Nitrogen cycling in shallow low oxygen coastal waters off Peru from nitrite and nitrate nitrogen and oxygen isotopes

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12

13 Abstract

O₂ deficient zones (ODZs) of the world's oceans are important locations for microbial 14 dissimilatory nitrate (NO₃⁻) reduction and subsequent loss of combined nitrogen (N) to biogenic 15 N₂ gas. ODZs are generally coupled to regions of high productivity leading to high rates of N-16 17 loss as found in the coastal upwelling region off Peru. Stable N and O isotope ratios can be 18 used as natural tracers of ODZ N-cycling because of distinct kinetic isotope effects associated 19 with microbially-mediated N-cycle transformations. Here we present NO_3^- and nitrite (NO_2^-) 20 stable isotope data from the nearshore upwelling region off Callao, Peru. Subsurface oxygen 21 was generally depleted below about 30 m depth with concentrations less than 10 µM, while 22 NO_2^- concentrations were high, ranging from 6 to 10 μ M, and NO_3^- was in places strongly depleted to near 0 µM. We observed for the first time, a positive linear relationship between 23 $NO_2^- \delta^{15}N$ and $\delta^{18}O$ at our coastal stations, analogous to that of $NO_3^- N$ and O isotopes during 24 NO_3^- uptake and dissimilatory reduction. This relationship is likely the result of rapid NO_2^- 25 26 turnover due to higher organic matter flux in these coastal upwelling waters. No such relationship was observed at offshore stations where slower turnover of NO₂⁻ facilitates 27 dominance of isotope exchange with water. We also evaluate the overall isotope fractionation 28 29 effect for N-loss in this system using several approaches that vary in their underlying assumptions. While there are differences in apparent fractionation factor (ϵ) for N-loss as calculated from the δ^{15} N of NO₃⁻, dissolved inorganic N, or biogenic N₂, values for ϵ are generally much lower than previously reported, reaching as low as 6.5‰. A possible explanation is the influence of sedimentary N-loss at our inshore stations which incurs highly suppressed isotope fractionation.

6

7 1 Introduction

8 Chemically combined nitrogen (N), e.g., nitrate (NO₃⁻), is an important phytoplankton nutrient 9 limiting primary productivity and carbon export throughout much of the ocean (e.g. Gruber, 10 2008). The marine nitrogen cycle involves a series of microbial processes, which transfer N between a number of chemical forms. These include N₂ fixation, nitrification (ammonium 11 (NH_4^+) and nitrite (NO_2^-) oxidation), and loss of combined N to N₂ via denitrification and 12 anaerobic ammonium oxidation (anammox). Of particular importance is the global balance 13 14 between sources of combined N (N2 fixation) and N-loss processes which ultimately control the combined N content of the ocean and thus its productivity and strength of the biological carbon 15 pump. N-loss typically occurs under nearly anoxic conditions where the first step, dissimilatory 16 17 NO₃⁻ reduction to NO₂⁻, active at oxygen (O₂) concentrations less than ~25 μ M (Kalvelage et 18 al., 2011), is used by heterotrophic microbes in lieu of O_2 for respiration. Canonically, the 19 denitrification pathway of successive reduction of NO₃⁻, NO₂⁻, nitric oxide (NO), and nitrous 20 oxide (N₂O) to N₂ was considered as the dominant pathway for N-loss. However, since the early 2000s, anammox (NO₂⁻ + NH₄⁺ \rightarrow N₂) was found to be widespread in the ocean (Kuypers 21 22 et al. 2003; 2005; Hamersley et al., 2007; Dalsgaard et al., 2012; Kalvelage et al., 2013). While 23 it is still a matter of debate whether denitrification or anammox is the dominant pathways for 24 N-loss in Oxygen Minimum Zones (OMZ's) (e.g., Lam et al., 2009; Ward et al., 2009), both N-25 loss processes have been shown to strongly vary spatially and temporally and are linked to organic matter export and composition (Kalvelage et al., 2013; Babbin et al., 2014). It follows 26 27 that there is still considerable uncertainty as to the controls on N-loss as well as the role for other linking processes such as DNRA (NO₃⁻ to NH₄⁺) and NO₂⁻ oxidation in the absence of O₂. 28 29 Marine N-loss to N₂ occurs predominately in reducing sediments and the O₂ deficient water columns of OMZ's as found in the Arabian Sea and Eastern Tropical North and South Pacific 30 31 (Ulloa et al., 2012; Lam et al., 2011 and references therein). NO₂⁻ is an important intermediate during N-loss and generally accumulates at concentrations up to ~10 µM in these regions 32

1 (Codispoti et al., 1986; Casciotti et al., 2013). The depletion of NO_3^- is typically quantified as a 2 dissolved inorganic N (DIN= $NO_3^- + NO_2^- + NH_4^+$) deficit relative to phosphate (PO_4^{-3}) 3 assuming Redfield stoichiometry and the accumulation of biogenic N₂ (when measured) is 4 detected as anomalies in N₂/Ar relative to saturation with atmosphere (Richards and Benson, 5 1961; Chang et al., 2010; Bourbonnais et al., 2015).

6 NO₃⁻ and NO₂⁻ N and O isotopes represent a useful tool to study N cycle transformations as 7 they respond to in situ processes and integrate over their charasteristic time and space scales. 8 Biologically mediated reactions are generally faster for lighter isotopes. For instance, both NO₃⁻ 9 uptake and dissimilatory NO₃⁻ reduction produce a strong enrichment in both ¹⁵N (δ^{15} N = 10 [(¹⁵N/¹⁴N_{sample})/(¹⁵N/¹⁴N_{standard})–1]×1000) and ¹⁸O (δ^{18} O = [(¹⁸O/¹⁶O_{sample})/(¹⁸O/¹⁶O_{standard})– 11 1]×1000) in the residual NO₃⁻ (Cline and Kaplan, 1975; Brandes et al., 1998; Voss et al., 2001; 12 Granger et al., 2004; 2008; Sigman et al., 2005).

Canonical values for the N isotope effect ($\epsilon \approx \delta^{15}N_{substrate} - \delta^{15}N_{product}$, without significant 13 14 substrate depletion) associated with microbial NO_3^- reduction during water-column denitrification range from 20 to 30% (Brandes et al., 1998; Voss et al., 2001; Granger et al. 15 2008). In contrast, the expression of the isotope effect of sedimentary denitrification is highly 16 suppressed as compared to the water-column (generally <3%) mostly due to near complete 17 consumption of the porewater NO_3^- and diffusion limitation (Brandes and Devol, 1997; 18 Lehmann et al., 2007; Alkhatib et al., 2012). The $\delta^{15}N$ and $\delta^{18}O$ of NO₃⁻ are affected in 19 fundamentally different ways during NO_3^- consumption and production processes. The ratio of 20 the ¹⁵N and ¹⁸O fractionation factors (${}^{18}\varepsilon$; ${}^{15}\varepsilon$) during NO₃⁻ consumption during denitrification or 21 assimilation by phytoplankton in surface waters is close to 1:1 (Casciotti et al., 2002; Granger 22 et al., 2004; 2008). While the $\delta^{15}N$ of the newly nitrified NO₃⁻ depends on the $\delta^{15}N$ of the 23 precursor molecule being nitrified, the O atom is mostly derived from water (with a δ^{18} O of ~0 24 %) with significant isotopic fractionation associated with O incorporation during NO₂⁻ and 25 NH₄⁺ oxidation (Casciotti, 2002; Buchwald and Casciotti, 2010; Casciotti et al., 2010). 26 27 Therefore, any deviation from this 1:1 ratio in the field has been interpreted as evidences that 28 NO₃⁻ regeneration is co-occurring with NO₃⁻ consumption (Sigman et al., 2005; Casciotti and McIlvin, 2007; Bourbonnais et al., 2009). NO₂ oxidation is associated with an inverse N 29 30 isotope effect (Casciotti, 2009), atypical of biogeochemical reactions, and can cause both lower and higher ratios for ${}^{18}\varepsilon$: ${}^{15}\varepsilon$ compared to pure NO₃⁻ assimilation or denitrification, depending on 31

- 1 the initial isotopic compositions of the NO_2^- and NO_3^- and the ¹⁸O added back (Casciotti et al.,
- 2 2013).

