ sensitivity of N₂O production by nitrification and denitrification are likely due to a combination of taxonomic variation as well as differences in sensitivity among the various enzymes of each pathway.

### 4.2 N₂O yields and hybrid N₂O formation from NH₄⁺

N₂O yields of AO were 0.15 – 2.07 % (N₂O-N mol/ NO₂–N mol = 1.5 x 10⁻³ – 20.7 x 10⁻³) which are at the higher end of most marine AOA culture or field studies (Hink et al 2017, Qin et al. 2017, Santoro et al. 2011, Stieglmeier et al. 2014). Only in 2015 off the coast of Peru a higher maximum yield of 3.14% was reported (Ji et al. 2018a). In the ETSP high N₂O yields from AO may be more common than previously thought. Not only high N₂O yields in low O₂ waters (< 6 µmol L⁻¹), but also higher yields at higher O₂ concentrations, 0.9 % at 30 µmol L⁻¹ O₂ compared to 0.06% at > 50 µmol L⁻¹ (Ji et al. 2018a) were found. In near coastal regions, higher N₂O yield...
at higher O2 concentrations expands the overall water volume where N2O production by AO contributes to high N2O concentration, which is more likely to be emitted to the atmosphere.

Insights into the production mechanism of N2O is gained from hybrid-N2O formation based on differentiating between production of single (45N2O) and double (46N2O) labelled N2O. If the production of 46N2O is higher than what is expected based on the binomial distribution, then an additional source of 14N can be assumed. 

In situ NH4+ is below detection in almost all water depths, hence in our incubations this pool is 99% labelled. As potential 14N substrates, NO2-, NH2OH and HNO are most likely. Whether hybrid N2O 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 N2O production from NO2- was variable with depth and oxygen, which can be explained by the different proportions of nitrifier versus denitrifier NO2- reduction to N2O. For example, in the interface sample S19 (892, 144 m, 3.69 µmol L-1 NO2-) N2O production from NO2- (0.72 ± 0.19 mmol L-1 d-1) was 20 times higher than from NH4+ (0.033 ± 0.0004 mmol L-1d-1) and no hybrid N2O formation from NO2- was found (Figure 5d). There, the major N2O 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 N2O production from NH4+ was independent of the rate at which N2O production took place and independent of the O2 concentration and varied little (70 – 86% of total N2O 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 N2O production like Fe(II), Mn, NO, NH2OH vary with depth and O2 concentration (Zhu-Barker et al. 2015, Kondo and Moffet 2015, Lutterbeck et al. 2018, Korth et al. 2019). Hence, any 14N which is integrated into N2O to produce a hybrid/single labelled N2O has to be passively or actively taken up by the cell first (Figure 9). There, it reacts with an intermediate product (15NO or 15NH2OH) 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 NH2OH 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 NO2- (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 N2O production is very likely, which always depends on the amount of NO produced in the first place. This could explain why we consistently find ~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 N2O production

