Sensitivity of the biological oceanic nitrogen cycle to changes in dissolved oxygen

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Content

Summary

- The largest source of nitrogen (N) is the atmospheric pool of molecular nitrogen (N_2), which is exclusively available to the ocean via biological N_2-fixation. Marine N_2-fixation has classically been ascribed to cyanobacteria such as *Trichodesmium* or *Crocosphaera* present at the sea surface and only to a minor part to non-phototrophic diazotrophs. In contrast to this classical view, we demonstrated the presence of 8 novel non-cyanobacterial clusters (P1-P8) by detecting the key functional marker gene for N_2-fixation *nifH*, in oxygen minimum zone (OMZ) waters off Peru. Those clusters were present in high abundances up to 10^5 *nifH* copies per L detected and quantified by cluster specific TaqMan- qPCR from surface waters down to > 4000 m and are distributed in specific patterns along vertical and horizontal gradients. The only cyanobacterial diazotroph detected in the OMZ off Peru was *Crocosphaera*, however, abundances were comparatively low (~ 10^2 *nifH* copies L^{-1}).
- Clusters P1 and P4 were dominating the diazotrophic community from the shelf to about 83°W at 10°S and to 77°W at 16°S. Thus, those clusters are considered highly important for N_2-fixation in this area. While the deep branching P1 cluster, which is closest related to spirochaeta, appears associated with deeper waters (100- 300 m) and thus lower oxygen (O_2) conditions, clusters P2, P3 and P4, were rather present in surface to sub-surface waters. Hence, we hypothesize that those newly identified clusters occupy different niches within the OMZ.
- Cluster P8 was present throughout the Peruvian OMZ with highest abundances up to10^6 *nifH* copies L^{-1} in the nutrient depleted open ocean region along a north-south transect dominating the respective diazotrophic community. Completely unexpected, this cluster was present down to > 4000 m depth; however, no cut-off with regard to depth has been defined.
- These most abundant clusters P1, P4 and P8 were detected at comparatively high nitrate (NO_3^-) concentrations up to ~40 µM, and moreover a significant correlation of P1 and nitrite (NO_2^-) has been determined. The abundant presence of those diazotrophs combined with N_2-fixation activity at high concentrations of those reactive N-compounds challenges the classical view that the habitats of marine diazotrophs are limited to N-depleted waters as those diazotrophs do not seem to be considerably inhibited by the present reactive N species (NO_3^-, NO_2^-,). Thus, we hypothesize that marine diazotrophic niches need to be extended towards low O_2/ high NO_3^- environments.
- The detection of active N_2-fixation in the presence of novel *nifH* clusters along 10°S in the OMZ off Peru points towards a key role of those novel clusters in re-generating the N-loss by denitrification or anaerobic ammonia oxidation (anammox).
- Moreover, some of those newly identified clusters were significantly triggered by glucose addition in incubation experiments; thus, we conclude that some of those diazotrophs are

heterotrophs. We interpret this as an important fact particularly with regard to ongoing eutrophication of the ocean.

- We demonstrated the co-occurrence of *nifH* and active N_2-fixation with key functional genes of nitrification, denitrification and anammox, pointing towards a close spatial coupling of N-input and N-loss processes in the OMZ off Peru, and most likely in other OMZs as well. The linear correlation between cluster P1 and nitrite and the key functional marker gene for anammox, *hzo*, has been detected in large parts of the OMZ off Peru.
- In addition, transient extreme anoxia as detected in coastal waters off Peru at a spontaneous sulphidic event (up to 3.5µM S^{2-} present in the water column) were demonstrated to trigger N_2-fixation massively over short time periods. At this S^{2-} event, we measured vertically integrated water column N_2-fixation rates of 840 µmol N d^{-1} m^{-2}, comparable to rates reported from areas dominated by major *Trichodesmium* blooms. Although the occurrence of anoxic events is temporally and locally limited, our results suggest that they contribute significantly to the N-input into the marine system. However, this term is difficult to quantify, it might provide an additional explanation for the deficit in fixed N in the ocean determined by model studies.
- Thus, we conclude that both findings, the presence of N_2-fixation in OMZ waters also below the photic zone and most likely even in deeper waters, as well as significant N_2-fixation during transient anoxic events consequently require an upward revision of the oceanic fixed N budget.
- The formation of oceanic nitrous oxide (N_2O) via nitrification has previously been exclusively ascribed to ammonia-oxidizing β- and *y*-proteobacteria (AOB). Our results state the first experimental evidence for oceanic N_2O production by ammonia-oxidizing archaea (AOA) in OMZs based on both field studies as well as pure culture experiments using the only cultivable archaeal ammonia-oxidizer *Nitrosopumilus maritimus* SCM1.
- We demonstrated that archaeal ammonia monooxygenase genes (*amoA)*, the key functional genes for ammonia oxidation, were abundant throughout the water column of the eastern tropical North Atlantic (ETNA) and large parts of the eastern tropical South Pacific Oceans. The maxima in abundance and expression of archaeal *amoA* genes (1.9×10^5 and 6×10^4 *amoA* copies mL^{-1}, respectively) correlated with the maximum in N_2O concentration and the oxygen minimum, most pronounced in the ETNA, whereas the abundances of bacterial *amoA* genes were negligible in both areas.
- The selective inhibition of archaea by *N*1-guanyl-1,7-diaminoheptane (GC7), specifically inhibiting the archaeal cell cycle in seawater incubations from the OMZ in the ETNA decreased the N_2O production in two experiments significantly. This finding points towards a major contribution of archaea to N_2O production, in this area.
- We further showed that archaea are able to produce N_2O in pure cultures as exemplarily demonstrated for the archaeal ammonia-oxidizer *Nitrosopumilus maritimus* SCM1. A

significant enhancement of N_2O production by *N. maritimus* at low oxygen concentrations as present in the oxycline off Mauritania (50- 100 µM) has been detected. This finding explains the accumulation of N_2O in the OMZ in the ETNA and in large parts of the OMZ off Peru. N_2O production from two AOB cultures (*Nitrosococcus oceani, Nitrosomonas marina*) was comparably low under similar conditions: While N_2O yields from NH_4^+ oxidation ranged from 0.002%-0.03% in *N. maritimus* cultures; AOB cultures produced 0.001%-0.006% N_2O during NH_4^+ oxidation.

- Combining our findings with the results of Kalvelage *et al.* (Kalvelage *et al.*, unpublished), which show by isotope pairing studies that aerobic ammonia oxidation takes place at much lower oxygen concentrations as previously assumed (down to 1 µM), we propose, that marine N_2O is largely produced by archaeal nitrification. Particularly under suboxic to hypoxic conditions, at which N_2O production was previously ascribed to denitrification, nitrification might contribute to a larger extent, as it has classically been suggested. This changes the classical view of N_2O production significantly. In the context of climate change and thus the ongoing deoxygenation of OMZs and increasing hypoxia of the world's oceans, these results are highly alerting.
- In summary, our observations demonstrated the overall importance of OMZs as hotspots of N-turnover processes. The presence of a newly identified source of N_2-fixation in OMZ waters performed by clusters, among which some are heterotrophs, has been demonstrated. Moreover, ammonia-oxidizing archaea were demonstrated to produce N_2O in the ocean and in pure cultures; a pronounced sensitivity towards dissolved oxygen concentrations in archaeal cultures was determined thus possibly explaining the high amounts of N_2O present in OMZs. The present low O_2 concentrations provide a niche, where apparently N-loss and N-input via N_2-fixation are tightly coupled. Thus, we conclude that understanding future changes occurring in these relatively small areas, compared to the global ocean, is crucial to estimate the marine N-budget.

Zusammenfassung

- Die größte globale Stickstoff (N)-Quelle ist die Atmosphäre, die zu 78% aus molekularem Stickstoff (N_2) besteht. Dieser molekulare Stickstoff ist dem Ozean ausschließlich über biologische N_2-Fixierung zugänglich. Bisher wurde die ozeanische N_2-Fixierung vorwiegend Cyanobakterien, die vor allem an der Meeresoberfläche vorkommen, so wie *Trichodesmium* und *Crocosphaera*, zugeschrieben. Der Beitrag von nicht- phototrophen Organismen in tieferen Gewässern zur ozeanischen N_2-Fixierung wurde als eher gering eingeschätzt. Dem entgegen wurden in dieser Studie in der Sauerstoffminimumzone (SMZ) vor Peru, auch in Gewässern unterhalb der euphotischen Zone, acht bisher unbekannte Cluster von N_2-Fixierern über das *nifH*-Gen, dass allgemein als funktioneller Marker für N_2-Fixierung benutzt wird, nachgewiesen. Diese Cluster wurden in hoher Abundanz bis zu 10^5 *nifH* Kopien pro L (mit Cluster-spezifischer TaqMan qPCR quantifiziert) detektiert. Sie waren horizontal und vertikal in der Wassersäule von der Oberfläche bis zu einer Tiefe von > 4000 m in spezifischen Mustern verteilt. Die einzige cyanobakterielle Sequenz, die in der SMZ vor Peru gefunden wurde, ist phylogenetisch mit *Crocosphaera* assoziiert, kommt aber in vergleichsweise geringer Abundanz vor (~ 10^2 Kopien L^{-1}).
- Cluster P1 und P4 dominierten unter den Diazotrophen vom Schelf bis ca. 83°W bei 10°S und bis zu 77°W bei 16°S. Daher wurden diese Cluster als wichtig in Hinblick auf die N_2-Fixierung erachtet. Das P1 Cluster, das sich phylogenetisch stark von den anderen detektierten Sequenzen unterscheidet, jedoch die größte Ähnlichkeit zu Spirochaeten zeigt, scheint vor allem in tieferen Wasserschichten (100 – 300 m) und daher auch bei niedrigeren O_2 Bedingungen vorzukommen. Dagegen kommen die Cluster P2, P3 und P4 eher in der Wasseroberfläche oder oberflächennahen Wasserschichten vor. Diese spezifischen Verteilungsmuster legen den Schluss nahe, dass diese neuen Cluster unterschiedliche Nischen in der SMZ einnehmen.
- Cluster P8 wurde in der gesamten SMZ vor Peru detektiert. Höchste Abundanzen von bis zu 10^6 *nifH* Kopien L^{-1} wurden entlang eines Nord-Süd Transekts im Nährstoff-verarmten offenen Ozean gefunden. Hier dominierte P8 und wurde völlig unerwartet bis zu einer Tiefe von > 4000 m gefunden.
- Die höchst abundanten Cluster P1, P4 und P8 und aktive N_2-Fixierung wurden zudem in Wassertiefen mit hohem Nitratgehalt bis zu ~ 40 µM NO_3^- detektiert, darüber hinaus wurde eine signifikante Korrelation zwischen P1 und Nitrit (NO_2^-) nachgewiesen. Dies lässt darauf schließen, dass bei diesen Organismen die N_2-Fixierung nicht durch die vorhandenen reaktiven N-Spezies (NO_3^-, NO_2^-) gehemmt wird. Dieser Befund bricht damit klassische Paradigmen der N_2-Fixierung; die ökologischen Nischen von N_2-Fixierern müssen somit neu definiert werden, wobei SMZ mit hohem Nitrat-Gehalt mit einbezogen werden müssen.

- Darüber hinaus wurde in Inkubationsexperimenten eine signifikante Verstärkung der N_2-Fixierung durch Glukosezugabe bei einigen dieser Cluster beobachtet, woraus wir schließen, dass diese Organismen heterotroph (im Gegensatz zu den vorher beschriebenen phototrophen N_2-Fixierern) sind. Im Hinblick auf die fortschreitende Eutrophierung der Ozeane wird dieses Ergebnis als ausgesprochen wichtig eingeschätzt.
- Zudem wurde das gleichzeitige Vorkommen des *nifH*-Gens und aktiver N_2-Fixierung zusammen mit Schlüsselgenen der Nitrifizierung, Denitrifizierung und Anammox in unseren Studien nachgewiesen, was auf eine enge räumliche Kopplung dieser Prozesse in der SMZ vor Peru und höchst wahrscheinlich auch in anderen ozeanischen SMZ schließen lässt. Eine lineare Abhängigkeit speziell des Clusters P1 von Nitrit und darüber hinaus von *hzo*, dem funktionellen Markergen für Anammox, wurde in großen Teilen der SMZ vor Peru nachgewiesen, was die Hypothese einer räumlichen Kopplung dieser Prozesse unterstützt.
- Zusätzlich zur N_2-Fixierung in tiefen Gewässern, könnten kurzlebige Anoxien, wie die, in Küstengewässern der SMZ vor Peru detektierte sulfidische (S^{2-}) Anoxie von Bedeutung für die N_2-Fixierung sein. Hier wurde über einen kurzen Zeitraum hinweg eine massive Verstärkung der N_2-Fixierung gemessen mit Fixierungsraten von 840 μmol N d^{-1} m^{-2}, vergleichbar mit Raten, die in Gewässern mit großen *Trichodesmium*-Blüten gemessen wurden. Trotz schwieriger Quantifizierbarkeit könnten diese Ereignisse eine zusätzliche Erklärung für das durch mathematische Modelle bestimmte ozeanische N-Defizit sein.
- Zusammenfassend erfordern sowohl der Nachweis der N_2-Fixierung unterhalb der euphotischen Zone, als auch die signifikante Verstärkung der Fixierungsraten an kurzlebigen anoxisch-sulfidischen Ereignissen eine dringliche Korrektur der N_2-Fixierung in mathematischen Modellen des ozeanischen N-Budgets nach oben hin.
- Die Bildung von Lachgas (N_2O) durch Nitrifizierung wurde früher ausschließlich den β- and *γ*-Proteobakterien zugeschrieben. Unsere Studien zeigen zum ersten Mal experimentell, dass die ozeanische N_2O-Bildung tatsächlich auf Archaeen zurückgehen könnte. Diese Erkenntnis basiert auf Feldstudien und Experimenten mit einer Reinkultur von *Nitrosopumilus maritimus* SCM1, dem einzigen kultivierbaren archaeellen Ammonium-Oxidierer.
- Wir konnten archaeelle Ammonium-Monooxygenasegene (*amoA*) in der ganzen Wassersäule im tropischen Nord-Ost Atlantik und in großen Teilen des tropischen Süd-Ost Pazifiks nachweisen. Maximale Abundanzen und die höchste Genexpression des archaeellen *amoA* Gens (je 1.9 x 10^5 und 6 x 10^4 Kopien mL^{-1}) korrelierten mit maximalen N_2O Konzentrationen und dem O_2 Minimum; diese Korrelation war am stärksten im tropischen Nord-Ost Atlantik nachweisbar. Entgegen hoher archaeeller *amoA* Abundanzen waren bakterielle *amoA* Gene in beiden Untersuchungsgebieten kaum detektierbar.
- Die selektive Hemmung des archaeellen Zellzyklus durch *N*1-guanyl-1,7-diaminoheptane (GC7) in Seewasserinkubationen mit Seewasserproben aus der SMZ des tropischen Nord-Ost Atlantiks, zeigte eine signifikante Verringerung der N_2O Produktion in zwei Experimenten.

Dies wird als starker Hinweis auf eine N_2O Produktion durch Archaeen in diesem Gebiet gedeutet.

- Unsere Studien zeigten darüber hinaus eine signifikante Verstärkung der N_2O-Bildung in Archaeen bei niedrigeren Sauerstoffkonzentrationen, wie sie in der Oxykline der SMZ vor Mauretanien vorkommen, was eine Erklärung für die Akkumulation von N_2O im tropischen Nord-Ost Atlantik und in großen Teilen der SMZ vor Peru bietet. Die N_2O Produktion durch zwei AOB Kulturen (*Nitrosococcus oceani, Nitrosomonas marina*) war vergleichsweise gering: Während in *N. maritimus* Kulturen 0.002%-0.03% N_2O aus durch Oxidation aus NH_4^+ gewonnen wurde, produzierten die AOB Kulturen nur 0.001%-0.006% N_2O bei der NH_4^+ Oxidation.
- Zieht man nun die Ergebnisse von Kalvelage *et al.* (Kalvelage *et al.*, unpublished) hinzu, die durch Isotopenstudien zeigen, dass Ammonium-Oxidation bei deutlich niedrigeren Sauerstoffkonzentrationen, als bisher vermutet, stattfinden kann (bis hin zu 1µM O_2), schlagen wir vor, dass archaeelle Ammonium-Oxidation für einen Großteil der ozeanischen N_2O Produktion auch unter suboxischen und hypoxischen Bedingungen verantwortlich ist, was die klassische Betrachtungsweise der ozeanischen N_2O Produktion durch Nitrifizierung stark verändert und ihr eine wichtigere Rolle im Vergleich zur Denitrifizierung zuschreibt. Im Hinblick auf den Klimawandel, der einhergeht mit der fortschreitenden Sauerstoffverarmung und Ausdehnung ozeanischer SMZ, die unserer Studie zur Folge die Produktion des Treibhausgases N_2O stark fördern, sind unsere Ergebnisse alarmierend.
- Zusammenfassend zeigt diese Studie die große Bedeutung der SMZ für verschiedene Prozesse im Stickstoffkreislauf. Neue Cluster von Stickstofffixierern in SMZ Gewässern wurden identifiziert, unter denen auch einige heterotrophe Cluster sind, und die sich von allen bisher bekannten Clustern stark unterscheiden. Die N_2-Fixierung in SMZ könnte maßgebend die Berechnungen des ozeanischen N-Budgets verändern, die bisher nur oberflächennahe N_2-Fixierung berücksichtigt. Darüber hinaus wurde N_2O Produktion durch ammonium-oxidierenden Archaeen in Kultur und in Umweltproben nachgewiesen, die stake Empfindlichkeit gegenüber Sauerstoff wurde gezeigt, die eine Erklärung dafür bietet, warum N_2O in maximalen Konzentrationen in und in der Nähe von SMZ vorhanden ist. Die niedrigen Sauerstoffkonzentrationen formen eine einzigartige ökologische Nische, in der N-verbrauch und N–eintrag (durch N_2-Fixierung) eng gekoppelt vorkommen. Daher ist es unabdingbar, diese Systeme zu verstehen, um zukünftige Veränderungen im Hinblick auf den marinen Stickstoffkreislauf in diesen, im Verhältnis zum globalen Ozean vergleichsweise kleinen Arealen, vorher zu sagen.

Introduction

Oxygen minimum zones in tropical ocean areas

Oxygen minimum zones (OMZs) are ocean areas with intermediate low oxygen layers (Fig.1) of various ranges, e.g. hypoxic in the tropical Atlantic and suboxic in the Indian ocean and eastern tropical Pacific ((Stramma et al. 2008; Stramma et al. 2010), Fig. 2). The total volume of OMZ in the ocean is according to current estimates ~40 x 10^{15} m^3, when considering oxygen (O_2) concentrations < 90 µM, the volume of suboxic ocean areas (O_2 < 4.5 µM) accounts for approximately 0.5 x 10^{15} m^3. Global warming leads to increased stratification of the upper ocean which in turn may cause a decrease of dissolved O_2 in tropical ocean areas with unknown impacts on marine biogeochemical processes such as the marine nitrogen (N) cycle (Bange et al. 2005; Codispoti 2010; Stramma et al. 2010).

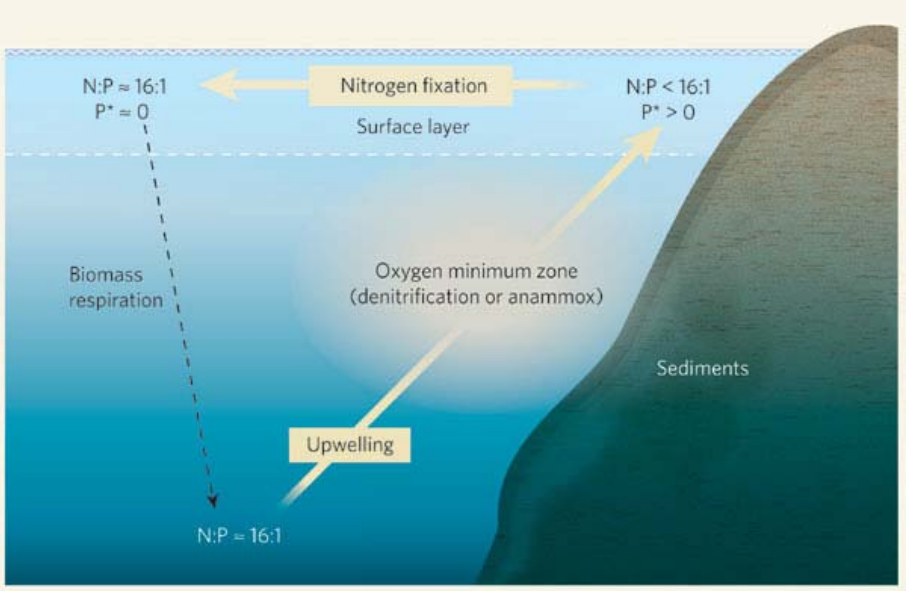


Fig. 1: Example of an oxygen minimum zone at an eastern boundary upwelling system with the classical vertical distribution of key processes involved in the marine N-cycle modified from Capone and Knapp, 2007: N-regeneration and -loss processes are assumed taking place within the OMZ, recycling the available nitrogen, this results in an nitrogen-to-phosphorus ratio is less than 16:1 in upwelling waters in contact with OMZs (excess P^* (P^* > 0)). Nitrogen fixation is assumed balancing the N:P ratio towards the open ocean (Capone and Knapp 2007).

The distribution of dissolved O_2 in the oceanic water body is strongly influenced and controlled by a combination of physical, chemical and biological processes all of which react to an unknown extent to global warming (Bopp et al. 2002; Karstensen et al. 2008; Stramma et al. 2008; Stramma et al. 2010). This results in an expansion and ongoing deoxygenation of OMZs as predicted by model studies

(Bopp et al. 2002) and by the statistical analysis of a 50 years time series (Stramma et al. 2008). Two examples of OMZs of different intensities are represented by the eastern tropical North Atlantic (ETNA) and the eastern tropical South Pacific (ETSP) Oceans. O_2 concentrations in the ETNA are commonly above 40 µmol L^{-1}, however, the very recent detection of a subsurface eddy near the islands of Cape Verde, nearly fully depleted in O_2 demonstrated that also in the ETNA O_2 concentrations can drop dramatically on a local scale (Brandt, Karstensen, unpublished). In the ETSP, a large and persistent OMZ with O_2 concentrations below the detection limit of conventional analytical methods (~2 µmol L^{-1}) is present off Peru and Chile (Fig. 2); with intermediate waters between ~75 and 600 m strongly depleted in O_2 (Stramma et al. 2008).

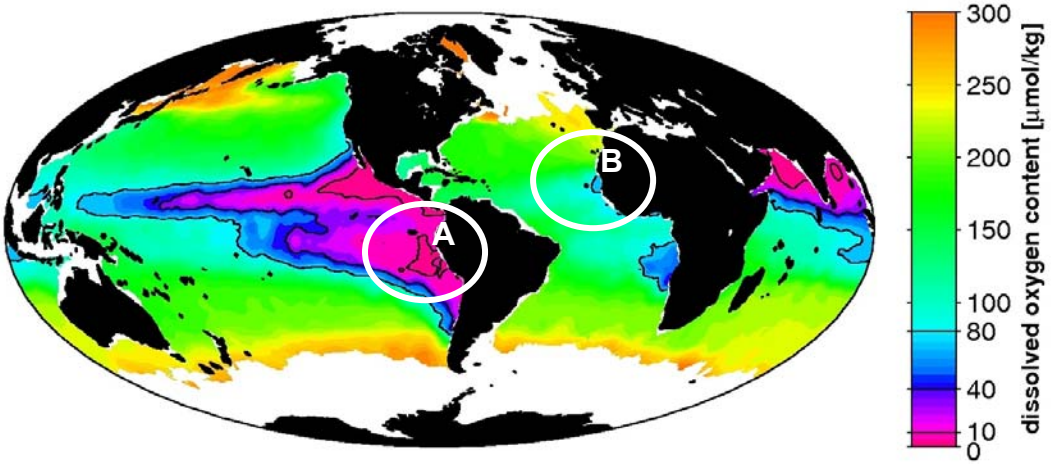


Fig. 2: Global distribution of O_2 at 400 m depth modified from Karstensen (unpublished): The major regions of low oxygen in the world ocean are all located in the tropical oceans, at shallow to intermediate depths. The area off Peru represents one of the most pronounced OMZs (Karstensen, unpublished). The investigated areas in (A) the eastern tropical South Pacific and (B) the eastern tropical North Atlantic Oceans are marked with circles.

Despite their relatively low contribution to the overall ocean volume (Codispoti 2010) the occurrence of hypoxic and anoxic zones is of particular importance with regard to the O_2 sensitivity of oceanic nutrient budgets, particularly with regard to the N-budget. Thus, the future development of the marine oceanic N-cycle will essentially depend on the extent of changing O_2 conditions in OMZs as relatively small key areas accounting for ~ 0.1 % of the oceans' volume (Capone 2008; Codispoti 2010).

Associated with wind-driven upwelling regions (Capone 2008), OMZ waters are fuelled with remineralized nutrients thus favouring N-turnover processes such as nitrification, anammox and denitrification. Current estimates ascribe 30–50% of the global N-loss to OMZs (Emery et al. 1955; Codispoti et al. 2001; Gruber 2004). Moreover, N-loss due to nitrate (NO_3^-) consumption by anammox and denitrification is supposed to result in low N/P ratios (leading to low N* values; i.e. excess nitrogen; (Fig. 3 (Deutsch et al. 2007)) which are proposed to promote N_2-fixation.

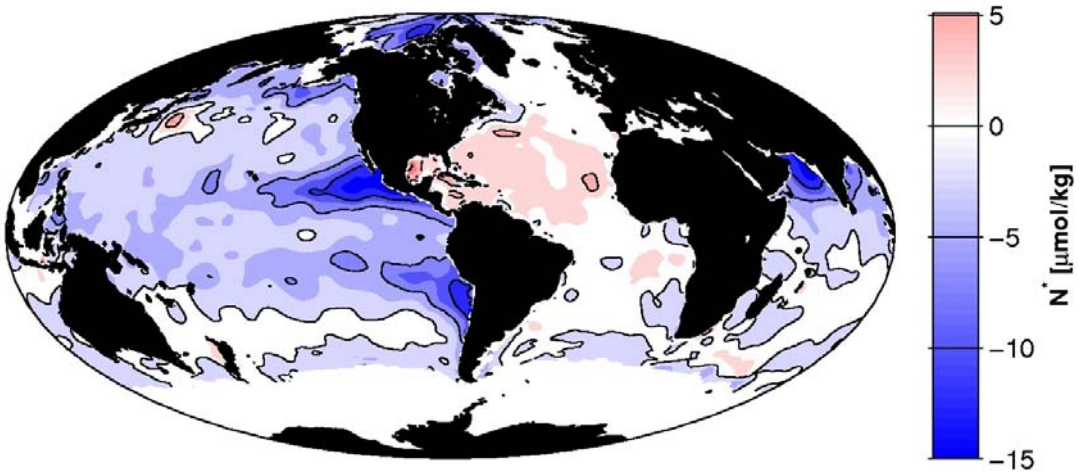


Fig. 3: Global distribution of N* at 400 m depth modified from Karstensen, unpublished:
N* is calculated according to: N* = (NO_3^- -16 PO_4^{3-} + 2.9) x 0.87 (µmol/kg) on the 26.6 isopycnal surface. The area off Peru is strongly depleted in reactive nitrogen, whereas the tropical Atlantic region is rather balanced with regard to the Redfiled ratio (N/P = 16/1).

Therefore, it has been suggested by model studies that N-loss and N-input via N_2-fixation are spatially linked in OMZs (Deutsch et al. 2007). However, direct evidence for such a linkage of N-loss and N_2-fixation in the ocean has been missing, so far, and the extent of a possible co-occurrence remains to be unravelled. Moreover, OMZs are predicted hotspots for the production of the climate relevant trace gas nitrous oxide (N_2O) by promoting biological formation via nitrification and denitrification at suboxic to anoxic conditions (Codispoti 2010; Naqvi et al. 2010).

The marine N- cycle

Nitrogen is an essential nutrient and a fundamental component of living organisms in general (Galloway 1996; Gruber and Sarmiento 1997; Gruber 2004). However, the atmospheric pool of dinitrogen (N_2) is only available to the ocean via biological N_2-fixation, which is restricted to a limited group of prokaryotes called diazotrophs (Capone 2008). During N_2-fixation, N_2 gas is reduced to ammonia; this energy consuming reaction is catalyzed by the O_2-sensitive enzyme nitrogenase (Fig. 4) and is performed as follows:

$$N_2 + 8H^+ + 16\ ATP + 8e^- \rightarrow 2\ NH_3 + H_2 + 16\ ADP + 16\ P_i$$

In environmental molecular studies used the *nifH* gene, encoding for the nitrogenase reductase enzyme (Fig. 4), as the functional genetic marker for N_2-fixation (Zehr et al. 1998).

The availability of fixed (i. e. bio-available inorganic) N is a limiting factor for oceanic primary productivity on a global scale (Gruber 2004) because oceanic N_2-fixation with a globally estimated rate of ~135 Tg N y^{-1} (Codispoti 2007) cannot balance the removal of fixed inorganic N-species by N-loss processes; consequently, current estimates propose an oceanic N-deficit of ~234 Tg N y^{-1} (Codispoti 2007). However, until recently, oceanic N_2-fixation was mainly attributed to phototrophic cyanobacteria, such as *Trichodesmium* or *Crocosphaera,* which are due to their light demand restricted to usually nutrient depleted surface to subsurface waters (Capone et al. 1997; Zehr and Turner 2001). Thus, estimates of N_2-fixation might be strongly biased as they focused exclusively on N_2-fixation by those cyanobacterial diazotrophs in the euphotic zone (Codispoti 2007).