Additional information on N-cycling processes can be obtained from the isotopic composition 3 4 of NO₂⁻. For example, because of its inverse N isotope effect, NO₂⁻ oxidation results in a lower $NO_2^- \delta^{15}N$ than initially produced by NH_4^+ oxidation and NO_3^- reduction (Casciotti, 2009; 5 Brunner et al., 2013). Logically, NO_2^- reduction would be expected to produce a positive 6 relationship between δ^{15} N-NO₂⁻ and δ^{18} O-NO₂⁻ though there are no quantitative observations in 7 8 the literature. Analogous to NO₃⁻ reduction, it also involves enzymatic breakage of the N-O bond. However, O-isotope exchange of NO_2^- with water (as a function of pH and temperature) 9 would reduce the slope of a NO₂⁻ δ^{18} O vs. δ^{15} N relationship toward zero. NO₂⁻ turnover time 10 11 can therefore be assessed from this observed relationship and in situ pH and temperature 12 (Buchwald and Casciotti, 2013) 13 It is still under discussion whether the global ocean N budget is in balance. Current estimates 14 from direct observations and models for N₂ fixation, considered the primary marine N source, range from 110-330 Tg N yr⁻¹ (Brandes and Devol, 2002; Gruber, 2004; Deutsch et al., 2007; 15 Eugster and Gruber, 2012; Großkopf et al., 2012). Estimates for major marine N-sinks, i.e., 16 denitrification and anammox in the water-column of oxygen deficient zones and sediments 17

account for 145-450 Tg N yr⁻¹ (Gruber, 2004; Codispoti, 2007; DeVries et al., 2012; Eugster 18 and Gruber, 2012). Large uncertainties are associated with this budget, mainly in constraining 19 20 the proportion of sedimentary denitrification which is typically estimated from ocean's N 21 isotope balance and the expressed isotope effects for water-column versus sedimentary NO_3^{-1} 22 reduction during denitrification (e.g. Brandes and Devol, 2002; Altabet, 2007; DeVries et al., 23 2012). Liu (1979) was first to suggest a lower ε for denitrification in the Peru ODZ as compared to the subsequently accepted canonical range for NO₃⁻ reduction of 20% to 30% 24 25 (Brandes et al., 1998; Voss et al., 2001; Granger et al., 2008). Ryabenko et al. (2012) provided 26 a more widely distributed set of data in support. Most recently, a detailed study in a region of 27 extreme N-loss associated with a Peru coastal mode-water eddy confirmed a ε value for N-loss of ~14‰ (Bourbonnais et al., 2015). Applying such a lowered value to global budgets would 28 29 bring the global N budget closer to balance.

30 Ryabenko et al. (2012) also suggested that ε values were even lower in the shelf region of the 31 Peru ODZ. To investigate further, we present here N and O isotope data for NO₂⁻ and NO₃⁻ 32 from shallow coastal waters near Callao, off the coast of Peru. These waters are highly productive as a consequence of active upwelling that is also responsible for shoaling of the oxycline. We determine the relationship between NO₂⁻ δ^{15} N and δ^{18} O and its implication for NO₂⁻ cycling in these shallow waters as compared to offshore stations. We finally derive isotope effects for N-loss and infer the likely influence of sedimentary N-loss, which incurs a highly suppressed isotope effect, at our relatively shallow sites.

6

7 2 Material and Methods

8 2.1 Sampling

9 The R/V Meteor 91 research cruise (M91) to the eastern tropical South Pacific Ocean off Peru in December 2012 was part of the SOPRAN program and the German SFB 754 project. It 10 11 included an along shore transect of seven inner shelf stations located between 12°S to 14°S that 12 were chosen for this study (Fig. 1). These stations had a maximum depth of 150 m except for 13 station 68 (250 m depth). We additionally sampled deep offshore stations during the M90 cruise in November 2012. Samples for NO₃⁻ and NO₂⁻ isotopic composition and N₂/Ar ratio 14 15 were collected using Niskin bottles mounted on a CTD/Rosette system, which was equipped with pressure, temperature, conductivity and oxygen sensors. O₂ concentrations were 16 determined using a Seabird sensor, calibrated using the Winkler method (precision of 0.45 17 μ mol L⁻¹) with a lower detection limit of 2 μ mol L⁻¹. Nutrients concentrations were measured 18 on board using standard methods as described in Stramma et al. (2013). 19

20 2.2 NO₂ and NO₃ isotope analysis

NO₂⁻ samples were stored in 125 ml HDPE bottles preloaded with 2.25 ml 6 M NaOH to 21 prevent microbial activity as well as alteration of δ^{18} O-NO₂ by isotope exchange with water 22 (Casciotti et al., 2007). Bottles were kept frozen after sample collection, though we have 23 24 subsequently determined in the laboratory that seawater samples preserved in this way can be kept at room temperature for at least a year without alteration of NO₂⁻ δ^{15} N or δ^{18} O 25 (unpublished data). Samples were analyzed by continuous He flow isotope-ratio mass 26 spectrometry (CF-IRMS; see below) after chemical conversion to N₂O using acetic acid 27 buffered sodium azide (McIlvin & Altabet 2005). Because of high sample pH, the reagent was 28 29 modified for NO₂⁻ isotope analysis by increasing the acetic acid concentration to 7.84 M. Inhouse (i.e., MAA1, $\delta^{15}N = -60.6\%$; MAA2, $\delta^{15}N = 3.9\%$; Zh1, $\delta^{15}N = -16.4\%$) and other 30

1 laboratory calibration standards (N23, $\delta^{15}N = 3.7\%$ and $\delta^{18}O = 11.4\%$; N7373, $\delta^{15}N = -79.6\%$ 2 and $\delta^{18}O = 4.5\%$; and N10219; $\delta^{15}N = 2.8\%$ and $\delta^{18}O = 88.5\%$; see Casciotti and McIlvin, 3 2007) were used for NO²⁻ $\delta^{15}N$ and $\delta^{18}O$ analysis.

NO₃⁻ samples were stored in 125 ml HDPE bottles preloaded with 1 ml of 2.5 mM sulfamic 4 5 acid in 25 % HCl to both act as a preservative and to remove NO₂⁻ (Granger and Sigman, 2009). Samples were also kept at room temperature and we have found that they can be stored 6 in this way for many years without alteration of NO₃⁻ δ^{15} N or δ^{18} O. Cadmium reduction was 7 used to convert NO_3^- to NO_2^- prior to conversion to N_2O using the "azide method" (McIlvin & 8 Altabet 2005) and IRMS analysis. Standards for NO₃⁻ isotope analysis were N3 ($\delta^{15}N = 4.7\%$) 9 and $\delta^{18}O = 25.6\%$), USGS34 ($\delta^{15}N = -1.8\%$ and $\delta^{18}O = -27.9\%$) and USGS35 ($\delta^{15}N = 2.7\%$ 10 and $\delta^{18}O = 57.5\%$) (Casciotti et al., 2007). The lowest concentration of NO₂⁻ or NO₃⁻ analyzed 11 for isotopic composition was 0.5 μ M, thus δ^{15} N-NO₃⁻ and δ^{15} N-NO₂⁻ could not be measured 12 13 below 37 m at station 63.

A GV Instruments IsoPrime Isotope Ratio Mass Spectrometer (IRMS) coupled to an on-line He continuous-flow purge/trap preparation system was used for isotope analysis (Sigman et al., 2001; Casciotti et al., 2002; McIlvin & Altabet 2005). N₂O produced by the azide reaction was purged with He from the septum sealed 20 ml vials and trapped, cryofocused and purified prior to transfer to the IRMS. Total run time was 700 s/sample (McIlvin & Altabet 2005). Isotopic values are referenced against atmospheric N₂ for δ^{15} N and VSMOW for δ^{18} O. Reproducibility was 0.2‰ and 0.5‰, respectively.