A positive stimulation of N2O production from denitrification by particulate organic matter was found, indicating carbon limitation of denitrification in the ETSP. The experimental POM amendments simulated a low POC export flux and represented a flux that happens over 2 - 15 days, assuming an export flux of 3.8 mmol m-2 d-1 and that 8% of the total POC pool is >50 µm (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 µm) used in the experiments is indicative of sinking particles. The stimulation of N2 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 N2O production from nitrite and nitrate 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. N$_2$O/N$_2$ production ratio can vary from 0 - 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$_3^-$ (118 mmol L$^{-1}$ d$^{-1}$) were found, indicating that denitrification was not carbon limited. This is consistent with previous studies on anti-cyclonic 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 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 hot spot 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. 2015b) and in soils (Weier et al. 1993), systems which are generally not carbon limited. Also, at the oxic - anoxic interface of 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$_2^-$/NO$_3^-$ concentrations (Figure S6). Intracellularly produced NO$_2^-$ does not seem to exchange with the surrounding pool, but ambient NO$_2^-$ is directly converted to N$_2$O, a process identified as “nitrite 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. 2015a, 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 AOA3 related to *Nitrosopelagicus brevis* (CN25) to be significantly correlated with the N₂O 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 AOA3 coincident with high nitrification rates and high N₂O production rates, we suggest that *Nitrosopelagicus brevis* related AOA likely play an important role in N₂O 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 N₂O production is consistent with the fact that *nirS* is not the enzyme directly synthesizing N₂O and *nirS* communities are sources as well as sinks for N₂O. 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 N₂O, 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 N₂O produced by nitrifiers and denitrifiers. However, *nirS* gene and transcript abundance correlated with N₂O production from NO₂⁻ making it a possible indicator for one part of reductive N₂O production. It is also worth noting that anammox related *nirS* genes and transcripts (*nirS* 166, 167) contribute up to 12% of the total copy numbers putting a wrinkle on *nirS* abundance as marker gene for denitrifiers only. The subtraction of the anammox 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 ¹⁵N tracer techniques and molecular techniques in order to investigate the factors that control N₂O production within the upper oxycline of the ODZ in the ETSP. Our results suggest that denitrification is a major N₂O source along the oxic - anoxic interface of the upper oxycline. Highest N₂O production rates from NO₂⁻ and NO₃⁻ were found at or below the oxic-anoxic interface, whereas highest N₂O production from AO was slightly shallower in the oxycline. Overall, *in situ* O₂ threshold below 8 µmol L⁻¹ favored nitrate and nitrite reduction to N₂O and high N₂O yields from AO up to 2.2%. A different pattern was observed for the community response to increasing oxygen, with highest N₂O production from NH₄⁺ and NO₃⁻ between 1.4 – 6 µmol L⁻¹ O₂ and high N₂O production from NO₂⁻ even at O₂ concentrations up to 22 µmol L⁻¹. This study highlights the diversity of N₂O production regulation and the need to conduct further experiments where single community members can be better constrained. Our experiments provide the first insights into N₂O regulation by particulate organic matter in the ETSP with particles greatly enhancing N₂O production (up to 5fold). Furthermore, the significant positive correlation between *Nitrosopelagicus brevis* (CN25) and N₂O produced from AO could indicate its importance in N₂O production and points out the great value of combining biogeochemical rate measurements with molecular analysis to investigate multifaceted N₂O cycling. This study shows that short term oxygen increase can lead to high N₂O production even from denitrification and extends the existing O₂ thresholds for high reductive N₂O production up to 22 µmol L⁻¹ O₂. Together with high N₂O yields from AO up to O₂ levels
of 30 µmol L⁻¹, an expansion of low oxygenated waters around ODZs predicted for the future can significantly increase marine N₂O production.

Regardless of which processes are responsible for N₂O production in the ODZ, high N₂O production at the oxic-anoxic interface of the upper oxycline sustains high N₂O concentration peaks with a potential for intense N₂O emission to the atmosphere. An average total N₂O production rate of 3.1 nmol N₂O L⁻¹ d⁻¹ in a 50 m thick suboxic layer with 0 – 20 µmol L⁻¹ O₂ leads to an annual N₂O efflux of 0.5 Tg N y⁻¹ in the Peruvian upwelling (2.22 ×10⁵ km², Arévalo-Martínez et al. 2015), which is within the estimates based on surface N₂O concentration measurements from 2012-2013 (Arévalo-Martínez et al. 2015, Bourbonnais et al. 2017). The importance of the Peru upwelling system for global N₂O emissions (5 – 22% of global marine N₂O emissions) is directly linked to the extreme N₂O accumulations in coastal waters. Coastal N₂O hotspots are well known (Bakker et al. 2014) and this study shows that they can be explained by considering denitrification as a major N₂O source. With the further parametrization of POM export as a driver for N₂O production from denitrification, models may be able to better predict N₂O emissions in highly productive coastal upwelling regions and to evaluate how fluxes might change with changing stratification and deoxygenation.