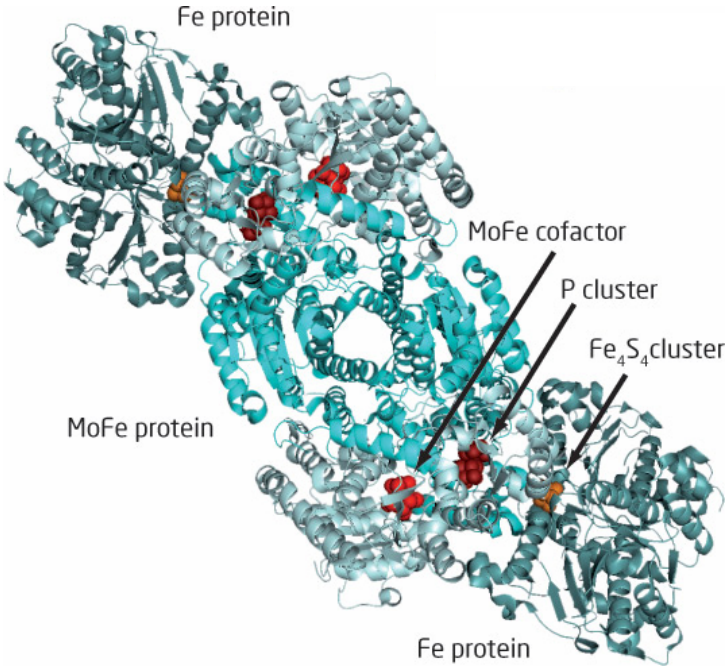


Fig. 4: Structure of the nitrogenase with co-factors
(source: http://www.case.dtu.dk/English/Research/Electrochemicaln2fixation)

Nevertheless, a broad diversity of non-cyanobacterial diazotrophs has been monitored in surface waters and OMZs, thus demonstrating the ubiquity of those diazotrophs in the ocean (Farnelid et al. 2011).

The environmental conditions leading to the distribution patterns of diazotrophs (Fig. 5) are still not fully understood, however temperature, iron and phosphorous supply and dissolved oxygen are regarded as key factors (as recently overlooked in Sohm *et al.*(2011)). An active involvement in N_2-fixation of non-cyanobacterial, potentially heterotrophic diazotrophs as a previously unrealized source of fixed N, particularly in the eastern tropical South Pacific, has been suggested ((Sohm et al. 2011), Fig. 5).

Further, the application of a novel method (Mohr et al. 2009) to determine *in situ* rates of N_2-fixation demonstrated that the apparent imbalance of N-loss and N-input might have partially been caused by a severe methodological-derived underestimation of N_2-fixation rates (Grosskopf *et al.*, submitted) by the previously used classical method (Montoya et al. 1996a). The major difference between the classic and the novel method consists of adding $^{15}N_2$ enriched filtered seawater instead of the direct injection of a $^{15}N_2$ gas bubble to the seawater incubations. Thus, upward revisions might most likely be required due to N_2-fixation by organisms other than previously considered ones and due to the described methodological underestimations.

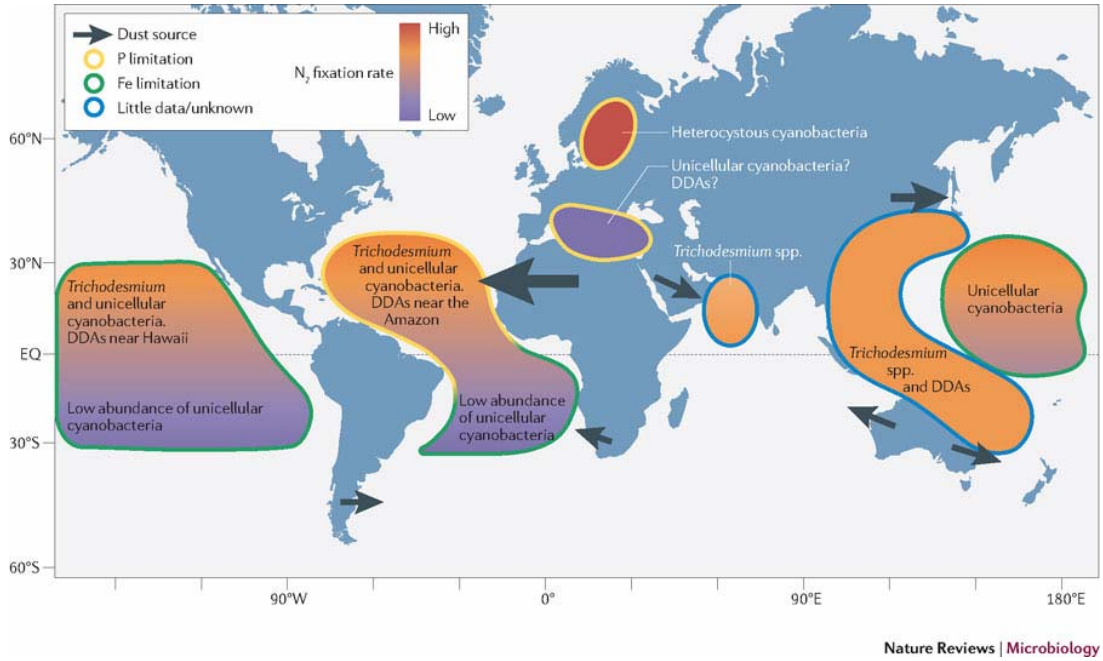


Fig. 5: Distribution of N_2-fixation rates, diazotrophs and nutrient limitation (Sohm et al. 2011): Relative N_2-fixation and the prevailing limiting nutrient are shown in each area by the fill colour and outline, respectively. Particularly, the low abundance of unicellular cyanobacteria in the eastern tropical South Pacific region is striking (DDA - diatom diazotroph association; EQ – equator).

N_2-fixation is highly sensitive towards dissolved O_2 (Dixon and Kahn 2004; Leigh and Dodsworth 2007), as the nitrogenase (Fig. 4), is inhibited by O_2 (Dixon and Kahn 2004). Thus, low oxygen conditions as present in OMZs sustain a potential niche for diazotrophs. However, high amounts of dissolved inorganic N compounds such as nitrate are commonly present in OMZs, which are classically regarded to inhibit N_2-fixation.

Nitrogen is continuously removed by **an**aerobic **amm**onium **ox**idation with nitrite (NO_2^-) to N_2 (anammox, (Thamdrup and Dalsgaard 2002; Kuypers et al. 2003; Kuypers et al. 2005; Francis et al. 2007)) which has been proposed the dominating N-loss process for instance in the OMZ waters off Namibia (Kuypers et al. 2005), Oman (Jensen et al. 2011), Peru (Hamersley et al. 2007) and Chile (Thamdrup et al. 2006). Moreover, N is lost or recycled via denitrification (the 4-step reduction of

nitrate (NO_3^-) to N_2 (Devol 2008)), DNRA (the **d**issimilatory **n**itrate **r**eduction to **a**mmonia, as hypothesized by Lam, *et al.* (Lam et al. 2009)) and nitrification (the aerobic oxidation of ammonia via NO_2^- to NO_3^- (Ward 2008), Fig. 6). The resulting overall net N-loss resulting from those processes to the ocean as well as the relative contribution of the respective processes is again strongly impacted by the present O_2 concentrations (Kalvelage *et al.*, unpublished), with decreasing O_2 concentrations favouring N-loss processes, in general. In OMZs enhanced N-loss (Deutsch et al. 2007) resulting in a N-deficit (low N*, see Fig. 3) and the simultaneous gain of phosphorous (P) from anoxic shelf sediments (resulting in high P*, i.e. excess P (Ingall and Jahnke 1994)), is proposed to provide niches for diazotrophs and thus may promote N_2-fixation. A spatial connection of N-loss and N-input via N_2-fixation in OMZs is therefore hypothesized. A very recently published study already demonstrated the co-occurrence of denitrification and N_2-fixation in an anoxic lake (Halm et al. 2009); however, evidence for a coupling of those processes in the ocean is currently missing.

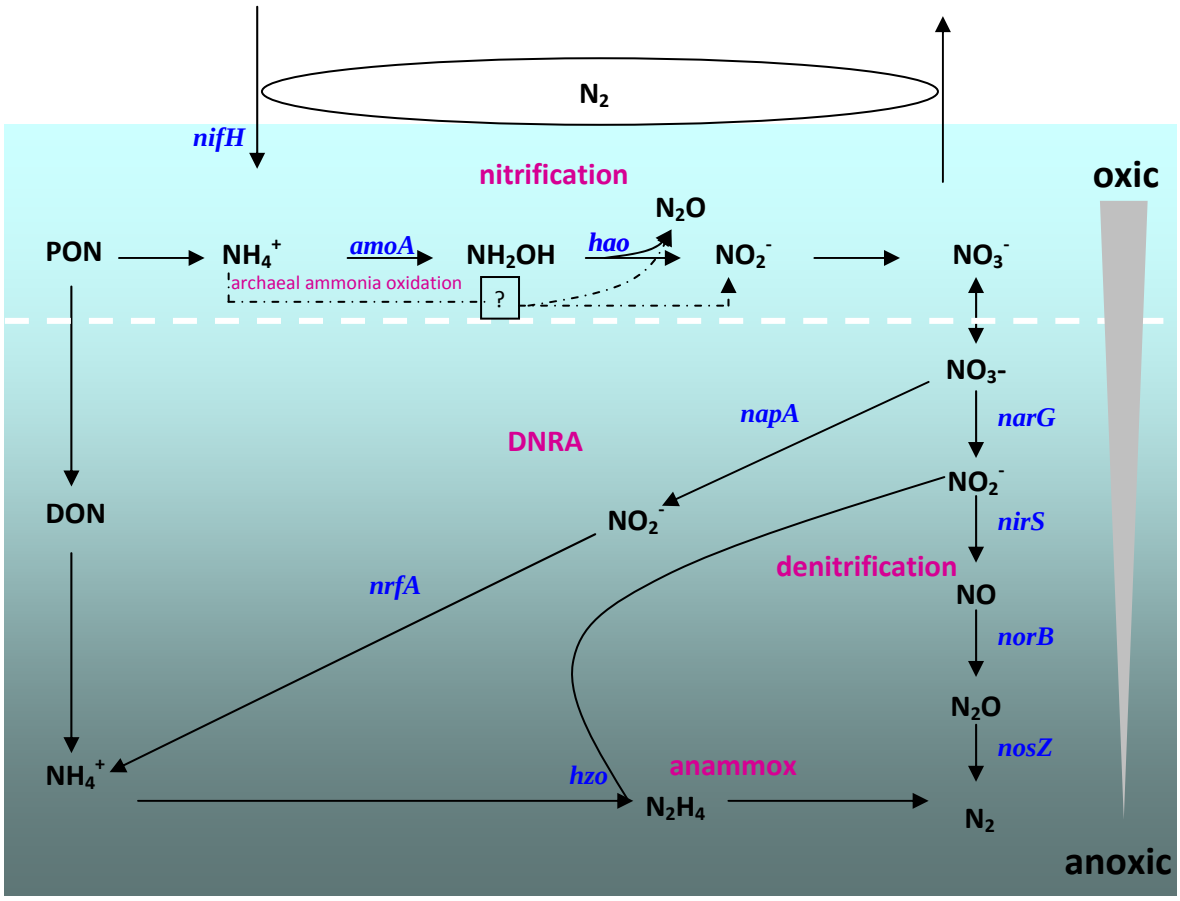


Fig. 6: The marine N-cycle with all known processes (pink) including key genes (blue) modified from Francis, *et al*. (Francis et al. 2007)

Besides the classical N-loss of fixed N via anammox and denitrification, the formation of the highly potent greenhouse gas and major ozone depleting compound N_2O (Denman 2007; Ravishankara et al. 2009) constitutes a minor N-loss term (~6 Tg N y^{-1}). Nevertheless, the production is of significant importance for both, the atmospheric chemistry and the Earth's climate (Bange 2006b; Codispoti 2007). The contribution of the marine N_2O emissions from the ocean (incl. coastal areas and rivers) to the atmosphere accounts for about 21% of the natural sources and 10% of the anthropogenic sources (Denman 2007). Thus, the ocean is a major source of N_2O to the atmosphere. Tropical upwelling regions such as the Arabian Sea and the south-eastern Pacific (off Peru and Chile, (Naqvi et al. 2005b; Cornejo et al. 2007)) show enhanced production of N_2O under low O_2 conditions as present at the boundaries of OMZs.

N_2O is biologically formed by prokaryotes via two major pathways (Fig. 5); both strongly sensitive to O_2: Under suboxic to hypoxic conditions, N_2O is mainly formed via denitrification. As an intermediate, N_2O can be produced and with further decreasing O_2 conditions consumed as well. In oxic to suboxic environments, N_2O is produced as a by-product during nitrification, which is the oxidation of ammonium via nitrite (NO_2^-) to NO_3^-. Until recently, N_2O production via nitrification was exclusively ascribed to ammonia-oxidizing bacteria (Goreau et al. 1980), however, the discovery of archaeal ammonia oxidation (Wuchter et al. 2006), their dominant abundance in various ocean regions (Molina and Farias 2009; Molina et al. 2010; Santoro et al. 2010) and the very recent publication of N_2O production detected in a archaeal enrichment cultures (Santoro et al. 2011) point towards a major role of those organisms in marine N_2O production. Moreover, a combination of both processes (denitrification and nitrification, connected via the intermediate product NO_2^-) has been demonstrated to enhance production of N_2O (Cantera and Stein 2007); however, information on the N_2O yield by the so called nitrifier-denitrification in the ocean is sparse. Additionally, production via DNRA and anammox is suggested (Kaspar and Tiedje 1981; Kaspar 1982; Welsh et al. 2001; Kartal et al. 2007; Lam et al. 2009) but the overall contribution of those processes to the marine N_2O budget has yet not been demonstrated. The yield of produced N_2O depends strongly on the present O_2 conditions (Codispoti et al. 2001; Codispoti 2010). Rapid changes of anoxic to oxic conditions, temporally and spatially, favour in general nitrification activity (Schweiger et al. 2007) and under suboxic conditions (1- 30 % O_2 saturation) N_2O production as well (Codispoti 2010). Sulphide pulse experiments indicate that during re-oxygenation partial nitrification (the oxidation of NH_4^+ to NO_2^-) associated with a significant formation of N_2O is triggered (Erguder et al. 2008; Yu et al. 2010). On the other hand, hypoxic conditions and spontaneous deoxygenation favour rapid N_2O production via denitrification (Naqvi et al. 2000; Codispoti 2010). Thus, highly dynamic systems as present in the OMZ off Peru with rapidly changing O_2 conditions are locations favouring N_2O formation by nitrification and denitrification, considerably. Hence, future oceanic net N_2O production will be substantially influenced by changing O_2 conditions in OMZs (Fig. 7, (Capone 2008; Codispoti 2010)), thus, the future development of OMZs is predicted to severely influence the future climate by critically impacting on N_2O production.

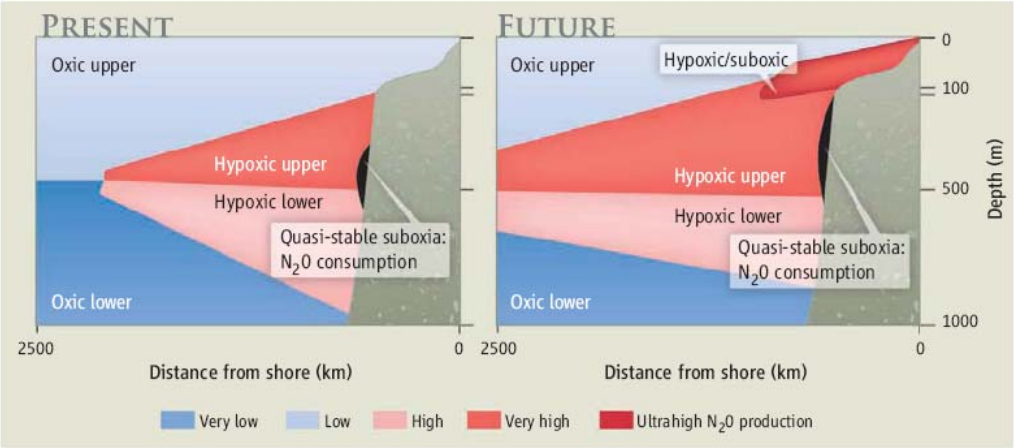


Fig. 7: A future perspective of a generic eastern boundary system in present-day and future oceans from Codispoti, 2010 (Codispoti 2010)**:** Suboxia can extend further offshore than shown here, but is absent from a large portion of the oceanic eastern boundary.

Marine Metagenomics/ -transcriptomics

The global N-cycle is predominantly driven by prokaryotes. A deeper understanding of the community structure and specific spatial distribution of microorganisms involved in the N-cycle is crucial. In marine suboxic environments with a high complexity of microbial N-transformations it is impossible to distinguish between the various processes using conventional chemical rate measurements.

To accomplish this task, the detection of functional genes of the N-cycle is of particular interest in revealing co-occurring processes and complex microbial community structures. The optimized high resolution monitoring of cluster specific distribution and expression patterns along horizontal and vertical gradients along with rates derived from onboard incubation experiments allow to assess the relative contribution of organism groups to N-turnover processes.

The inability to cultivate the majority of microorganisms present in the environment (99% according to current estimates (Amann et al. 1995)), particularly in the ocean demonstrates the need for culture-independent approaches. A variety of methods developed over the recent years achieves to overcome this bottleneck (Handelsman 2004; Weiland et al. 2010). In order to assess the present genetic and metabolic potential, novel techniques such as pyrosequencing of DNA and cDNA libraries, TaqMan-based quantitative real time PCRs and microarrays for gene abundance and expression studies are available, now, thus allowing an in-depth analysis of complex communities. The comparison of traditional Sanger sequencing to pyrosequencing is expected to show a higher saturation by this novel technique; hence, the broader coverage compared to conventional methods demonstrates the need for those novel applications. Moreover, microarrays allow fast screening of large numbers of samples for target genes and their expression (Taroncher-Oldenburg et al. 2002). High resolution gene

quantification by qPCR allow a higher precision compared to clone library abundances (Langlois et al. 2005a) and further enable an absolute quantification of gene and transcript copy numbers.

Open questions and major goals of this project

The potential future changes of the marine N-cycle are a topic of emerging importance in biogeochemical studies, particularly in the context of climate change. However, the microbial key processes in OMZs, the O_2 tolerance or sensitivity of the contributing organisms and the spatial and temporal variation of the various N-turnover processes and contributing organisms remain to be unravelled. Field studies on the tolerable O_2 ranges of the various N-turnover processes have previously been missing. Potential overlaps and co-occurrences of N-cycle processes have yet not been described. Additionally, the discrepancy between N-loss and N-input by N_2-fixation in the ocean has still not been explained; investigations on N_2-fixation below the euphotic zone are not available. Moreover, the organisms responsible of the formation of oceanic N_2O have not directly been identified.

Hence, this thesis aimed to investigate the effects of O_2 on microbial communities involved in N-loss, N_2-fixation (Chapter 1) and N_2O production (Chapter 2, 3) in OMZ waters. High resolution measurements of nutrients, O_2, N_2O and key functional genes involved in the N-cycle were used to identify regions of active N-transformations and key players of the N-cycle in and near the investigated OMZs (see Fig. 2). Rate measurements of N_2-fixation and N_2O production were conducted at various O_2 concentrations via on-board incubation experiments, the involved microorganisms were identified using molecular techniques and in case of N_2O production by performing inhibitor experiments. To assess the abundances of organisms involved in the N-cycle and their active involvement, a set of key genes has been used as functional markers (Fig. 5). The gene abundance and expression determined in high resolution along vertical and horizontal gradients in the water column has been used to estimate the genetic potential and active contribution of the various pathways of the N-cycle. Thus, key organisms and their reaction towards changes in dissolved O_2 were identified in order to estimate (i) future changes of the community structure and their active involvement in the N-cycle, (ii) the ability of microorganisms to react on changing O_2 conditions and (iii) the overall impact on biologically driven N-cycle processes.

Chapter 1: Nitrogen fixation in the oxygen minimum zone off Peru

Summary

Marine dinitrogen (N_2) fixation is quantitatively the most important source of new nitrogen (N) to the ocean (Duce et al. 2008). It is of global importance because the availability of fixed N controls primary production in large areas of the surface ocean. Classically, marine N_2-fixation is regarded as a process predominantly ascribed to cyanobacteria present in the euphotic zone (Capone et al. 2005). However, assuming that the oceanic N-inventory is at steady-state (Codispoti 2007), cyanobacterial N_2-fixation in surface waters alone cannot balance oceanic nitrogen losses. Thus, current estimates of the oceanic N budget led to the proposal that oceanic oxygen minimum zones (OMZ) may provide additional niches for N_2-fixing organisms, possibly balancing oceanic N-loss terms.

Here, we report a high diversity of diazotrophs, including novel potentially heterotrophic clades, exhibiting specific distribution patterns and actively contributing to N_2-fixation throughout the water column in the OMZ off Peru, thus extending the habitat of marine diazotrophs to low oxygen/ high NO_3^- areas. Moreover, the co-occurrence of the functional marker gene for N_2-fixation, *nifH,* and active N_2-fixation with key functional genes of nitrification, anammox and denitrification suggests that a close spatial coupling of N-input and N-loss processes exists in the OMZ off Peru. Further, N_2-fixation in OMZs and adjacent surface waters is proposed to significantly contribute to the oceanic N-budget, thus stating a substantial paradigm shift of the traditional view of the oceanic N-cycle. Particularly with regard to ocean deoxygenation as a result of global warming, our results demonstrate the increasing importance of OMZs as hotspots of N_2-fixation.

Introduction

The atmospheric pool of dinitrogen (N_2) is made available to the ocean by biological N_2-fixation, a process exclusively performed by diazotrophs, a special group of prokaryotes (Capone 2008; Moisander et al. 2010). Global estimates, mainly based on N_2-fixation by photoautotrophic diazotrophs in surface waters (~150 Tg N y^{-1}) cannot balance the nitrogen (N) loss (~ 400 Tg N y^{-1}) (Karl et al. 2002; Codispoti 2007) resulting from microbial processes such as anammox (the anaerobic oxidation of ammonium with nitrite to N_2 (Dalsgaard et al. 2003; Kuypers et al. 2003)) and denitrification (the 4-step reduction of nitrate (NO_3^-) to N_2 (Falkowski 1997; Codispoti 2007)). However, all those processes, including N_2-fixation, are strongly sensitive towards dissolved oxygen (O_2). Hence, oxygen minimum zones (OMZs), such as present in the Arabian Sea and the Pacific Ocean (Stramma et al. 2008), particularly the large and persistent OMZ off Peru with O_2 concentrations below the detection limit of conventional methods, are suggested to provide niches for organisms involved in the N- cycle and are proposed hotspots of N-turnover (Capone 2008; Codispoti

2010). Low O_2 concentrations, excess phosphorous (P) (Deutsch et al. 2007), resulting from enhanced N-loss and from the release of reactive phosphate into the water column from the sediment (Ingall and Jahnke 1994), and the availability of dissolved iron in OMZs are environmental conditions generally proposed to favour N_2-fixation; however, the diazotrophic communities detected in OMZ waters by recent molecular studies differ strongly from the classic marine diazotrophic communities (Fernandez et al. 2011; Hamersley et al. 2011) (Zehr et al. 2000; Zehr and Turner 2001; Falcon et al. 2002; Langlois et al. 2005a). Further, information on the spatial distribution of diazotrophs present in OMZs and their relative contribution to the fixed N budget in the ocean are currently missing.

According to the low O_2 conditions and the present excess P (Deutsch et al. 2001; Deutsch et al. 2007), we propose that N_2-fixation in OMZs and adjacent surface waters has significantly been underestimated (partially due do a lack of measurements) and might at least partially balance the apparent deficit in fixed N (~200 Tg N y^{-1}, (Codispoti 2007)).

Here, we present a dataset of two cruises to the eastern tropical North Pacific (ETSP) off Peru (M77/3 and M77/4 on the German research vessel *Meteor*, Dec. 2008- Feb. 2009). By combining molecular tools with *in situ* rate measurements using ^{15}N- incubations, seven novel *nifH* clusters have been identified only distinctly related to previously described marine diazotrophs, some potentially heterotrophic as suggested by glucose addition experiments. The cluster specific *nifH* abundance and expression, along with active N_2-fixation throughout the water column and with the presence of key genes of N-loss processes, was determined. Further, the co-occurrence of N_2-fixation and N-loss processes has been estimated; sustaining a novel niche for diazotrophs in OMZs.

Results

Diversity of novel *nifH* clusters in the OMZ off Peru

Unexpectedly, high diazotrophic diversity in *nifH* sequences was detectable in the OMZ off Peru. From a total of 600 DNA and cDNA sequences from various depths, seven novel *nifH* clusters (further referred to as P1- P7) were identified from *nifH* clone libraries of several stations during two cruises (M77/3, M77/4, 2008/2009) to the OMZ off Peru (Fig. 1.1, map). Those clusters, which have previously not been described, were in large parts amplified from OMZ waters below the euphotic zone, which points towards a non-phototrophic metabolism in those organisms. None of those clusters present in our clone libraries was affiliated to the filamentous non-heterocystous cyanobacterium *Trichodesmium sp.*, to the diatom endosymbiont *Richelia sp.* or to group A, all of which were previously considered key diazotrophs in the ocean occurring at high abundances (Zehr and Ward 2002; Montoya et al. 2004; Church et al. 2005a). Sequences of group B closest related to *Crocosphaera* (Zehr et al. 1998) were the only cyanobacterial sequences recovered to a small extent from some of our libraries (1 of ~300 sequences). P8, previously identified in other studies (Fernandez et al. 2011; Halm et al. 2011), was mainly present in clone libraries from M77/4 along the North-South transect at 85.83°W (Fig. 1.1) down to more than 4000 m.

The presence of a diazotrophic community largely differing from those previously described, strongly points towards the fact that the current understanding of marine N_2-fixation needs to be reassessed. Clusters P1 and P2, which are phylogenetically closest related to spirochaeta and archaea, respectively, do not contain many marine diazotrophic representatives. The presence of those deep branching clusters demonstrates that other groups than previously considered might significantly contribute to oceanic N_2-fixation. Present throughout the water column of the OMZ off Peru, cluster P1 was amplified from all clone libraries, thus dominating large parts of the system. Besides those deep branching clusters, clusters P3-P7 are related to different branches of the proteobacteria, showing some similarity to *nifH* genes amplified from hypoxic basins in the Californian Bight and the OMZs off Peru and Chile (Fernandez et al. 2011; Hamersley et al. 2011). Although none of them was identical to previously published sequences, proteobacterial diazotrophs seem to be abundant in OMZs and thus might be of importance with regard to expanding OMZs (Stramma et al. 2008).

The phylogenetic analysis of *nifH* genes demonstrated a surprisingly broad diversity of sequences falling into clusters previously not considered abundant or important for N_2-fixation in the ocean. The presence of those clusters indicates a potential to fix N_2 in OMZ waters. Thus, current budgets of oceanic N_2-fixation mainly based on photoautotrophic diazotrophs in the photic zone (Codispoti 2007) might have led to a significant underestimation of oceanic N_2-fixation; however, the present N-deficit indicates that N_2-fixation within the OMZ does not fully balance the present N-deficit (Deutsch et al. 2007).

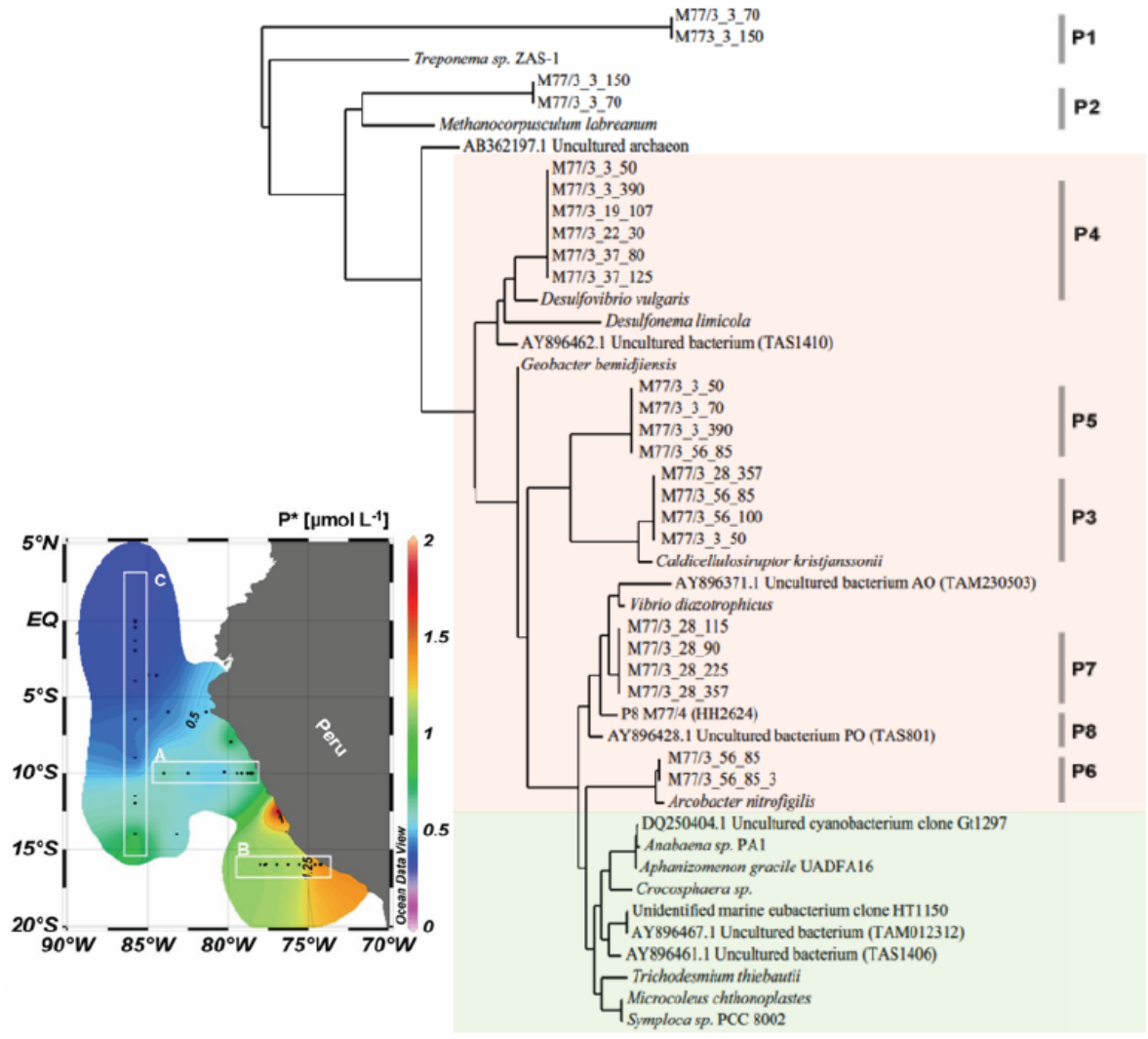


Fig. 1.1: **Phylogenetic tree of *nifH* sequences:** sequences were derived from DNA *nifH* clone libraries from the OMZ of Peru, sequences highlighted in light green are related to cyanobacteria, and red highlighted sequences are proteobacterial. The depth integrated P* distribution throughout the OMZ is shown on the map, the three main transects along (A) 10°S, (B) 16°S and (C) 85.83°W are indicated by black boxes.