21 2.3 N₂/Ar IRMS analysis and calculation of biogenic N₂ and δ^{15} N biogenic 22 N₂

23 The accumulation of biogenic N₂ from denitrification and anammox can be measured directly 24 from precise N₂/Ar measurements (see above; Richards and Benson, 1961; Chang et al., 2010; 25 Bourbonnais et al., 2015). As described in Charoenpong et al. (2014), N₂/Ar samples were collected from Niskin bottles using 125 ml serum bottles, and all samples were treated with 26 HgCl₂ as a preservative and filled without headspace. When cavitation bubbles formed from 27 28 cooling of warm, near-surface samples, these bubbles were collapsed and reabsorbed by 29 warming samples in the laboratory in a 30-35°C water bath before analysis. N₂/Ar was 30 measured using an automated dissolved gas extraction system coupled to a multicollector IRMS (Charoenpong et al., 2014). Excess N₂ was calculated first from anomalies relative to 31

N₂/Ar expected at saturation with atmosphere at *in situ* temperature and salinity. Locally produced biogenic N₂ was obtained by subtracting excess N₂ at the corresponding density surface for waters outside of the ODZ (O₂ >10 μ M) not affected by N-loss (Chang et al., 2010; Bourbonnais et al., 2015). δ¹⁵N biogenic N₂ was calculated from the δ¹⁵N-N₂ anomaly as in Bourbonnais et al. (2015). Reproducibility was better than 0.7 μ M for excess N₂ and 0.03 ‰ for δ¹⁵N-N₂. δ¹⁵N of biogenic N₂ was calculated by mass balance as in Bourbonnais et al. (2015).

8 2.4 Isotope effect (ε) calculations

9 Isotope effects are estimated using the Rayleigh equations describing the change in isotope 10 ratio as a function of fraction of remaining substrate. The following equations are used for a

11 closed system (Mariotti et al., 1981):

12
$$\delta^{15}$$
N-NO₃⁻ = δ^{15} N-NO₃⁻ (f=1) - $\epsilon \times \ln[f_1]$ or (1)

13
$$\delta^{15}$$
N-DIN =

$$\delta^{15} \text{N-DIN} (f=1) - \varepsilon \times \ln[f_2]$$
(2)

- 14 where f_1 is the fraction of remaining NO₃⁻ and f_2 is the fraction of remaining DIN (NO₃⁻ + NO₂⁻ 15 concentrations). δ^{15} N-DIN is the average δ^{15} N for NO₃⁻ and NO₂⁻ weighted by their 16 concentrations. The fraction of remaining DIN is a better estimation of the overall effective 17 isotope effect for N-loss (Bourbonnais et al., 2015), while using NO₃⁻ as the basis to calculate 18 ϵ specifically targets NO₃⁻ reduction. See below for details of f value calculation.
- 19 The overall isotope effect for N-loss can also be estimated from the $\delta^{15}N$ of biogenic N₂ 20 produced:
- 21 δ^{15} N-biogenic N₂ = δ^{15} N-DIN (f=1) + $\epsilon \times f_2 / [1 f_2] \times \ln[f_2]$ (3)
- 22 Whereas the closed system equations assume no addition or loss of substrate or product,
- 23 corresponding steady-state open system equations can account for such effects (Altabet, 2005):

24
$$\delta^{15}$$
N-NO₃⁻ = δ^{15} N-NO₃⁻ (f=1) + ϵ [1 - f₁] or (4)

25
$$\delta^{15}$$
N-DIN = δ^{15} N-DIN (f=1) + $\epsilon \times [1 - f_2]$ (5)

26
$$\delta^{15}$$
N-biogenic N₂ = δ^{15} N-DIN (f=1) - $\varepsilon \times f_2$ (6)

- 27 For all equations, the slope represents ε and the y-intercept is the initial $\delta^{15}N$ prior to N-loss.
- For calculations using equations 3 and 6 we only used $\delta^{15}N$ values associated with biogenic N₂
- 29 greater than 7.5 µM because of increasing noise below this level due to the large atmospheric
- 30 dissolved N₂ background (typically up to \sim 500 μ M).

Since the closed system equations assume no loss or resupply of substrate or production in a water parcel, they are appropriate where there is little mixing and/or advection is dominant over mixing. The open system equations take into account supply from or loss to surrounding water parcels, e.g. mixing dominance. Both cases represent extreme situations. In the next section, we will estimate and compare ε using both sets of equations.

To do so, we need to estimate the fraction of NO₃⁻ or DIN remaining (f). The assumption of
Redfield stoichiometry (as in eq. 9) in source waters is typically made:

8
$$f_{1p} = [NO_3^-] / Np_{expected} \text{ or}$$
 (7)

9 $f_{2p} = ([NO_3^-] + [NO_2^-]) / Np_{expected}$ (8)

10 Np_{expected} =
$$15.8*([PO_4^{3-}] - 0.3)$$
 (9)

11
$$N_{observed} = [NO_3^-] + [NO_2^-] + [NH_4^+]$$
 (10)

where Np_{expected} is the concentration expected assuming Redfield stoichiometry. Equation 9 was derived in Chang et al. (2010) from stations to the west of the ETSP ODZ (143-146°W) and takes into account preformed nutrient concentrations. In our study, NH_4^+ generally did not significantly accumulate, except at station 63, and was thus not included. This has been the traditional approach to quantify N-loss in ODZ's (N deficit, Np_{def}), by comparing observed DIN concentrations (N_{observed}) to Np_{expected}:

$$18 Np_{def} = Np_{expected} - N_{observed} (11)$$

However, the assumption of Redfield stoichiometry may not be appropriate in this shallow environment due to preferential release of PO_4^{3-} following iron and manganese oxyhydroxide dissolution in anoxic sediments (e.g., Noffke et al., 2012). An alternative method of calculating f makes use of our biogenic N₂ measurements to estimate expected N prior to N-loss (N_{expected})

23 bio N_2) and f values based on it:

24
$$N_{\text{expected bio } N_2} = [NO_3^-] + [NO_2^-] + 2 \times [Biogenic N_2]$$
 (12)
25 $f_{1\text{bio}N^2} = [NO_3^-] / N_{\text{expected bio } N_2}$ or (13)

$$\frac{1}{1000} = \frac{1}{100} \frac$$

- 26 $f_{2bioN2} = [NO_3^{-} + NO_2^{-}] / N_{expected} bio N_2$ (14)
- A third way to estimate f is to use NO_3^- or DIN concentrations divided by observed maximum NO₃⁻ or DIN concentrations for the source of the upwelled waters (see red rectangles in Fig. 2).
- 29

1 3 Results

2 **3.1** Hydrographic characterization

3 During the study period, there was active coastal upwelling especially at station 63 as seen by 4 relatively low satellite sea surface temperatures, higher chlorophyll a concentrations, and a 5 shallow oxycline (Fig. 1). A common relationship and narrow range for T and S were found, 6 comparable to T/S signatures for offshore ODZ waters between ~100 and 200 m depths 7 (Bourbonnais et al. 2015), indicating a common source of water upwelling at these inner shelf 8 stations (Fig. 2). This is expected in these shallow waters, where upwelling of the Peru Coastal 9 Current with low O_2 and high nutrients plays a dominant role (Penven et al., 2005). O_2 10 increased only in warmer near-surface waters as a consequence of atmospheric exchange. There was a change in surface water temperature from 15 °C to 20 °C (Fig. 1 B) with distance along 11 12 the coast (from 12.0°S to 14.0°S, about 222 km) that indicates corresponding changes in upwelling intensity. Stronger local wind forcing likely brought up colder deep water near 13 14 station 63.

