Dear editor,

Please find attached the responses to the reviewers' comments, as well as a track changed version of our manuscript. We believe that addressing the critical comments of both reviewers improved our manuscript substantially.
In addition, we now added the Pangaea doi numbers of the metadatasets (oxygen, nutrients, physical parameters), as well as the final Genbank accession numbers for the sequencing results.

All the best,
Carolin Löscher and co-authors

Response to Referee #1

We thank referee 1 for this thorough review of our manuscript. The comments and thoughts provided by Referee 1 were particularly helpful to improve this manuscript. Questions and comments were addressed as described in the following; modifications are highlighted in yellow in the main text:

Referee Comment (RC) 1: Why is carbon fixation higher at the deeper depths and associated with N2 fixation? Authors concluded that N2 fixation co-occurred with N loss process. So how was carbon fixation sustained (during the incubations) without reactive nitrogen? In addition, Fig. 3 suggests that C fixation was very high in the center of eddy B at above 100m depth, but there was no reactive nitrogen at that depth either. So what sustained C fixation? My guess is that all these higher carbon fixation rates are sustained by reactive nitrogen from N2 fixation. If the authors have ammonium (released from the fixed nitrogen) data then my hypothesis can be (dis)proved.

Author comment (AC) 1: The fact that C fixation is very high at deeper depths may relate to so-called 'dark carbon fixation' as previously shown to be important in this region (Schunck et al., 2013), the Cariaco basin (Taylor et al., 2001) and lake environments with enhanced N-loss and N_2 fixation activity (Camacho et al., 2001). If I should speculate, I would also suggest that N_2 fixation greatly drives C fixation, where it is high. While nitrate and nitrite data are present, ammonia data are unfortunately not available from those samples therefore one part of the reactive N pool is missing. It is thus not entirely possible to show what drives C fixation and a real prove (or disprove)of this hypothesis cannot be given at that time but should be subject of follow-up studies.

RC 2: Was there sufficient light to sustain carbon fixation at around 300 m depth (Fig. 3).

AC 2: In order to answer this question, we analyzed the available fluorescence data (Krahmann, 2014). From this data, light should be unavailable at 300 m depth (# 1639, eddy A: fluorescence < 0.04 factory calibrated units or approx. µg L^{-1} at 300 m, compared to 2,59 µg L^{-1} in the upper Chl maximum at 40 m). A distribution of chlorophyll A is also given in Stramma et al. (2013, Fig. 4), which shows disappearance of Chl a between 80 and 100 m. Therefore, C fixation may rather be ascribed to dark C- fixers as previously described to significantly contribute to primary production in OMZ waters (see above). Although not much information about the character of dark C-fixers or their importance in the environment is presently available, this topic itself is highly interesting and it is tempting to address it by a separate study.

Minor comments:

RC 3: Page 18948, Line 2: “Nitrogen.....ocean (Codispoti, 1989)” is slightly incorrect statement. It is reactive nitrogen (NH4, NO3) that limits primary production, as ocean has plenty of N in the form of dissolved gas. “is limiting” should be replaced as “limits”

AC3: We agree and modified the term to ‘Reactive nitrogen’.

RC 4: Page 18948, Line 25-29: “In addition.....reported for this region (Chaigneau et al., 2009).” is confusing. Does the last part of the paragraph mean that the no of eddies are increasing, or no. of reported eddies are increasing?

AC 4: Chaigneau et al. (2009) compared different upwelling systems (Peru-Chile, California, Canary, Benguela) based on a 15 year record of satellite altimetry data. Among those systems, eddy frequency (eddies/week) was the second highest in the upwelling off Peru. In order to clarify, we rephrased the sentences:

In addition to its remarkable biological activities, the physically dynamic character of the ETSP in the upwelling system off Peru favors mesoscale activities (Chelton et al., 2011). Compared to other upwelling regions (e.g. off California, Benguela), enhanced frequency of eddies has been reported for this region (Chaigneau et al., 2009).

RC 5: Page 18950, Line 11-13: How was the age of eddies estimated?

AC 5: The age has been estimated by Stramma et al. 2013 based on satellite monitoring of sea level height anomalies. This information, as well as the reference, was added:

The age of the eddy was determined by Stramma et al. (2013) bases on satellite monitoring of sea level height anomaly data.

RC 6: Page 18951, Line 2: Why only autotrophic? Why cannot positive P* stimulate heterotrophic N2 fixation?

AC 6: This is actually true- why shouldn’t it stimulate N2 fixation in general? Therefore, ‘autotrophic’ has been removed.

RC 7: Page 18951, Line 8: Replace “N2/Ar-1” by “N2/Ar”

AC 7: Changed as suggested.

RC 8: Page 18951, Line 13: But anammox can occur at [O2] > 10µM

AC 8: Indeed, e.g. Kalvelage and colleagues (2011) showed that anammox is still measureable at O_2> 10 µM. However, anammox rates were also found to sharply drop at O_2 levels of 10-15 µM (see Fig. 3 in Kalvelage et al., 2011). O_2 was always > 10 µM at the reference station. Further, we argue, that anammox is not significant at this offshore station because biogenic N_2 did not significantly accumulate there, i.e., there was no N_2 excess (or biogenic N_2) peak corresponding to the O_2 minimum (Figure SI in Bourbonnais et al., 2015, attached).

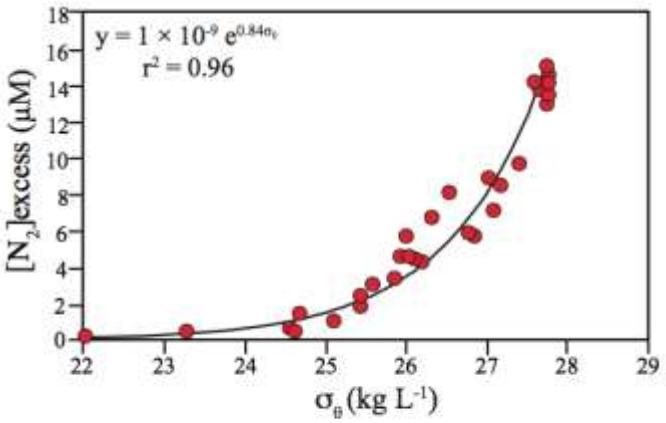

Figure S1. $[N_2]_{excess}$ versus σ_θ at a background station unaffected by N-loss located north of the OMZ (1.67°N, 85.83°W) sampled in November 2012 (M90 cruise).

RC 9: Page 18951, Line 13: M90 does not have any locations in the northern hemisphere.
So location 1.67°N seems to be incorrect.

AC 9: M90 had several stations on the Northern hemisphere that are not shown or discussed in that study, but, for your reference, I attached the cruise track to this rebuttal letter. Therefore, the position is correct.

RC 10: Page 18951, Line 18: Replace "N2 Ar-1" by "N2/Ar"

AC 10: Changed as suggested.

RC 11: Page 18952, Line 10-12: "For each.....of the rates". Then mention the range of measured enrichment values.

AC 11: We included the range of $^{15}N_2$ enrichment as a range (2.4 ± 0.144 atom%, p. 18952, line 4 of the Discussion paper)

RC 12: Page 18952, Line 25: "-" should be replaced by > or ~, whichever applicable.

AC 12: This is a vacuum filtration, therefore the '-' refers to the fact that the pressure is negative. A '—' is therefore correct.

RC 13: Page 18956, Line 7-10: "A coastal origin.....signals" How could the movement of eddy C be the reason for lower N loss? Explain.

AC 13: Eddy C did not stay at the coast as eddies A and B did. Thus, it was less exposed to 'coastal' conditions compared to the other eddies, e.g. organic matter load of the water mass or trace metal availability. As strong N

loss is mainly present at the coast it may be considered a coastal feature and what we measure in eddies A and B would therefore be a result of their residence time at the coast.

RC 14: Page 18957, Line 5: Previous studies that the authors have mentioned also presented quantitative rates. Then why the authors say "first quantitative rates of N_2 fixation?