Data availability: The data presented here were archived in the SFB754 database (www.sfb754.de). The N₂O data are also available from the Marine Methane and Nitrous Oxide (MEMENTO) database (https://memento.geomar.de/de/n2o).

Author contributions: CF, HWB and BW conceptualized the study. CF and MS performed experiment. CF and ELP analyzed samples. RX and EA collected POM. DLAM sampled and measured N₂O concentrations. AJ performed qPCR. SO supported mass spectrometer analysis. CF analyzed data and led the writing effort, with substantial contributions from all co-authors.

Competing interests: Authors declare no competing interests.

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Table 1: Overview of characteristics of samples. bd - below detection limit of Winkler method and seabird sensor (2 µmol L\(^{-1}\)), x - analysis includes qPCR and microarray with qPCR products, x* - only qPCR, no microarray

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Table 2: Quality (N/C), quantity (Addition µmol L⁻¹) and origin (station and depth) of added, autoclaved and non-autoclaved particulate organic matter (POM) and increase in NH₄⁺ concentration after autoclaving.

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Figure Legends:

Figure 1: Study site, showing transect and station numbers, in the Eastern Tropical South Pacific during cruise M138.

Figure 2: Depth profiles of $O_2$, nutrients and $N_2O$ in the upper 400 m for all stations. Panel numbers 1 - 4) refer to the transect numbers.

Figure 3: (I) Profiles of AO (a, e, I), $N_2O$ production rates from $NH_4^+$ (b, f, j), archaeal amoA gene (c, g, k) and transcript copy numbers mL$^{-1}$ (d, h, l). (II) Profiles of nitrate reduction rates (a, f, k, o), $N_2O$ production rates from $NO_3^-$ (b, g, l, p) and $NO_2^-$ (c, h, m, q) and nirS gene (d, I, n, r) and transcript copy numbers mL$^{-1}$ (c, j, m, s). 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 ($0 = \text{interface}$). Shaded area indicates the anoxic zone. Note different scale for $N_2O$ production rates.

Figure 4: $O_2$ dependence of $N_2O$ production rates from $NH_4^+$ (a, d), $NO_2^-$ (b, e) and $NO_3^-$ (c, f). Upper panel (a-c) is $N_2O$ production along natural $O_2$ gradient from all stations. Lower panel (d-f) is $N_2O$ production in manipulated $O_2$ experiments with water from oxic - anoxic interface from slope station 892 (S11, 0 µmol L$^{-1}$ $O_2$, 145m) and shelf station 894 (S19, 0 µmol L$^{-1}$, 120 m). Note different scale for $N_2O$ 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).

Figure 5: $O_2$ dependency of hybrid $N_2O$ formation from $NH_4^+$ (a, c) and $NO_2^-$ (b, d) along the natural $O_2$ gradient (a, b) and for the $O_2$ manipulations (c, d) from sample S11 (0 µmol L$^{-1}$ $O_2$, 145m) and S19 (894, 0 µmol L$^{-1}$, 120 m).

Figure 6: Yields (%) of $N_2O$ production during $NH_4^+$ oxidation (a, c) and during nitrate 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$^{-1}$ $O_2$, 145m) and S19 (894, 0 µmol L$^{-1}$, 120 m). Error bars present ± SD calculated as error propagation.

Figure 7: Bar plots of 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).

Figure 9: Scheme illustrating the possible reactions for hybrid $N_2O$ formation. The ellipse represents an AOA cell.
Figure 1
Figure 2
Figure 3
Figure 4
Figure 5
Figure 6
Figure 7

(a) N₂O production from NO²⁻ to N₂O (nmol L⁻¹ d⁻¹) for control, POM1, POM2, and POM3 treatments.

(b) NO₃⁻ to N₂O production (nmol L⁻¹ d⁻¹) for the same treatments.
Figure 8
Figure 9