15 **3.2** Dissolved O₂ and nutrient concentrations

As a consequence of active upwelling sourced from the offshore ODZ, the oxycline was very 16 17 shallow at our in-shore stations. O₂ was generally depleted below 10 to 20 m (Fig. 3 A) and 18 was always less than 10 µM below 30 m. Because we are focusing on N-transformations that 19 occur in the absence of O₂, our data analyses will be mainly restricted to samples where O₂ 20 concentration is below this value. Whereas a recent study indicates that denitrification and 21 anammox are reversibly suppressed at nanomolar O₂ levels (Dalsgaard et al., 2014), CTD deployed Seabird O₂ sensors are not sufficiently sensitive to detect such low concentrations and 22 23 hence our choice of a 10 µM threshold. In contrast, NO₂⁻ oxidation, an aerobic process, was shown to occur even at low to non-detectable O_2 (Füssel et al., 2012). 24

Both Si(OH)₄ and PO₄³⁻ concentrations had very similar vertical and along section distributions (Fig. 3 C and D). Concentrations were at a minimum at the surface, presumably due to phytoplankton uptake, and increased with depth to up to 46 μ M and 3.7 μ M, respectively. Station 63 had the highest near-bottom concentrations, a likely result of release from the sediments, which is futher supported by high near-bottom NH₄⁺ concentrations (up to ~4 μ M) as compared to the other stations (Fig. 3 B, C, and D). In contrast to other nutrients, NO_3^- and NO_2^- concentrations were lowest near-bottom at station 63, only reaching their maxima above 60 m. Across most of our stations, NO_3^- concentration was 22 μ M at 20 to 40 m depth but decreased to near zero deeper within the O₂-depleted zone due to microbially mediated NO_3^- reduction (Fig. 4 A). NO_2^- concentrations correspondingly ranged from 6 to 11 μ M for O₂ concentrations less than 10 μ M (Fig. 4 B). The highest $NO_2^$ concentration (11 μ M) was found at around 50 m (station 64), but only reached 6 μ M at all other stations.

8 3.3 NO₂⁻ and NO₃⁻ isotope compositions

As a consequence of kinetic isotope fractionation during N-loss, the N and O isotope composition of NO₃⁻ and NO₂⁻ varied inversely with NO₃⁻ and NO₂⁻ concentrations, with maximum δ^{15} N and δ^{18} O values near the bottom at each station. δ^{15} N-NO₃⁻ increased from about 10‰ in surface waters to up to 50‰ in the O₂-depleted zone (Fig. 4 C), with near bottom values at station 64 significantly higher (50‰) than at the other stations which ranged from 20 to 30‰. δ^{15} N-NO₂⁻ varied from -25 to about 10‰ (Fig. 4 D), with maximum values also in deeper waters at station 64.

As expected for NO₃⁻ reduction, δ^{18} O-NO₃⁻ positively co-varied with δ^{15} N-NO₃⁻ and ranged 16 from 12 to 46‰. We observed an overall linear relationship between δ^{15} N-NO₃⁻ and δ^{18} O-NO₃⁻ 17 with a slope of 0.86, which was significantly different than 1 (p-value<0.05), and a y-intercept 18 of 1.90 ($r^2 = 0.996$, see Fig. 5 A). NO₃⁻ δ^{15} N and δ^{18} O have been shown to increase equally 19 (ratio 1:1) during assimilatory and dissimilatory NO₃⁻ reduction (Casciotti et al., 2002; Granger 20 21 et al., 2004; 2008). However, deviations from this trend have been observed in the ocean and interpreted as evidence for co-occurring NO_3^- production processes (Sigman et al. 2005; 22 Casciotti and McIlvin, 2007 ; Bourbonnais et al., 2009; 2015). In this study, we observed a 23 $NO_3^- \delta^{18}O$ vs $\delta^{15}N$ relationship less than 1, likely originating from NO_2^- re-oxidation to NO_3^- in 24 25 our environmental setting as in Casciotti and McIlvin (2007). We also observed, for the first time, a significant correlation between δ^{15} N-NO₂⁻ and δ^{18} O-NO₂⁻ in the ODZ for our in-shore 26 27 water stations (Fig. 5 B). As in prior studies (Casciotti and McIlvin 2007; Casciotti et al., 28 2013), no such relationship was observed by us for a nearby set of offshore stations (see Fig. 5 C) where longer NO_2^{-1} turnover times likely facilitated O isotope exchange with water. We will 29 30 discuss implications of this unique finding in the next section.

1 3.4 The δ^{15} N difference between NO₃⁻ and NO₂⁻

The difference in δ^{15} N between NO₃⁻ and NO₂⁻ ($\Delta\delta^{15}$ N) reflects the combined isotope effects of 2 3 simultaneous NO₃⁻ reduction, NO₂⁻ reduction, and NO₂⁻ oxidation. For NO₃⁻ reduction alone, 4 highest $\Delta \delta^{15}$ N values would be around 25‰ at steady-state (Cline and Kaplan, 1975; Brandes et al., 1998; Voss et al., 2001; Granger et al., 2004; 2008). The effect of NO₂⁻ reduction would be 5 to increase the $\delta^{15}N$ of the residual NO₂⁻, thus decreasing $\Delta\delta^{15}N$. In contrast, NO₂⁻ oxidation is 6 associated with an inverse kinetic isotope effect (Casciotti, 2009) which acts to decrease the 7 residual $\delta^{15}N$ of NO₂⁻ and hence overall increases the $\Delta\delta^{15}N$. Therefore, following NO₂⁻ 8 oxidation, $\Delta \delta^{15}$ N may be larger than expected from NO₃⁻ and NO₂⁻ reduction alone, especially 9 if the system is not at steady-state (Casciotti et al., 2013). $\Delta \delta^{15}$ N ranged from 15% to 40% 10 11 (average = 29.78‰ and median = 32.5‰) for samples with $O_2 < 10 \mu$ M. These results confirm 12 the presence of NO_2^- oxidation for at least some of our depth intervals.

13 3.5 N deficit, biogenic N₂ and δ^{15} N-N₂

N deficits, biogenic N₂ concentrations, and δ^{15} N-N₂ anomalies relative to equilibrium with 14 atmosphere were overall greater in the O2-depleted zone reaching highest values near the 15 bottom of station 63 (Fig. 7). N deficit, calculated assuming Redfield stoichiometry (Eqs. 9 to 16 11), ranged from 17 to 59 µM in this region. The concentration of biogenic N in N₂ ranged 17 from 12 to 36 μ M-N and, as expected, was strongly linearly correlated with N deficit (r² = 0.87; 18 Fig. 8 C). However, the slope of 0.45 for the linear relationship shows biogenic N in N₂ to be 19 only half that expected from Np_{def}, a possible consequence of benthic PO_4^{3-} release. The linear 20 relationship ($r^2 = 0.91$) observed between biogenic N in N₂ and DIN (Fig. 8 A) supports a 21 22 single initial DIN value for the source waters to our stations and hence validates using this as a basis for calculating f. The slope of the correlation (0.74) is much closer to 1 as compared to 23 the correlation with Np_{def}, further supporting excess PO_4^{-3} as a contributor to the latter. 24 However this value is still significantly less than 1, suggesting that biogenic N in N₂ may also 25 26 be underestimated. Because our data are restricted to O₂-depleted depths, it is unlikely that biogenic N2 was lost to the atmosphere. Alternatively, mixing of water varying in N2/Ar can 27 28 result in such underestimates of biogenic N2 when N2/Ar anomalies are used to calculate excess 29 N_2 (see Charoenpong et al., 2014). As seen below, our estimates of ε are rather insensitive to 30 choice of Np_{def}, biogenic N in N₂, or DIN concentration changes as the basis for calculation of 31 f.

The δ^{15} N-N₂ anomaly, i.e., the difference between the δ^{15} N-N₂ observed and at equilibrium, 1 2 derived as in Charoenpong et al. (2014), ranged from -0.2 to 0.1‰ (Fig. 7 C). The corresponding range in δ^{15} N biogenic N₂ at O₂ < 10 μ M was from -9.0 to 3.2‰. Negative δ^{15} N-3 N_2 anomaly (i.e., lower δ^{15} N-biogenic N_2) is produced at the onset of N-loss, because 4 extremely depleted ¹⁵N-N₂ is first produced. At a more advanced N-loss stage, we expect δ^{15} N-5 N_2 anomaly and $\delta^{15}N$ -biogenic N_2 to increase, which we observed in this study, as heavier ^{15}N 6 is added to the biogenic N₂ pool. The δ^{15} N-N₂ anomaly signal appears small when compared to 7 the isotopic composition of NO_3^- and NO_2^- but is 1) analytically significant and 2) the result of 8 9 dilution by the large background of atmospheric N_2 (400 to 500 μ M N_2).