AC 14: To our knowledge, this is the first study presenting N_2 fixation measurements from that region using the method developed by Mohr et al., 2010. Other previous studies, including our own, used the bubble addition method, which has been shown to largely underestimate N_2 fixation rates. The quantification in those studies was therefore biased and not fully quantitative.

RC 15: Page 18958, Line 18: Replace "consistence" with "consistency"

AC 15: Changed as suggested.

RC 16: Page 18959, Line 24-27: "As previous……eddy C.". How? Then N2 fixation should have also occurred at the surface in the eddies A and B.

AC 16: This is true and this is also in accordance with our observations. We therefore replaced eddy C by 'the eddies'.

RC 17: Page 18966: Caption of Table 1. Replace "biogeochemical properties" by "biogeochemical parameters"

AC 17: Changed as suggested.

RC 18: Page 18967: Caption of Fig. 1. Acronym SSHA does not go together with Aviso sea level anomaly

AC 18: This is true; SSHA refers to sea surface height anomaly, derived from the Aviso satellite. We modified the caption to 'Aviso satellite-derived altimeter sea surface height anomaly data (SSHA)'.

RC 19: Page 18968: Caption of Fig. 2. Delete "Hydrographic……16.45'S."

AC 19: Deleted.

RC 20: Page 18969: Enlarge the legend and axes title of Fig. 3

AC 20: We enlarged the legend and axes titles.

RC 21: Page 18971: Caption of Fig. 5: Does eddy A stand for eddy A1 or A2?

AC 21: This refers to eddy A1, we added this information to the caption.

RC 22: Page 18972: Caption of Fig. 6: “Strongest negative correlations are present between N2 fixation and O2 and N2 fixation and O2.” should be replaced by “Strongest negative correlations are present between N2 fixation and O2

AC 22: Actually, a negative correlation with O2 and with temperature was present; therefore, the second ‘O2’ was replaced by ‘temperature’.

References:

Bourbonnais, A., Altabet, M. A., Charoenpong, C. N., Larkum, J., Hu, H., Bange, H. W., and Stramma, L.: N-loss isotope e_ects in the Peru oxygen minimum zone studied using a mesoscale eddy as a natural tracer experiment, Global Biogeochem. Cy., 29, 793–811, doi:10.1002/2014GB005001, 2015

Camacho A, Erez J, Chicote A, Florin M, Squires MM, et al. (2001) Microbial microstratification, inorganic carbon photoassimilation and dark carbon fixation at the chemocline of the meromictic Lake Cadagno (Switzerland) and its relevance to the foodweb. Aquat Sci 63: 91–106. doi: 10.1007/pl00001346

Kalvelage T, Jensen MM, Contreras S, Revsbech NP, Lam P, Günter M, et al. (2011) Oxygen Sensitivity of Anammox and Coupled N-Cycle Processes in Oxygen Minimum Zones. PLoS ONE 6(12): e29299. doi:10.1371/journal.pone.0029299

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Taylor GT, Iabichella M, Ho TY, Scranton MI, Thunell RC, et al. (2001) Chemoautotrophy in the redox transition zone of the Cariaco Basin: A significant midwater source of organic carbon production. Limnol Oceanogr 46: 148–163. doi: 10.4319/lo.2001.46.1.0148

Response to Referee #2

We thank referee #2 for recommending our manuscript for publication in Biogeoscience. The comments and thoughts provided by referee greatly helped to improve this paper, we addressed the suggestions as described in the following, changes in the text are highlighted in the main text.

Material and Methods

RC 1: Page 18952, line 26 the authors state" The filtrations were stopped after one hour since high particle load in surface waters often lead to a clogging of the filters." Please provide detail on how much volume has passed the filter by then (Table in Suppl). Likewise, how do the authors consider the error introduced by stopping the filtration abruptly e.g. considering the sedimentation of particles on the filter. Why did the authors not adjusted the filtration volume accordingly, and why did they choose one hour? Did they filter the rest onto a second filter and measure both?

AC 1: The filtration volumes were already reported as a range in the methods part (p.18952, l. 23 in the Discussion paper). The filtration was performed in a stepwise manner. After rotating the bottles, 0.5 L was filtered. Afterwards, if filters were not clogged, bottles were again rotated and additional 0.2-0.5 L were filtered and so on. Therefore, an error from sedimentation cannot occur. One hour was not actively chosen but was the average filtration time before filters clogged. The rest of the incubation water was used for chlorophyll filtration and DNA/RNA filtration.

RC 2: Page 18950, line 11: How was the age of the eddy determined?

AC 2: The age has been estimated by Stramma et al. 2013 based on satellite monitoring of sea level height anomalies. This information, as well as the reference, was added to this sentence.

RC 3: Page 18951, line 2: Does P* also stimulate heterotrophic N2 fixation?

AC 3: This could indeed happen; therefore the term 'autotrophic' has been removed.

RC 4: Page 18951, Line 8, also line 18: Do the authors mean N2/Ar?

AC 4: Yes, this has been changed.

RC 5: Page 18952, Line 10-12: Please add the exact initial enrichments for each bottle, e.g. in a table or give a range.

AC 5: We included the range of $^{15}N_2$ enrichment as a range (2.4 ± 0.144 atom%, p. 18952, line 4 of the Discussion paper)

RC 6: Page 18952, Line 25: Replace "-" by "∼".

AC 6: The '-' refers to the negative pressure resulting from vacuum filtration and is thus correct.

Results and Discussion

RC 7: Figure 3- What sustained C-fixation in A1 below 200 m and was there any light available at that depth?

AC 7: Light was unavailable at this depth and enhanced C fixation may rather be due to so-called 'dark carbon fixation' as previously shown to be important in this region (Schunck et al., 2013), and e.g. the Cariaco basin (Taylor et al., 2001) or lake environments with enhanced N-loss and N_2 fixation activity (Camacho et al., 2001). A sentence stating that dark C fixation is likely the reason for enhanced C fixation at deeper depths has been added to the manuscript.

RC 8: Page 18957, Line 5: There are already quantifications so the authors are not the first who provide quantitative N2 fixation rates. Please correct.

AC 8: To our knowledge, this is the first study presenting N2 fixation measurements from that region using the method developed by Mohr et al., 2010. Other studies cited here, used the bubble addition method that underestimates rate measurements. The quantification was therefore biased and not fully quantitative.

RC 9: Page 18967: Fig. 1. SSHA is not an acronym for Aviso sea level anomaly- please correct.

AC 9: We agree and modified to 'Aviso satellite-derived altimeter sea surface height anomaly data (SSHA)'.

RC 10: Page 18969, Fig. 3: Please enlarge numbers and legends- it's hard to read.

AC 10: We modified the figure as suggested.

RC 11: Page 18972, Fig. 6. Please delete repetition of between "N2 fixation and O2 and N2 fixation and O2"

AC 11: We modified the sentence to 'N2 fixation and O2 and N2 fixation and temperature'.

N_2 fixation in eddies of the eastern tropical South Pacific Ocean

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Short title: N_2 fixation in ETSP eddies

Abstract

Mesoscale eddies play a major role in controlling ocean biogeochemistry. By impacting nutrient availability and water column ventilation, they are of critical importance for oceanic primary production. In the eastern tropical South Pacific Ocean off Peru, where a large and persistent oxygen deficient zone is present, mesoscale processes have been reported to occur frequently. However, investigations on their biological activity are mostly based on model simulations, and direct measurements of carbon and dinitrogen (N_2) fixation are scarce.

We examined an open ocean cyclonic eddy and two anticyclonic mode water eddies: a coastal one and an open ocean one in the waters off Peru along a section at 16°S in austral summer 2012. Molecular data and bioassay incubations point towards a difference between the active diazotrophic communities present in the cyclonic eddy and the anticyclonic mode water eddies.