Spatial distribution and abundance of novel *nifH* clusters

The vertical and horizontal distribution of the *nifH* gene of those novel diazotrophic clusters throughout the water column of the OMZ off Peru from surface waters down to 1000 m (P8 at transect C, Fig. 1.1 map, along 85.833°W) and most likely even deeper, points towards an important contribution of N_2-fixation below the photic zone to the marine N-budget. The detected diazotrophic clusters were distributed in specific patterns with high *nifH* gene copy numbers (Fig. 1.2). Clusters P1 and P4 were present in significant abundances up to 10^5 copies per L (detected by cluster specific TaqMan- qPCR), thus dominating the diazotrophic community from the shelf to about 83°W at 10°S and to 77°W at 16°S. While the P1 cluster appears associated with deeper waters (100- 300 m), and lower O_2 conditions other clusters (P2, P3, P4 Fig. 1.2 and Fig. S1) were present in surface to sub-

surface waters. Hence, those novel identified clusters appear to occupy different niches within the OMZ.

In contrast to the general assumption that diazotrophs thrive mainly in N-depleted waters, clusters P1, P4 and P8 were present at nitrate (NO_3^-) concentrations up to ~40 μM (Fig. 1.2), indicating that the expression and activity of the nitrogenase is apparently not inhibited by those NO_3^- concentrations, in case the nitrogenase is active. In contrast to P1 and P4 with highest abundances at coastal stations, cluster P8, although present throughout the Peruvian OMZ (Fig. 1.2, 1.3), dominated the diazotrophic community and had highest abundances up to 10^6 *nifH* copies L^{-1} in the nutrient depleted open ocean region along the north-south transect (transect C, Fig. 1.1,1.3). Thus, P1 with highest abundances on the shelf and P8 with highest abundances off-shore, are spatially inversely correlated (Fig. S2)

Crocosphaera (CR) a cyanobacterium, commonly regarded as one of the most important diazotrophs in the ocean (Zehr et al. 2001; Church et al. 2005b), was mainly present off-shore in waters with high NO_3^- concentrations. At 16°S, *nifH* genes affiliated to CR were only detected in copy numbers up to 1.8×10^2 copies L^{-1} on the shelf (Fig. 1.2). The distribution of CR suggests that parameters other than NO_3^- influence the presence of CR. Abundances of CR are generally rather low compared to other detected diazotrophs and it remains unclear if CR is actively fixing N_2 in the OMZ off Peru.

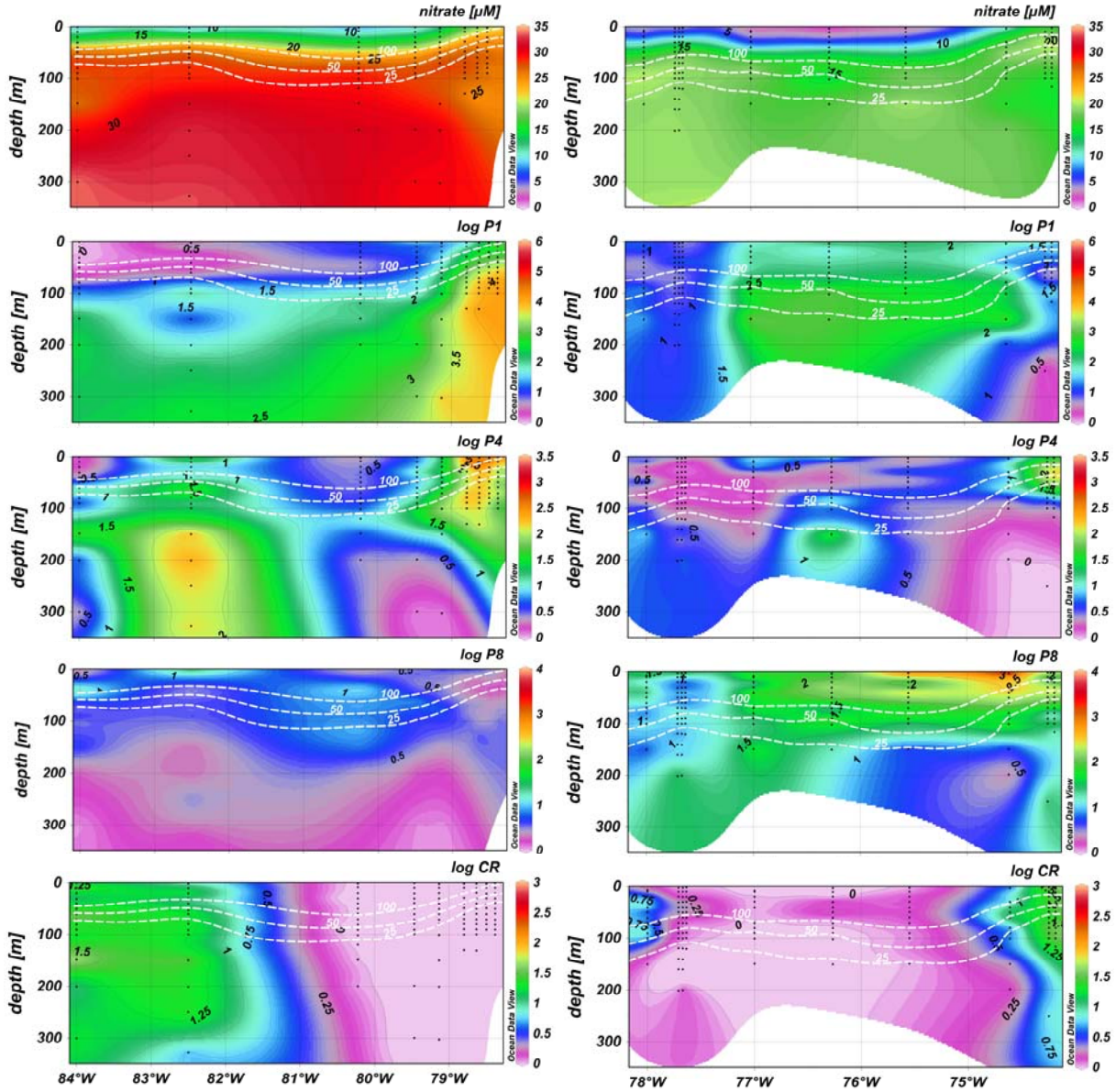


Fig. 1.2: Distribution of NO_3^-, O_2 and the newly identified *nifH* clusters: NO_3^- (µM), *nifH* clusters P1, P4, P8 and *Crocosphaera* (log_{10} copies L^{-1}), the oxycline (µM O_2) is indicated with white contour lines along (A) 10°S and (B) 16°S.

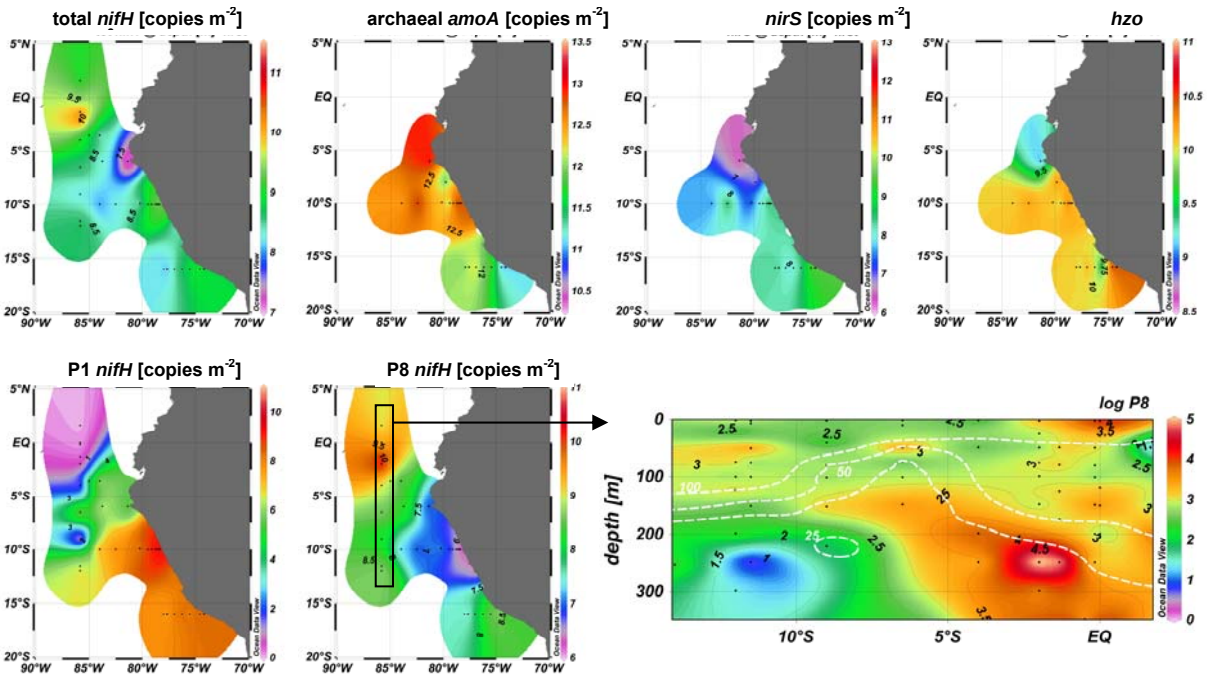


Fig. 1.3: Depth integrated horizontal distribution of functional key genes of N-loss processes and N_2-fixation in the upper 350 m in the Peruvian OMZ: archaeal *amoA* (functional marker for archaeal ammonia oxidation), *nirS* (functional marker for denitrification), *hzo* (functional marker for anammox), *nifH* for diazotrophs clusters P1, P8 and total *nifH* (log_{10} copies per L). The section shows the vertical distribution of the dominant *nifH* cluster P8 (log_{10} copies L^{-1}, measured by qPCR) along a North-South transect at 85.83°W as indicated by the black box on the map, the oxycline (µM O_2) is indicated as contour lines.

In situ N_2-fixation

We demonstrated active N_2- fixation in the presence of novel *nifH* clusters detected along 10°S pointing towards a key role of the newly identified clusters for N_2-fixation in this area. A broad peak of N_2-fixation extending into the OMZ (beginning at ~ 40 m) could be observed offshore from 79.134°W to 81.361°W (Fig. 1.4, Fig. S3). Analysis of endpoints of $^{15}N_2$-incubation experiments (Fig. 1.4) demonstrated the exclusive presence of P1, P4 and P7 *nifH* genes in the different treatments (Fig. 1.4, Fig. S4 shows a similar experiment at a more coastal station). In those experiments, N_2-fixation was promoted by the addition of 10 µM glucose and further more by the combined addition of oxygenated water (2 µM O_2) and glucose. This suggests that heterotrophic diazotrophs present in the upper portion of the OMZ are limited by the availability of reduced carbon compounds (see also Fig. S4).

While the addition of glucose stimulated the growth of clusters P1, P4 and P7, the combined addition of glucose and oxygen promoted exclusively the growth of P7, whereas P1 and P4 were not significantly affected. Highest N_2-fixation rates in this experiment were present along with an increase of *nifH* copy numbers of a combination of P1, P4 and P7 when no O_2 was added (in situ O_2 concentrations ~ 1.85 µM), pointing towards an active contribution of those clusters at the present low O_2 conditions in the ETSP. The additional supply of O_2 suggests a potential switch from a

combination of clusters towards P7 when O_2 is transported (e.g. by O_2 intrusions or lateral mixing) into the OMZ, demonstrating the capability of the diazotrophic community to react to rapidly changing O_2 conditions.

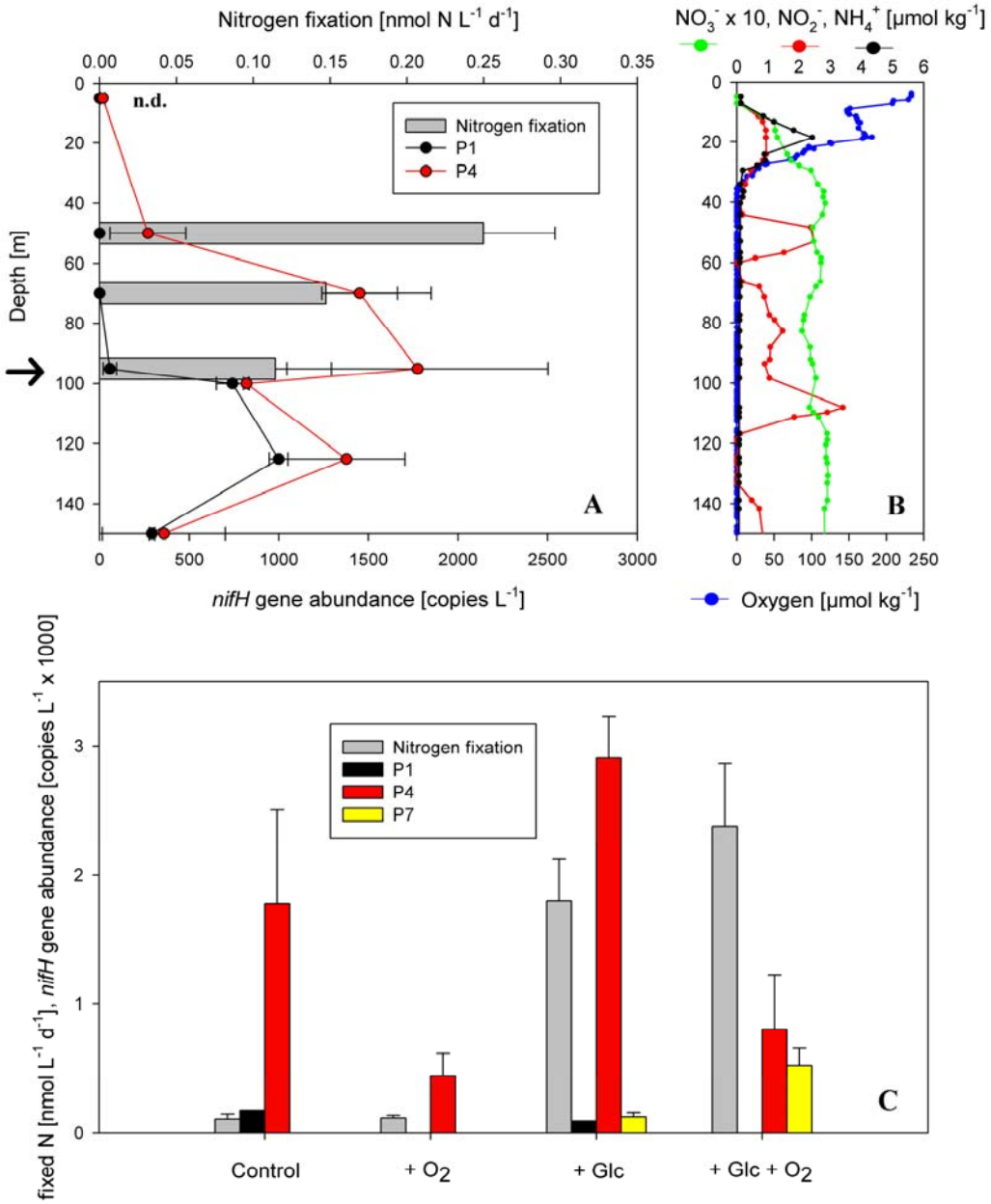


Fig. 1.4: **Vertical profiles of N_2- fixation**: (A) Water column N_2- fixation determined by 24h $^{15}N_2$- incubation experiments along the initial vertical distribution of *nifH* clusters P1 and P4 at station #3 (Fig. 1.1, map, 10°S/ 81.3°W) and (B) along O_2 and nutrient gradients. (C) The effect to the addition of glucose (2µM) and oxygen (10 µM) on N_2-fixation as well as on the *nifH* gene abundances of the detectable clusters P1, P4 and P7 (both as end-point measurements in 24 h incubations, samples from 95m depth indicated by an arrow in panel A)) were determined.

Further south, N_2-fixation rates increased, showing higher activity in surface waters than at depth, consistent with low N/P ratios (resulting in high P*), there. Highest N_2-fixation rates of 24.8 ± 8.4 nmol N d^{-1} L^{-1} were measured at a sulphidic station (12.37°S/ 77°W) in surface waters, where *nifH* of CR and P1 was actively expressed (samples specifically collected for mRNA purification, Fig. 1.5). At this station, large parts of the water column were fully anoxic (Fig. 1.5), and hydrogen sulfide (H_2S) was present. Below 30 m depth, the water column was depleted in NO_3^- and NO_2^-, the key substrates for anammox and denitrification, thus N-loss processes were partially hindered. However, ammonia was present in concentrations of 2-4 µM below the oxycline. Integrated water column N_2-fixation rates exceeded 1 mmol N d^{-1} m^{-2} comparable to rates reported from major *Trichodesmium* blooms (Capone et al. 2005) (although possibly underestimated, as very recently demonstrated (Grosskopf; Mohr et al. 2010)). The peak in *nifH* expression of P1 present at 80 m along with a maximum in N_2-fixation indicates an active involvement of this cluster in N_2-fixation, at this depth (Fig. 1.5). Moreover, surface N_2-fixation might mainly result from P5, which was the only cluster present in cDNA clone libraries, here, although the *nifH* gene and active gene expression of P5 was only detectable from 15-30 m depth (Fig. S5). The measured high N_2-fixation rate detected at this sulphidic station demonstrates that, in addition to an underestimation of oceanic N-input due to deep N_2-fixation, spontaneous sulphidic events, previously been reported to occur in intense OMZs (Naqvi et al. 2000), might sporadically trigger significant N_2-fixation, potentially providing an important N-input to the ocean. In addition to a spatial underestimation of the diazotrophic habitat, those temporally restricted events sustain hotspots of N_2-fixation in OMZs and adjacent surface waters. Hence, N_2-fixation in and near OMZs is proposed to significantly contribute to the oceanic fixed N budget; yet, further studies have to follow focusing on quantifying this source.

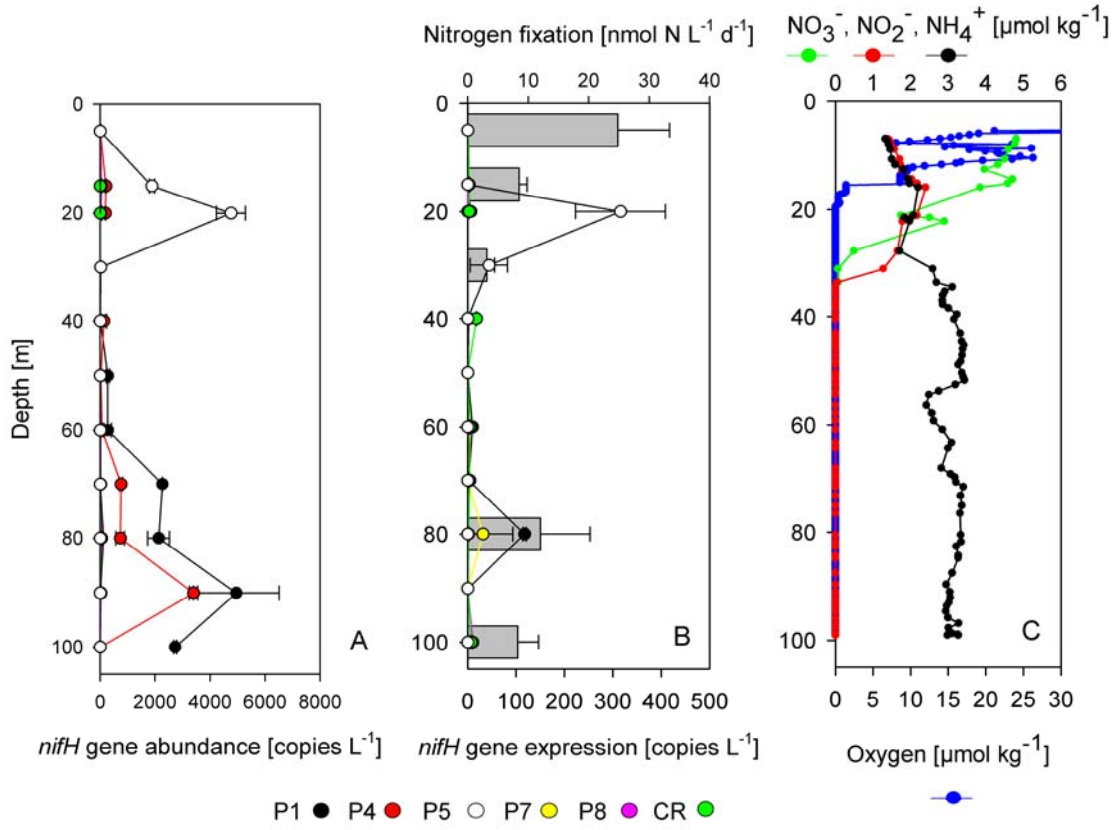


Fig. 1.5: Vertical distribution of (A) the *nifH* gene abundance, (B) the measured N_2-fixation and *nifH* gene expression (dashed line, second x- scale, samples have specifically collected for RNA extraction), and (C) chemical parameters (O_2, NO_3^-, NO_2^-, NH_4^+) at a coastal sulphidic station (M77/3, #19, 12.37°S/ 77°W)

Co-occurrence of N_2-fixation and N-loss processes

High *nifH* gene abundances of cluster P1 coincided with maxima in abundance of key genes of archaeal ammonia- oxidizers (archaeal *amoA*, coding for the ammonia monooxygenase), denitrifiers (*nirS*, coding for the cd1-containing nitrite reductase) and anammox bacteria (*hzo*, coding for the hydrazine oxidoreductase), suggesting a close spatial coupling between N_2-fixation and N-loss (Fig. 1.3), as previously proposed by the model prediction of Deutsch *et al.* (Deutsch et al. 2007). N_2-fixation may be responsible for the progressive increase in N: P ratio from the inshore waters to the open ocean. A linear correlation of P1 and NO_2^- was indicated by a principal component analysis (Fig. S6), which is most pronounced along 10°S (n = 112, r = 0.799, Fig. S6). This finding is in line with the significant correlation of P1 and *hzo* (n = 113, r = 0.591) and P1 and archaeal *amoA* (n = 237, r = 0.56), at 10°S. Total microbial cell numbers (Fig. S7) show that archaeal ammonia oxidizers largely dominate the system, making up to 80 % of total microbial cells, while the abundance of diazotrophs is ~ 3 orders of magnitude lower, assuming one *nifH* or one *amoA* gene copy per cell. Anammox has been demonstrated to be the major active process in N-loss during the sampling period in the OMZ off Peru (Kalvelage *et al.*, unpublished), thus a co-occurrence of N_2-fixation and anammox rather than denitrification, is likely. Evidence for the co- occurrence of N-loss and N_2-fixation mainly ascribed to

Chlorobium-like diazotrophs has previously been documented for an anoxic lake (Halm et al. 2009) and is proposed to play a major role in marine N_2-fixation, as well. We hypothesize, that hotspots of N-loss processes, such as OMZs, provide niches for N_2-fixers. Consequently, the marine N-budget might essentially depend on the future development of OMZs.

Conclusions

At the time of sampling, the OMZ off Peru supported a diverse community of diazotrophs, with selected phylotypes reaching abundances that suggest they play a role in N_2- fixation. This has been demonstrated by the detection of several novel *nifH* clusters, their presence and expression along with active N_2-fixation. The abundance of *nifH* throughout the water column down to 1000 m points towards a significant contribution to N_2-fixation in OMZ waters and most likely even deeper; sustaining additional N-input in addition to surface N_2-fixation. We conclude that the apparent deficit in oceanic fixed N might largely result from underestimating deep N_2-fixation, as current estimates of the marine N-budget exclusively focus on photoautotrophic N_2-fixation in the photic zone. Moreover, high N_2-fixation during transient events, such as described, here, might significantly contribute to the oceanic N-input and might require additional upwards revision of the oceanic fixed N term. Further, we obtained evidence that the presence of reactive N-compounds (NO_3^-, NO_2^-) in the Peruvian OMZ does not negatively impact N_2-fixation, thus significantly challenging the current view of N_2-fixation in high N environments.

A major contribution of heterotrophic diazotrophs to N_2-fixation among the novel clusters is suggested, as demonstrated in fertilization experiments with glucose. The co-occurrence of cluster P1 with key functional genes of mainly nitrification and anammox suggests a spatial coupling of N-input and N-loss processes in the OMZ off Peru, normalizing the N- deficit present on the shelf towards the open ocean. Detected at highest N_2-fixation rates, a particular importance of this cluster is indicated. Those findings represent major paradigm shifts in understanding oceanic N_2-fixation, by extending the niches of diazotrophs towards high nitrogen and low oxygen environments, demonstrating the co-occurrence of N_2-fixation and N-loss and indicating a major dependency of diazotrophic clusters on O_2 and reduced carbon compounds.

In the context of eutrophication (Duce et al. 2008) and expanding OMZs (Stramma et al. 2008), understanding the importance of N_2-fixation in OMZs and of diazotrophs affiliated to those preferably anoxic heterotrophic clusters is crucial for estimating future changes in the marine N-budget.

Methods summary

Hydrographic parameters and nutrients

Samples for salinity, O_2 concentrations and nutrients were taken from a 24-Niskin- bottle rossette equipped with a CTD sensor or a pump-CTD. Oxygen concentrations were determined according to the Winkler method; salinity and nutrient concentrations were determined as decribed (Grasshoff 1999) .

Molecular genetic methods

Samples for the extraction of DNA/ RNA were taken by filtering a volume of about 2 L (exact volumes were determined and recorded continuously) of seawater through 0.2 µm polyethersulfon membrane filters (Millipore, Billerica, MA, USA). The filters were immediately frozen and stored at -80°C. Specific RNA samples were taken by filtering recorded volumes of seawater for a time intervall not exceeding 20 min.

DNA and RNA was extracted using the Qiagen DNA/RNA All prep Kit (Qiagen, Hilden, Germany) according to the manufacturers protocol. Nucleic acid concentrations were determined using PicoGreen and RiboGreen (Invitrogen, Carlsbad, CA) measurements.

Residual DNA was removed from the purified RNA by a Dnase I treatment (Invitrogen, Carlsbad, CA), purity of RNA was checked by 16S rDNA PCR amplification prior reverse transcription. The extracted RNA was gene specifically reverse transcribed to cDNA using the Superscript III First Strand synthesis Kit (Invitrogen, Carlbad) following the manufacturers' protocol.

NifH was amplified by PCR with primers and probes according to (Zani et al. 2000; Langlois et al. 2005a). For the novel *nifH* clusters, qPCR primers and probes were designed with the Primer Express software package, oligonucleotide sequences and qPCR conditions are given in Tab.1. *AmoA* PCRs and quantitative PCRs were performed as described in Loescher (submitted 2011); *nirS* and *hzo* were amplified according to Lam (2007) and Schmid (2010).

Cloning of PCR amplicons was performed using the Topo TA Cloning®Kit (Invitrogen, Carlsbad, CA) according to the manufacturers' instructions. Sanger sequencing was carried out by the Institute of Clinical Molecular Biology, Kiel. Sequences were analyzed using the ClustalW multiple alignment tool on a 321 bp fragment for *nifH*, sequence differences were set on a minimum of 5%, phylogenetic trees were made using distance-based neighbour-joining analysis.

Pure cultures of *Azotobacter vinelandi* (DSMZ 332), *Klebsiella pneumoniae* M5A1 and *Methanosarcina mazei* Gö1 were grown under N2-fixing conditions, mRNA was purified using Qiagen DNA/RNA All prep Kit and consequtively used for RT-qPCR using the Qiagen Quantitect SybrGreen RT Kit (both from Qiagen, Hilden, Germany).

$^{15}N_2$ seawater incubations

Seawater incubations were performed in triplicates at 6 stations in the OMZ off Peru (M77/3) as previously described (Montoya et al. 1996b, 1996a; Capone 2001). P* was calculated according to Deutsch *et al.* (Deutsch et al. 2007).

Acknowledgments

We thank the authorities from Peru for the permission to work in their territorial waters. We acknowledge the support of the captain and crew of the R/V Meteor, as well as the chief scientists Martin Frank and Lothar Stramma. Moreover, we thank I. Grefe for sampling during M77/4; we further thank K. Stange, F. Malien, V. Leon and P. Fritsche for oxygen and nutrient measurements and G. Klockgether for assisting with the mass spectrometry. We thank H. Bange and V. Bertics for helpful discussion and input. Financial support for this study was provided by the DFG Sonderforschungsbereich 754 (www.sfb754.de). G.L. was funded by the MPI Bremen.

Tab 1: Primers and PCR conditions. For real-time qPCR, the initial denaturing step was 10 min at 95°C, annealing temperatures were the same as in the end point PCRs, no final extension step took place, 40 cycles were performed followed by melting curve analysis.

Cluster	reference sequence	Forward primer 5'-3'	Reverse primer 5'- 3'	Probe	annealing temperature [°C]
P1	M773_3_70_4	GGACTACATTCGGACTAG	GTCGTAACCACGATCTAG	TCTTCAAAATCCCGCGTCCCG (antisense)	60
P2	M773_3_150_7	GGTGTTCTATGTGTTGAA	GTAGGAGTTACGAATTGG	TCGCCTAGCACATCATAGATCAC (antisense)	50
P3	M773_56_100_1	CACAGTTAGAGAGGTAGG	CAAGGTCGTCAGTAAAAG	AGCTCGACAAGGTAATGTTCACA (sense)	54
P4	M773_22_30_1	CTCGCACAGAAATCAGTG	GCATGTTAATGGAAGTGATG	ACGTCGAACTCGAAGACATCCG (sense)	60
P5	M773_56_100_11	GGAAGTCTTACTTGAAGG	CACCATTTTCCTCAAGAA	ATTGCTGTAATAACGCCTCTGC (antisense)	60
P6	M773_56_85_3	GCTCAATCTACAATTATGC	GCTGTAATAACTCCTCTAC	ACCACCTGACTCAGTACAATTAATGT (antisense)	50
P7	M773_28_115_1	GGTTCTGTTGAAGACATC	CGAAGTCTAAGTCTTCTTC	ATCGCTGTGATTACACCACGAC (antisense)	52
P8	M774_800m_11 B14a	ACTCGTCTGACTTCAC	TTAATACATCGTTCCA	AAAGCACAGAATCATG	52

Supplemental material

Distribution of additional novel *nifH* clusters

Although clusters P2, P3, P6 and P7 showed lower *nifH* abundance than the previously discussed clusters, they show a specific distribution along vertical and horizontal gradients. Generally, those clusters appear in higher abundance at 16°S, where N/P ratios were rather low. C P3 was present in highest abundances in surface waters, potentially favouring present high P* value.