10 **3.6** Isotope effect (ε)

11 Isotope effects were calculated using eqs. 1 to 6 to compare closed versus open system 12 assumptions as well as different approaches to estimating f. Examples of plots of the closed 13 system equations with f calculated using biogenic N2, are shown in Fig. 6. Comparison of 14 results using all 3 approaches for calculating f (i.e. Redfield stoichiometry, biogenic N₂ and 15 observed substrate divided by maximum "upwelled" concentration, (see section 2.4)) are shown in Table 1 (closed system) and 2 (open system). In the case of the closed system, ε values were 16 in all cases lower than canonical ones, ranging narrowly from ~6‰ for changes in the $\delta^{15}N$ of 17 DIN to ~14‰ for changes in δ^{15} N-NO₃⁻ (Table 1). For the open system equations, estimated ϵ 18 19 was higher and covered a large and unrealistic range from $\sim 12\%$ for changes in the biogenic N₂ to ~63‰ for changes in the δ^{15} N of NO₃. For our inshore water stations, where we observed a 20 21 single water mass (Fig. 2), a closed system should be a more realistic approximation of ε . The Rayleigh equations' y-intercepts, where f=1, represent the initial $\delta^{15}N$ of NO₃⁻ or DIN, and 22 23 varied from -0.5 to 3.7‰ and -18.4 to 6.2‰ for closed and open systems, respectively. The 24 higher end of this range is more realistic based on prior isotopic measurements for source 25 waters (e.g., Bourbonnais et al., 2015).

1 4 Discussion

2 4.1 Behavior of NO₂

3 NO₂⁻ is an important intermediate during either oxidative or reductive N-cycle pathways and can accumulate at relatively high concentrations through the ocean. While NO_2^- is generally 4 5 elevated at the base of the sunlit euphotic zone (i.e. primary NO_2 maximum; Dore and Karl, 6 1996; Lomas and Lipschultz, 2006), highest concentrations are found in ODZ's as part of the 7 secondary NO₂⁻ maximum (Codispoti and Christensen 1985; Lam et al. 2011). Accordingly, high NO₂⁻ concentrations ranging from 7.2 to 10.7 µM were observed at 50-75 m depth in 8 9 coastal O₂-depleted waters in this study as a likely consequence of dissimilatory NO₃⁻ reduction 10 (e.g., Lipschultz et al., 1990; Lam et al., 2009; Kalvelage et al., 2013).

11 To assess the influence of the various N cycle processes that have NO₂⁻ as either a substrate or product, we first examined the relationship between the $\delta^{15}N$ and $\delta^{18}O$ of NO₂⁻. Several 12 processes can influence the isotopic composition of NO₂⁻. NO₃⁻ reduction to NO₂⁻ is associated 13 with a ε of 20 to 30‰ (Cline and Kaplan, 1975; Brandes et al., 1998; Voss et al., 2001; Granger 14 et al., 2004; 2008) and acts to produce NO₂⁻ depleted in ¹⁵N and ¹⁸O. In contrast, NO₂⁻ 15 reduction as part of either anammox, denitrification or DNRA increases both the δ^{15} N and δ^{18} O 16 of residual NO₂, with laboratory and field estimates for ε clustering around 12‰ to 16‰ 17 (Bryan et al., 1983; Brunner et al., 2013; Bourbonnais et al., 2015). However, NO₂⁻ oxidation to 18 NO_3^- at low or non-detectable O_2 has been shown to be an important sink for NO_2^- in ODZs 19 20 (e.g. Füssel et al., 2012). Anammox bacteria can also use NO₂⁻ as an electron donor during CO₂ 21 fixation under anaerobic conditions (Strous et al., 2006).

22 Nitrite oxidation has its own unique set of isotope effects (Casciotti, 2009; Buchwald and

23 Casciotti, 2010). Nitrite oxidation incurs an unusual inverse N isotope effect varying from -

24 13‰ for aerobic (Casciotti, 2009) to -30‰ for anammox-mediated NO₂⁻ oxidation (Brunner et

al., 2013), resulting in lower $\delta^{15}N$ for NO₂⁻ as it is oxidized to NO₃⁻, and increasing $\Delta\delta^{15}N$.

- 26 Moreover, enzyme catalysis associated with NO₂⁻ oxidation is readily reversible (Friedman et
- al., 1986) also causing O isotope exchange between NO_2^- and water (Casciotti et al., 2007). O
- atom incorporation during both NH_4^+ and NO_2^- oxidation have also been shown to occur with
- 29 significant isotope effect, such that the δ^{18} O of newly microbially produced NO₃⁻ in the ocean
- 30 range from -1.5‰ and 1.3‰ (Buchwald and Casciotti, 2012).

Past studies have found NO₂⁻ δ^{18} O values in ODZ's in isotope equilibrium with water as a 1 likely consequence of relatively long turnover time (e.g., Buchwald and Casciotti, 2013; 2 3 Bourbonnais et al., 2015). O isotope exchange involves the protonated form, HNO₂, but 4 because of its high pKa as compared to NO_3^- , this process can occur even at neutral to alkaline ocean pH on a time scale of 2 to 3 months at environmentally relevant temperatures (Casciotti 5 et al., 2007). NO₂⁻ δ^{18} O isotopic composition at equilibrium with water is a function of the δ^{18} O 6 7 of water and temperature (+14‰ for seawater at 22 °C) (Casciotti et al., 2007; Buchwald and Casciotti, 2013) and is independent of its $\delta^{15}N$ value such that plots of NO₂⁻ $\delta^{18}O$ vs $\delta^{15}N$ 8 usually have a slope of near zero. This is seen in our NO₂⁻ data from offshore stations occupied 9 10 during M90 (Fig. 5 C).

We observed, for the first time, a significant linear relationship for $NO_2^- \delta^{18}O$ vs. $\delta^{15}N$ at our 11 inshore stations (slope = 0.64 ± 0.07 , $r^2 = 0.59$, p-value= $3x10^{-6}$) where $O_2 < 10 \ \mu M$ (Fig. 5 B). 12 Coupled δ^{15} N and δ^{18} O effects for NO₂⁻ have not been as well studied as NO₃⁻. Nevertheless, if 13 NO_2^- turnover was faster than equilibration time with water, NO_3^- and NO_2^- reduction whether 14 as part of the denitrification, anammox or DNRA pathways, should also produce a positive 15 relationship between NO₂⁻ δ^{15} N and δ^{18} O. In contrast to our offshore stations (Fig. 5 C), this 16 positive relationship thus demonstrates that the oxygen isotopic composition of NO₂⁻ is not in 17 equilibrium with water due to both rapid NO₂⁻ turnover and the dominance of NO₂⁻ reduction 18 over oxidation in Peru coastal waters. Higher rates for aerobic NH₄⁺ and NO₂⁻ oxidation, as 19 well as anaerobic NO_3^- reduction to NO_2^- , and further reduction to NH_4^+ (DNRA) or N_2 have 20 21 been reported in shallow waters off Peru presumably due to increased coastal primary 22 production and organic matter supply to the in-shore OMZ (e.g. Codispoti et al., 1986; Lam et al., 2011; Kalvelage et al., 2013). However, as our observations are restricted to anoxic waters, 23 24 only high rates of N-loss could explain this more rapid NO₂⁻ turnover.

In principal, we can estimate NO₂⁻ turnover time from knowledge of rates for exchange with 25 water and assumptions of the δ^{18} O vs δ^{15} N slope expected in the absence of exchange. 26 Unfortunately, the slope of the relationship between NO₂⁻ δ^{18} O vs δ^{15} N expected in the absence 27 of equilibration with water is not yet known. An upper limit for turnover time for NO_2^- can be 28 estimated based on equilibration time as a function of in situ pH and temperature (Buchwald 29 30 and Casciotti, 2013). During the M91 cruise in December, subsurface temperature was 13 to 15°C along our transect and corresponding pH was near 7.8 (Michelle Graco, unpublished 31 data). Assuming the NO_2^- pool is in steady-state, we estimated an equilibration time of up to 32

 \sim 40 days for pH near 7.8 (estimated from equation 1 and Fig. 2 in Buchwald and Casciotti, 2013). A turnover time of up to 40 days implies a flux of N through the NO₂⁻ pool of at least 0.21 μM d⁻¹, as estimated from the maximum NO₂⁻ concentration observed in this study divided by this estimated turnover time. Assuming steady-state, this range also approximates the rates of NO₃⁻ reduction as well as NO₂⁻ oxidation plus production of N₂ from NO₂⁻. This estimated flux is consistent with measured high NO₃⁻ reduction and NO₂⁻ oxidation rates of up to ~1 μM d⁻¹ in Peru coastal waters (<600 m depth, Kalvelage et al.; 2013).