In the cyclonic eddy, highest rates of N_2 fixation were measured in surface waters but no N_2 fixation signal was detected at intermediate water depths. In contrast, both anticyclonic mode water eddies showed pronounced maxima in N_2 fixation below the euphotic zone as evidenced by rate measurements and geochemical data. N_2 fixation and carbon (C) fixation were higher in the young coastal mode water eddy compared to the older offshore mode water eddy. A co-occurrence between N_2 fixation and biogenic N_2, an indicator for N loss, indicated a link between N loss and N_2 fixation in the mode water eddies, which was not observed for the cyclonic eddy. The comparison of two consecutive surveys of the coastal mode water eddy in November 2012 and December 2012 revealed also a reduction of N_2 and C fixation at intermediate depths along with a reduction in chlorophyll by half, mirroring an aging effect in this eddy. Our data indicate an important role for anticyclonic mode water eddies in stimulating N_2 fixation and thus supplying N offshore.

1 Introduction

Reactive nitrogen (N) limits primary production in large parts of the ocean (Codispoti, 1989). Biological dinitrogen (N_2) fixation is an important external input of N, representing more than 60-80% of the new N provided to the Ocean (Codispoti, 2007;Duce et al., 2008), and can partially relieve N limitation. For decades, N_2 fixation was conventionally thought to occur mainly in nutrient-depleted surface waters such as found in the subtropical gyres (Sohm et al., 2011). However, some recent modeling studies have suggested a close spatial link between fixed N loss, i.e. N_2 production via anammox and/or denitrification, occurring in oxygen deficient zones (ODZs), and N_2 fixation taking place in the adjacent surface ocean with the consequence that the potential habitat of N_2 fixing organisms is larger than previously thought (Deutsch et al., 2007). Furthermore, as both processes are favored under oxygen depleted conditions and as some organisms responsible for these processes do not need light, their coupling in ODZ waters would damp excursions in the oceanic N inventory and promote stability of the global N budget.

In the recent years, efforts have been placed on investigating N_2 fixation in the eastern tropical South Pacific (ETSP) (Dekaezemacker et al., 2013;Fernandez et al., 2015;Löscher et al., 2014) and the results of those field studies have significantly advanced our understanding of diazotrophy in low-O_2 regions of the ocean. They indeed confirmed the frequent occurrence of N_2 fixation in denitrified waters and below the euphotic zone. A biogeochemical significance of non-cyanobacterial diazotrophs (i.e., microbes capable of N_2 fixation) has been described, and their enormous potential to fix N_2 in the ETSP seems also to depend on organic matter supply (Fernandez et al., 2015).

In addition to its remarkable biological activities, the physically dynamic character of the ETSP in the upwelling system off Peru favors mesoscale activities (Chelton et al., 2011). Compared to other upwelling regions (e.g. off California, Benguela) enhanced frequency of eddies has been reported for this region (Chaigneau et al., 2009). Mesoscale eddies are physical structures with horizontal scales of less than 100 km and timescales of around one month. These features can transport physical and chemical properties from the coast towards the open ocean (Klein and Lapeyre, 2009) and impact on the ocean by modulating nutrient availability (Fong et al., 2008;Altabet et al., 2012). Cyclonic and mode water eddies can inject nutrients to the euphotic zone through vertical displacement of isopycnal surfaces, which increases surface primary production (McGillicuddy et al., 2007). Overall, investigations on the impact of mesoscale eddies on N_2 fixation are scarce. Fong et al. (2008) reported a stimulation of N_2 fixation in a mode water eddy of the oligotrophic North Pacific. Another study showed increased abundances of *Trichodesmium* in mesoscale eddies of the Western South and North Atlantic associated with strong temporal variations (Olson et al., 2015).

In the ODZ off Peru, mesoscale eddies have previously been identified as N loss hotspots (Altabet et al., 2012;Bourbonnais et al., 2015), but to date no detailed surveys on their relevance for N_2 fixation in this

region are available. The spatial connection between N loss and N_2 fixation that has been proposed for this region (Fernandez et al., 2011) may, however, indicate a potential for N_2 fixation associated to eddies in the ODZ off Peru.

The major goal of this study was to advance our understanding of eddy-related N_2 fixation by surveying one cyclonic and two anticyclonic mode water eddies along a 16.45°S transect during the R/V Meteor cruises M90 and M91 in November-December 2012. During the survey of these three eddies, we measured both N_2 fixation rates and abundances of *nifH*, a key functional molecular marker gene. Additionally, N_2 fixation was compared to N loss signals in the water column to investigate their coupling in the eddy systems. One particular eddy was surveyed twice (in November 2012 and December 2012), allowing monitoring the temporal development of N_2 fixation and primary production in an aging eddy.

2 Material and Methods

2.1 Sampling description and biogeochemical parameters

Selection of sampling stations and identification of eddy cores and edges were based on sea level height anomaly data from Aviso (http://aviso.altimetry.fr) and followed the criteria defined by Stramma et al. (2013). Briefly, the eddies were tracked during the R/V Meteor cruises M90 and M91 in November-December 2012. Three eddies were detected in area extending from the Peruvian coast to ~84°W and from 15°S to 18°S (Fig. 1, Stramma et al., 2013). Two eddies (further referred to as eddy A centered at about 16°S, 76°W and eddy B centered in the open ocean at about 17°S, 83°W) were mode water eddies and one was cyclonic (further referred to as eddy C, centered in the open ocean at 16°S, 80°W, Fig. 1). The age of the eddy was determined by Stramma et al. (2013) bases on satellite monitoring of sea level height anomaly data. At the time of the survey, the near-coastal eddy A was about 2 months old (3 months during the second survey), while the open-ocean eddy B was 5 months and the cyclonic open-ocean eddy C was 2 months old.

Samples for salinity, O_2 concentrations and nutrients (nitrate, NO_3^-; nitrite, NO_2^-; phosphate, PO_4^{3-} and ammonium, NH_4^+) were taken from a 24-Niskin- bottle rosette equipped with a conductivity-temperature-depth (CTD) sensor or from a pump-CTD (Friedrich et al., 1988). O_2 concentrations were determined using a Seabird sensor, calibrated to the Winkler method (precision of 0.45 µmol L^{-1}; the lower detection limit was 2 µmol L^{-1}; (Stramma et al., 2013)). Nutrient concentrations were determined as previously described (Grasshoff, 1999) using a QuAAtro auto-analyzer (SEAL Analytical GmbH, Germany; precision for NO_2^-, NO_3^-, and PO_4^{3-} were ± 0.1 µmol L^{-1}, ±0.1 µmol L^{-1}, ±0.02 µmol L^{-1}, respectively). Excess PO_4^{-3}, P* (i.e., the anomaly in P relative to expected stoichiometry with N) was calculated from

dissolved inorganic nitrogen (DIN= NO_3^- + NO_2^-) and PO_4^{3-} measurements according to Deutsch et al. (2007):

$$P^* = PO_4^{3-} - DIN / r_{16:1},$$

where $r_{16:1}$ is the ratio of nitrate to phosphate at Redfield conditions. Positive P* has been thought to stimulate N_2 fixation

2.2 N_2/Ar, Biogenic N_2 measurements

High precision measurements of N_2/Ar were made on septum sealed samples using on-line gas extraction system coupled to a multicollector continuous flow-IRMS as described in Charoenpong et al. (2014). N_2 excess ($[N_2]_{excess}$), i.e. the observed $[N_2]$ minus the equilibrium $[N_2]$ at in-situ temperature and salinity, was calculated based on the N_2/Ar ratio with daily calibration against seawater standards equilibrated with air at fixed temperatures (5°C, 15°C and 25°C). Precision (standard deviation) for duplicate measurements was generally better than ±0.7 μmol L^{-1} for $[N_2]_{excess}$.

We calculated biogenic $[N_2]$ ($[N_2]_{biogenic}$), the $[N_2]$ produced by denitrification or anammox, by subtracting the $[N_2]_{excess}$ at a background station ($[N_2]_{excess\_bkgd}$) unaffected by N loss ($[O_2]$>10 μmol L^{-1}) located north of the ODZ (1.67°N, 85.83°W, M90 cruise) from the observed $[N_2]_{excess}$ at corresponding σ_θ (as described in Bourbonnais et al., 2015):

$$[N_2]_{excess\_bkgd}\,(\mu mol\ L^{-1}) = 1 \times 10^{-9}\, e^{0.84\sigma_\theta}$$

This corrects for non-local biological N loss as well as physically-produced deviations in equilibrium N_2/Ar (Hamme and Emerson, 2002).