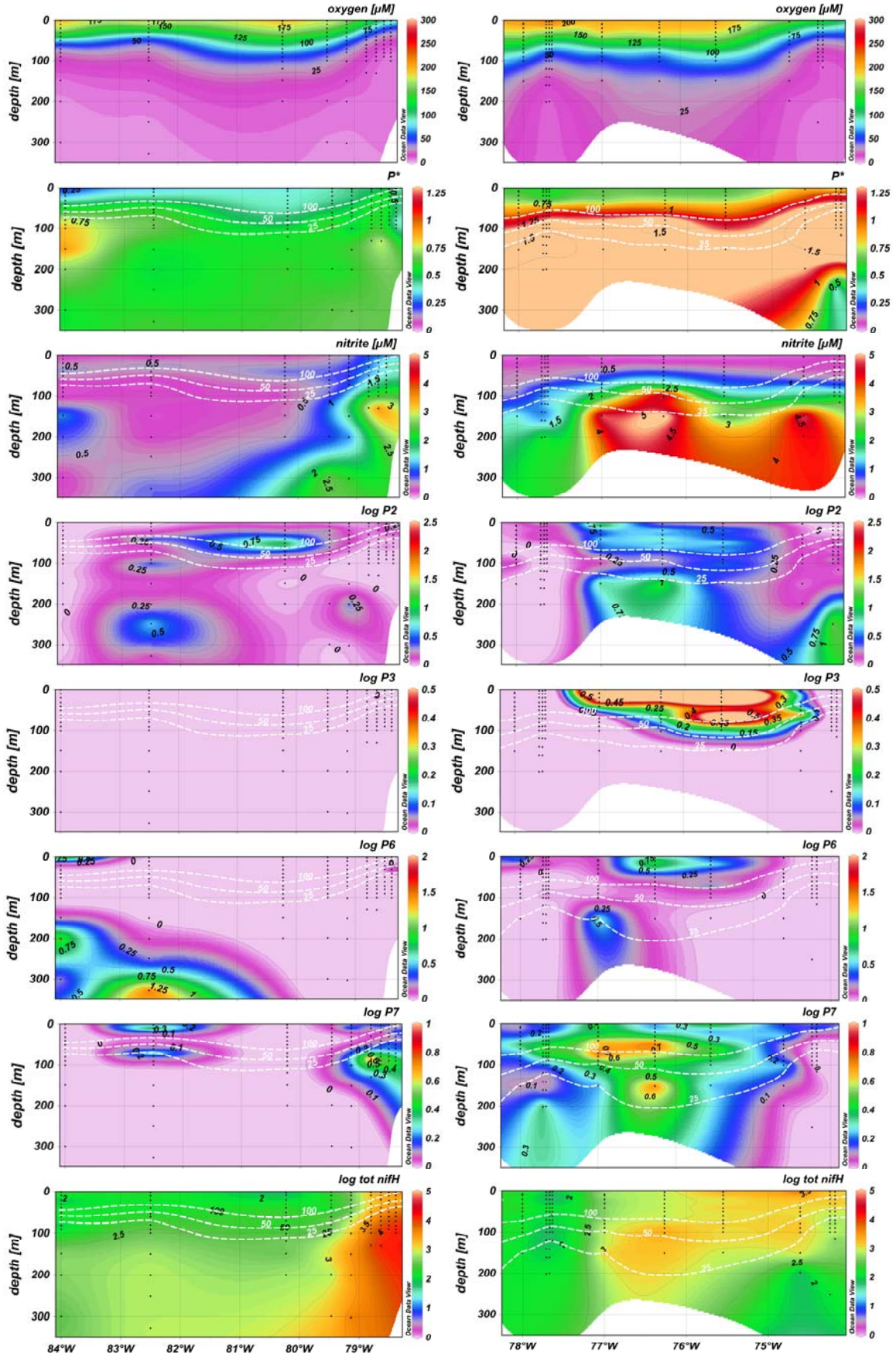


Fig. S1: Distribution of P*, NO_2^-, novel *nifH* clusters and total *nifH*: P*, NO_2^- (µM), *nifH* clusters P2, P3, P6 and P7 and total *nifH* (log_{10} copies L^{-1}, total *nifH* = tot *nifH*, sum of all detected clusters)), the oxycline (µM O_2) is indicated with white contour lines along (A) 10°S and (B) 16°S.

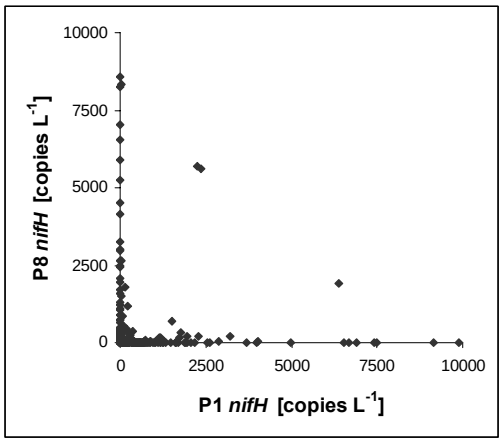


Fig. S2: P1 *nifH* gene abundance versus P8 *nifH* gene abundance

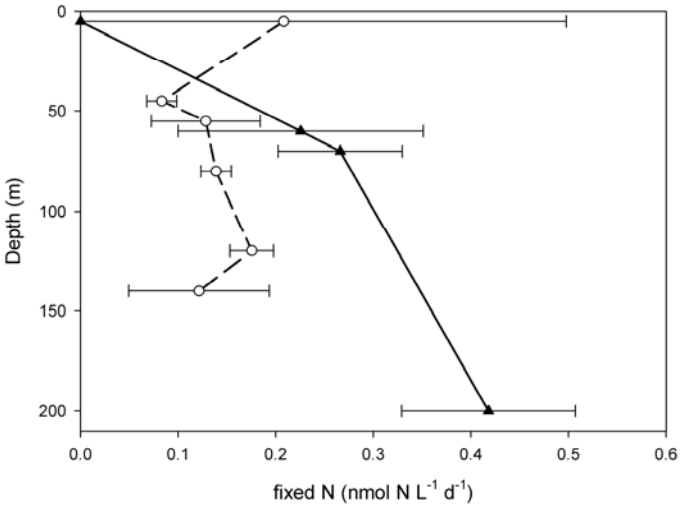


Fig. S3: **Vertical profiles of N_2- fixation**: 24h $^{15}N_2$- incubation experiments at two stations at 10°S (# 805 at 79.134°W is marked with black triangles, and # 811 at 81.361°W with open circles).

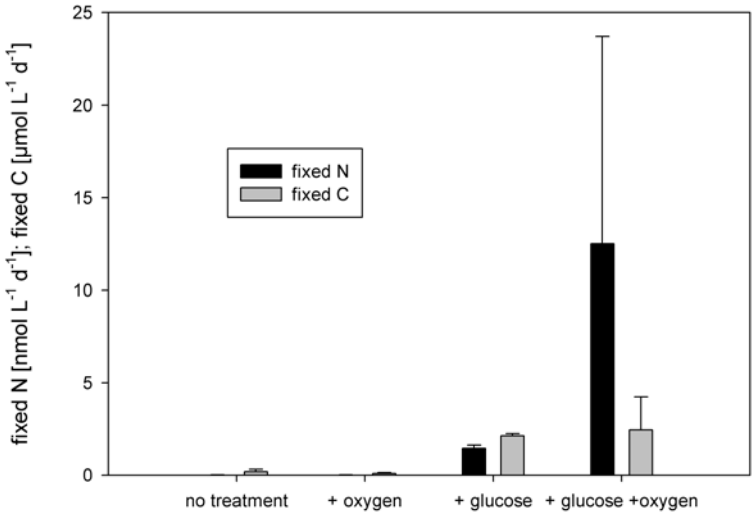


Fig. S4: N_2-fixation measured in seawater incubation experiments: N_2-fixation was triggered by glucose and glucose/ oxygen addition (#807, 20 m depth).

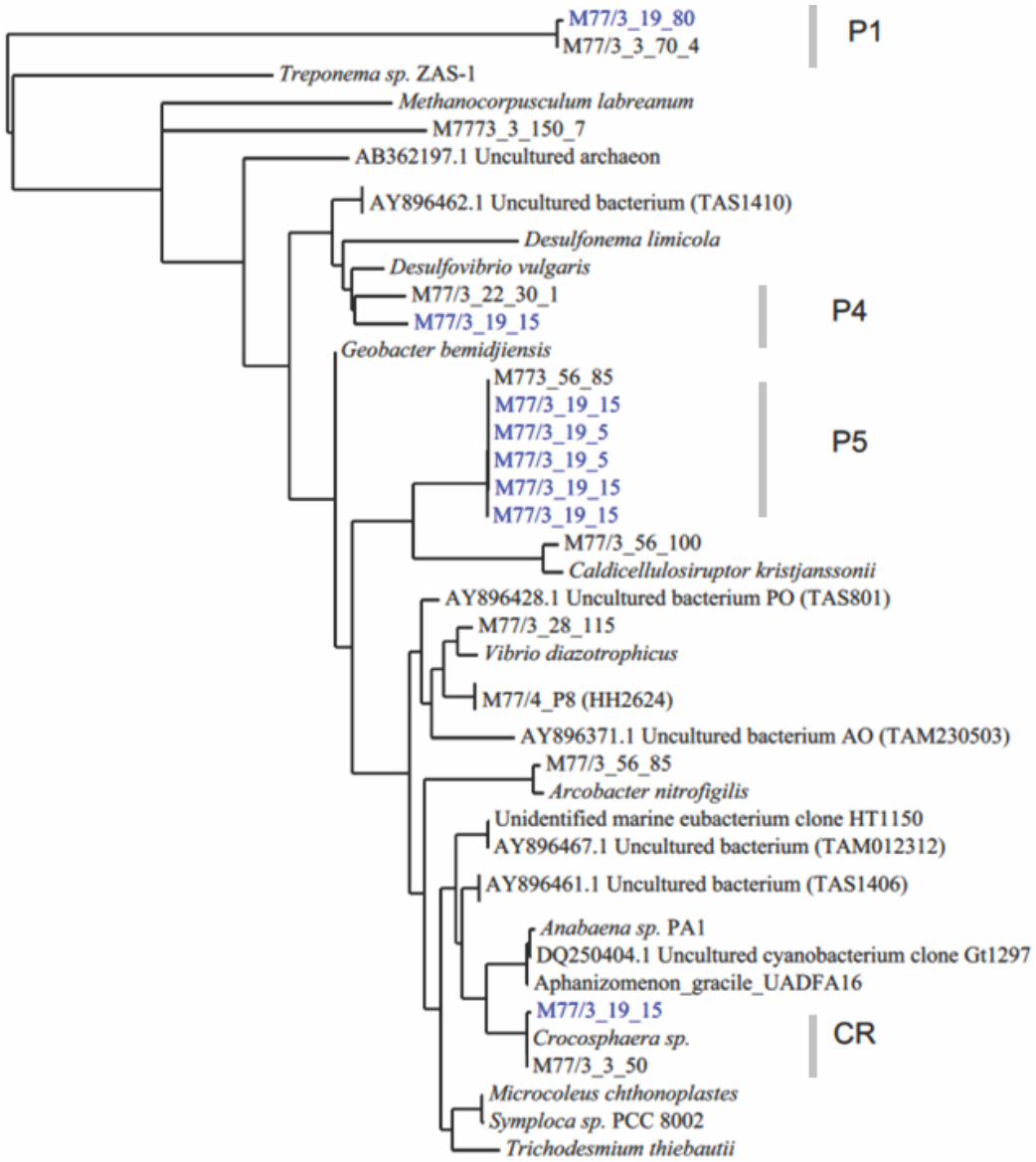


Fig. S5: Phylogenetic tree of *nifH* sequences: blue sequences were derived from cDNA *nifH* clone libraries from #19 (H_2S present), black sequences are derived from DNA *nifH* clone libraries.

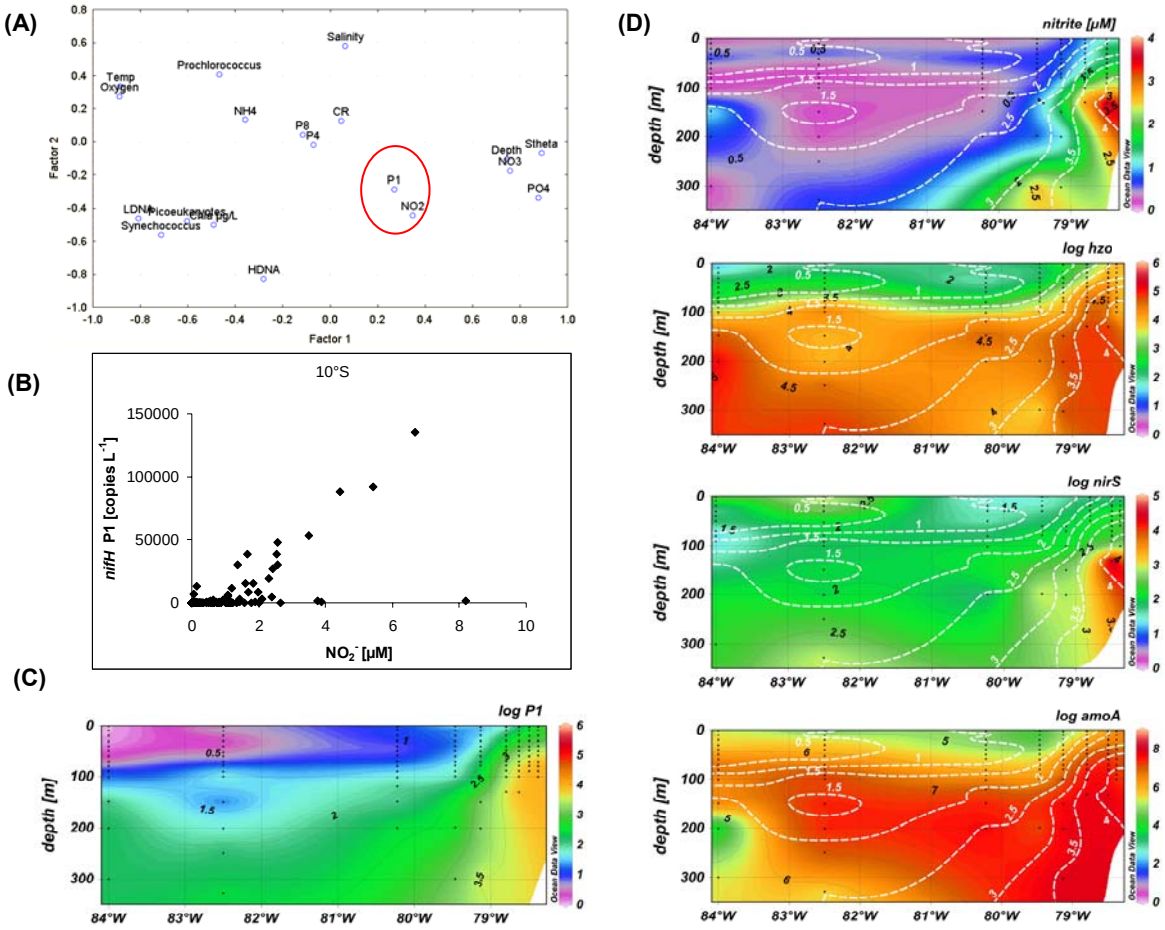


Fig. S6: Correlation of *nifH* P1 and NO_2^- : (A) The principle component analysis shows a linear correlation of cluster P1and NO_2^-, indicated by the red circle. (B) A significant correlation of P1 and NO_2^- is detected along 10°S (black dots). (C) P1 [$\log_{10}$ copies L^{-1}] and (D) NO_2^- [µM], *nirS*, *hzo* and *amoA* sections section overlaid by P1 [$\log_{10}$ copies L^{-1}] indicated by dashed white contour lines.

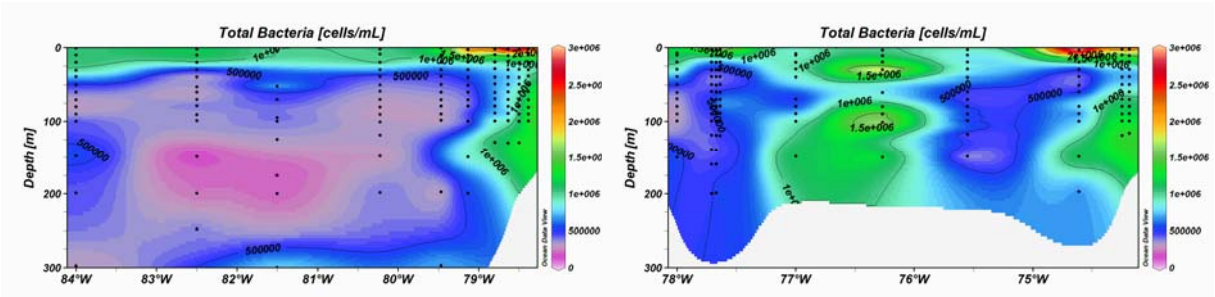


Fig. S7: Distribution of total non-green microorganisms along (A) 10°S and (B) 16°S.

O_2 dependency of the degradation of *nifH* transcripts

Compared to the sulphidic station, *nifH* gene expression was rather low or below the detection limit at other stations and in the non-sulphidic surface sample at this station; although, active N_2-fixation was detected. This discrepancy might be potentially due to the fact, that the presence of H_2S might have maintained the anoxic character of the seawater over the filtration period, thus protecting the *nifH* transcripts from degradation in samples with H_2S present. Degradation of *nifH* transcripts in other samples might have been due to a possible regulatory effects in response to O_2 on the transcription of the *nifH* gene or due to low stability of the mRNA in the presence of O_2 ($T_{1/2}$ = 2.4 min) as previously reported for mRNAs in *Prochlorococcus* (Steglich et al. 2010). We observed in pure culture studies with two diazotrophic bacteria, *Azotobacter vinelandi* (DSMZ 332), *Klebsiella pneumoniae* M5A1 and the diazotrophic archaeon *Methanosarcina mazei* Gö1 (of which the first is microaerophilic, while the latter both are anaerobic organisms) a significant decrease in *nifH* transcripts (~ 25%) after an exposure time to air of 20 min. After 60 min, no *nifH* transcripts were detectable in cultures of *A. vinelandi* and *M. mazei*, in *K. pneumoniae* cultures, no decrease compared to samples taken after an exposure time of 20 min was detectable (Fig. S8). In the context of the described discrepancy in *nifH* genes and transcripts, we propose that this is a result of exposure of the cells to O_2 during the filtration period. Short mRNA half-life times were considered to allow rapid responses to changing environmental conditions and might therefore provide advantages in highly dynamic systems. Therefore, we hypothesize the low *nifH* expression being due to the conditions during filtration rather than to the absence of transcripts.

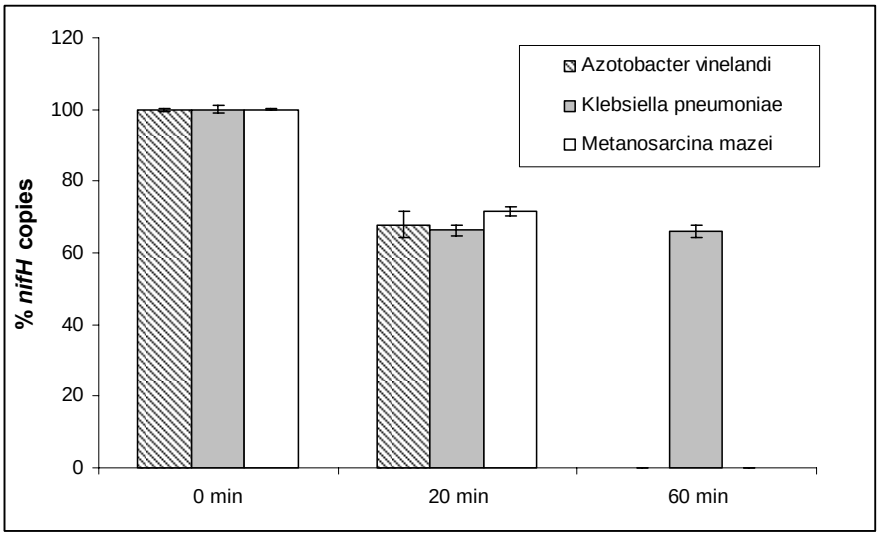


Fig. S8: Decrease in *nifH* transcripts over time after exposing cells to O_2, measured by RT-qPCR and normalized to the initial sample T_0, error bars denote the standard deviation of six technical replicates

Chapter 2: Marine pathways to nitrous oxide

Introduction

There is no doubt that oceanic N_2O emissions play a major role in the atmospheric N_2O budget. The quantification of the oceanic N_2O emissions and the identification of the marine pathways of N_2O formation and consumption have received increasing attention during the last few decades. The very first study of oceanic N_2O (in the South Pacific Ocean) was published by Craig and Gordon (Craig and Gordon 1963), followed by studies in the North Atlantic Ocean by Junge and Hahn during the late 1960s and early 1970s (Junge and Hahn 1971; Junge 1974). Junge and Hahn were the first to quantify the oceanic source of atmospheric N_2O. In 1976 Yoshinari published his now 'classical' study of N_2O profiles in the Sargasso and Caribbean Seas, which turned out to be groundbreaking because it was the first study to report the inverse correlation between N_2O and O_2 concentrations in the water column (Yoshinari 1976). He also introduced the term ΔN_2O (for a definition see below) as a measure of the 'apparent N_2O production' and found a linear correlation between ΔN_2O and AOU (apparent oxygen utilization) (Yoshinari 1976). Based on this, he suggested that 'N_2O production in the sea is related in some way to the oxidation sequence of organic matter', which was an early hint of nitrification (i.e. microbial oxidation of NH_4^+ to NO_3^-) as a major N_2O formation process in the ocean. Cohen and Gordon (Cohen and Gordon 1978), Cohen (Cohen 1978) and Elkins et al. (Elkins et al. 1978) were the first to report a significant N_2O consumption in the oxygen minimum zone in the subsurface waters of the eastern tropical Pacific Ocean and the anoxic waters of the Saanich Inlet basin (off Vancouver Island). They attributed the N_2O loss to microbial reduction of N_2O to N_2 (i.e. denitrification). In order to verify the marine pathways to N_2O, isotope studies have been introduced in recent years: first, measurements of the $\delta\,^{15}N$ value of dissolved N_2O were presented by Yoshida et al. (Yoshida et al. 1984) and nine years later Kim and Craig (Kim and Craig 1993) published the first measurement of the dual isotope signature ($\delta^{15}N$ and $\delta\,^{18}O$) of oceanic N_2O. This was followed by the publication of the isotopomeric signature of N_2O (which makes it possible to distinguish the position of ^{15}N within the asymmetric N_2O molecule: NNO) by Popp et al. (Popp et al. 2002) and Toyoda et al. (Toyoda et al. 2002).

In this chapter we present a short overview of the current knowledge about the role of the ocean as a source of N_2O and a short description about oceanic N_2O distribution. It is followed by a discussion of the major marine pathways to N_2O. In the concluding section we discuss possible consequences of climate change for both the marine pathways of N_2O and the oceanic emissions of N_2O. More information about N_2O in the ocean can be found in a recently published overview article by Bange (Bange 2008).

The role of the ocean for the global budget of atmospheric nitrous oxide

The oceanic N_2O emissions play a major role in the atmospheric N_2O budget (see for example Bange, (Bange 2006a)). In the 4th assessment report of the IPCC, mean annual N_2O emissions (ranges are given in parenthesis) of 3.8 (1.8–5.8) × 10^{12}g (Tg) N and 1.7 (0.5–2.9) Tg N were attributed to the open ocean and coastal areas (including rivers), respectively (IPCC 2007). According to the IPCC report, open ocean and coastal areas (including rivers) represent about 21 per cent and 10 per cent of the total natural and anthropogenic N_2O sources of 17.7Tg N yr^{-1}, respectively (IPCC, 2007). There are various reasons for the considerable ranges of uncertainty in the global N_2O emission estimates (Bange et al. 2008): (1) different methodological approaches (empirical models versus extrapolation of measurements), (2) the application of different air–sea exchange models and (3) the fact that the applied classification of coastal areas is not uniform.

Nitrous oxide in the ocean

Concentrations of dissolved N_2O are usually expressed as nmol $litre^{-1}$ or nmol kg^{-1}. The degree of N_2O saturation (given in per cent) is defined as the ratio of the measured N_2O concentration to the theoretical N_2O equilibrium concentration. The equilibrium concentration in turn depends on the water temperature, salinity, ambient air pressure and the atmospheric N_2O dry mole fraction at the time when the water mass was last in contact with the atmosphere (Weiss and Price 1980). An N_2O surface saturation of 100 per cent indicates that the water phase is in equilibrium with the overlying atmosphere. N_2O saturation values <100 per cent indicate undersaturation (i.e. uptake of N_2O into the water phase when measured in the ocean surface layer) whereas saturation values >100 per cent stand for supersaturation (i.e. N_2O release from the water phase to the atmosphere when measured in the ocean surface layer). The N_2O excess (or N_2O anomaly) is defined as the difference between the measured N_2O and the theoretical N_2O equilibrium value. It can be expressed either as a difference in concentration units, $[\Delta N_2O]$, or as a difference in partial pressures, ΔpN_2O.

Surface ocean

Nevison et al. (Nevison et al. 1995) calculated a global mean N_2O surface saturation of 103.5 per cent, which indicates that the ocean, on a global scale, is supersaturated with N_2O and acts as a net source of N_2O to the atmosphere. N_2O saturations in the ocean surface layer are not uniform and can show considerable seasonal variability (Nevison et al. 1995). However, the current data coverage does not make it possible to decipher the seasonality in most parts of the ocean. Global maps of ΔpN_2O in the upper 10m of the world's oceans have been computed by Nevison et al. (Nevison et al. 1995) and Suntharalingam and Sarmiento (Suntharalingam and Sarmiento 2000). Common features of both maps

(Fig. 2.1) are: (1) enhanced N_2O anomalies in the equatorial upwelling regions of the eastern Pacific and Atlantic Oceans, enhanced N_2O anomalies along coastal upwelling regions such as along the west coasts of North and Central America, off Peru, off Northwest Africa and in the north-western Indian Ocean (Arabian Sea); and (2) N_2O anomalies close to zero (i.e. near equilibrium) in the North and South Atlantic Ocean, the South Indian Ocean and the central gyres of the North and South Pacific Oceans.

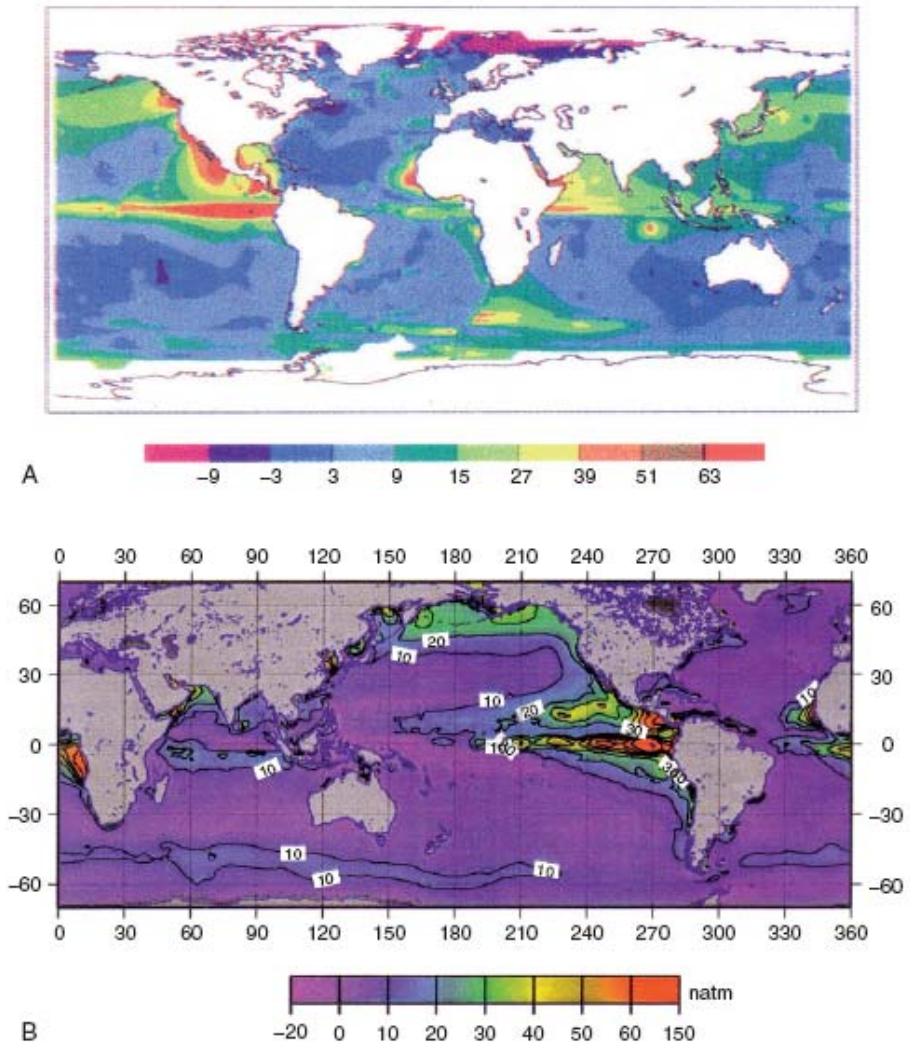


Fig. 2.1: Maps of ΔpN_2O (in natm) in the surface layer of the world's oceans: (A) map by Nevison et al. (1995) and (B) map by Suntharalingam and Sarmiento (2000). Please note that the colour coding is non-linear and different for both maps. Differences in the two maps result mainly from different computation methods. Additionally, both maps are biased by insufficient data coverage in some parts of the ocean (for example in the Indian and western Pacific Oceans).

Coastal areas

In general, enhanced N_2O emissions in coastal areas are found in upwelling systems and nitrogen-rich estuaries (Seitzinger et al. 2000; Nevison et al. 2004). However, as in the case of the open ocean emissions, flux estimates from coastal areas are heavily biased by a seasonal variability, which is, in the majority of the studies, only inadequately resolved.