8 NO₂ oxidation is a chemoautotrophic process that requires a thermodynamically favorable 9 electron acceptor such as O₂. As mentioned above, NO₂⁻ oxidation appears to occur in ODZ's at low or non-detectable O₂ (e.g. Füssel et al. 2012) despite lack of knowledge of its 10 thermodynamically favorable redox couple. The difference in $\delta^{15}N$ between NO₂⁻ and NO₃⁻ 11 $(\Delta \delta^{15} N = \delta^{15} N - NO_3^{-} - \delta^{15} N - NO_2^{-}$ see Section 3.3) is further evidence for the presence of NO₂⁻ 12 oxidation in the ODZ (e.g. Casciotti et al., 2013). At steady-state, and in the absence of NO₂⁻ 13 oxidation, $\Delta \delta^{15}$ N should be no more than the ε for NO₃⁻ reduction (20 to 30%) minus the ε for 14 NO₂⁻ reduction by denitrifying or anammox bacteria (12-16‰; Bryan et al., 1983; Brunner et 15 al., 2013; Bourbonnais et al., 2015) or 8-18‰. Our results range from 15-40‰ and average 16 29.8 % for samples with O_2 concentrations <10 μ M. 17

18 The inverse kinetic isotope effect associated with NO₂⁻ oxidation is likely responsible for these 19 high $\Delta\delta^{15}N$ values (e.g. Casciotti and Buchwald, 2012; Casciotti et al., 2013). Taking all 20 isotope effects into account, the following equation can be derived to estimate $\Delta\delta^{15}N$ at steady-21 state:

22
$$\Delta \delta^{15} N \text{ (steady state)} = \varepsilon_{\text{NO3-red}} - (1-\gamma) x \varepsilon_{\text{NO2-red}} - \gamma x \varepsilon_{\text{NO2-oxid}}$$
 (15)

where γ is the fraction of NO₂⁻ oxidized back to NO₃⁻. Highest values (over 30‰) are found between 50 and 100 m, implying greater importance for NO₂⁻ oxidation in deeper waters.

Given that $\varepsilon_{NO2-oxid}$ has been reported to be -13‰ for aerobic NO₂⁻ oxidation and using the literature ranges for $\varepsilon_{NO3-red}$ and $\varepsilon_{NO2-red}$ above, our observed $\Delta\delta^{15}N$ implies that up to 100 % of NO₂⁻ produced by NO₃⁻ reduction could be oxidized back to NO₃⁻. This estimate is higher than ratios of NO₂⁻ oxidation/NO₃⁻ reduction of up to 54% for the Peruvian coastal ODZ derived from direct rate measurements (Lam et al., 2009; Kalvelage et al., 2013), and should thus be considered as an upper limit. Alternatively, NO₂⁻ oxidation also occurs as part of the overall metabolism of anammox bacteria (Strous et al., 2006) which can be the dominant N₂ producers in the Peru ODZ (Kalvelage et al., 2013). A large inverse kinetic ε for NO₂⁻ oxidation of ~-30‰ has been observed for anammox bacteria in culture (Brunner et al., 2013). If this is the sole pathway for NO₂⁻ oxidation, our data suggest NO₂⁻ oxidation up to only ~80% of total NO₃⁻ reduction. However, anammox bacteria only oxidize a minor fraction of NO₂⁻ to NO₃⁻ in culture. At the same time, estimates of NO₂⁻ oxidation (8.48 to 928 nM d⁻¹) are significantly higher than N-loss rates by anammox (2.84 to 227 nmol N l⁻¹ d⁻¹) on the Peruvian shelf (Kalvelage et al., 2013), clearly indicating non-anammox related nitrite oxidation.

The deviations from a 1:1 relationship for NO₃⁻ δ^{18} O and δ^{15} N can also be indicative of NO₂⁻ 8 oxidation. During NO₃⁻ uptake or dissimilative NO₃⁻ reduction, NO₃⁻ δ^{15} N and δ^{18} O increase 9 10 equally with a ratio of 1:1 (Granger et al., 2004; 2008). We observed a slope of about 0.86 (Fig. 5 A) for the NO₃⁻ δ^{18} O versus δ^{15} N relationship in the in-shore Peru ODZ, similar to recent off-11 12 shore observations (Bourbonnais et al., 2015). Prior reports of deviations toward higher values for the slope were indicative of addition of newly nitrified NO₃⁻ from a relatively low $\delta^{15}N$ 13 14 source (e.g. see Sigman et al., 2005; Bourbonnais et al., 2009). Our observed deviation toward slopes < 1 can instead be explained by the addition of newly nitrified NO₃⁻ with a lower δ^{18} O-15 NO₃, mostly derived from water (Andersson and Hooper, 1983), relative to the high ambient 16 δ^{18} O-NO₃⁻ values. In fact, a slope for δ^{18} O: δ^{15} N of either greater or less than 1 can be observed, 17 depending on initial environmental NO₃⁻ isotopic composition relative to any in-situ sources 18 19 (Casciotti et al., 2013). Casciotti and Buchwald (2012) showed model results where NO₂⁻ oxidation generally produces a slope <1 for the NO₃⁻ δ^{18} O versus δ^{15} N relationship, when the 20 $NO_3^- \delta^{15}N$ and $\delta^{18}O$ are higher than ~15‰ as observed in Casciotti et al. (2013) and 21 22 Bourbonnais et al. (2015).

23 4.2 Isotope effects for N-loss

As described above, the Rayleigh fractionation equations (Eqs. 1 to 6) are used here to estimate ϵ values (Mariotti et al., 1981; Altabet, 2005) and examine the significance of calculations using a) different approaches for calculating f (Eqs. 7 and 14), b) changes in the δ^{15} N of substrate (DIN) versus changes in the δ^{15} N of product, and c) closed versus open system equations. This approach provides redundancy in our estimates of ϵ and tests implied assumptions including N and ¹⁵N balance between NO₃⁻ or DIN loss and the accumulation of biogenic N₂.

Linear regression coefficients for ε calculated using the different approaches presented in 1 2 section 2.4 are listed in Tables 1 and 2. For illustration, example Rayleigh closed system plots for δ^{15} N-NO₃⁻, δ^{15} N-DIN, or δ^{15} N biogenic N₂ as a function of f_{2bioN2} are shown in Fig. 6. 3 Surprisingly, ε values estimated from the slope of these relationships are not sensitive to choice 4 5 of method for calculating f despite the lack of 1:1 correspondence between different bases (Np_{expected}, biogenic N₂, or [NO₃⁻]/[NO₃⁻]_{max}). In the case of ε calculated from changes in δ^{15} N-6 DIN, ε ranged narrowly with choice of f from 6.3 to 7.4 with standard errors on the slope of 7 8 <0.6 (Table 1). As there was no significant difference between bases for calculating f, it 9 appears that all three of our approaches are valid for this purpose.

However, ε for N-loss (closed system) does vary significantly between calculations using 10 changes in δ^{15} N-NO₃, δ^{15} N-DIN, or δ^{15} N biogenic N₂. ϵ is largest for changes in δ^{15} N-NO₃ 11 (~14 ‰) and smallest for changes in δ^{15} N-DIN (~7‰). ε based on δ^{15} N biogenic N₂ is 12 intermediate (~11‰). The latter two, using DIN or biogenic N₂ as the basis to calculate ε , are 13 more representative of N-loss. Calculations based on changes in δ^{15} N-NO₃⁻ are affected by 14 NO_2^- accumulation and isotope effects of NO_2^- oxidation (see above). The 4‰ difference in ε 15 calculated from changes in $\delta^{15}N$ of biogenic N₂ vs. $\delta^{15}N$ of DIN may arise from the 16 contribution of NH₄⁺ derived from organic matter to biogenic N₂ via the anammox process. 17 Supporting this hypothesis, NH_4^+ accumulation (5.3-7.5 μ M) associated with a relatively low 18 δ^{15} N-NH₄⁺ of 3.8‰ to 6.1‰ was observed at 125 and 200 m bottom water depths at shallow 19 20 stations located in the studied area (~12.3°S and 77.3°W) in January 2013 (unpublished results). A contribution of NH_4^+ from organic material and consumption by anammox could 21 therefore supply comparatively lower $\delta^{15}N$ to the biogenic N₂ pool, increasing ε that must be 22 23 larger to account for the observed isotopic enrichment.