2.3 N_2/C-fixation rate measurements

Sample seawater was taken from the Niskin bottles or from the pump-CTD and filled into 4.5 L polycarbonate bottles (Nalgene, Thermo Fisher, Waltham, Massachusetts, USA) capped with Teflon-coated butyl rubber septum. Incubations were performed as previously described (Grosskopf et al., 2012) with the method developed by Mohr et al. (2010). In contrast to the traditionally used bubble addition method (Montoya et al., 1996), $^{15}N_2$ gas (Cambridge Isotopes, Lot no.: I-16727) was dissolved in degassed water from the same sampling depth in order to guarantee a high dissolution and a stable enrichment in

$^{15}N_2$. Each incubation bottle was supplemented with 100 mL of $^{15}N_2$-enriched seawater containing defined amounts of 98% $^{15}N_2$ gas in order to reach final and constant $^{15}N_2$ enrichment of 2.4 ±0.144 atom%. A recent study reported a slight potential contamination of $^{15}N_2$ gas with 0.024 ± 0.006 µmoles $^{15}N\text{-}NO_3^-$ /NO_2^- and 0.014 ± 0.004 µmoles $^{15}N\text{-}NH_4^+$ per mole $^{15}N_2$ (Dabundo et al., 2014). According to Dabundo et al. (2014), however, low concentrations of contaminants in Cambridge-$^{15}N_2$ gas do not significantly inflate N_2 fixation rates such as those presented here. In addition, we examined the $^{15}N_2$ gas used in our incubations following the hypobromide oxidation method (Warembourg, 1993) and no contamination has been detected. For each bottle, the initial enrichment of $^{15}N_2$ has been determined and considered for the calculation of the rates.

For carbon fixation measurements, $NaH^{13}CO_3$ (98 atom% ^{13}C, Sigma-Aldrich, St. Louis, Mo, USA) was dissolved in sterile MilliQ water (1g/ 50 mL). 1 ml was added to the incubations with a syringe (~3.5 atom% final in 4.5 L bottles). In order to investigate the contribution of heterotrophic vs. autotrophic diazotrophs to N_2 fixation, glucose addition experiments were performed with ^{13}C-labelled glucose (Sigma-Aldrich, St. Louis, Mo, USA), dissolved in MilliQ water (1.44 g L^{-1}), and the concentrated solution was added through the septum with a syringe to yield a final concentration of 2 µmol L^{-1} glucose. Bottles from surface water were kept in a seawater-cooled on-deck Plexiglas incubators covered with blue light foil (blue-lagoon, Lee filters, Andover, Hampshire, UK) that mimics the ambient irradiance at around 10 m depth. Samples from the ODZ were stored at 12°C in the dark. After 24 hours of incubation, 0.7 – 2.5 L of seawater were filtered onto pre-combusted (450°C, 5 hours) 25 mm diameter GF/F filters (Whatman, GE healthcare, Chalfont St Gile, UK) under gentle vacuum (-200 mbar). The filtrations were stopped after one hour since high particle load in surface waters often lead to a clogging of the filters. Filters were oven dried (50°C) for 24 hours and stored over desiccant until analysis. Environmental samples of 2 L untreated seawater were filtered and prepared in the same way to serve as blank values for natural abundance. For isotope analysis, GF/F filters were acidified over fuming HCl overnight in a desiccator to remove inorganic C. Filters were then oven-dried for 2 hours at 50°C and pelletized in tin cups. Samples for particulate organic carbon and nitrogen (POC and PON) and isotopic composition were analyzed on an Elemental Analyzer Flash EA 1112 series (Thermo Fisher, Waltham, Massachusetts, USA) coupled to a mass spectrometer (Finnigan Delta Plus XP, Thermo Fisher, Waltham, Massachusetts, USA). Measurements were calibrated using reference gases between each sample and caffeine every 6 samples. A table of N_2 and C fixation rate measurements is given in the supplemental material.

Possible correlations between environmental parameters and N_2 fixation rates were explored by principal component analysis (PCA) based on 58 cases. Computations were performed in PAST version 3.07 (Hammer et al., 2001). Metadatasets for M90 and M91were deposited at PANGAEA

(doi:10.1594/PANGAEA.830245, doi:10.1594/PANGAEA.857751, doi:10.1594/PANGAEA.817193, doi:10.1594/PANGAEA.817174).

2.4 Molecular methods

For molecular analysis, nucleic acid samples were collected by filtering up to 1 L of seawater (exact volumes were recorded and the filtration time was shorter than 20 min) onto polycarbonate membrane filters with a pore size of 0.2 µm (Millipore, Darmstadt, Germany). Immediately after collection, samples were flash frozen in liquid nitrogen and stored at -80 °C until extraction. Nucleic acids were extracted using DNA/RNA AllPrep Kit (Qiagen, Hildesheim, Germany) with minor changes in the protocol (Löscher et al., 2014).

For cDNA library construction, residual DNA was removed from the purified RNA by a DNase I treatment (Life Technologies, Carlsbad, CA, USA). The extracted RNA was gene specifically reverse transcribed to cDNA using the Superscript III First Strand synthesis Kit (Life Technologies, Carlsbad, CA, USA) following the manufacturers' protocol and *nifH* cluster specific no-template qPCRs were performed to assure the purity of RNA. Quantitative PCRs were performed with cDNA as described, before (Löscher et al., 2014); however, a ViiA7 qPCR system (Life Technologies, Carlsbad, CA, USA) was used and the reaction volume was reduced to 12.5 µl. The detection limit of the qPCRs was deducted from non-template controls. No amplification was detected after 45 cycles, setting the theoretical detection limit to one copy L^{-1}. As the detection limit depends on the sample and elution volumes, we calculated a detection limit of 40 copies L^{-1}. For *nifH* transcript diversity analysis, a PCR based amplification of the *nifH* gene was performed followed by Topo TA cloning and sequencing using established protocols (Lam et al., 2007;Langlois et al., 2005). Sequences were submitted to GenBank (accession numbers: KX090448-KX090515).

Phylogenetic analysis of *nifH* transcripts was conducted using a Muscle alignment on a 321 bp fragment with the Mega 6.0 package (Tamura et al., 2013), sequence differences were set at a minimum of 5%, neighbor joining trees were constructed as previously described (Löscher et al., 2014).

3 Results and Discussion

The investigated eddies originated from the shelf-slope region of the Peru margin (however, the exact origin of eddy B could not be determined). While eddy A remained close to the coast during the two

months period, eddies B and C propagated further offshore. Key hydrographical properties are specified below but an extensive description can be found in Stramma et al. (2013).

Eddy A, a nearshore mode water eddy, showed a pronounced O_2 minimum from 100 m downwards with lowest concentrations close to the detection limit of the Winkler method (~ 2 μmol O_2 kg^{-1}). The influence of the coastal upwelling was visible from the lifting of the upper isopycnal towards the shore (Arévalo-Martínez et al., 2015). Below the oxycline at ~ 100 m depth, nutrient concentrations were generally higher in the ODZ relative to surface waters. While PO_4^{3-} concentrations were not considerably different in the eddy compared to surrounding waters, NO_3^- concentrations showed a pronounced decrease in the ODZ of eddy A compared to surrounding waters (see figure S1 for individual sections through eddies A, B and C). This decrease correlated with an increase in NO_2^- concentrations at the same depth ($r^2 = 0.76$, n= 52 below the oxycline). A comparison to biogenic N_2 as indicator for active or past N loss processes showed a maximum along with the NO_2^- maximum thus supporting the view of ongoing N loss in eddy A (Fig. 2, see Bourbonnais et al. (2015) for details on N loss processes in eddy A). As a result of this N loss, we observed large values for excess P*, which is classically considered to promote N_2 fixation in surface waters (Karl et al., 2002).