The narrow bands of coastal upwelling systems such as those found in the northwestern Indian Ocean (Arabian Sea) and in the southeastern Pacific Ocean (off central Chile) have been identified as 'hot spots' for extremely high N_2O anomalies with N_2O saturations of up to 8250 per cent and 2426 per cent, respectively (Naqvi et al. 2005a; Cornejo et al. 2007). In nitrogen-rich estuarine systems, high N_2O anomalies are usually only found in the estuaries themselves, whereas the adjacent shelf waters, which are not influenced by the river plumes, are close to equilibrium with the atmosphere. Bange (Bange 2006b), for example, computed mean N_2O saturations of 113 per cent and 467 per cent for European shelf and estuarine systems, respectively.

Nitrous oxide distribution in the water column

The shapes of N_2O profiles generally fall into three categories:

[b]** Cat. I profiles from oceanic regions with dissolved oxygen concentrations $[O_2]$ >10µmol litre^{-1} throughout the water column (for example in the Atlantic Ocean, the South Indian Ocean and the central North Pacific and central South Pacific Oceans);

** Cat. II profiles from regions with sub-oxic environments (0 <$[O_2]$ <2–10µmol litre^{-1}, (Codispoti et al. 2005)) such as found in intermediate water depths from about 200m to about 800m in the Arabian Sea and the eastern North/South Pacific Ocean;

** Cat. III profiles from regions with anoxic deep water masses with $[O_2]$ = 0µmol litre^{-1} and hydrogen sulphide present. Anoxic water masses are found only in a few regions of the world's oceans. Perennial anoxic environments occur in the Black Sea and the Cariaco Basin off Venezuela. Temporarily occurring anoxic conditions have been reported from the deep basins of the central Baltic Sea.

Typical N_2O profiles illustrating Cat. I-III profiles are shown in Figure 2.2 (additional examples and references can be found in Bange, 2008). It is obvious that the shapes of the N_2O profiles undergo a significant change when $[O_2]$ falls below the threshold for sub-oxic conditions. For instance, the one-peaked profiles (Cat. I) observed in the southern Arabian Sea turn into two-peaked profiles in the central Arabian Sea where sub-oxic conditions are found in the intermediate layers (Bange et al.

2001). Cat. III show no pronounced N_2O peak at the boundary of the oxic and anoxic water masses (Hashimoto et al. 1983; Walter 2006; Westley et al. 2006).

The characteristics of the profiles described above are valid for 'static' oceanic systems under steady-state conditions with turnover times much longer than one year. Some coastal areas, however, show a dynamic behaviour, with a rapid seasonal overturning from oxic via sub-oxic to anoxic conditions and vice versa (for example the shelf off West India, the western Baltic Sea, the shelf off Chile, an upwelling area off southwest Africa and the Gulf of Mexico). In these kinds of transient systems, significant amounts of N_2O can accumulate temporarily during the short transition time when the system is about to change its oxygen regime. Interestingly, the timing of the N_2O accumulation occurs at different transition stages and seems to be characteristic for different coastal systems: in the south-western Baltic Sea, N_2O only accumulates when the system is shifting from anoxic to oxic conditions (Figure 2.3, (Schweiger et al. 2007)), whereas N_2O accumulates when the systems are shifting from oxic to sub-oxic (off central Chile) or to anoxic (off West India) conditions (Naqvi et al. 2006; Cornejo et al. 2007) . During the transition stages, the accumulation of N_2O does not occur in the anoxic zones itself but at the oxic/anoxic boundaries. In anoxic zones, N_2O is usually found at very low or even undetectable concentrations.

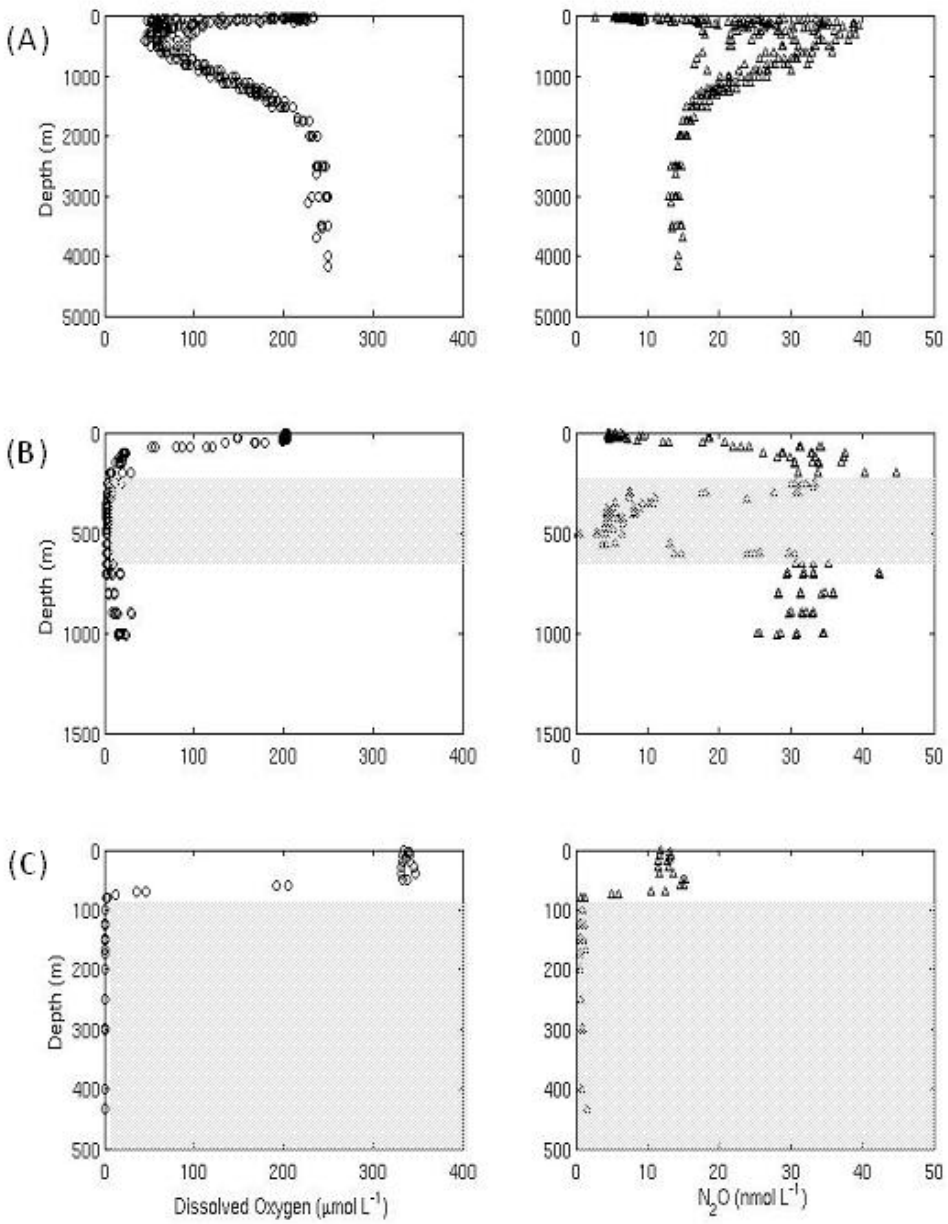


Fig. 2.2: Typical N_2O profiles (right column) and dissolved O_2 (left column): (panel A) Cat. I profiles from the tropical North Atlantic Ocean; (panel B) Cat. II profiles from the Guinea Dome in eastern Tropical North Pacific Ocean; (panel C) Cat. II profile from the Landsort Deep in the western Gotland Basin (central Baltic Sea)

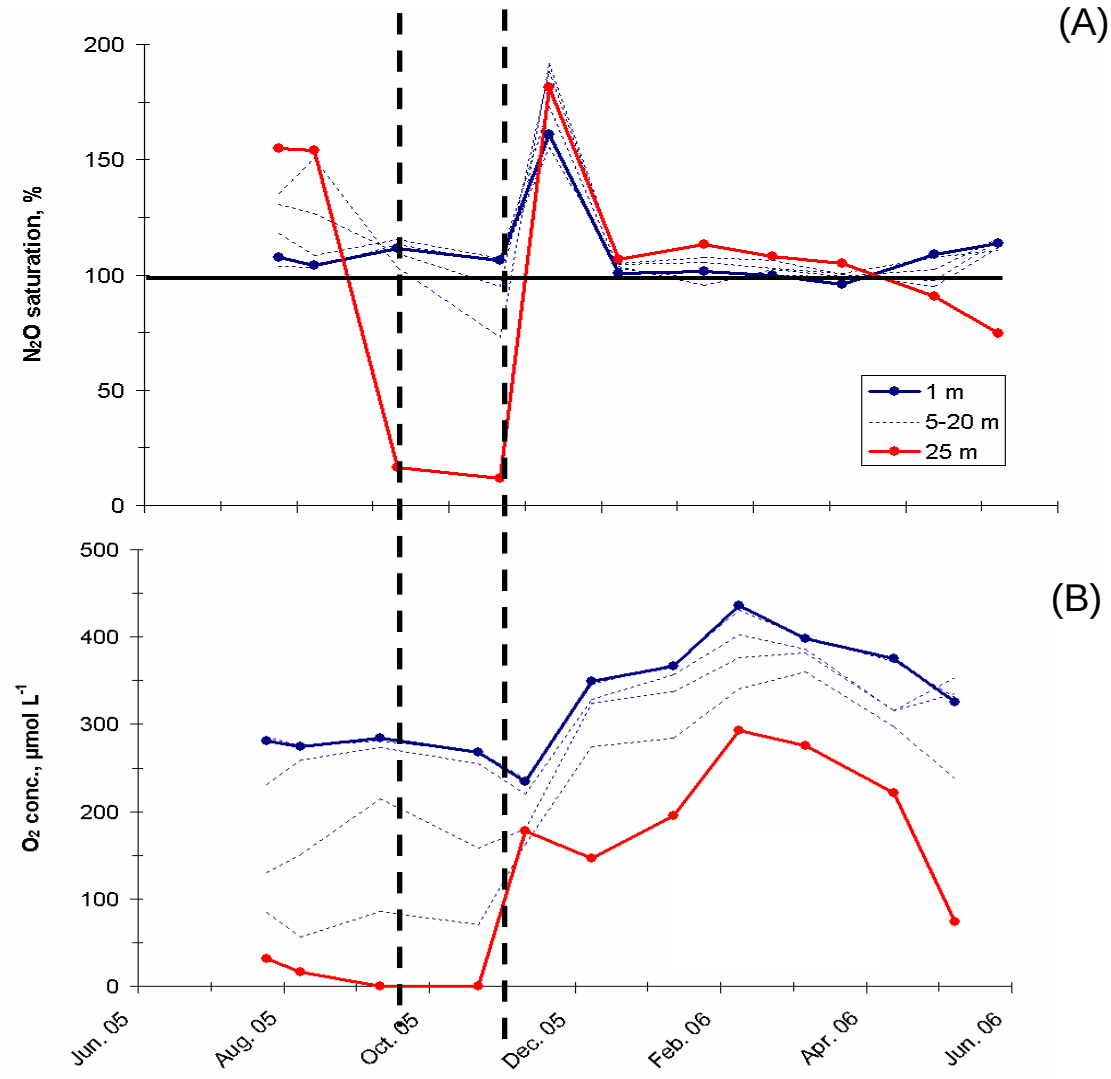


Fig. 2.3: N_2O saturations (A) and O_2 concentrations (B) at the time series station Boknis Eck (south-western Baltic Sea, 54°31'N, 10°02'E, max. depth 28m) measured on a monthly basis from July 2005 to May 2006

Major pathways

Today's prevailing view is that there are only two dominating microbial processes, i.e. bacterial nitrification and bacterial denitrification, during which oceanic N_2O is formed either as a by-product or as an intermediate (Figures 2.4 and 2.5). The global budget of oceanic N_2O sources and sinks given in Bange and Andreae (Bange and Andreae 1999) indicates that about 35 per cent of the oceanic N_2O is produced during denitrification, with the rest resulting from nitrification.

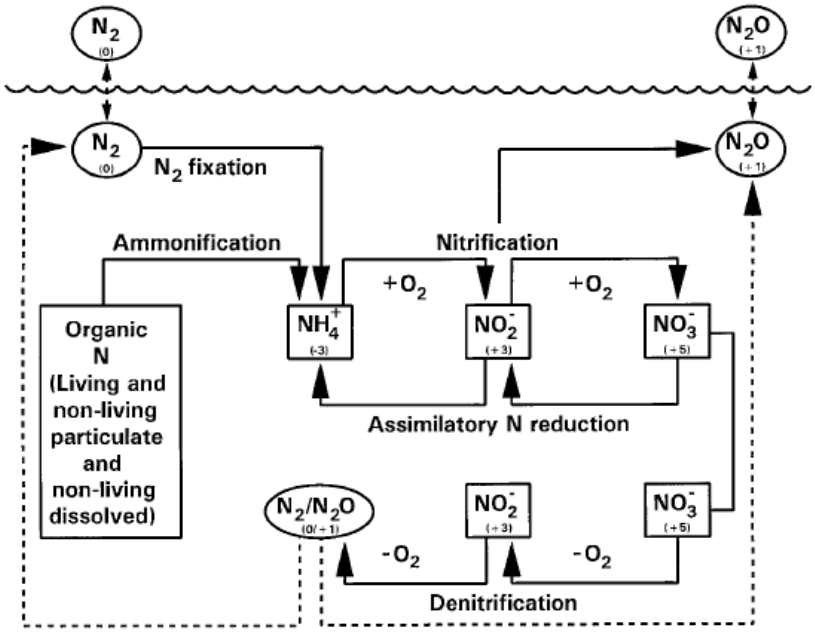


Fig. 2.4: Simplified sketch of the oceanic nitrogen cycle

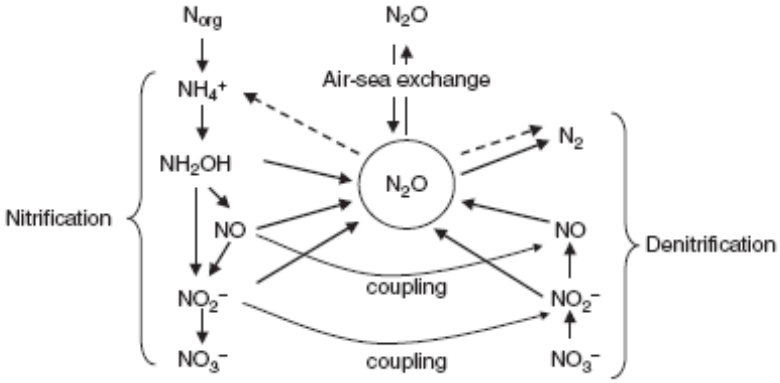


Fig. 2.5: Overview of processes, which influence the N_2O distribution in the ocean.

Bacterial denitrification

Denitrification results in a loss of bio-available (fixed) nitrogen in the form of gaseous products such as N_2O and N_2 (for details on denitrification see the overview article by (Devol 2008)):

$$NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2.$$

As can be seen from the denitrification reaction sequence, N_2O is an intermediate, with its concentration at any time determined by the balance between production and consumption to N_2. The net accumulation of dissolved N_2O depends on the dissolved O_2 concentrations (see below). Under extreme O_2 depletion (such as found in the intermediate depths of the eastern tropical Pacific Ocean and the Arabian Sea, see above) there is a net N_2O consumption during denitrification, resulting in low N_2O concentrations. Denitrification is a well-known feature of many different bacteria species in terrestrial and oceanic environments. Denitrifiers are facultative anaerobic bacteria, which can reduce

NO_3^- when oxygen becomes limiting. Thus the occurrence of denitrification is favoured under sub-oxic ($0 < O_2 < 2–10 \mu mol\ litre^{-1}$, (Codispoti et al. 2005)) conditions. Denitrification does not occur under anoxic conditions ($O_2 = 0 \mu mol\ litre^{-1}$, hydrogen sulphide present).

Bacterial nitrification

Nitrification is the oxidation of ammonium, NH_4^+, to NO_3^- via hydroxylamine, NH_2OH, and nitrite, NO_2^-. For details about nitrification see the overview article by Ward (2008). Autotrophic nitrification represents the final step of the remineralization of nitrogen containing organic matter and is performed in two steps by AOB (for example *Nitrosomonas, Nitrosospira and Nitrosococcus*) and nitrite-oxidizing bacteria (NOB) (for example *Nitrobacter* and *Nitrospira*), respectively:

$$\text{AOB:}\quad NH_4^+ \rightarrow NH_2OH\ (\rightarrow NO) \rightarrow NO_2^-$$

$$\text{NOB:}\quad NO_2^- \rightarrow NO_3^-$$

NO is not known to be an obligatory intermediate during ammonium oxidation. It can be produced by AOB but the mechanism is not well understood. During autotrophic nitrification N_2O can be formed by AOB either via the pathways $NH_2OH \rightarrow N_2O$ and $NO \rightarrow N_2O$ or via the pathway $NO_2^- \rightarrow NO \rightarrow N_2O$ (the latter is part of the so-called nitrifier–denitrification process). Nitrification is an aerobic process, however, under low-oxygen (sub-oxic) conditions, N_2O yields are enhanced. Alternatively, N_2O can be formed during heterotrophic nitrification (i.e. nitrification linked to aerobic denitrification) via the reaction $NO_2^- \rightarrow NO \rightarrow N_2O$ as well, but the enzymes involved in the heterotrophic reaction sequence are different from those involved in the autotrophic pathway. Under oxic conditions, N_2O yields from heterotrophic nitrification are higher than those from autotrophic nitrification. However, the relevance of heterotrophic nitrification in the marine environment is not known yet.

Both nitrification and denitrification as sources and sinks of oceanic N_2O have been described in the water column, in the sediments and in association with suspended particles (for example (Schropp et al. 1985; Seitzinger 1990; Michotey and Bonin 1997; Nevison et al. 2003; Codispoti et al. 2005)). N_2O yields from nitrification range from 0.004 per cent to 0.4 per cent, whereas the N_2O yield from the denitrifying sub-oxic zone in the Arabian Sea was estimated to be about 2 per cent (see overview in Bange (Bange et al. 2008)). Culture studies with strains of nitrifiers revealed that the N_2O yield from nitrification is significantly enhanced (up to 10 per cent) under sub-oxic conditions (Goreau et al. 1980). N_2O yields from sedimentary denitrification range from 0.1 per cent to 0.5 per cent, with values up to 6 per cent in nutrient-rich regions (see overview in (Seitzinger and Kroeze 1998)).

Nitrous oxide–oxygen gas relationship

The relationship between oceanic N_2O production/consumption and dissolved O_2 concentrations is shown schematically in Figure 2.6. While the influence of O_2 concentrations on the N_2O production via nitrification is still lacking a mechanistic explanation, the influence of O_2 on denitrification and thus N_2O production results from two factors: (1) the redox potential of NO_3^- respiration favours denitrification under reduced O_2 concentrations (see for example (Falkowski et al. 2008)) and (2) the enzyme involved in N_2O consumption, N_2O reductase, is sensitive to O_2 concentrations (Firestone and Tiedje 1979). For example, Naqvi *et al.* (Naqvi et al. 2000) attributed the accumulation of N_2O off West India to the onset of denitrification at low O_2 concentrations, with the assumption that the activity of the N_2O reductase could not be established because of frequent aeration of the shallow shelf waters (so-called stop-and-go denitrification).

The apparent oxygen utilization (AOU) is a measure of the amount of O_2 consumed during organic matter remineralization (oxidation) in the ocean. Because nitrification is part of the organic matter oxidation sequence, plots of ΔN_2O versus AOU have been used to identify the prevailing formation and consumption processes of N_2O in the water column. The overwhelming majority of the Cat. I profiles (see above) show positive linear ΔN_2O/AOU relationships, suggesting that nitrification is the main N_2O formation process in most parts of the oceans (Bange and Andreae 1999). This is supported by the fact that in most oxic water columns N_2O is positively correlated with dissolved nitrate (NO_3^-), the final product of nitrification (see for example (Walter 2006)). However, there are caveats against a straightforward interpretation of the linear ΔN_2O/AOU relationship as an indicator for N_2O formation via nitrification because a linear ΔN_2O/AOU relationship may not necessarily result from nitrification alone: most recently, based on N_2O isotopomeric data (see below), Yamagishi *et al.* (Yamagishi et al. 2005) argued that net N_2O formation in the oxygen minimum zone (OMZ) of the western North Pacific Ocean mainly results from denitrification with only a small contribution from nitrification.

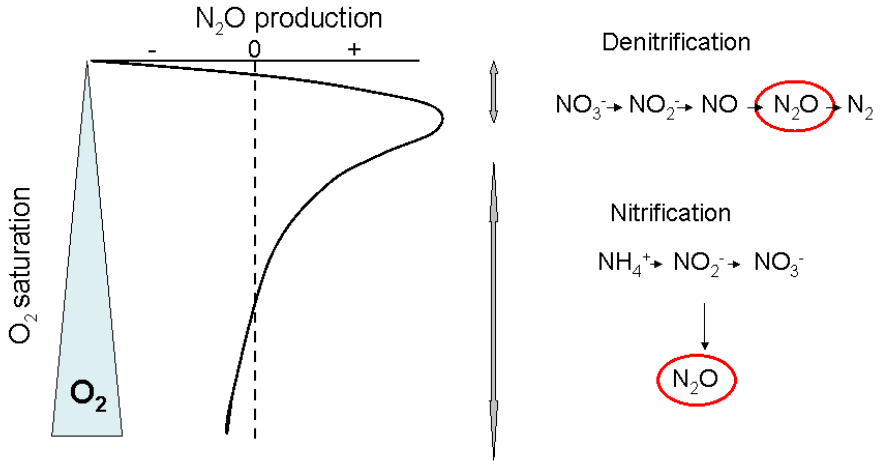


Fig. 2.6: N_2O production versus O_2 saturation in the ocean, modified from Codispoti *et al.* (Codispoti et al. 1992)

They showed that this N_2O, when diffusing into deep waters, produces a reasonably linear ΔN_2O/AOU relationship. Moreover, by applying a two end-member mixing model, Nevison *et al.* (Nevison et al. 2003) showed that isopycnal mixing of water masses with different preformed N_2O and O_2 concentrations can result in a linear ΔN_2O/AOU relationship, which can mask the 'true' biological N_2O production. They state:

> We find that the biological N_2O yield per mole O_2 consumed cannot be calculated with great confidence from cross-plot correlation slopes. The essential problem is that the N_2O yield is spatially variable. As a result, strong mixing gradients exist in the data that can overwhelm more subtle N_2O production terms (Nevison et al. 2003).

A linear ΔN_2O/AOU relationship does not exist in sub-oxic and anoxic water masses (i.e., Cat. II and Cat. III profiles, see above) indicating a complex interplay between N_2O formation and consumption during denitrification and/or a coupling of nitrification and denitrification at the upper boundary of the sub-oxic zones (see for example (Bange et al. 2005; Walter et al. 2006; Westley et al. 2006; Farías et al. 2007; Yamagishi et al. 2007)).

Isotope studies

The isotope ratio $^{15}N/^{14}N$ of N_2O is expressed as $\delta\,^{15}N_{atm}$ relative to atmospheric N_2:

$$\delta\,^{15}N_{atm}\ (\text{sample})\ [‰] = ((^{15}N/^{14}N)_{sample} / (^{15}N/^{14}N)_{std} - 1) \times 1000.$$

In the same way, the isotope ratio $^{16}O/^{18}O$ of N_2O is usually expressed as $\delta\,^{18}O_{VSMOW}$ relative to Vienna standard mean ocean water (VSMOW). However, in some cases $\delta\,^{18}O_{atm}$ relative to O_2 in the atmosphere is reported; $\delta\,^{18}O_{VSMOW}$ can be converted to $\delta\,^{18}O_{atm}$ with the equation (Kim and Craig 1993):

$$\delta\,^{18}O_{atm} = -23.0 + \delta\,^{18}O_{VSMOW} / 1.0235$$

Mean $\delta\,^{15}N_{atm}$ and $\delta\,^{18}O_{VSMOW}$ of N_2O in the troposphere are 6.72 ± 0.12 per ml and 44.62 ± 0.21 per mil, respectively (Kaiser et al. 2003).

The isotopic composition of oceanic N_2O is determined by its atmospheric imprint, the isotopic signals of biological sources and sinks, and mixing processes within the ocean. This, in turn, implies that there are characteristic signals of enrichment or depletion (so-called fractionation), which can be attributed to different biological processes as well as physical processes. The isotopic signature of biologically

derived N_2O depends on the isotopic composition of the substrates such as NO_3^- (denitrification) and NH_4^+ (nitrification) and the isotopic depletion/enrichment during these processes. An overview of the isotopic depletion/enrichment of N_2O from culture experiments is shown in Figure 2.7. It is obvious that the range of the resulting nitrogen depletion in N_2O during denitrification and nitrification is similar. The isotopic signal of oxygen in N_2O produced during nitrification is introduced by the $\delta\,^{18}O$ value of both dissolved O_2 and H_2O (Ostrom et al. 2000). The isotopic signal resulting from air–sea exchange is small compared to the biological processes; therefore, biological N_2O formation should yield a clear isotopic signature in oceanic N_2O. However, the identification of nitrification or denitrification as N_2O producing processes strongly depends on the knowledge of the isotopic signatures of the substrates, which can vary temporarily and spatially. A detailed overview of studies of the isotopic signature of oceanic N_2O is given in (Bange 2008). The main results of actual N_2O isotopic studies are summarized in the following sections.

Repeated measurements of N_2O depth profiles at the Hawaii ocean time series station ALOHA in the subtropical North Pacific Ocean revealed that $\delta\,^{15}N$ and $\delta\,^{18}O$ of N_2O were in equilibrium with tropospheric N_2O at the ocean surface and steadily decreased from the ocean surface to minimum values at about 100–300m depth at the base of the euphotic zone, followed by an increase to maximum values at 800m. The depletion of both ^{15}N and ^{18}O was attributed to nitrification (Dore et al. 1998; Ostrom et al. 2000; Popp et al. 2002). A more detailed study at ALOHA that included measurements of $\delta\,^{18}O$ in dissolved O_2 and H_2O, revealed that N_2O might be formed by two different pathways: first, by nitrification via NH_2OH or NO at most depths and, second, by nitrifier–denitrification via reduction of NO_2^- (between 350 and 500m (Ostrom et al. 2000)).

The situations in the central Arabian Sea and the eastern tropical North Pacific Ocean are more complex. N_2O was found to be strongly enriched in both ^{15}N and ^{18}O in the denitrifying oxygen minimum zone, whereas N_2O in the surface layer was depleted in ^{15}N but slightly enriched in ^{18}O compared to tropospheric N_2O (Yoshinari et al. 1997; Naqvi et al. 1998b; Naqvi et al. 1998a). N_2O in the core of the oxygen minimum zone was obviously formed by denitrification, since the final reduction step to N_2 should result in enriched N_2O. However, the 'light' N_2O found above the OMZ might be explained by a coupled nitrification-denitrification pathway where NO is formed during nitrification which is then reduced to N_2O during denitrification (Yoshinari et al. 1997; Naqvi et al. 1998b; Naqvi et al. 1998a).

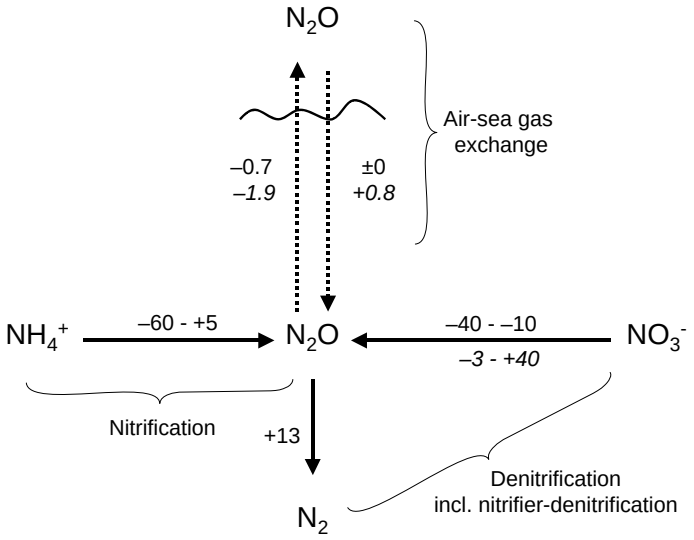


Fig. 2.7: Isotopic depletion/ enrichment for nitrogen and oxygen in N_2O relative to the substrates NO_3^- and NH_4^+, and the product N_2; Note: Values are given in ‰, Source: Bange (2008).

As mentioned in the introduction, N_2O is an asymmetrical molecule and therefore it is possible to distinguish so-called isotopomers according to the position of ^{15}N within the N_2O molecule (the corresponding δ notation is given in parenthesis): $^{14}N^{15}NO$ ($\delta\ ^{15}N^{\alpha}$) and $^{15}N^{14}NO$ ($\delta\ ^{15}N^{\beta}$) (Toyoda and Yoshida 1999). The ^{15}N site preference (SP_{N2O}) in N_2O is given as $\delta\ ^{15}N^{\alpha} - \delta^{15}N^{\beta}$. Measurements of SP_{N2O} should allow for the identification of the mechanisms of N_2O formation according to the different microbial pathways (Sutka et al. 2003, 2004). Based on the results of a study with cultures of AOB, nitrifier–denitrifiers and denitrifiers, Sutka et al. (Sutka et al. 2006) concluded that the characteristic SP_{N2O} of nitrification and denitrification (including nitrifier–denitrification) are generally ~33 per mil and ~0 per mil, respectively. Thus, isotopomers might be used to distinguish between N_2O produced during oxidation (nitrification) and reduction (denitrification and nitrifier–denitrification) processes; however, it seems that isotopomers cannot be used to reveal subtle differences such as that between nitrifier–denitrification and denitrification (Schmidt et al. 2004; Sutka et al. 2006). So far, the oceanic distributions of N_2O isotopomers have been determined at a few stations in the North and South Pacific Oceans(Popp et al. 2002; Toyoda et al. 2002; Yamagishi et al. 2005; Charpentier et al. 2007), in the sub-oxic eastern tropical North Pacific Ocean and Gulf of California (Yamagishi et al. 2005; Yamagishi et al. 2007), and in the anoxic Black Sea (Westley et al. 2006). In general, the conclusions from the SP_{N2O} distribution are in overall agreement with the ΔN_2O/AOU relationships and bulk isotopic signatures of N_2O as described above. In the North Pacific Ocean SP_{N2O} values of up to 35 per mil were determined, indicating that nitrification is the main N_2O formation process throughout the water column (Popp et al. 2002; Toyoda et al. 2002).