The different approaches for estimating the ε for N-loss can also be evaluated by examining the initial substrate δ^{15} N predicted where f = 1 for each set of regressions. In the case of changes in δ^{15} N-DIN and using Np_{expected} or biogenic N₂ as bases for f, realistic values are found consistent with the source of upwelled waters of 6 to 7‰ (Table 1; also see Ryabenko et al., 2012). For regressions based on changes in δ^{15} N-biogenic N₂, initial δ^{15} N values are somewhat lower (~3 ‰), possibly due to a source from organic N decomposition.

30 Estimates of ε using open system equations are generally much higher than for closed system 31 equations particularly for changes in δ^{15} N-NO₃⁻ with unrealistically high values (39-63‰; 1 Table 2). However, values for both closed and open systems tended to converge for estimates 2 based on changes in δ^{15} N-DIN or δ^{15} N-biogenic N₂ with the latter having no significant 3 difference. Estimates of substrate initial δ^{15} N using the open system equations range widely and 4 do not consistently reflect realistic values (Table 2).

Closed system estimates of ε are likely more reliable in our setting because of low likelihood of 5 6 mixing between water masses of contrasting characteristics on the shelf. Temperature and 7 salinity in the ODZ at our stations narrowly ranged from 13.5 to 15 °C and 34.88 to 34.98 (Fig. 8 2), similar to T/S signatures from offshore source waters (Bourbonnais et al., 2015), and 9 suggestive of a single water mass. Accordingly, as in Bourbonnais et al. (2015), we view the closed system equations as most reliable with a value of ~6.5% for ε based on changes in δ^{15} N 10 DIN as the likely best estimate. However, given the overlap with the results of open system 11 12 equations for changes in δ^{15} N of biogenic N₂, an upper bound of ~11‰ appears appropriate. 13 This range in ε for N-loss falls below the results of Bourbonnais et al. (2015) for a near-coastal 14 eddy in the same region and time period ($\sim 14\%$) and is much less than the canonical range of 15 20 to 30‰ (Brandes et al., 1998; Voss et al., 2001; Granger et al., 2008). As discussed in 16 Bourbonnais et al. (2015), a lower overall ε for net N-loss could help resolve any imbalance in 17 the oceanic N-budget, by decreasing the ratio of sedimentary and water-column N-loss necessary to account for the observed $\delta^{15}N$ of mean ocean NO₃. 18

19 There are several reasonable explanations for these relatively low ε values in coastal waters. 20 These include higher microbial growth rates associated with higher productivity, which would 21 shift biochemical rate limitation away from enzyme reactions to membrane transport with low fractionation potential (e.g. Wada and Hattori, 1978). Another is greater influence from benthic 22 23 N cycling processes in our relatively shallow inshore system as compared to deeper waters. 24 Sediment N-loss has been shown to incur low ε due to, in analogous fashion to the affect of 25 microbial growth rate, dominance of substrate transport limitation through the sediment (Brandes and Devol, 1997). This possibility will be explored further in the next section. 26 27 Unlikely explanations for our relatively low ε values for N-loss include the effects of decreasing NO_3^- concentration (Kritee et al., 2012) and contributions from organic N via 28 29 anammox to biogenic N₂. Lack of curvature in the Rayleigh plots demonstrates a lack of 30 dependence of substrate concentration (Fig. 6 A & B) as the range in f corresponds to a large 31 range in NO₃⁻ or DIN concentrations. The possible effects of contributions from organic N to

1 biogenic N₂ has already been taken into account in calculations based on changes in the δ^{15} N of

2 biogenic N₂, as discussed above.

3 4.3 Using ε values for estimating sediment N-loss

The low ε value we observe for water column N-loss at our inshore stations may be explained
by contributions from sediment N-loss (e.g. see Sigman et al., 2003). If so, observed ε for Nloss in the water-column should be the weighted average of the actual ε values for N-loss in the
water column and sediments:

8
$$\epsilon_{obs} = \epsilon_{wc} \times (1 - P_{sed}) + \epsilon_{sed} \times P_{sed}$$
 (16)

9 where ε_{wc} and ε_{sed} are the isotope effect of water column and sediments and P_{sed} is the proportion of water column and sedimentary N-loss, respectively. We take $6.8 \pm 0.5\%$ as the 10 11 value for ε_{obs} (Fig. 6, Table 1), a value of 13.8 ± 1.3‰ for ε_{wc} as estimated for offshore waters 12 by Bourbonnais et al. (2015), and a ε_{sed} of 1.5 ‰ as in Sigman et al. (2003). From these 13 numbers, we estimated that the proportion of N-loss due to sedimentary N-loss could be up to ~60% (48 to 64%) at our coastal stations, which is in the same range than previously reported 14 for other marine coastal environments, e.g. Saanich Inlet (also up to 60%; Bourbonnais et al., 15 2013). Our estimate is higher than the 25% of benthic vs. total N-loss from a reaction-diffusion 16 17 model and direct flux measurements for the same coastal region off Peru (Kalvelage et al., 18 2013). However, our comparison to direct measurements of fluxes should be considered 19 tentative as they are made at single locations over relatively short time periods are thus subject 20 to considerable spatial and temporal heterogeneity.

21

22 5 Conclusions

The inshore Peru ODZ is distinguished from offshore by its high productivity as a consequence of coastal upwelling as well as possible greater influence from benthic processes. To examine impact on N-loss processes and their isotope effects, we investigated the dynamics of N and O isotope of NO_2^- and NO_3^- at 6 coastal stations off Peru.

We found that N-loss representing the net effect of partial denitrification, anammox and nitrification produced in sum large variations in isotopic composition. $NO_2^- \delta^{15}N$ ranged from – 20 to 10‰ and $NO_3^- \delta^{15}N$ ranged from 10 to 50‰. Generally, NO_3^- and NO_2^- isotope values varied inversely with their concentrations as expected for Rayleigh-like fractionation effects. Isotope values were usually higher in low-O₂ near bottom waters where N species
 concentrations were also relatively low.

We observed, for the first time, a positive linear relationship between $NO_2^{-1} \delta^{15}N$ and $\delta^{18}O$ at our inshore stations. In offshore ODZ waters, such a relationship has never previously been observed as $NO_2^{-1} \delta^{18}O$ reflected equilibration with water in these regions (Buchwald and Casciotti, 2013). Our results suggest a turnover time for NO_2^{-1} faster than the equilibration time with water and the effect of NO_2^{-1} oxidation over NO_2^{-1} reduction in these highly productive coastal waters. We estimated a NO_2^{-1} turnover time of up to ~40 days from our data.