Eddy A, which was estimated to exist for two months at the time of the first survey, was sampled again one month later; the first observation is further referred to as "eddy A1", the second survey is referred to as "eddy A2". Since the first observation, a decrease in O_2 (table 1) and NO_3^- (Arévalo-Martínez et al., 2015;Stramma et al., 2013) has been observed indicating ongoing respiration and N loss. These signals of enhanced ongoing N loss weakened over time as eddy A aged (Stramma et al., 2013). An extensive characterization of N loss signals in this eddy revealed a complete consumption of NO_3^- (Bourbonnais et al., 2015), possibly due to nutrient subduction via organic matter export out of the anticyclonic eddy (Omand et al., 2015). Most intense N loss signals were observed near the core of eddy A, where the ODZ is in direct contact with the euphotic zone via uplifting of isopycnals (Bourbonnais et al., 2015) thus supporting the impact of freshly produced organic matter on N loss (Babbin et al., 2014).

Eddy B, an offshore mode water eddy, was characterized by slightly deeper oxycline and nutriclines compared to eddy A (at ~200 m water depth). Although less pronounced than in eddy A, NO_3^- concentrations decreased within the ODZ in the eddy along with an increase in NO_2^- and biogenic N_2 between 200 and 300 m depth, again indicating N loss. P* was slightly higher in the O_2 depleted core waters, however, to a lesser extent and slightly deeper compared to eddy A. Stramma et al. (2013) observed weaker signals for N loss in eddy B, which were also mirrored by lower N_2O production (Arévalo-Martínez et al., 2015). This weakening may result from less organic matter export into the core of the eddy (Fig. 2).

Eddy C, was the investigated offshore cyclonic eddy (Fig. 2) (Stramma et al., 2013). NO_3^- did not show the same pronounced decrease in the core of the eddy as detected in eddies A and B, but NO_2^- and biogenic N_2 were found slightly enriched between 200 and 300 m water depth, possibly from the onset of N loss at this location or a left-over signal from enhanced N loss within the coastal upwelling as previously described for this region (Kalvelage et al., 2013), and confirmed by the excess of P compared to N in its core waters. A coastal origin of eddy C has been described; however, compared to the mode water eddies A and B, eddy C moved westward without staying in the shelf/slope region (Stramma et al., 2013), which may be one reason for the lower N loss signals.

3.2 Patterns of N_2 and C fixation in the three eddies

N_2 fixation was strongly associated with intermediate waters of the mode water eddies A and B, while the cyclonic eddy C showed maximum rates of N_2 fixation in surface waters but no detectable N_2 fixation in the O_2 depleted core waters.

In eddy A1, intense N_2 fixation was detected between 200 and 350 m water depth in the eddy center with maximum rates of 4.4 nmol N L^{-1} d^{-1} at 250 m depth (Fig. 3). At the same depth, carbon fixation was the highest reaching 0.51 µmol C L^{-1} d^{-1}, coinciding with elevated N_2 fixation. High carbon fixation associated to the center of eddy A extended, however, deeper down to 400 m. High carbon fixation in the absence of light (compare chl a data in Stramma et al., 2013) is likely attributed to dark carbon fixation as previously described to take place in this and other OMZs (Schunck et al., 2013, Taylor et al., 2001). Similarly, eddy B showed high rates of N_2 fixation in its center with maxima of 1.89 nmol N L^{-1} d^{-1} at 350 m depth. In eddy B, the maximum in carbon fixation was exclusively present above the upper isopycnal, and thus not in direct contact with the N_2 fixation zone. However, a smaller peak could be observed at ~380 m depth. Maximum N_2 fixation rates in eddy C (0.51- 1.48 nmol N L^{-1} d^{-1}) were detected in surface waters (Table S1). Carbon fixation in eddy C was lower compared to eddy A and B, however also mostly present towards the rim, while close to the detection limit in the center.

Compared to previous studies in this area, N_2 fixation rates for eddies A and B are generally 1-2 orders of magnitude higher (e.g. Dekaezemacker et al. (2013): 0.01-0.88 nmol N L^{-1} d^{-1}; Löscher et al. (2014): 0.01- 0.4 nmol N L^{-1} d^{-1}); here, it must be noted that we used the improved method by Mohr et al. (2010) which allow us to present here the first quantitative rates of N_2 fixation in this area, while previous studies may have underestimated N_2 fixation rates (Grosskopf et al., 2012). An aging effect was mirrored by a decrease in N_2 and C fixation below 200m in the center of Eddy A when comparing the measurements from eddy A1 sampled in November 2012 with eddy A2 surveyed a month later in December 2012. C fixation rates increased towards the eddy edge. This may be attributed to biological consumption or export

of nutrients needed for biological activities within the eddy that are still available due to lower consumption or diffusion through the rim.

Observations of higher N_2 fixation rates in accordance with our dataset suggest an overall stimulation of N_2 fixation associated with anticyclonic mode water eddies (Fong et al., 2008). N_2 fixation rates of 8.6 nmol N L^{-1} d^{-1} have been measured in surface waters of an eddy in the oligotrophic North Pacific Ocean (Fong et al., 2008). N_2 fixation was only measured in surface water samples (5 m depth) in Fong et al. (2008), thus a direct comparison with N_2 fixation within the O_2-depleted eddy core waters, as measured in this study, is not possible.

The occurrence of enhanced N_2 fixation associated with intermediate water depths is in accordance with our previous study from that region, where we detected a variety of non-cyanobacterial diazotrophs compared to relatively minor numbers of cyanobacterial diazotrophs related to *Crocosphaera* (Löscher et al., 2014). In order to characterize the expression of the key functional marker gene for N_2 fixation, *nifH*, we conducted a phylogenetic study on *nifH* diversity in the transcript pool. Similar to the previous study, most of the detected *nifH* transcripts were affiliated to non-cyanobacterial diazotrophs (P1-P8) with some cyanobacterial *Crocosphaera*-related (UCYN-B) *nifH* sequences present at much lesser extent (Fig. 4, table S2). Quantification of *nifH* transcripts related to the detected clusters showed maximum abundances associated with the maxima in N_2 fixation in eddy A, B and C (Fig. 4). A potential for heterotrophic N_2 fixation was deducted from glucose fertilization experiments with water samples from the cores of eddies A and B. Here, glucose addition greatly enhanced N_2 fixation from 0.86 ± 0.1 nmol N L^{-1} d^{-1} to 39.19 ± 4.31 nmol N L^{-1} d^{-1} at 100 m depth in eddy A and from 0.251 ± 0.03 nmol N L^{-1} d^{-1} to 62.18 ± 1.9 nmol N L^{-1} d^{-1} at 125 m depth in eddy B, respectively. However, no increase in N_2 fixation by glucose addition could be achieved in eddy C (100 m, Fig. 5), which may result from different diazotrophic communities (i.e. cyanobacterial UCYN-B *nifH* sequences present). Therefore, the availability of reduced carbon compounds may essentially control N_2 fixation in modewater eddies. Assuming that organic matter export is limiting for N loss as previously suggested (Babbin et al., 2014;Ogawa et al., 2001;Bianchi et al., 2014) and that deep water N_2 fixation is a non-cyanobacterial (i.e., heterotrophic) process as shown by the diversity of the diazotrophs and the stimulation of N_2 fixation rates after glucose addition, the interplay between both may be even closer as previously thought.

3.3 Co-occurrence of N_2 fixation and N loss in mode water eddies

Largely consistent with the distribution of NO_2^-, biogenic N_2 showed pronounced maxima below the mixed layer depth in eddies A and B, and a less pronounced maximum in eddy C. A similar distribution has been determined for P* (Fig. 2). The consistency of those parameters indicates either ongoing N loss or its left-over signal as already reported for the upwelling off Peru (Kalvelage et al., 2013). In an earlier study from that region (i.e., Löscher et al., 2014), we found a close spatial coupling between N loss, or a

relic signal as suggested by Kalvelage et al. (2013), and N_2 fixation for the same upwelling region off Peru. The strongest signals for both N_2 fixation and N loss were tightly linked to a coastal sulphidic plume (Schunck et al., 2013). The westward propagation of mesoscale eddies implies that properties of the waters which were "trapped" within its center at the time of formation are transported offshore (Chelton et al., 2007). Enhanced N_2 and C fixation rates, as well as high N deficit, as depicted by P*, and biogenic N_2 concentrations in eddy B indeed suggest that this coupling can be transported far offshore.