An additional significant contribution by nitrifier–denitrification within particles was suggested for the pycnocline at 250–350m in the central South Pacific subtropical gyre because a SP_{N2O} minimum of only 10–12 per mil was found at that depth range (Charpentier et al. 2007). In contrast, Yamagishi et al. (Yamagishi et al. 2005) suggested a net N_2O formation in the oxygen minimum zone of the western North Pacific Ocean, which mainly results from both formation and consumption during denitrification, with only a minor contribution by nitrification. SP_{N2O} data from sub-oxic (eastern tropical North Pacific Ocean, Gulf of California) and anoxic (Black Sea) environments indicate that N_2O production via nitrification and N_2O production/consumption via denitrification can be coupled and might even be concurrent at the oxic to sub-oxic/anoxic interfaces (Westley et al. 2006; Yamagishi et al. 2007).

Marine pathways to nitrous oxide and climate change

The lesson from the past

The atmospheric history of N_2O is illustrated by the ice core records which now reach back to 650,000 years before the present (yr BP) (Spahni et al. 2005). It seems that atmospheric N_2O concentrations followed glacial climate changes but in a complex way (Spahni et al. 2005). The significant variability of the atmospheric N_2O concentrations have been attributed to concurrent changes in both the terrestrial source and in the oceanic source (Sowers et al. 2003; Flückiger et al. 2004). However, the ice core data do not allow for identification of the key parameters responsible for the abrupt changes of the N_2O sources. More recently, coupled climate/biogeochemistry models were applied to investigate the role of the oceanic N_2O production during fast climate changes such as the Younger Dryas cold period (~12,000 yr BP) (Goldstein et al. 2003) and the Heinrich event H5 (~48,000 yr BP) (Schmittner and Galbraith 2008). In both models the oceanic N_2O production was parameterized as a function of AOU. The model results of Goldstein et al. (Goldstein et al. 2003) suggested that the variability of the oceanic N_2O source was the main but not the sole contributor to the observed changes of atmospheric N_2O. The model results of Schmittner and Galbraith (Schmittner and Galbraith 2008) showed that the abrupt changes of atmospheric N_2O during the Heinrich event H5 could have been caused by variabilities of the oceanic sources alone. They proposed that changes of the ocean circulation results in fast adjustments of the oxygen concentrations in the thermocline, which in turn drives the oceanic N_2O production via nitrification (Schmittner and Galbraith 2008).

Another line of argument is derived from $\delta\ ^{15}N$ records from sediments underlying sub-oxic denitrification zones in the open ocean: several studies showed that the temporal variations of the denitrification signal in both the Arabian Sea and the eastern tropical Pacific Ocean during the last 23,000 years is paralleled by the reconstructed atmospheric N_2O concentration from ice core records (Figure 2.8) (Suthhof et al. 2001; Thunell and Kepple 2004; Agnihotri et al. 2006; Pichevin et al.

2007). These results imply that variations in the amount of the water column denitrification might have led to changes in the magnitude of N_2O formation and its subsequent release to the atmosphere.

On the basis of the model results and sedimentary $\delta^{15}N$ records introduced above we can conclude that the rapid changes observed in the paleorecord of N_2O concentration might be dominated by changes in the oceanic N_2O production (nitrification and/or denitrification) via pronounced changes of the dissolved oceanic oxygen concentrations.

The ongoing rapid increase in atmospheric N_2O, which started during the 19th century, is mainly attributed to the increase of agricultural activities (Kroeze et al. 1999; Ishijima et al. 2007), which in turn was caused by the expansion of agricultural land and industrialization that came along with the increasing availability of agricultural fertilizers triggered by the development of the Haber-Bosch process. A potential indirect contribution by oceanic sources (for example increased N_2O emissions as a result of eutrophication of coastal areas) has not been quantified yet.

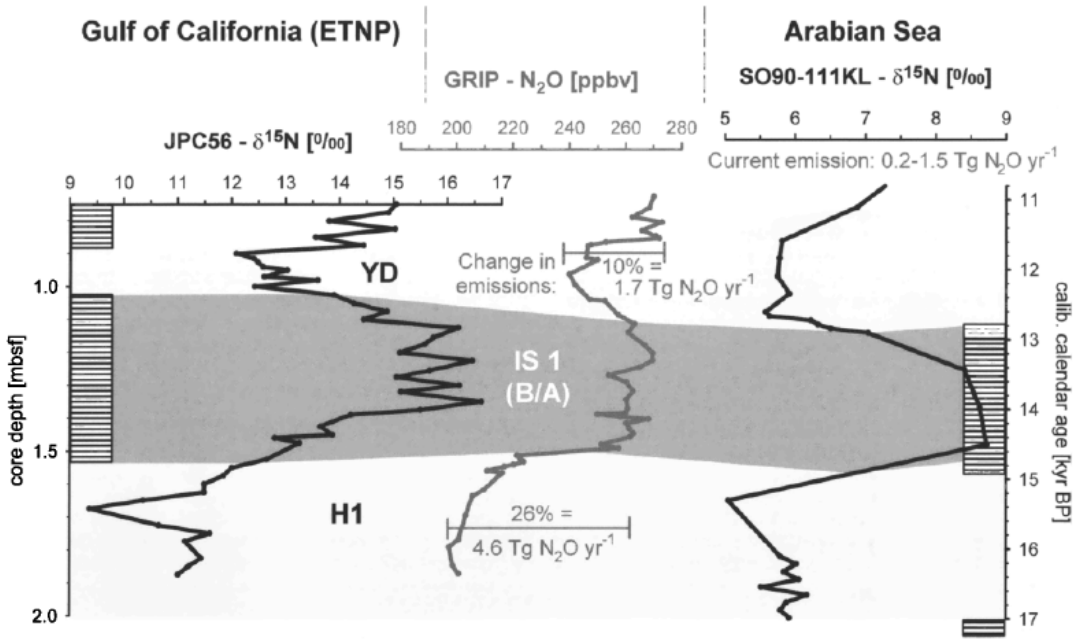


Fig. 2.8: $\delta^{15}N$ profiles from sediment cores in the Gulf of California/eastern tropical North Pacific, ETNP (core no. JPC56) and Arabian Sea (core no. SO90-111KL) compared to reconstructed atmospheric N_2O data from the GRIP (Greenland Ice Core Project) ice core

Present day and future scenarios

Coastal Ocean

As mentioned above, oceanic N_2O is exclusively produced by biological processes, thus, its production is indirectly linked to the biological productivity of the coastal and open oceans (Figure 2.9). This, in turn, implies that we have to understand how eutrophication of the coastal areas and fertilization of the open ocean influences the productivity and the resulting O_2 depletion during the remineralization (oxidation) of organic material. Both nitrification and denitrification are involved in the remineralization process and the N_2O yield of both processes depends on the prevailing O_2 concentrations (see above).

On the basis of N_2O measurements on the shelf of the west coast of India, Naqvi et al. (Naqvi et al. 2000) cautioned that the N_2O emissions from shallow sub-oxic/anoxic coastal systems might increase in the future due to the fact that the number of coastal regions with severely depleted dissolved oxygen concentrations is currently increasing worldwide (UNEP 2004; Díaz and Rosenberg 2008). Eutrophication can also significantly stimulate the sedimentary N_2O formation by denitrification, which was demonstrated by Seitzinger and Nixon (Seitzinger and Nixon 1985) in microcosm experiments. This is in line with observations that N_2O release from mangrove ecosystems appear to be very sensitive to eutrophication: N_2O release across the mangrove sediment/atmosphere interface can be enhanced up to 2800 times when adding NH_4^+ and/or NO_3^- (Bauza et al. 2002; Muñoz-Hincapié et al. 2002; Kreuzwieser et al. 2003). Therefore, it seems realistic to expect that the N_2O emissions from shallow sub-oxic/anoxic coastal systems (including mangrove ecosystems) will increase in the near future due to increasing nutrient inputs caused by the ongoing industrialization and intensification of agricultural activities.

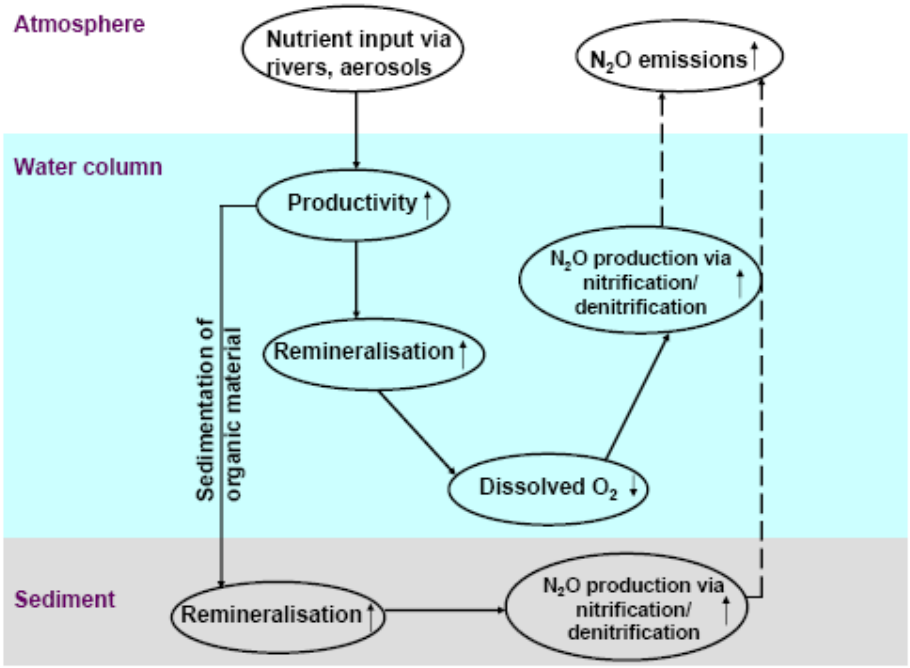


Fig. 2.9: Simplified scheme of processes leading to enhanced N_2O formation in coastal areas.

Open Ocean

Only recently, Stramma et al. (Stramma et al. 2008) showed that the oxygen minimum zones of the intermediate layers (300 to 700m water depth) in various regions of the ocean are expanding and have been losing oxygen with rates ranging from 0.09 ± 0.21µmol kg^{-1} $year^{-1}$ (in the eastern equatorial Indian Ocean) up to 0.34 ± 0.13µmol kg^{-1} $year^{-1}$ (in the eastern tropical Atlantic Ocean) during the last 50 years. In order to assess the maximum associated N_2O formation, we may roughly estimate the additional long-term N_2O formation in the tropical Atlantic Ocean: assuming a mean $\Delta N_2O/AOU$ ratio of 10^{-4} (Walter et al. 2006) we compute an additional N_2O concentration of 1.7nmol kg^{-1}. This translates into a contribution of 6 per cent of the actual mean N_2O background concentration of about 30nmol kg^{-1} at 500m depth in the tropical North Atlantic Ocean (Walter et al. 2006). However, an N_2O accumulation at intermediate water depths in the open ocean will not lead to an immediate release of N_2O to the atmosphere because these waters are not in direct contact with the atmosphere. A major fraction of the accumulated N_2O will be subsequently released to the atmosphere from other oceanic regions when the water masses are brought back to the ocean surface.

A future increase in N_2O emissions have been suggested as indirect results of enhanced productivity via increases in nitrogen or iron (Fe) aerosol deposition (Fuhrman and Capone 1991; Jin and Gruber 2003; Duce et al. 2008), N_2 fixation (Karl 1999) and riverine nutrient inputs (Naqvi and Jayakumar 2000) (Figure 2.10).

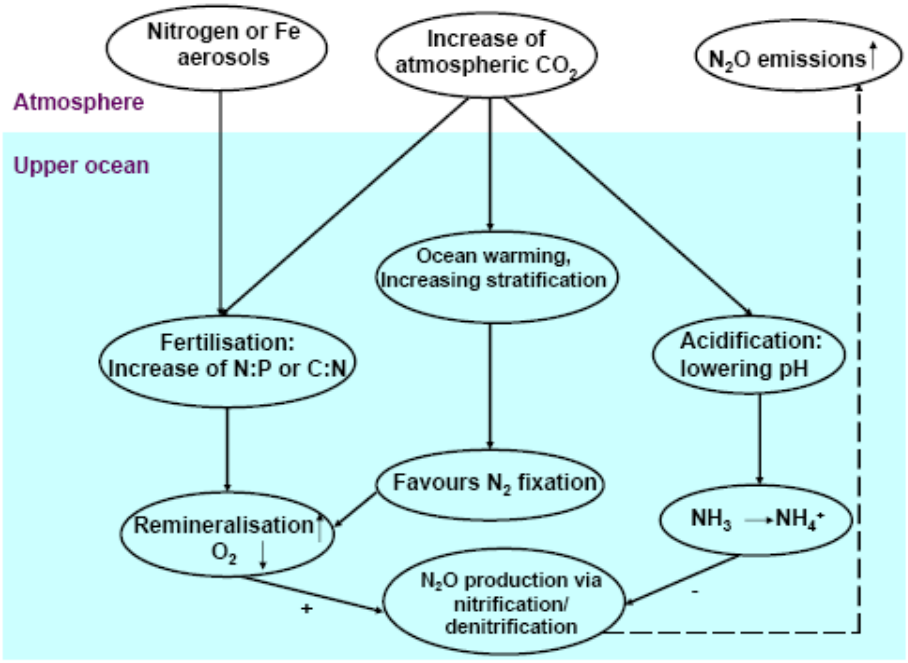


Fig. 2.10: Simplified scheme of processes leading to future N_2O formation and/or consumption. Up and down arrows within the ovals stand for increase or decrease, respectively. The plus sign (+) stands for a potential enhancement of N_2O production, whereas the minus sign (–) stands for a decrease in N_2O production.

Results from a coupled global climate/ocean-circulation/-biogeochemistry model applying a business-as-usual CO_2 emission scenario indicate that the sub-oxic areas and N_2O production in the open ocean are not likely to change significantly during the next 100 years (Schmittner et al. 2008). The picture changes when the model is run for the next 4000 years: then, indeed, a 64 per cent increase of the oceanic N_2O production because of decreasing O_2 concentrations in the open ocean is predicted (Schmittner et al. 2008). A more dramatic short-term expansion of the open ocean sub-oxic areas ($[O_2]$ <5 µmol litre^{-1}) during the next 90 years was predicted with the same model when using unusually high C: N ratios (for the remineralized organic material) that were derived from mesocosm experiments simulating future high atmospheric CO_2 concentrations (Oschlies et al. 2008). One would expect that this also changes the near-future N_2O production and emissions, however, the effect of high C:N ratios on the N_2O production was not investigated in the study by Oschlies et al. (Oschlies et al. 2008).

Ocean acidification (Raven et al. 2005), caused by increasing atmospheric CO_2 ($CO_2 + H_2O \rightarrow HCO_3^- + H^+$), could lead to a counteracting effect because it shifts the oceanic NH_3/NH_4^+ equilibrium towards NH_4^+ with consequences for AOB because they preferably take up NH_3 and not NH_4^+ (Ward 2008). Therefore, an overall decrease of the oceanic NH_3 concentrations might lead to a decrease in nitrification (Huesemann et al. 2002) and has the potential to decrease N_2O production via nitrification as well.

Nitrous oxide emissions and winds

The final release or uptake of N_2O across the ocean/atmosphere interface depends on physical processes such as wind-driven air–sea gas exchange (Wanninkhof 2007) and wind-driven oceanic circulation/mixing processes (coastal upwelling, storm events). For example, (Naik et al. 2008) showed that a storm can deepen the mixed layer considerably, thereby entraining N_2O from the subsurface maximum to the surface layer, where it easily escapes to the atmosphere. Therefore, N_2O emissions triggered by strong wind events may contribute significantly to both the regional and global oceanic N_2O emissions (Patra et al. 2004; Bange et al. 2008). In view of the fact that the strongest tropical cyclones, especially in the northern Indian Ocean, have been increasing in intensity in recent years (Elsner et al. 2008), we might expect an increase of N_2O emissions triggered by storm events. Therefore, any long-term changes of the atmospheric circulation that alter the wind fields with respect to the wind speeds and the wind field patterns might lead to changes in the N_2O emissions.

Possible alternative pathways to nitrous oxide

Nitrous oxide from anammox?

During the last years, anaerobic ammonium oxidation (anammox, $NO_2^- + NH_4^+ \rightarrow N_2$) has received increasing attention as an additional, previously not recognized, significant loss process of fixed nitrogen in the ocean (see for example (Francis et al. 2007; Devol 2008)). N_2O has been found to be formed only in small amounts during nitric oxide detoxification ($NO_2^- \rightarrow NO \rightarrow N_2O$) that seems to be performed by the anammox bacterium *Kuenenia stuttgartiensis* as side reaction of the anammox reaction (Kartal et al. 2007). Anammox has been found in the sub-oxic zones of eastern tropical South Pacific Ocean, in the upwelling off Namibia (Kuypers et al. 2005; Thamdrup et al. 2006; Hamersley et al. 2007) as well as in the central Baltic Sea (Hannig et al. 2007), but not in the sub-oxic zones of the central Arabian Sea (Nicholls et al. 2007). The question of whether anammox is involved in the production and/or consumption of N_2O in the ocean should be the subject of further investigation.

Nitrous oxide from nitrification and denitrification by archaea?

Archaea have been detected in almost all oceanic regions throughout the water column and in the sediments (see for example (Karner et al. 2001; Sinninghe Damsté et al. 2002; Francis et al. 2005; Teira et al. 2006; Varela et al. 2008)). The successful isolation of a NH_4^+ -oxidizing archaeon (Könneke et al. 2005) raised the question whether we have overlooked the role of ammonium-oxidizing archaea (AOA) in the oceanic nitrogen cycle. Meanwhile the gene amoA, which is commonly used as a marker gene for the ammonium-oxidizing enzyme ammonia monooxygenase in

Crenarchaeota (the dominant group of mesophilic archaea in the ocean), has been detected in the North Atlantic Ocean, the North Sea, the Black Sea and in sediments (see for example (Francis et al. 2005; Wuchter et al. 2006; Lam et al. 2007)). On the basis of the dominant abundance of the AOA amoA compared to the bacterial amoA, it has been suggested that *Crenarchaeota* in the uppermost 1000m of the North Atlantic Ocean were mainly responsible for NH_4^+ oxidation (i.e. the first step of nitrification), whereas nitrifying bacteria seem to play only a minor role (Wuchter et al. 2006). Similar results were found in estuarine sediments where AOA seem to play a major role as NH_4^+ oxidizers (Caffrey et al. 2007). Archaea are also capable of performing the classical denitrification pathway, including N_2O formation and its subsequent reduction to N_2 (see for example the overview article by Cabello et al. (Cabello et al. 2004), and references therein). Despite the fact that archaea perform the same nitrogen transformation processes as bacteria, there are 'significant differences in the structure and regulation of some enzymes involved in the nitrogen metabolism in archaea' as stated by Cabello et al. (Cabello et al. 2004). This might be especially important for interpreting N_2O isotopic signatures. However, N_2O production and/or consumption by archaea have not been studied yet.

Concluding remarks

Based on the points discussed in the sections above, it is clear that the marine pathways to N_2O (which are exclusively biological processes) have been and will be sensitive to ongoing environmental changes. We do not know, however, how the oceanic N_2O pathways will be altered and it is even more difficult to predict how the future oceanic N_2O emissions will be affected. Our rather poor ability to predict the future oceanic N_2O cycling (and to explain the N_2O paleorecord) is due to the fact that we still have an only rudimentary knowledge about both the oceanic distribution of N_2O and the mechanisms of its major production processes. Emerging new aspects such as possible N_2O formation during anammox or by archaea might have the potential to change our traditional view of the oceanic N_2O pathways in the near future.

Chapter 3: Production of oceanic nitrous oxide by ammonia- oxidizing archaea

The recent finding that microbial ammonia oxidation in the ocean is performed by archaea to a greater extent than by bacteria has drastically changed the view on oceanic nitrification. The numerical dominance of archaeal ammonia-oxidizers (AOA) over their bacterial counterparts (AOB) in the ocean leads to the hypothesis that AOA rather than AOB could be the key organisms for the oceanic production of the strong greenhouse gas nitrous oxide (N_2O). Very recently, enrichment cultures of marine ammonia-oxidizing archaea have been described to produce N_2O (Santoro et al. 2011). Here, we demonstrate that archaeal ammonia monooxygenase genes (*amoA)* were detectable throughout the water column of the eastern tropical North Atlantic and eastern tropical South Pacific Oceans. The maxima in abundance and expression of archaeal *amoA* genes correlated with the N_2O maximum and the oxygen minimum, whereas the abundances of bacterial *amoA* genes were negligible. Moreover, selective inhibition of archaea in seawater incubations from the ETNA decreased the N_2O production significantly. Studies with the marine archaeal ammonia-oxidizer *Nitrosopumilus maritimus* SCM1 provided the first direct evidence for N_2O production in a pure culture of AOA, thus excluding the involvement of other microorganisms as possibly present in enrichments. *N. maritimus* showed high N_2O production rates under low oxygen concentrations comparable to concentrations existing in the oxycline of the ETNA, whereas the N_2O production from two AOB cultures was comparably low under similar conditions. Based on our findings, we hypothesize that the observed production of N_2O in tropical ocean areas results mainly from archaeal nitrification and is largely impacted by the decrease of dissolved oxygen in the ocean.

Atmospheric nitrous oxide (N_2O) is a strong greenhouse gas and a precursor of stratospheric ozone depleting radicals . The ocean is a major source of N_2O contributing approximately 30% of the N_2O in the atmosphere . Oceanic N_2O is exclusively produced during microbial processes such as nitrification (under oxic to suboxic conditions) and denitrification (under suboxic conditions) . The formation of N_2O as a by-product of nitrification was reported for AOB (oxidation of ammonia (NH_4^+) via hydroxylamine (NH_2OH) to nitrite (NO_2^-) and in case of nitrifier-denitrification further reduction to nitric oxide (NO) and N_2O. The accumulation of oceanic N_2O is favored in waters with low oxygen (O_2) concentrations, which is attributed to an enhanced N_2O yield during nitrification. The frequently observed linear correlation between ΔN_2O (i.e., N_2O excess) and the apparent oxygen utilization (AOU) is usually taken as indirect evidence for N_2O production via nitrification (Yoshida et al. 1989).
The traditional view that oceanic NH_4^+ oxidation is only performed by ammonia-oxidizing bacteria (AOB) has been challenged by (i) the frequent presence of archaeal *amoA* genes in metagenomes of various environments (Venter et al. 2004b; Venter et al. 2004a; Schleper et al. 2005; Treusch et al. 2005; Lam et al. 2009), (ii) the successful isolation of the NH_4^+ oxidizing archaeon *N. maritimus* and (iii) the fact that archaea capable of NH_4^+ oxidation have been detected in all oceanic regions

throughout the water column and in sediments . Moreover, *N. maritimus* appears to be adapted to perform NH_4^+ oxidation even under the oligotrophic conditions that dominate in large parts of the open ocean. These observations point towards an important role of AOA (now constituting the novel archaeal lineage of *Thaumarchaeota* (Brochier-Armanet et al. 2008; Spang et al. 2010)) for the oceanic nitrogen cycle, which has been overlooked until recently . Archaeal N_2O production has been proposed to contribute significantly to the upper ocean N_2O production in the central California Current and has recently been demonstrated to occur in two AOA enrichment cultures (Santoro et al. 2011). However, the capacity of AOA to produce N_2O in the ocean has yet not been demonstrated directly in a pure culture.

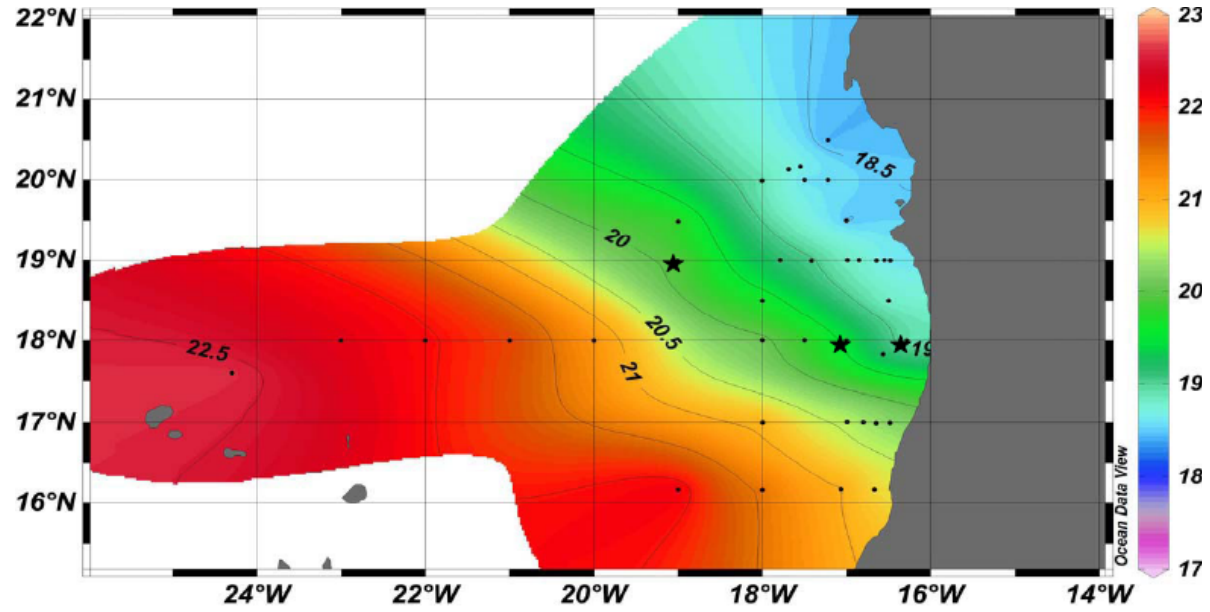
The eastern tropical North Atlantic (ETNA) and the eastern tropical South Pacific (ETSP) Oceans represent two contrasting oceanic O_2 regimes: While O_2 concentrations in the ETNA are commonly above 40 µmol L^{-1}, the ETSP regime is characterized by a pronounced depletion of O_2 in intermediate waters between ~75 and 600 m resulting in a oxygen minimum zone (OMZ) with O_2 concentrations close or even below the detection limit (~2 µmol L^{-1}) of conventional analytical methods.

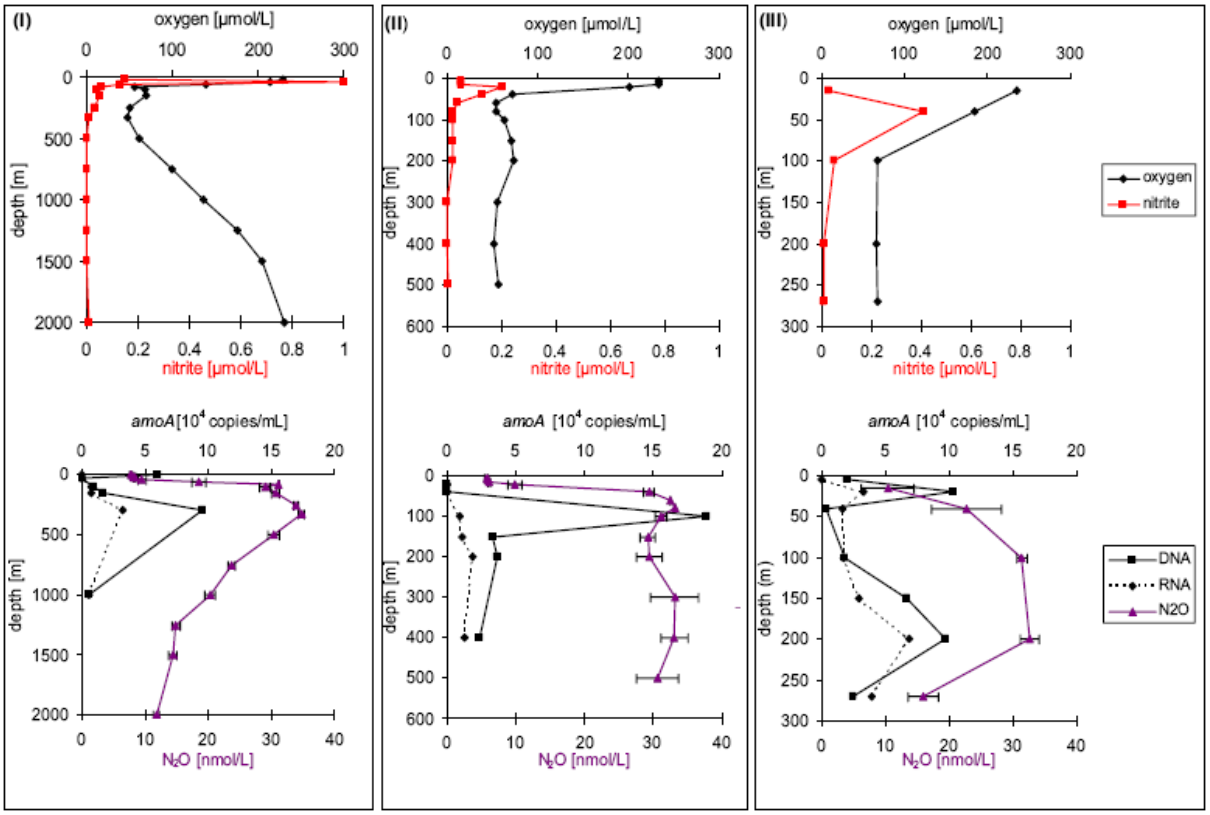
The *amoA* gene coding for the alpha subunit of the ammonia monooxygenase is present in archaea as well as in β- and *γ*-proteobacterial ammonia-oxidizers and is commonly used as a functional biomarker for this physiological group (Venter et al. 2004a; Schleper et al. 2005; Treusch et al. 2005; Hallam et al. 2006). Thus, in order to identify whether archaeal or bacterial *amoA* was associated with the maximum in N_2O concentration in the ocean, we determined the archaeal and bacterial *amoA* gene abundances and expression in relation to N_2O concentrations along vertical profiles during three cruises (in February 2007, February 2008, and June 2010) to the ETNA and one cruise (in January 2009) to the ETSP. We further demonstrate the N_2O production in a pure culture of *N. maritimus* SCM1, establish the O_2 sensitivity of archaeal N_2O production which is of highest impact at times of ocean deoxygenation (Stramma et al. 2010). N_2O production from pure cultures of the two marine nitrifying bacteria *Nitrosococcus oceani* NC10 and *Nitrosomonas marina* NM22 is low compared to the rates achieved by the archaeal isolate.