The difference in δ^{15} N between NO₃⁻ and NO₂⁻ ($\Delta\delta^{15}$ N) was high, reaching up to 40% in deeper 9 10 waters, and was greater than expected from NO₃⁻ and NO₂⁻ reduction only. The influence of NO₂⁻ oxidation is consistent with this observation due to its inverse fractionation effect 11 (Casciotti, 2009). Additional evidence for NO₂⁻ oxidation is found in the relationship between 12 $NO_3^- \delta^{15}N$ and $\delta^{18}O$. NO_3^- reduction alone is expected to produce a 1:1 relationship (Granger et 13 al., 2008). While we observed a linear relationship between NO₃⁻ δ^{15} N and δ^{18} O, the slope of 14 0.86 is indicative of simultaneous addition of NO₃⁻ with relatively low δ^{18} O, also consistent 15 16 with a role for NO_2^- oxidation at our coastal sites. However, a favorable thermodynamic couple for NO_2^- oxidation in the absence of O_2 in these waters remains unknown. 17

18 A number of different approaches for estimating ε for N-loss were compared including choice of N form for changes in δ^{15} N (NO₃⁻, DIN, or biogenic N₂), closed vs open system Rayleigh 19 equations, and the basis for calculating the denominator in f (Npexpected, biogenic N2, or 20 maximum NO₃⁻). For the latter, there was little difference in estimated ε despite discrepancies 21 22 between the removal of NO₃⁻ and appearance of N₂ estimated from them. Observation of a 23 single water mass (T-S plot) in our coastal region as well as more realistic ranges for derived ε and initial $\delta^{15}N$ indicated that closed system assumptions were more realistic. Using closed 24 system equations, relatively low ε values were calculated; ~7 % for changes in the δ^{15} N of DIN 25 and ~11 ‰ for changes in the δ^{15} N of biogenic N₂. As in Bourbonnais et al. (2015), ε calculated 26 from changes in the δ^{15} N of NO₃⁻ alone was not representative of the ε for overall N-loss in 27 consideration of the build up of NO₂⁻ with distinct δ^{15} N. These estimates for ε for net N-loss are 28 lower than recently reported for a nearby offshore eddy with intense N-loss (~14‰; 29 30 Bourbonnais et al., 2015). This lower ε may be attributed to the influence of sedimentary Nloss on the Peruvian shelf (e.g., Bohlen et al., 2011) with a highly suppressed ε on the overlying 31

1 water column at our shallow stations. Given this assumption, we estimate that sedimentary N-

2 loss (by both denitrification and anammox) could account for up to 60% of the total N-loss in in

3 shore Peru ODZ waters.

4 Our results further support geographical variations in the ε of N-loss in ODZ'S, possibly related to the effects of varying primary productivity and microbial growth rates on the expression of ε 5 6 and partitioning between water-column and sedimentary denitrification. These variations need 7 to be considered in future global isotopic N budget (e.g. see Brandes and Devol. 2002). 8 potentially bringing the global N budget more in balance. This is further supported by the 9 relatively lower ε for N-loss of ~14‰ recently observed offshore in the ETSP ODZ by 10 Bourbonnais et al. (2015). A lower water-column ε for N-loss also decreases the fraction of sedimentary denitrification needed to balance the global isotopic N budget (Brandes and Devol. 11 12 2002; Altabet, 2007).

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Table 1. ϵ for NO₃⁻ reduction and net N loss estimated from both DIN consumption and produced biogenic N₂ using Rayleigh closed system equations (eqs. 1-3). Results are calculated for f based on either Np_{expected} (eqs. 7-9), biogenic N₂ (eqs. 12-14) and measured substrate divided by maximum (upwelled) substrate concentrations (see text, section 2.4). The standard error of the slope (ϵ) is shown.

	Basis for f	ε	y-intercept	r ²
	Npexpected	13.9 ± 0.7	3.74	0.92
δ^{15} N-NO ₃ ⁻	N ₂ Biogenic	14.3 ± 0.9	3.71	0.95
	[NO ₃ ⁻]/[NO ₃] _{max}	14.7 ± 0.6	-0.55	0.95
	Np_{expected}	6.3 ± 0.3	7.20	0.92
δ^{15} N-DIN	N ₂ Biogenic	6.6 ± 0.4	6.71	0.94
	DIN/DIN _{max}	7.4 ± 0.6	10.90	0.91
δ ¹⁵ N- Biogenic	$Np_{expected}$	10.5 ± 1.5	2.94	0.70
N ₂	N ₂ Biogenic	10.6 ± 1.5	3.04	0.72

Table 2. ε for NO₃⁻ reduction, and net N loss estimated from both DIN consumption and produced biogenic N₂ using Rayleigh open system equations (eqs. 4-6). Results are calculated for f based on either Np_{expected} (eqs. 7-9), biogenic N₂ (eqs. 12-14) and measured substrate divided by maximum (upwelled) substrate concentrations (see text, section 2.4). The standard error of the slope (ε) is shown.

	Basis for f	ε	y-intercept	r ²
	Npexpected	63.0 ± 4.5	-18.42	0.86
δ^{15} N-NO ₃ -	N ₂ Biogenic	66.30 ± 6.2	-21.92	0.87
	[NO ₃ ⁻]/[NO ₃ ⁻] _{max}	38.9 ± 2.7	6.19	0.87
	Npexpected	17.4 ± 1.2	3.26	0.88
δ^{15} N-DIN	N ₂ Biogenic	20.0 ± 1.8	1.72	0.89
	DIN/DIN _{max}	13.2 ± 0.9	8.45	0.91
δ ¹⁵ N-	Npexpected	12.3 ± 1.9	1.94	0.67
Biogenic				
N_2	N ₂ Biogenic	14.15 ± 2.1	2.25	0.68

1 Figures



3 Figure 1. Station map with satellite data from <u>http://disc.sci.gsfc.nasa.gov/giovanni/</u>. A: sea

- 4 surface chlorophyll *a* concentrations (mg/m³), B: night time sea surface temperature (°C).
- 5



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Figure 2. Temperature vs. salinity plots. In A, color indicates O_2 concentration (μ M). In B, color indicates NO_2^- concentration (μ M). Black dots in B mean no NO_2^- concentration data are available. Points in red rectangle at bottom of each plot belong to station 68 for depths greater than 150m.



Figure 3. O_2 and nutrient distribution along the transect. A: O_2 concentration (μ M) with isotherm overlay, B: NH₄⁺ concentration (μ M), C: Si(OH)₄ concentration (μ M) and D: PO₄³⁻ concentration (μ M). Grey region represents bathymetry. The depth for station 68 is 253m.





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Figure 4. Transects off the Peru coast for A: NO_3^- concentration (μ M) with O_2 overlay, B: NO_2^- concentration (μ M), C: $\delta^{15}N-NO_3^-$ (‰) and D: $\delta^{15}N-NO_2^-$ (‰). Grey region represents approximate bathymetry. No isotopic data are available for the deeper samples collected at station 63, because NO_3^- and NO_2^- concentrations were below analytical limits (<0.5 μ M).



Figure 5. Relationships between δ¹⁵N and δ¹⁸O for NO₃⁻ and NO₂⁻, respectively, for
O₂≤10µM. A: δ¹⁸O-NO₃⁻ vs δ¹⁵N-NO₃⁻ for station 62 to 68. B: δ¹⁸O-NO₂⁻ vs δ¹⁵N-NO₂⁻
for station 62 to 68. C: δ¹⁸O-NO₂⁻ vs δ¹⁵N-NO₂⁻ for M90 offshore stations 51, 59, 106
and 111 (see text, section 3.3). For each plot, overall linear regressions are shown.
Significant correlation coefficients at a 0.05 significance level are denoted by *.



Figure 6. Raleigh relationships used to estimate ε (slope) and initial δ^{15} N-substrate (yintercept) assuming a closed system. A: for NO₃⁻ reduction (Eq. 1 and text, section 2.4), B: for N-loss calculated from the substrate (DIN) consumption (Eq. 2 and text, section 2.4) and C: for N-loss calculated from the δ^{15} N of biogenic N₂ (Eq. 3 and text, section 2.4). In C, only samples with O₂ concentrations less than 10 µM and biogenic N₂ values >7.5 µM were considered. Significant correlation coefficients at a 0.05 significance level are denoted by *.



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Figure 7. N deficit, biogenic N in N₂ and δ^{15} N-N₂ anomaly with O₂ overlaid. A: N deficit calculated using PO_4^{3-} (µM) (N_{pdef}) and assuming Redfield stoichiometry (see 3 Eqs. 9, 10 and 11, section 2.4). B: biogenic N in N₂ (μ M). C: δ^{15} N-N₂ anomaly relative 4 to equilibrium with atmosphere (‰). Biogenic N_2 or $\delta^{15}N-N_2$ anomaly were not 5 6 measured at stations 62, 64 and 66.



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Figure 8. Cross-plots of biogenic N in N₂ versus DIN (A), NO₃⁻ (B) and Np_{def} (C), see Eqs. 9-11 in text). All plots have the overall linear regression overlaid. All the points are restricted to O₂ concentrations less than 10 μ M. Biogenic N₂ was not measured for stations 62, 64 and 66. Significant correlation coefficients at a 0.05 significance level are denoted by *.