A coupling between N loss and N_2 fixation is indicated by (i) the concurrent deepening of the maxima in N loss (i.e. maximum in biogenic N_2) and N_2 fixation from the coast to the open ocean, (ii) the concurrent decrease in both biogenic N_2 concentrations and N_2 fixation rates over time and (iii) the co-occurrence between NO_2^- (either resulting from NO_3^- reduction or from remineralization of organic matter through NH_4^+ oxidation) and N_2 fixation rates, and between biogenic N_2 and N_2 fixation rates (Fig. 2, 3). We observed lower biogenic N_2 signals and N_2 fixation in eddy B compared to eddy A1 with the maximum in N_2 fixation located deeper (~350 m) in the water column, compared to eddy A1 (~250 m). While we detected enhanced carbon fixation in eddy A at the same depth as N_2 fixation, this coupling was far less pronounced in eddy B. Still P* was lower in eddy B compared to eddy A, which points towards a normalization of N:P ratio *via* N_2 fixation.

Although, our results provide evidence for a coupling of N_2 fixation and N loss, statistical analysis of our dataset, however, did not confirm N loss as exclusive control on N_2 fixation, but displays a dependence of N_2 fixation on O_2 and temperature, as well (Fig. 6). The dependency on O_2 would explain the difference of N_2 fixation between the modewater eddies A and B, and the cyclonic eddy C, which was in its ODZ slightly less anoxic. Several studies suggested primary production to be limited by Fe availability in the upwelling system off Peru (Bruland et al., 2005;Hutchins and al., 2002;Messie and Chavez, 2015). Baker et al. (2015) report in their study from the same cruise series excess Fe supply (with respect to N supply) via atmospheric deposition to the southern part of this region (~15-16°S). As previous studies identified iron (Fe) to generally (co-)limit N_2 fixation (Mills et al., 2004;Moore and Doney, 2007;Moore et al., 2009), atmospheric Fe sources may promote surface water N_2 fixation, which may explain enhanced N_2 fixation in surface waters of the eddies. Other studies emphasize the comparably higher importance of the benthic Fe source in these waters (Chever et al., 2015;Scholz et al., 2014), which may be particularly important in eddies A and B due to their longer residence time at the coast. Enhanced N_2 fixation in the mode-water eddies A and B may, besides a coupling to N loss, be additionally promoted by Fe availability possibly from benthic sources.

4. Conclusions

We conducted the first detailed survey of N_2 fixation in three eddies off the coast of Peru in the ETSP. Our results demonstrated enhanced N_2 fixation rates connected to two anticyclonic mode water eddies off Peru, while N_2 fixation was not increased in the cyclonic eddy. N_2 fixation rates were highest in the ODZ of the two anticyclonic mode water eddies. This is in agreement with recent findings, which demonstrated that N_2 fixation is not only present in oligotrophic surface waters but also widely distributed throughout the water column. N_2 fixation co-occurred with N loss processes, which in combination with low O_2 concentrations may largely explain the presence of N_2 fixation in ODZ waters. Taken together, our results point towards an important role for eddies in supplying fixed N compounds to the open ocean via enhanced N_2 fixation. Although our data do not allow quantification of the overall impact of eddies on N_2 fixation in the ETSP off Peru, they clearly underscore the importance of high-resolution surveys for understanding the biogeochemistry of N cycle processes in eddies.

Acknowledgements

We thank the Peruvian authorities for the permission to work in their territorial waters. We further thank the captains, crews and chief scientists of R/V Meteor during the M90 and M91 cruises. We acknowledge the technical assistance of T. Baustian, V. Len, V. Lohmann, N. Martogli, G. Krahmann, K. Nachtigall, M. Philippi, H. Schunck and J. Larkum. We further thank L. Stramma, D. Arevalo-Martinez, S. Thomsen, C. Callbeck and J. Karstensen for helpful discussion of the results. The cruise M91 was funded by the BMBF project SOPRAN with grant # FKZ 03F0662A. This study is a contribution of the DFG-supported collaborative research center SFB754 (http://www.sfb754.de) and was supported by NSF grants OCE 0851092 and OCE 115474 to M.A.A. and a NSERC Postdoctoral Fellowship to A. B..

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Tables

Table 1. Vertically integrated biogeochemical parameters of the three eddies in the ETSP during the M90 and M91 cruises. N_2 and C fixation rates as well as O_2; concentrations are expressed as integrated concentrations/abundances over the upper 500 m of the water column (data taken from the eddy centers). Chl *a* concentrations are taken from Stramma et al. (2013) and represent maximum concentrations in the subsurface maximum.

	A (M90)	A (M91)	B (M90)	C (M90)
N_2 fixation (µmol N m^{-2} d^{-1})	628.7	490.8	245.0	150.6
C fixation (mmol C m^{-2} d^{-1})	64.4	3.9	42.8	6.7
O_2 (mol m^{-2})	37.5	27.6	37.7	45.2
chl *a* max. (µg L^{-1})	6.1	2.5	2.5	2.8

Figures

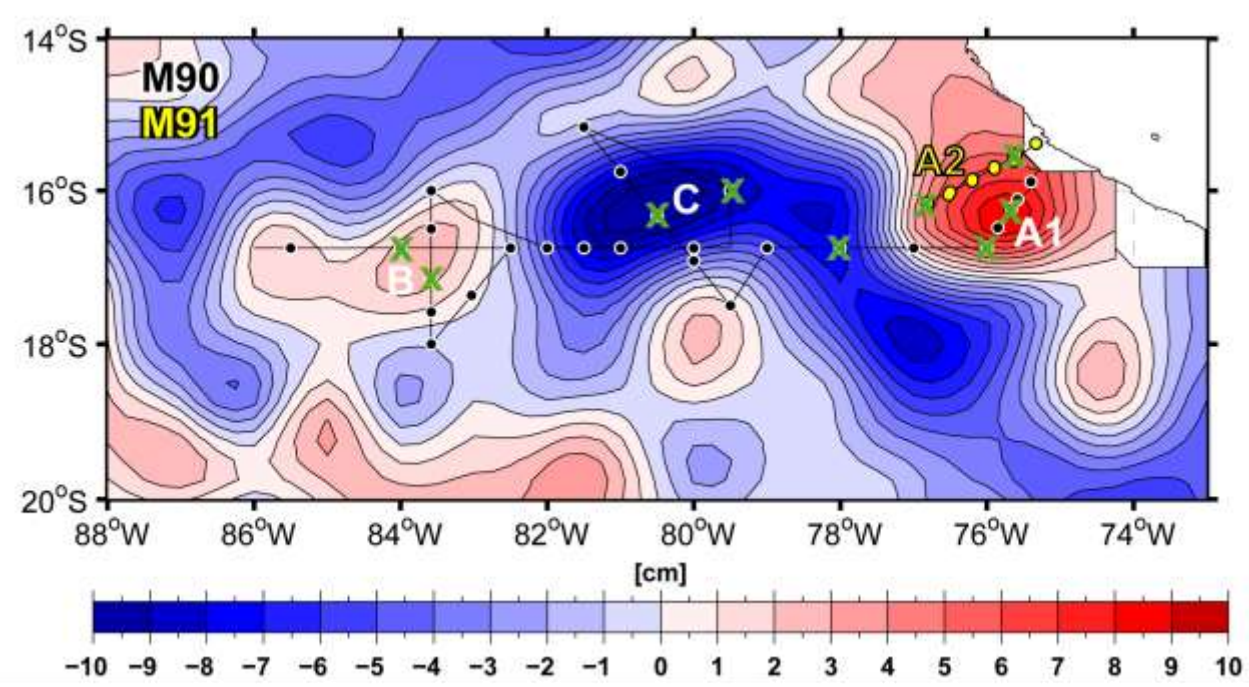

Figure 1: Distribution of Aviso satellite-derived sea surface height anomaly (SSHA) distribution as described by Stramma et al. (2013) on 21 November 2012. Eddies are labelled in white (A and B denote the coastal and the open ocean mode water eddies, respectively; C denotes the cyclonic eddy). The cruise track from the M90 cruise is shown in black, CTD-bottle stations are indicated with black dots, green

crosses denote stations sampled for N_2 fixation. The cross section through the aged coastal mode water eddy during the consecutive cruise M91 is denoted with yellow dots (A2).