Vertical distribution of AOA and AOB along N_2O depth profiles

Vertical profiles of N_2O showed a distribution with concentrations between 10 and 35 nmol L^{-1} in the ETNA whereas the N_2O concentrations in the ETSP were in the range from 10 to 374 nmol L^{-1} (Fig. 3.1). In the majority of the sampled stations in the ETNA and ETSP, the accumulation of dissolved N_2O was associated with minimum O_2 concentrations as expected (Codispoti 2010). Maximum N_2O concentrations in the ETNA were generally lower compared to the ETSP because O_2 concentrations in the ETSP were extremely depleted below 75 m resulting in enhanced N_2O accumulation (Suntharalingam et al. 2000; Codispoti 2010).

(A) **Sea surface temperature - Eastern tropical North Atlantic (ETNA)**





(B) Sea surface temperature - Eastern tropical South Pacific (ETSP)

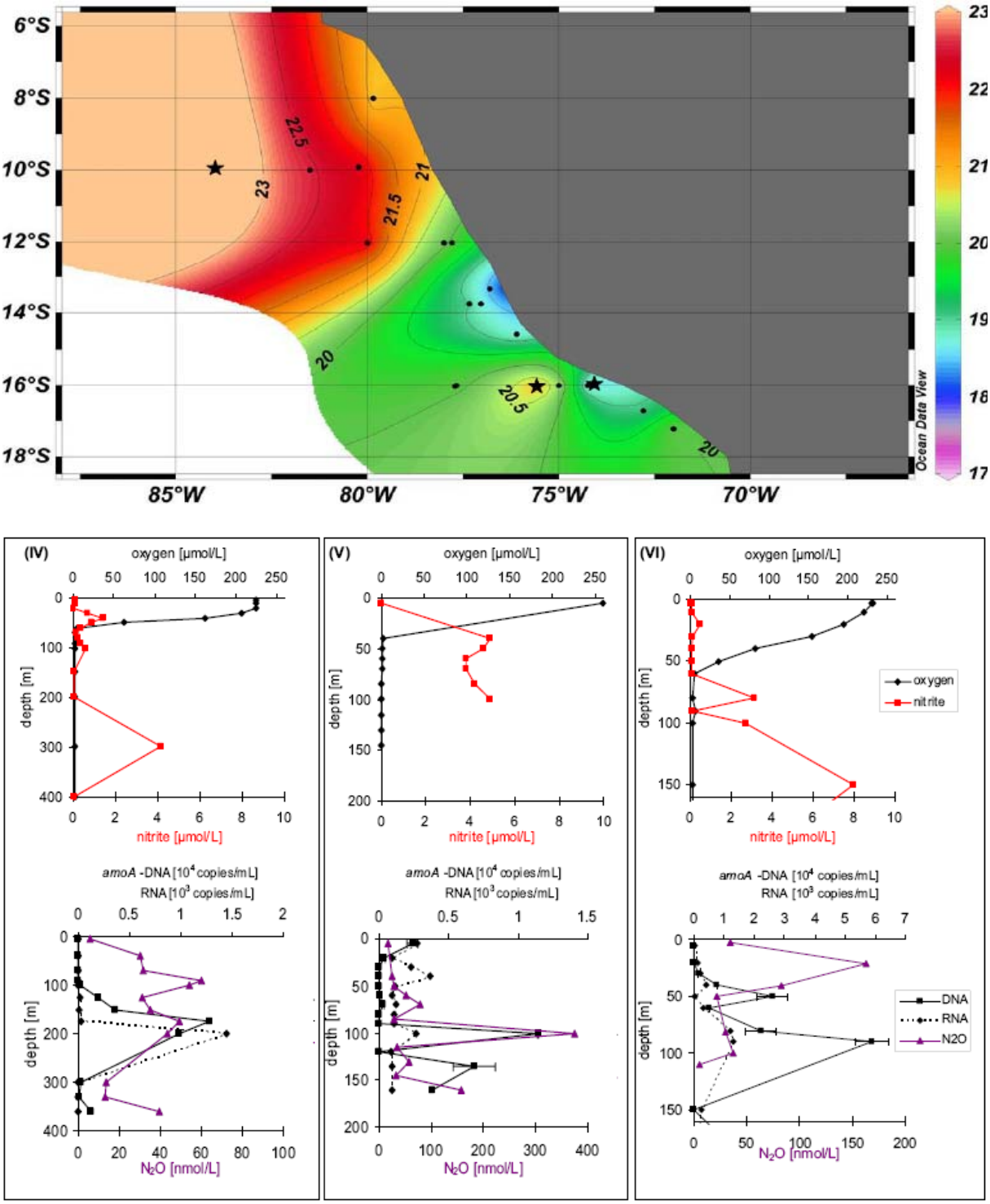


Fig. 3.1: Maps of sea surface temperatures (A) from the eastern tropical North Atlantic Ocean and (B) from the eastern tropical South Pacific Ocean. The locations of sampling stations are indicated with dots in the maps. Selected vertical profiles (I-VI) of O_2, NO_2^-, N_2O (measured in triplicates) and archaeal *amoA* (measured in duplicates by qPCR) are shown; (I) and (IV) are located offshore, (II) and (V) are located on the continental slope, and (III) and (VI) are onshore/coastal stations.

The well-established linear correlation between ΔN_2O and AOU as well as NO_3^- was found for the ETNA (Fig. 3.2) indicating that nitrification was the likely pathway for N_2O production. High copy numbers of archaeal *amoA* genes and high N_2O concentrations co-occurred in the ETNA suggesting a tight coherence between AOA abundance and N_2O accumulation in the layers with low O_2 (Fig. 3.3). A coherence of N_2O and archaeal *amoA* was detected at some stations in the ETSP, but was not a general feature (Fig. 3.1) possibly resulting from N_2O production via other processes such as denitrification, nitrifier-denitrification or anammox (Kartal et al. 2007) at present suboxic conditions. Gene abundances of archaeal *amoA* in the ETNA and ETSP were detectable throughout the water column and reached values of up to 1.9 x 10^5 and 6 x10^4 copies mL^{-1}, respectively (Fig. 3.1). Gene abundances of β- and ɣ-proteobacterial *amoA* were much lower (up to 950 and 735 copies mL^{-1} in the ETNA and ETSP, respectively) and in comparison seem to be negligible. This is in line with previous studies reporting 1-4 orders of magnitude higher abundances of AOA than AOB in various oceanic regions (Francis et al. 2005; Wuchter et al. 2006; Church et al. 2009; Lam et al. 2009; Santoro et al. 2010). Thus, we propose that N_2O production occurs via archaeal nitrification in the ETNA and might also be present in the ETSP despite the fact that we did not find the ΔN_2O/AOU correlation in the ETSP. A difference of one order of magnitude between *amoA* copies in RNA and in DNA is present in vertical profiles of the ETSP, with copy numbers up to 7 x 10^4 copies mL^{-1} present in the DNA and up to 1.5 x 10^3 copies mL^{-1} in the RNA. A similar tendency is detectable in the ETNA, however, the difference is less pronounced compared to the ETSP. This discrepancy, already reported by Lam *et al*. 2009 (Lam et al. 2009), is hypothesized to be due to a diurnal cycle of ammonia-oxidation and therefore variable *amoA* expression. Moreover, a sampling bias due to comparably long filtration times (up to 30 min) might have led to RNA degradation; as previous studies reported transcript half-lives of down to 0.5 min in *Prochlorococcus* (Steglich et al. 2010).

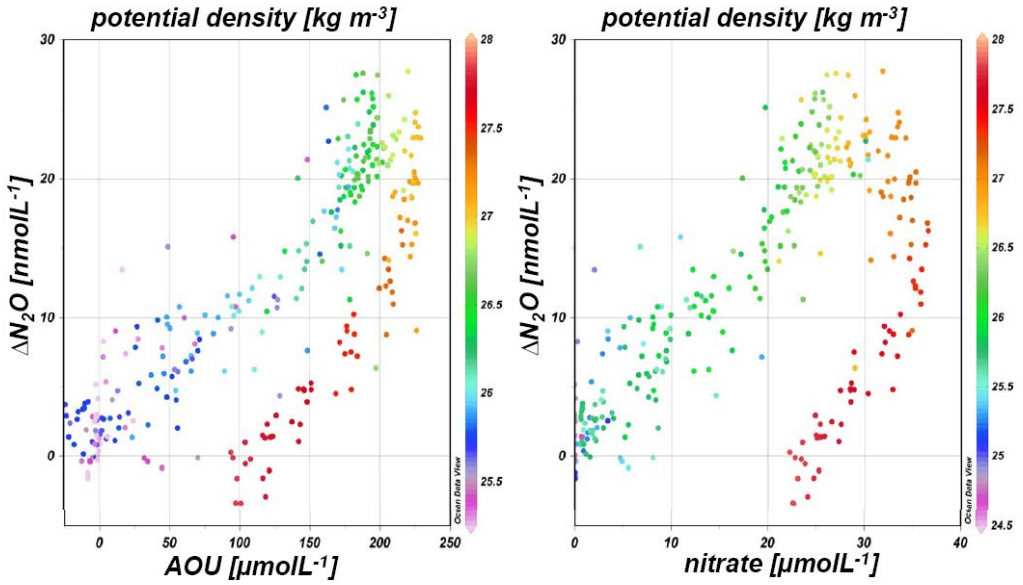


Fig. 3.2: ΔN_2O versus the apparent oxygen utilization (AOU) and nitrate in the ETNA (data from cruises ATA03 and P348), the potential density is colour–coded

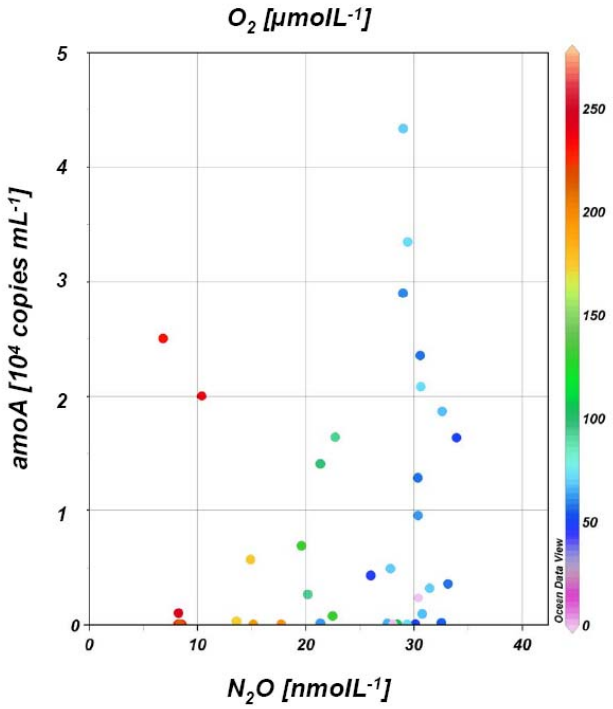


Fig. 3.3: Archaeal *amoA* versus N_2O and O_2 in the ETNA (data from the cruises ATA03, P348 and P399/2). The O_2 concentration is colour-coded.

Phylogenetic diversity of archaeal *amoA*

The diversity of AOA present in the ETNA was determined based on the analysis of 106 *amoA* sequences from 7 stations of 2 cruises (P348, ATA03). Sequences were derived from 10 depths between the ocean surface and 1000 m. The sequences split into two main clusters, with sequences from the O_2 minimum clustering mainly in cluster B (Fig. 3.4). Only 2.7% of sequences derived from samples from the O_2 minimum fall into cluster A. Sequences derived from depths between the surface and the upper oxycline were distributed over both clusters (Fig.3.4). In the ETSP, sequences from within as well as from depths above the OMZ separated into both clusters, with the majority of absolute sequence numbers from the OMZ affiliating with cluster B (Fig. 3.4), as already observed for the sequences from the O_2 minimum in the Atlantic Ocean.

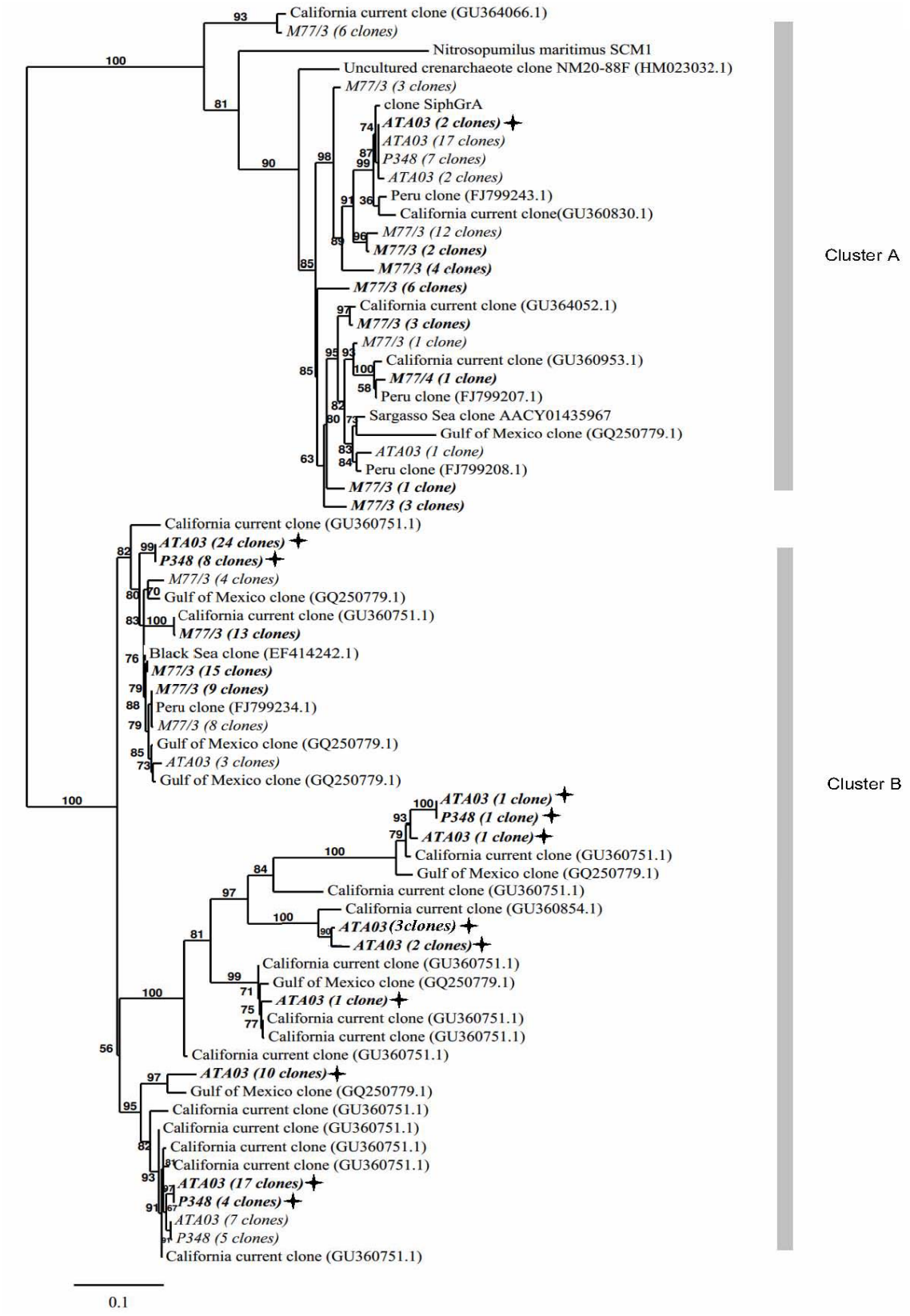


Fig. 3.4: Distance-based neighbour-joining phylogenetic tree of archaeal *amoA* sequences from the ETNA (cruises ATA03 and P348) and ETSP (cruise M77/3). Sequences derived from the oxygen minimum zone (OMZ) of the ETNA are in italics, bold and marked with solid stars, sequences from above the OMZ are in italics. Sequences from the OMZ of the ETSP are in italics and bold; sequences from above the OMZ are in italics.

Potential importance of cluster B affiliated Thaumarchaeota for N_2O production

The distribution of archaeal *amoA* genotypes along vertical profiles in the ETNA with the majority of cluster B sequences present in clone libraries from the OMZ suggest a production of N_2O by *Thaumarchaeota* affiliated with cluster B, which was previously reported to be a deep marine cluster (Hallam et al. 2006) associated mainly with O_2 and NH_4^+ poor waters (Molina et al. 2010). These findings suggest a niche separation based on O_2 concentrations of cluster B affiliated AOA, which is consistent with our data from the ETSP. Regarding the potential decrease in dissolved O_2 concentrations in tropical ocean areas (Stramma et al. 2010), we hypothesize that cluster B affiliated AOA might dominate the production of N_2O and the balance between reduced and oxidized nitrogen species in the ocean, gradually.

N_2O production in the ETNA

In two out of three 24h seawater incubations performed at three different stations in the ETNA at the depth of the OMZ, N_2O production was significantly lower in samples treated with N^1-guanyl-1,7-diaminoheptane (GC_7), a hypusination inhibitor shown to selectively inhibit the cell cycle of archaea (Jansson et al. 2000) (Fig. 3.5). In the third experiment a similar trend was observed, however it was not statistically significant. These findings support the hypothesis that N_2O production in large parts of the ETNA occurs within the OMZ and is mainly carried out by archaea.

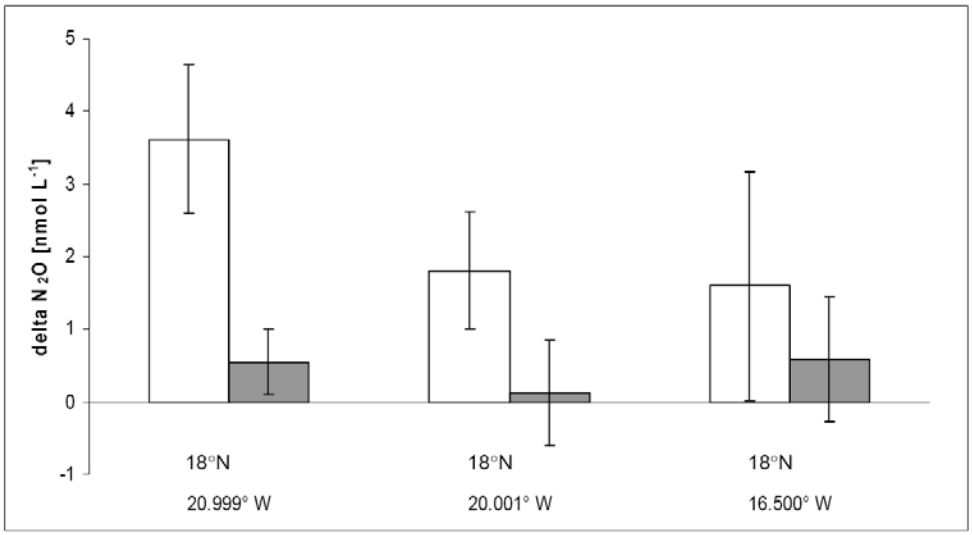


Fig. 3.5: N_2O production determined from seawater incubations at three different stations (1-3) from the ETNA (P399). Delta N_2O was calculated as the difference of N_2O concentrations over the incubation time (i.e. 24h). Open columns represent samples with no inhibitor, filled columns represent samples with the archaeal inhibitor GC_7. Error bars indicate the standard deviation of three technical replicates

N_2O production in *N. maritimus* pure culture experiments

A direct evidence for N_2O production in archaea was detected in experiments with pure cultures of *N. maritimus*. Production of N_2O in *N. maritimus* cultures was inversely correlated to O_2 concentrations (Fig. 3.6) which were chosen according to the O_2 concentrations present in the ETNA (112, 223 and 287 µmol L^{-1}, Fig. 3.1). *N. maritimus* cultures grew at comparable rates under the varying O_2 conditions and showed similar nitrification rates. Thus, the observed variation of the measured N_2O production can be assumed to exclusively depend on the prevailing initial O_2 conditions. N_2O production rates from the two AOB cultures (*Nitrosomonas marina* NM22 and *Nitrosococcus oceani* NC10) were considerably lower compared to the N_2O produced by *N. maritimus* (Fig. 3.6, Tab S1). The N_2O yields from NH_4^+ oxidation ranged from 0.002%-0.03% in the culture of *N. maritimus* to 0.001%-0.006% in the AOB cultures. The N_2O production rates derived from our AOB experiments are in accordance to those reported by Goreau *et al.* (Goreau et al. 1980), even though a different experimental setup was used. Culture experiments such as those presented here, are performed with AOB cell densities (~10^5 cells mL^{-1}) which are much higher than usually found in the ocean (10^2-10^3 cells mL^{-1}) (Wuchter et al. 2006; Lam et al. 2009). Thus, the N_2O production rates from the AOB cultures are probably overestimated and not representative as N_2O production per cell by AOB is also depending on the present cell densities (Frame 2010). In contrast, the AOA cell densities in our culture experiment (~10^5 -10^7 cells mL^{-1}) were comparable to those present in the oceanic environment (~10^5 cells mL^{-1}) and thus seem to be reasonably representative.

Using the results from culture experiments as an approximation, the observed archaeal N_2O production rate for low O_2 conditions (140 nmol L^{-1} d^{-1}; normalized to 10^6 cells mL^{-1} yielding ~24 nmol L^{-1} d^{-1}, see Tab. S1) translates roughly estimated into a maximal potential oceanic production rate of about 14 nmol m^{-2} s^{-1} under the assumption of a low O_2 layer thickness of about 50 m as typically found in the ETNA. Compared to estimates of N_2O emissions from the ETNA to the atmosphere of up to 2 nmol m^{-2} s^{-1} (Wittke et al. 2010), potential oceanic archaeal N_2O production might be indeed significant.

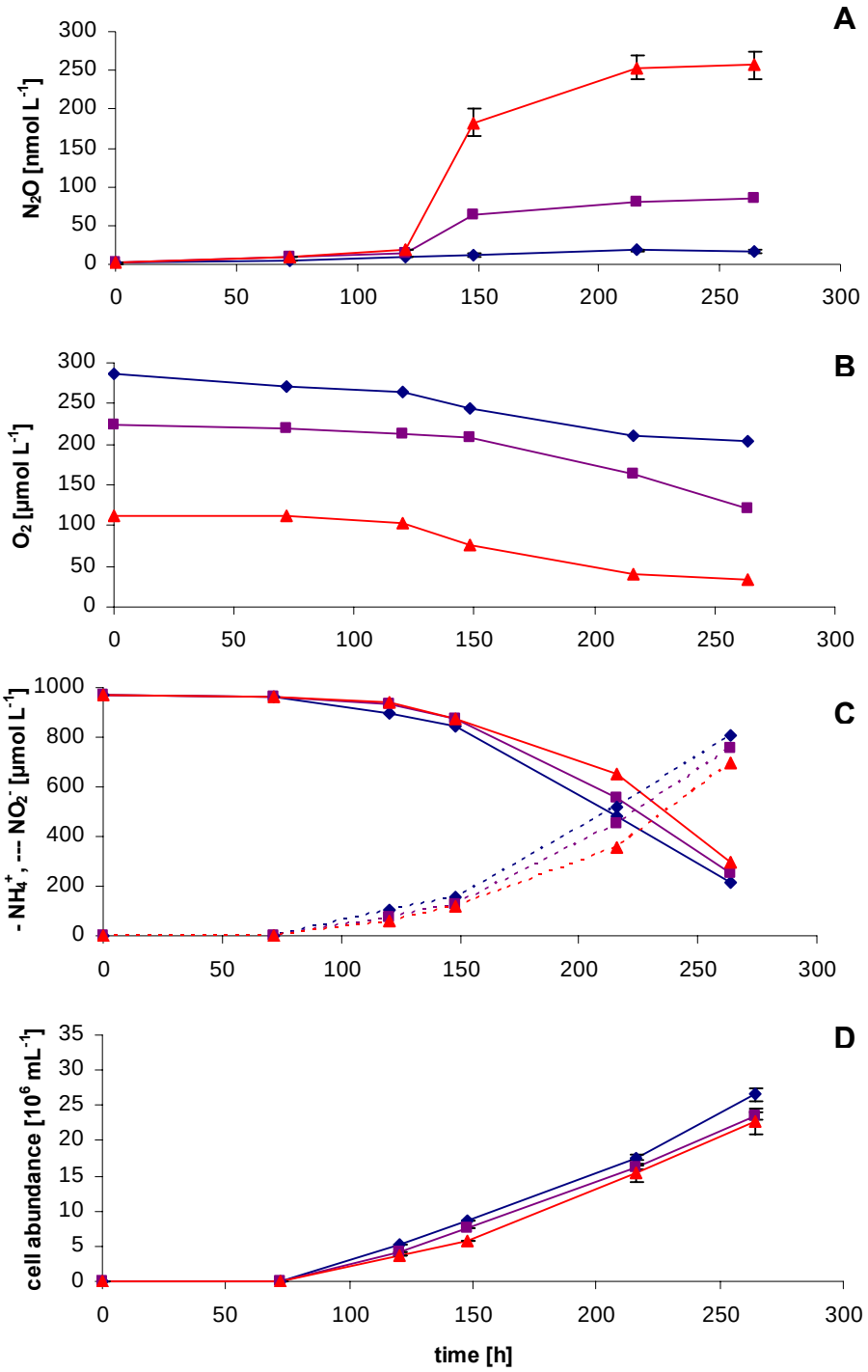


Fig. 3.6: N_2O (A), O_2 (B), NH_4^+ and NO_2^- (C) as well as cell abundances (D) determined from incubation experiments with pure cultures of *N. maritimus*. Experiments are colour-coded according to their initial O_2 concentrations: red (112 µmol L^{-1}); violet (223 µmol L^{-1}); and blue (287 µmol L^{-1}). N_2O and cell abundances were measured in triplicates and the associated error ranges are indicated (please note that in the most cases the error bars are too small to be visible in the figure).

Potential pathways for archaeal N_2O production

AOB can produce N_2O from NH_2OH during nitrification or from NO_2^- during nitrifier- denitrification (Shaw et al. 2006; Kool et al. 2010). In AOA however, no equivalent to the hydroxylamine-oxidoreductase, which catalyzes the oxidation of NH_2OH to NO_2^- during nitrification, has been identified (Könneke et al. 2005; Martens-Habbena et al. 2009). In contrast, the detection of the nitrite reductase gene *nirK* in the sequenced genomes of cultured *Thaumarchaeota* (Könneke et al. 2005; Martens-Habbena et al. 2009) led to the theory that AOA might produce N_2O by nitrifier-denitrification. To identify the origin of N_2O formation isotopomeric studies were performed with *N. maritimus* pure cultures. A ^{15}N site preference (SP_{N2O}) in N_2O of 34 ± 12 ‰ was detected, consistent with results from AOA enrichments (Santoro et al. 2011), which is in agreement with the SP_{N2O} of ~33‰ typically found in AOB cultures performing NH_4^+ oxidation (Sutka et al. 2006) (for comparison: nitrifier-denitrification of AOB results in a SP_{N2O} of about 0‰). Thus, a production via the oxidation of NH_4^+ to NO_2^-, potentially via an unknown intermediate, is suggested, whereas N_2O production via nitrifier-denitrification is unlikely.

Summary

Taken together, the dominating abundance of archaeal *amoA* relative to AOB, the coherence of N_2O accumulation and *amoA,* the inhibition of N_2O production in seawater experiments in the presence of the archaeal inhibitor GC_7 as well as the N_2O production by *N. maritimus* point to the fact that, in large parts of the ocean, N_2O is produced by archaeal, rather than by bacterial, nitrification. Further, the archaeal N_2O production appears to be highly sensitive to the dissolved O_2 concentration, with highest N_2O production rates at low O_2 concentrations such as those present in the OMZ of the ETNA. The expansion of OMZs in the future in many parts of the ocean (Stramma et al. 2008) may lead to an enhanced N_2O production in the ocean and therefore may have severe consequences for the budget of N_2O in the atmosphere as well.

Acknowledgements

We thank the authorities of Cape Verde, Mauritania and Peru for the permission to work in their territorial waters. We acknowledge the support of the captains and crews of R/V Poseidon, R/V L'Atalante, and R/V Meteor as well as the chief scientists of ATA03, A. Körtzinger, and M77/3, Martin Frank. Moreover, we thank T. Kalvelage for sampling during P348, and T. Großkopf and H. Schunck for sampling during M77/3; we further thank K. Stange, F. Malien, M. Lohmann, V. Leon and P. Fritsche for oxygen and nutrient measurements. We thank A. Pommerening-Röser for providing cultures of *N. oceani* NC10 and *N. marina* NM22 and C. Fehling for performing the isotopomeric studies. Financial support for this study was provided by the DFG Sonderforschungsbereich 754 (www.sfb754.de) and the BMBF Verbundprojekt SOPRAN

(www.sopran.pangaea.de; SOPRAN grants 03F0462A and 03F0611A). MK was financially supported by the DFG.

Methods summary

Hydrographic parameters and nutrients

Samples for salinity, O_2 concentrations and nutrients were taken from a 24-Niskin- bottle rossette. Oxygen concentrations were determined according to the Winkler method, salinity and nutrient concentrations were determined as decribed in Grasshoff *et al.* (Grasshoff 1999) .

Determination of dissolved N2O concentrations

Triplicate samples for N_2O analysis were taken from CTD/rosette casts during the cruises P348 (February 2007), ATA03 (February 2008), P399 (June 2010) to the ETNA and M77/3 (January 2009) to the ETSP. N_2O was measured according to the method described in Walter *et al.* (Walter et al. 2006). ΔN_2O and AOU were calculated accordingly.

Molecular genetic methods

Sampling

Samples for the extraction of DNA and RNA were rapidly taken by filtering (~30min filtration time) a volume of about 2 L (exact volumes were determined and recorded continuously) of seawater through 0.2 µm polyethersulfon membrane filters (Millipore, Billerica, MA, USA). The filters were immediately frozen and stored at -80°C.

Nucleic acid purification

DNA and RNA was extracted using the Qiagen DNA/RNA All prep Kit (Qiagen, Hilden, Germany) according to the manufacturers protocol with a small modification. A lysozyme treatment (50 µg mL^{-1} for 10 min at room temperature) followed by a proteinase K treatment was performed prior starting the extraction. Extracts of DNA and RNA were quantified fluorometrically using a NanoDrop 2000 (Thermo Scientific Fischer). A treatment with Dnase I (Invitrogen, Carlsbad, CA) was performed with the extracted RNA to remove any residual DNA, purity of RNA was checked by 16S rDNA PCR amplification before reverse transcription.