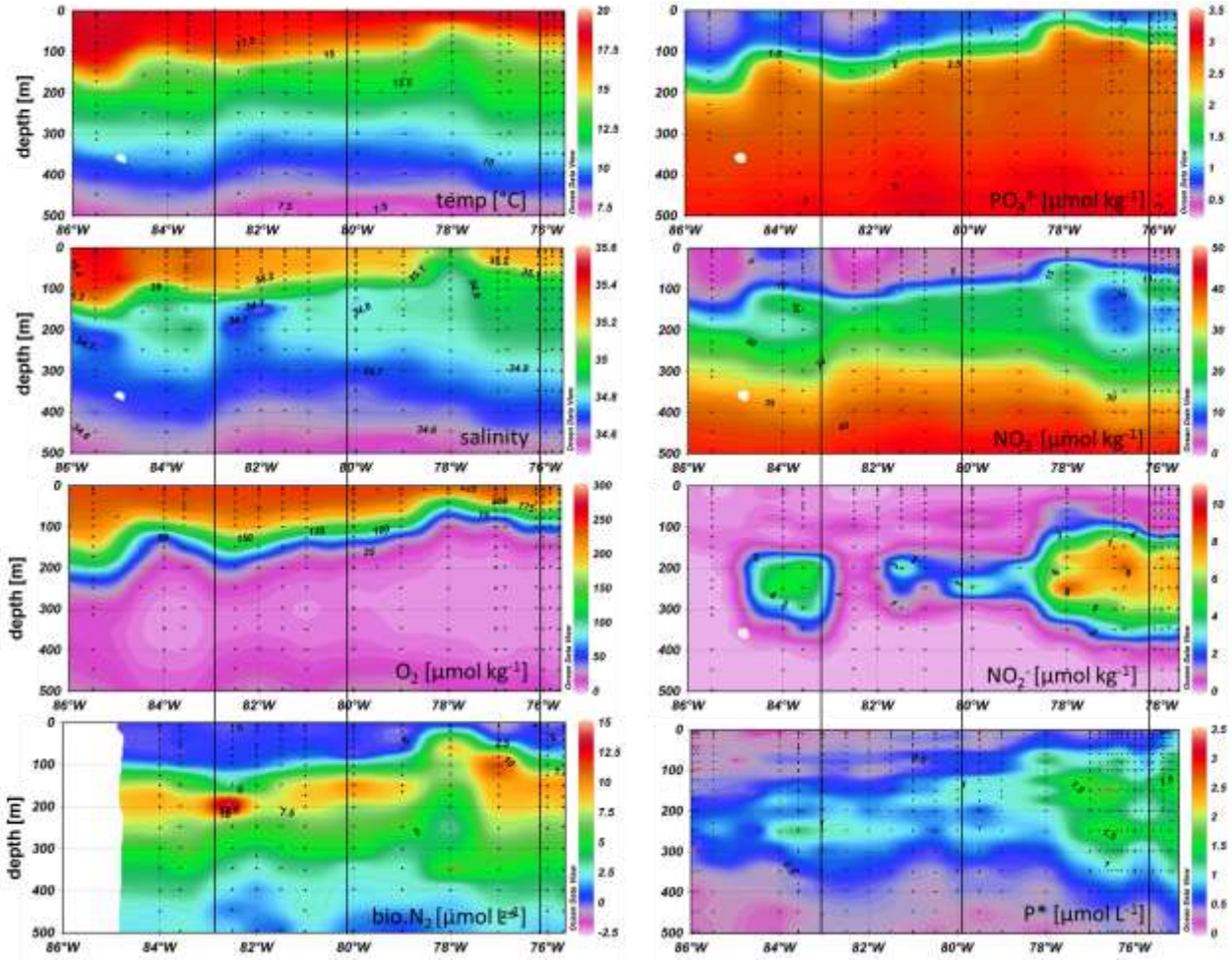

Figure 2: Temperature, salinity and oxygen, phosphate, nitrate, nitrite, biogenic N_2 and P* for eddies A, B and C along a cross section at 16°45'S during the M90 cruise are shown. The black lines indicate the eddy centers at ~76°W (eddy A), ~80.1 °W (eddy C) and ~83.3°W (eddy B).

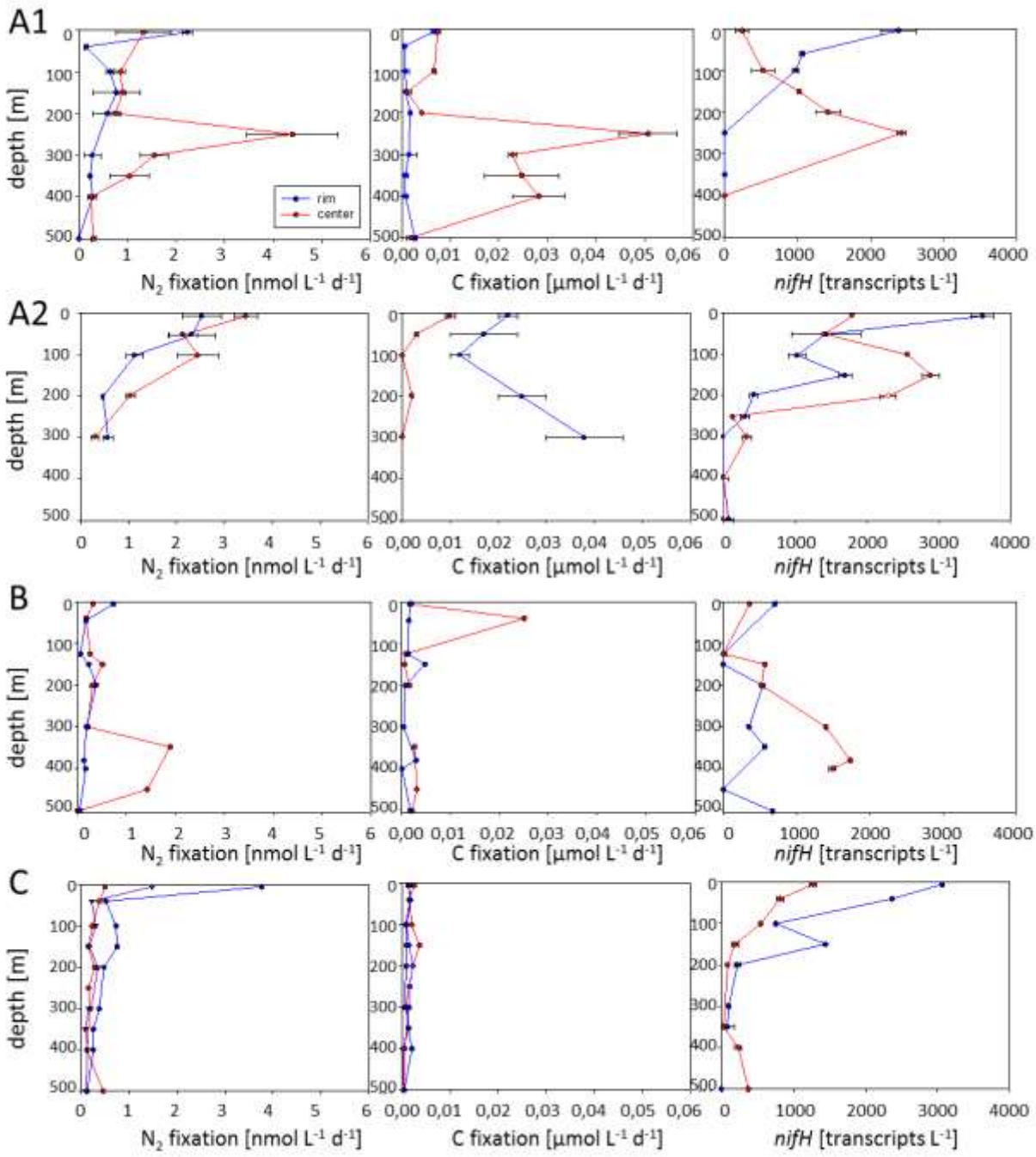

Figure 3: Vertical distributions of N_2 and C fixation rates and *nifH* transcript abundance (sum of detected clusters P2, P7 and *Crocosphaera*-like diazotrophs as quantified by qPCR) in eddy A1 (M90), eddy A2 (M91), eddy B and eddy C.

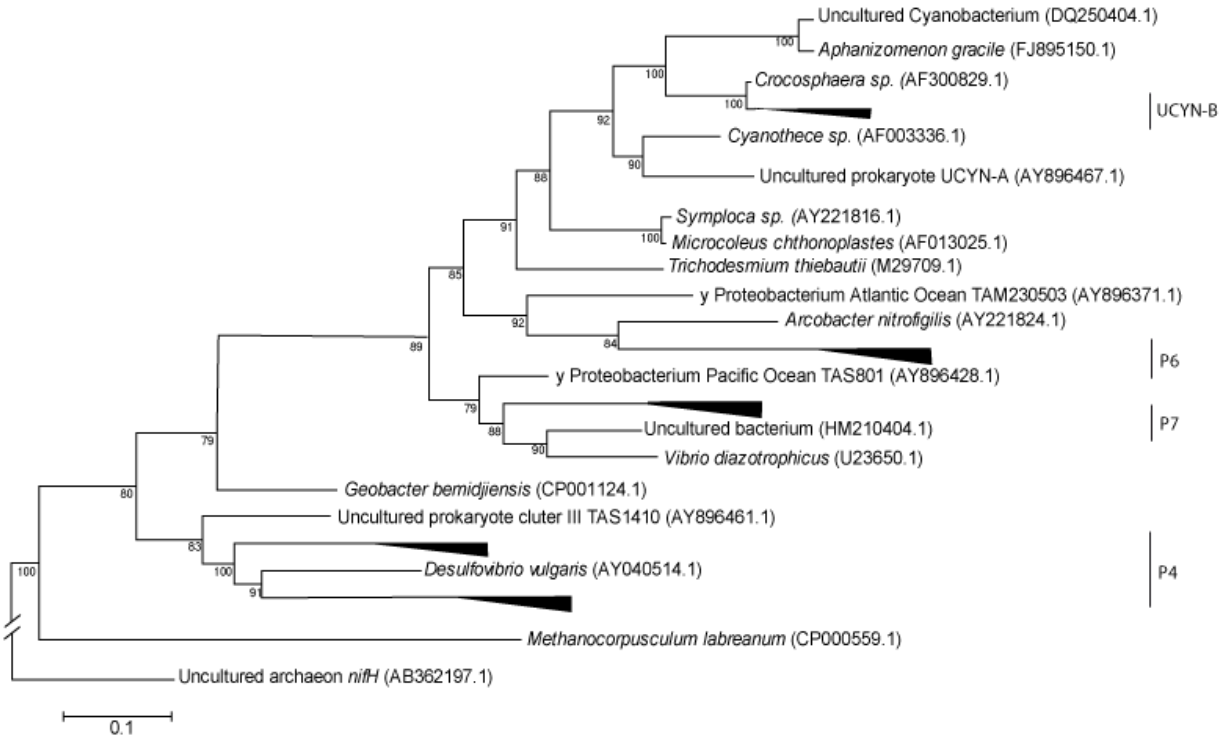

Figure 4: Phylogenetic diversity in *nifH* cDNA libraries, black triangles denote detected clusters present in samples from M90 and M91. The tree was constructed from a ClustalW alignment as a neighbor joining tree, bootstrap values are given (% of 1000 bootstraps) below branches, P4, P6 and P7 are clusters previously identified in that region (Löscher et al., 2014).

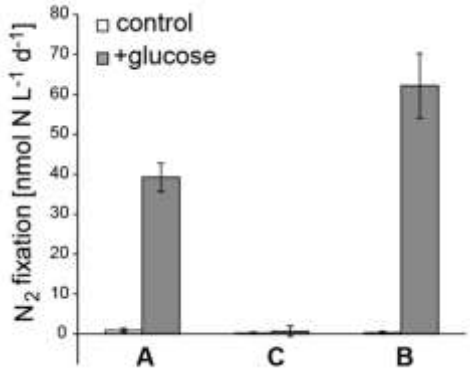

Figure 5: N_2 fixation in response to glucose fertilization experiment performed in eddy A1 (100 m), B (125 m) and C (100 m); samples were derived from the eddy center stations water depth.

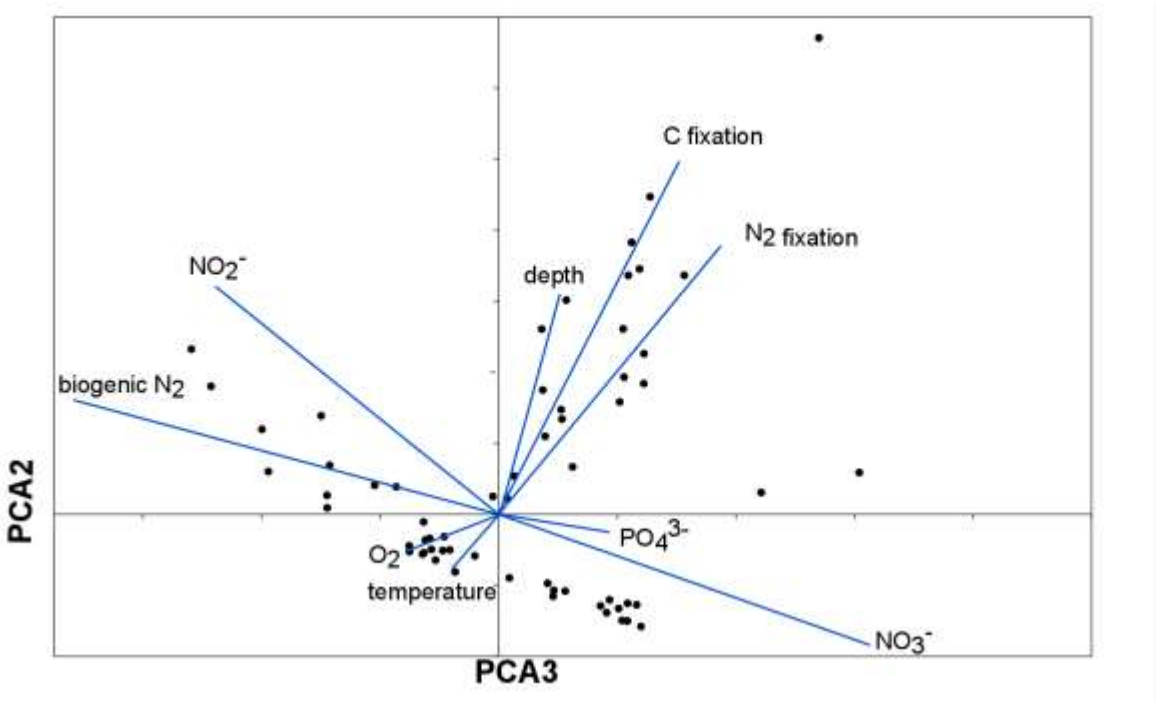

Figure 6: Principal component analysis correlation biplot shows relations between N_2 fixation and environmental variables. Strongest negative correlations are present between N_2 fixation and O_2 and N_2 fixation and temperature. N_2 fixation is positively correlated with C fixation as indicated by the direction of vectors. Black dots denote single samples (n=58).