PCR and quantitative PCR

The extracted RNA was reverse transcribed to cDNA by using the QuantiTect®reverse transcription Kit (Qiagen, Hilden, Germany) following the manufacturers' protocol.

Bacterial and archaeal *amoA* as marker genes for nitrification, bacterial *nirS, nirK* and *nosZ* as marker genes for denitrification were PCR- amplified from DNA and cDNA. PCR and quantitative PCR

conditions and primers are listed in Tab. S2. PCRs were performed using 0.1 µL FlexiTaq (Promega Corporation, USA).

Absolute quantification of bacterial and archaeal *amoA* was performed with standard dilution series; quantification was performed in duplicates. Standards for quantitative PCRs were obtained from *Nitrosococcus oceani* NC10, *Nitrosomonas marina* NM22 and NM51 for bacterial *amoA* and from an environmental clone for crenarchaeotal *amoA* (GenBank accession number JF796147). Reactions were performed in a final volume of 25µL using 0.5µL of each primer, 6.5µL nuclease free water and 12.5µL SYBR qPCR Supermix W/ROX (Invitrogen, Carlsbad, CA) . Reactions were performed using an ABI 7300 Real Time PCR system (Applied Biosystems, Carlsbad, CA) according to Lam *et al.* (Lam et al. 2007).

Construction of clone libraries and phylogenetic analysis

Cloning of PCR amplicons was performed using the Topo TA Cloning®Kit (Invitrogen, Carlsbad, CA) according to the manufacturers' instructions.

Sequencing was carried out by the Institute of Clinical Molecular Biology, Kiel. Sequences for archaeal *amoA* were analyzed using the ClustalW multiple alignment tool on a 495 bp fragment (sequences were submitted to GenBank under accession numbers JF796145- JF796179), sequence differences were set on a minimum of 5%, phylogenetic trees were made using distance-based neighbour-joining analysis (Saitou and Nei 1987).

Seawater incubations

Seawater incubations were performed at three different stations in the ETNA (cruise P399). 25mL serum bottles were filled with seawater from 200- 250m depth from the CTD, closed with an air-tight butyl rubber stopper and aluminium crimp-capped. Triplicate samples were taken to determine the initial N_2O concentration, six bottles were incubated, one triplicate as a control and one triplicate was treated with 1mM of the hypusination inhibitor *N*1-guanyl-1,7-diaminoheptane (GC_7) (Jansson et al. 2000).

Incubations were kept for the duration of the experiment (24h) in the dark at 8°C. The experiment was stopped by $HgCl_2$ addition, followed by the determination of the final N_2O concentrations.

Culture experiments

Pure cultures of *Nitrosopumilus maritimus* SCM1, *Nitrosococcus oceani* NC10 and *Nitrosomonas marina* NM22 were grown in triplicates in 125 mL serum bottles according to Goreau *et al.* and Koenneke *et al.* (Goreau et al. 1980; Könneke et al. 2005). Serum bottles were closed with an air-tight butyl rubber stopper and aluminium crimp-capped. Cultures were kept for the duration of the experiment in the dark. Cell abundances were monitored microscopically after staining with the fluorescent DNA-binding dye 40 ,6 0-diamidino-2-phenylindole (DAPI) and with by flow cytometry (FACScalibur, Becton, Dickinson) after staining with Sybr Green I (Invitrogen, Carlsbad). Cultures

were checked for heterotrophic contaminants microscopically and by 16S rDNA analysis.

N_2O concentrations were measured by gas chromatography using the headspace method as described above, oxygen concentrations were determined using Winkler titration. NH_4^+ and NO_2^- concentrations were determined at several time points over the complete incubation time frame (Grasshoff 1999). In order to exclude chemical N_2O production from the medium cultures toxified with mercury chloride were measured in parallel; no N_2O production could be detected.

Isotopomeric studies were performed with cultures of 0.5L volume, grown in Serum bottles, supplemented with $^{15}NH_4^+$ (10% of total NH_4^+). Measurements were performed as described in Fehling *et al.* (Fehling and Friedrichs 2010).

Tab S1: N_2O production in culture experiments: Mean O_2 and N_2O concentrations (in triplicate samples) of pure cultures of *N. maritimus*, *N. marina* and *N. oceani* after 264 h incubation, the initial NH_4^+ concentration (~1 mmol L^{-1}) was completely converted to NO_2^- in the end of the experiment in AOB cultures, NO_2^- was below the detection limit at the initial time point of the incubation

Culture	**N_2O [nmol L^{-1}]**	**O_2 [µmol L^{-1}]**	**ratio N_2O / NH_4^+ [%]**	**max. N_2O production [nmol L^{-1} day^{-1} 10^{-6} cells]**
Nitrosopumilus maritimus SCM1	254.75 ± 16.86	33.5	0.026	24.27
	82.63 ± 1.89	121.1	0.009	5.6
	15.57 ± 2.38	203.2	0.002	0.44
***Nitrosomonas marina* NM22**	41.71 ± 0.039	44.7	0.006	4.17
	14.4 ± 0.4	142.9	0.003	1.44
***Nitrosococcus oceani* NC10**	36.78 ± 1.33	49.8	0.005	3.68
	11.91 ± 0.33	163.7	0.001	1.21

Tab S2: Primers and PCR conditions. For real-time qPCR the initial denaturing step was 10 min at 95°C, annealing temperatures were the same as in the end point PCRs, no final extension step took place, 40 cycles were performed followed by melting curve analysis. A fragment of 175 bp was amplified in qPCRs of archael *amoA*.

Target organism	**Target gene**	**oligonucleotide**	**Sequence (5' → 3')**	**PCR conditions**	**reference**
β-proteobact. ammonia-oxidizers	*amoA*	amoA1F' amoA2R amoA-1F (qPCR) amoAR_new (qPCR)	GGGGTTTCTACTGGTGG CCTCKGSAAAGCCTTCTTC GGGGTTCTACTGGTGGT CCCCTCGGCAAAGCCTTCTTC	94°C for 5 min, 30 x (94°C for 20 s, 55°C for 1 min, 72°C for 1min), 72°C for 10 min	(Rotthauwe et al. 1997)
γ-proteobact. ammonia-oxidizer	*amoA*	amoA3F amoA4R A189 (qPCR) A682 (qPCR)	GGTGAGTGGGYTAACMG GCTAGCCACTTTCTGG GGCGACTGGGACTTCTGG GAACGCCGAGAAGAACGC	94°C for 5 min, 30x (94°C for 20s, 48°C for 1 min, 72°C for 1 min), 72°C for 10 min	(Purkhold et al. 2000)
archaeal ammonia-oxidizers	*amoA*	Arch-AmoAF Arch-AmoAR AamoA_for (qPCR) AamoA_rev (qPCR)	STAATGGTCTGGCTTAGACG GCGGCCATCCATCTGTATGT GGGCGACAAAGAAGAATAAACACTCGC ACCTGCGGTTCTATCGGCGT	95°C for 5 min, 30x (94°C for 45s, 50°C for 1 min, 72°C for 1 min), 72°C for 10min	(Francis et al. 2005) this study

Chapter 4: Construction and screening of marine metagenomic libraries

Abstract

Marine microbial communities are highly diverse and have evolved during extended evolutionary processes of physiological adaptations under the influence of a variety of ecological conditions and selection pressures. They harbor an enormous diversity of microbes with still unknown and probably new physiological characteristics. Besides, the surfaces of marine multi-cellular organisms are typically covered by a consortium of epibiotic bacteria and act as barriers where diverse interactions between microorganisms and hosts take place. Thus microbial diversity in the water column of the oceans and the microbial consortia on marine tissues of multi-cellular organisms are rich sources for isolating novel bioactive compounds and genes. Here we describe the sampling, construction of large insert metagenomic libraries from marine habitats and exemplarily one function based screen of metagenomic clones.

Key Words: Isolation of metagenomic DNA, 16S rDNA phylogenetic analysis, fosmid library, function-based screen

Introduction

Current estimates indicate that more than 99 % of the microorganisms present in many natural environments are not readily cultivable with conventional approaches (Amann et al. 1995). To overcome the difficulties and limitations associated with cultivation techniques, several DNA-based molecular methods have been developed in order to explore the diversity and potential of microbial communities (Streit and Schmitz 2004; Lorenz and Eck 2005; Pham et al. 2007; DeLong 2009). The new and rapidly developing field of so called ‘metagenomics’ tries to analyze the complex genomes and genomic information of microbial communities present in the different environmental habitats. Primarily employed to study non-cultivable microbiota for a better understanding of global microbial ecology in different environmental niches (Handelsman 2004), metagenomic data also provide information on the functional role of the different microbes within the community. This is emphasized by several recent examples, e.g. the discovery of a new bacterial rhodopsin, proteorhodopsin (Beja et al. 2000; Beja et al. 2001; de la Torre et al. 2003) and the recent insights into symbiosis between a marine oligochaete and its microbial community (Woyke et al. 2006). In recent years, efficient DNA isolation techniques for various habitats and vector systems for cloning large metagenomic DNA fragments (such as cosmids, fosmids or BACs) allowing to screen large clone libraries for functional activities have been established and are available as commercial kits (Shizuya and Kouros-Mehr 2001; Wild et al. 2002).

The oceans are the largest ecological systems on earth (Azam 1998) harbouring marine microorganisms with an average cell density of approximately 5 x 10^5 cells/ml, leading to the estimation that the oceans are a living space for approximately 3.6 x 10^{28} microorganisms (DeLong and Karl 2005). Marine microbial communities are highly diverse and have evolved during extended evolutionary processes of physiological adaptations under the influence of a variety of ecological conditions and selection pressures. They harbour an enormous diversity of metabolically complex microbes with still unknown and probably new physiological characteristics and are thus rich sources for isolating novel bioactive compounds and genes (Karl 2007). Microbes are also known to form symbiotic relationships with various marine invertebrates, e.g. sponges, corals and squids, and are thus suspected to produce particular biologically active and pharmacologically valuable natural products (Kennedy et al. 2007). Furthermore, the microbial consortia on marine multicellular organisms are attractive model systems to understand the complex interplay between microbes and their host cells that may be also relevant to the human barrier organs and its microbiota providing insight into the development of human diseases and identification of new drug targets.

Materials

1. Sampling

1.1. Marine water sampling

1. membrane pump with respective membranes (polycarbonate or polyvinylidenfluoride membrane filters of 10 µm and 0.22 µm pore size) or a **C**onductivity-, **T**emperature-, **D**epth sensor (CTD) equipped with a 24 Niskin 10 L bottle rosette
2. peristaltic pump to accelerate the filtration
3. *in situ* pumps for marine deep water sampling
4. liquid nitrogen to freeze the filters for long term storage at -80°C

1.2. Sampling from marine invertebrates

1. equipment for sampling marine organisms, e.g. clean buckets, bottles, a dip net
2. autoclaved seawater to wash away loosely attached microorganisms
3. sterile Petri dishes and sterile cotton-tipped applicators to swab microorganisms from the surfaces of the marine eukaryote
4. liquid nitrogen to freeze the filters for long term storage at -80°C

2. Isolation of metagenomic DNA

1. 37 °C and 65 °C incubator, centrifuge
2. DNA extraction buffer: 100 mM Tris/HCl pH 8.0, 100 mM sodium-EDTA, 100 mM sodium-phosphate, 1.5 M NaCl, 1 % CTAB (vol/vol)

3. TE buffer: 10 mM Tris/pH 8.0, 1 mM EDTA
4. 20 mg/ml Proteinase K (Fermentas, St. Leon-Rot), 50 mg/ml Lysozyme (Roth, Karlsruhe), RNase A (Qiagen, Hilden), 20 % SDS, chloroform, 100 % isopropanol, 70 % ethanol

3. 16S rDNA phylogenetic analysis

1. reaction tubes, pipettes, thermocycler
2. Bacteria-specific primer 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and the universal primer 1492R (5'-GGTTACCTTGTTACGACTT-3')
3. archaea-specific primer set 20F (5`-TTCCGGTTGATCCCTGCCAGG-3`) and 958R (5`-TCCGGCGTTGAACTCCAATT-3`)
4. 10 x Taq reaction buffer, 25 mM $MgCl_2$, 10 mM dNTPs, Taq polymerase (e.g. Fermentas, St. Leon-Rot), sterile water
5. Gel extraction and Purification Kit (e.g. Macherey-Nagel, Düren)
6. TA cloning Kit (e.g. Invitrogen, Karlsruhe)
7. Sequencing Reaction Kit (BigDye® Terminator v3.1 Cycle Sequencing Kit, Applied Biosystems, Darmstadt) and a capillary sequencer

4. Construction of a metagenomic large insert library

1. CopyControl™ Fosmid Library Production Kit (Epicentre, Madision, WI)
2. TE buffer: 10 mM Tris/pH 8.0, 1 mM EDTA
3. 0.025 µm cellulose filters type VS from Millipore (Schwalbach)
4. Phage-dilution buffer: 10 mM Tris/HCl pH 8.3, 100 mM NaCl, 10 mM $MgCl_2$
5. LB containing 10 mM $MgSO_4$ for growth of EPI300-T1R host cells
6. LB plates supplemented with 12.5 µg/ml chloramphenicol
7. microtiter plates (96 wells) containing 150 µl LB supplemented with 12.5 µg/ml chloramphenicol
8. Dimethylsulfoxid (DMSO)

5. Screening metagenomic libraries for cellulose degrading enzymatic activity

1. CMC agar plates: 0.4 % carboxymethyl cellulose (CMC) is dissolved in water by short time heating. LB-plates supplemented with 0.2 % CMC are prepared by adding the 0.4 % CMC solution to the dry ingredients of the medium prior to sterilisation by autoclaving.
2. 48er or 96er steel stamps
3. Congo Red solution (Roth, Karlsruhe)
4. 1 M NaCl solution
5. 1 M HCl

Methods

1. Sampling procedures

1.1. Marine surface water sampling

Surface water can be collected by either membrane pumps or any other highly effective clean pumping system on board. Further, samples can also be taken by a **C**onductivity-, **T**emperature-, **D**epth sensor (CTD), equipped with a 24 Niskin 10 L bottle rosette (Fig. 4.1). Samples from the potentially high productive surface layer around chlorophyll maxima should exceed a volume of 100 L but do not necessarily need to be larger than 200 L, due to the high abundance of microorganisms there. After collecting, pre-filtration with filters of 10 µm pore size is performed directly followed by a consecutive filtration with polycarbonate or polyvinylidenfluoride membrane filters of 0.22 µm pore size (see Note 3). To carry out this large volume filtration in an appropriate time frame, an efficient pumping system is requested, for example a peristaltic pump (see Note 2). Filters are immediately frozen and stored at -80°C (see Note 4).

1.2. Marine deep water sampling

Samples from below the euphotic zone, where not much cell material is present, should be collected in larger volumes of at least 200 L. A CTD equipped with a 24 Niskin 10 L bottle rosette can be used for the collection of such samples; filtration is then carried out as described above. As this sampling method is limited to a certain volume, mostly 240 L, it is highly time consuming, and may lead to stress responses due to dramatically changing environmental conditions during the filtration time on board (light, temperature, pressure). In this case, a sample collection by *in situ* pumps should be preferred. Those pumps can be set at the depth of interest, depending on the cable length of the ships' winch (Fig. 4.2A); this method further allows simultaneous deployment of several pumps at different depth. Therefore, the use of *in situ* pumps is highly time saving, and additionally leads to a higher conservation and consequently to a more realistic image of the microbial community (see Note 1). Moreover, a filtration of higher volumes of water is possible, depending on the pump type up to 5.000 L. Filtration is also conducted using carbonate membrane filters of 0.22 µm pore size, and a pre-filtration is not required. After recovering the pumps, filters are immediately removed from the pumps (Fig. 4.2B), frozen and stored as described above.

1.3. Sampling from marine invertebrates

After sampling, the marine organisms are thoroughly rinsed with filtered (0.22 µm) and autoclaved seawater to remove loosely attached microorganisms. If possible the organisms are then placed in sterile Petri dishes and an area of approximately 2 - 5 cm² (depending on the amounts of microbes and down stream applications) is swabbed with a sterile cotton-tipped applicator. In case of a fragile organism, the complete animal can be extracted for DNA isolation, resulting in a mixture of prokaryotic and eukaryotic DNA of unknown ratio. In this case, enrichment of prokaryotic cells, e.g. by fractionated centrifugation can be applied prior to DNA extraction. For comparative phylogenetic analysis, ambient seawater should be sampled and filtered as described above.

Fig. 4.1: CTD sampling: CTD equipped with a 24 Niskin 10 L bottle rosette on German research vessel Meteor

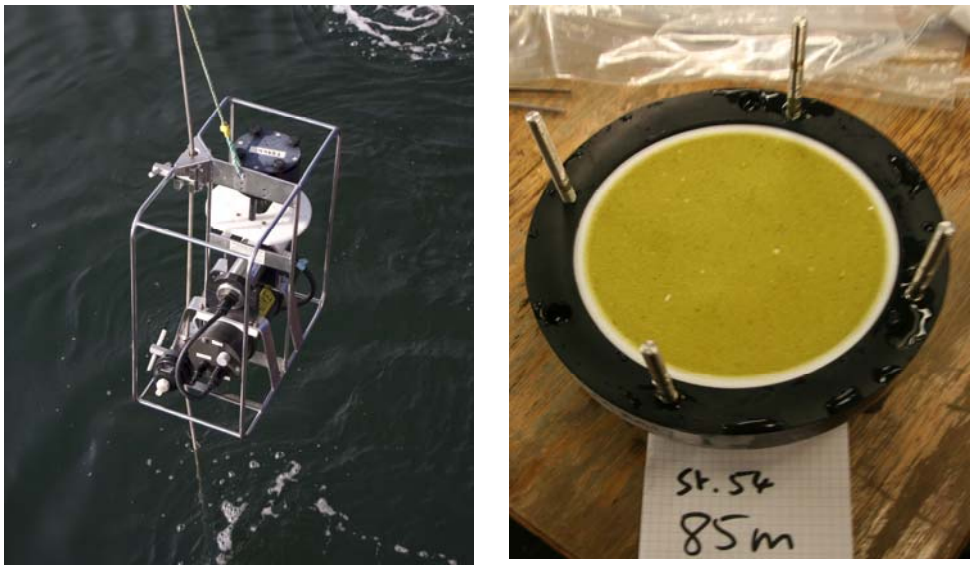


Fig. 4.2: Sample collection: deployment of an *in situ* pump from RV Meteor (left panel), filter-holder with filter of an *in situ* pump (right panel)

2. Isolation of metagenomic DNA

DNA from filters or swabs is commonly extracted by a direct lysis of the microorganisms. Additional steps prior the lysis may be required to isolate DNA from inhibitor-contaminated habitats or enrich prokaryotic cells in order to minimize co-extraction of eukaryotic DNA (Gabor et al. 2003). The following modified protocol of Henne *et al.* (Henne et al. 1999) describes the genomic DNA isolation based on direct lysis of the microorganisms from filter or swab samples. The volumes are appropriate for 2.5 cm^2 of a filter and should be adjusted according to the filter or sample size.

1. 1.35 ml DNA extraction buffer (see Note 7), supplemented with 20 µl Proteinase K (20 mg/ml) and 200 µl lysozyme (50 mg/ml) are added to the sample followed by an incubation at 37 °C for 30 min; optional shaking (150 rpm).
2. 1.5 µl (17,000 U) RNase A are added followed by further incubation at 37 °C for 30 min.
3. 150 µl 20 % SDS are added followed by an incubation for 2 h at 65 °C and subsequent centrifugation at 4,500 x g for 10 min.
4. Chloroform extraction of the supernatant followed by precipitation of the nucleic acids with isopropanol (0.7 vol) for 1 h at room temperature and subsequent centrifugation for 45 min at 16,000 x g and 4 °C.
5. The DNA precipitate is washed with 70 % ethanol, dried and solved in 25 µl TE buffer.

This extraction protocol uses enzymatic methods to remove cell walls, resulting in sphaeroplasts or protoplasts. The use of sodium dodecyl sulfate (SDS) disrupts mainly tertiary or quartary protein structures; cetyl trimethylammonium bromide (CTAB) additionally removes polysaccharides and remaining proteins. An increase from 1 % to 5 % CTAB in the DNA extraction buffer allows an improved lysis of archaeal cell walls which significantly differ from the bacterial cell walls (Sogin et al. 2006; De Corte et al. 2009) (see Note 5). In some cases, e.g. DNA extraction of samples containing high amounts of Gram positive bacteria, initial mechanical cell lyses might be necessary, e.g. using a bead beater with small glass, ceramic, zirconium, or steel beads (Treusch et al. 2004) (see Note 6). Finally, the isolated metagenomic DNA is analyzed by gel electrophoresis and should contain large fragments (Fig. 4.3) in case of constructing a metagenomic large insert library.

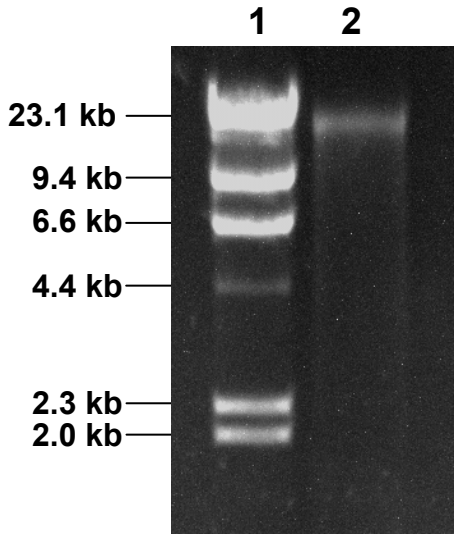


Fig. 4.3: Gel electrophoretic analysis of metagenomic high molecular weight DNA

3. 16S rDNA phylogenetic analysis

Bacterial and archaeal 16S rRNA genes present in the metagenomic DNA are commonly PCR amplified using the Bacteria-specific primer 27F and the universal primer 1492R (Lane et al. 1991) or the archaea-specific primer set 20F and 958R (DeLong 1992), 2-10 ng of extracted DNA (see Note 8) and a standard amplification protocol; e.g. 5 min at 95 °C followed by 30 cycles of 30 s at 94 °C, 45 s at 55 °C, and 1.5 min at 72 °C. The resulting 1,500 bp bacterial or 1,000 bp archaeal PCR fragments (Fig. 4.4) are purified and cloned into a sequencing vector, e.g. a TA cloning vector allowing an efficient cloning (Mead et al. 1991), followed by independent and complete DNA sequence analyses for both strands using the primers 27F and 1492R or 20F and 958R or universal primers complementary to the flanking vector regions (Pages and Holmes 1998; Suarez-Diaz and Anaya-Munoz 2008). The 16S rDNA analysis not only allows insight into present community structure of the respective habitat, it also points out the likely potential of the habitat to detect new biotechnological relevant enzymes. In addition to the knowledge gained on the actual microbial diversity, additional PCR amplifications can be performed using specific primer sets in order to analyze the presence of functional genes, e.g. the *nifH* gene for diazotrophes, encoding a structural gene of nitrogenase, the key enzyme of nitrogen fixation (Langlois et al. 2005b, 2005a; Langlois et al. 2008).

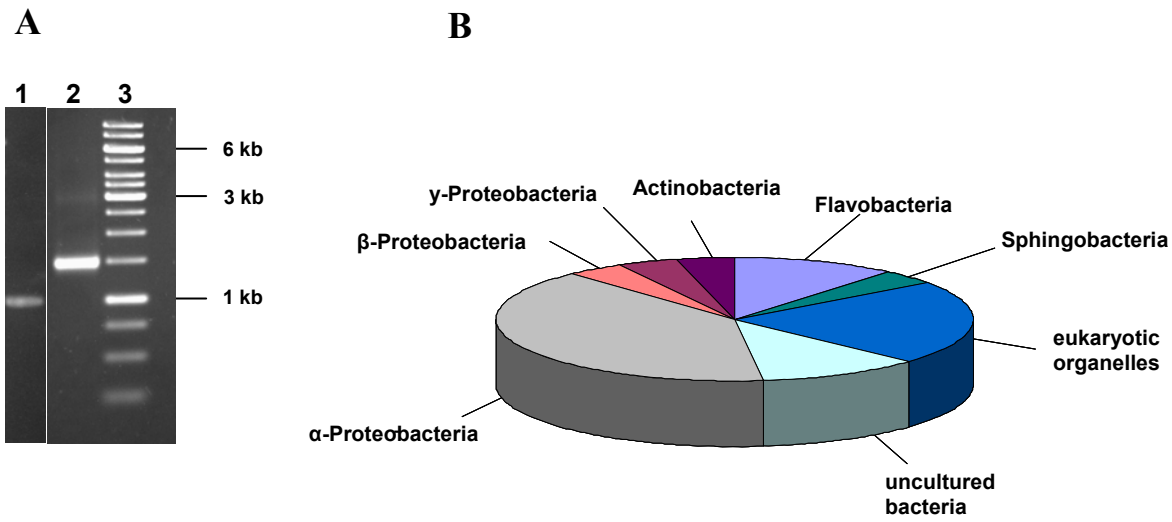


Fig. 4.4: 16S rDNA phylogenetic analysis of a marine habitat. (A) 16S rDNA gene amplification of metagenomic DNA using universal bacterial (line 2) and archaeal (line 1) primers. (B) Respective phylogenetic composition of the marine habitat based on 16S rDNA sequencing analysis.

4. Construction of a metagenomic large insert library

Fosmid and **B**acterial **A**rtificial **C**hromosome (BAC) vectors have been developed to clone large genomic DNA fragments of up to 40 kb and ~ 120 kb, respectively. These vectors replicate using the single-copy F-factor replicon and show high stability carrying large inserts (Wild et al. 1996). Recently, novel large insert vectors have been developed carrying both, the single-copy and an additional inducible high copy number origin of replication (Wild et al. 2002). This ensures on the one hand insert stability and successful cloning of encoded and expressed toxic proteins and unstable DNA sequences, and on the other hand allows increased DNA yields in vector preparations and functional screens of clone libraries by induction to high copy numbers (Sektas and Szybalski 1998). Thus, BACs and fosmids have become standard tools for constructing genomic clone libraries. Genomic library construction kits are commercially available that pursue blunt-end cloning strategies resulting in complete and unbiased libraries. The 'Copy Control™ Fosmid Library Production Kit' (e.g. with pCC1FOS) (Epicentre, Madison/USA) combines all advantages to stable insert large DNA fragments into the vector with little expenditure of time (Fig. 4.5). In the following the corresponding protocol according to the manufacture's instructions is presented:

Preparation of DNA: High molecular weight (meta)-genomic DNA is isolated as described above and diluted in TE buffer at a concentration of ~ 500 ng/µl (see Note 9)

Shearing: DNA fragments in the range of 20-40 kb are obtained by multiple pipetting the DNA solution using a 1000 µl pipette tip

End-Repair of the DNA fragments: The end-repair reaction described below generates blunt-ended, 5'-phosphorylated DNA fragments and can be scaled up or down depending on the amount of available DNA, followed by incubation at room temperature (RT) for 45 min (see Note 10).

Sterile water	x µl
10 x End-Repair buffer	8 µl
2.5 mM dNTPs	8 µl
10 mM ATP	8 µl
Up to 20 µg sheared DNA	x µl
End-Repair enzyme mix	4 µl
Total reaction volume	80 µl

Dialysis: The End-Repair reaction mix is dialyzed for 30 min at RT against sterile water to remove interfering salts. This step can be performed e.g. by using 0.025 µm cellulose filters type VS from Millipore (Schwalbach) placed on the surface of sterile water in a Petri dish, on which the reaction mix is placed.

Ligation: The ligation reaction is mixed in a 10:1 molar ratio of **CopyControl pCC1FOS vector** to insert DNA and incubated for 2 hours at RT followed by overnight incubation at 16 °C (see Note 11). The following reagents are combined in the order listed.

Sterile water	x µl
10 x Fast-Link ligation buffer	1 µl
10 mM ATP	1 µl
CopyControl pCC1FOS vector (0.5 mg/ml)	1 µl
Insert DNA (0.25 µg of 40 kb DNA)	x µl
Fast-Link DNA ligase	1 µl
Total reaction volume	10 µl

Packaging reaction: 10 µl of the ligation reaction are added to one-half of the provided **MaxPlax Lambda Packaging extract** (25 µl) in a reaction tube being kept on ice. The packaging reaction is incubated at 30 °C. After 90 min the remaining 25 µl of **Lambda Packaging Extract** are added and the reaction is incubated for additional 90 min at 30 °C. Following the incubation, the **Phage-Dilution buffer** is added to 1 ml final volume and mixed gently. For storage at 4 °C, 25 µl of chloroform are added.

Titration of the packaged CopyControl fosmid library: Prior transducing the complete packaging reaction it is recommended to determine the phage particle titer (e.g. CopyControl Fosmid clones). 10 µl of the packaging reaction is added to 100 µl of exponentially growing EPI300-T1^{R} host cells (LB containing 10 mM $MgSO_4$) followed by incubation at 37 °C for 20 min. Aliquots of the transduced EPI300-T1^{R} cells are plated on LB plates supplemented with 12.5 µg/ml chloramphenicol and incubated overnight at 37 °C to select for the CopyControl Fosmid clones. Colonies are counted and the phage particles titer is calculated.

Transduction and plating the CopyControl fosmid library: According to the titration and the estimated number of clones required, the volume of the packing reaction (fosmid library) required for the construction of the respective clone library is calculated. The transduction into EPI300-T1^{R} host cells is performed as described above in several parallel reactions using the volumes mentioned above. Appropriate aliquots of the infected bacteria are plated on LB plates supplemented with 12.5 µg/ml chloramphenicol for selection and incubated overnight at 37 °C. Fosmid clones obtained are grown in microtiter plates (96 wells) and subsequently stored at -70 °C in the presence of 8 % DMSO.

Induction to higher copy numbers: The fosmid clones of a library can be induced to reach higher fosmid copy numbers in order to achieve high fosmid DNA yields for sequencing, fingerprinting or other downstream applications. Induction to higher copy numbers is also recommended for direct

function-based screening assays of the clone library e.g. on plates. The induction can be achieved in any desired culture volume depending on the downstream application. In general, LB medium is supplemented with chloramphenicol and 1:1000 of induction solution and 1:10 of the respective overnight culture followed by incubation for 5 h at 37 °C with agitation.

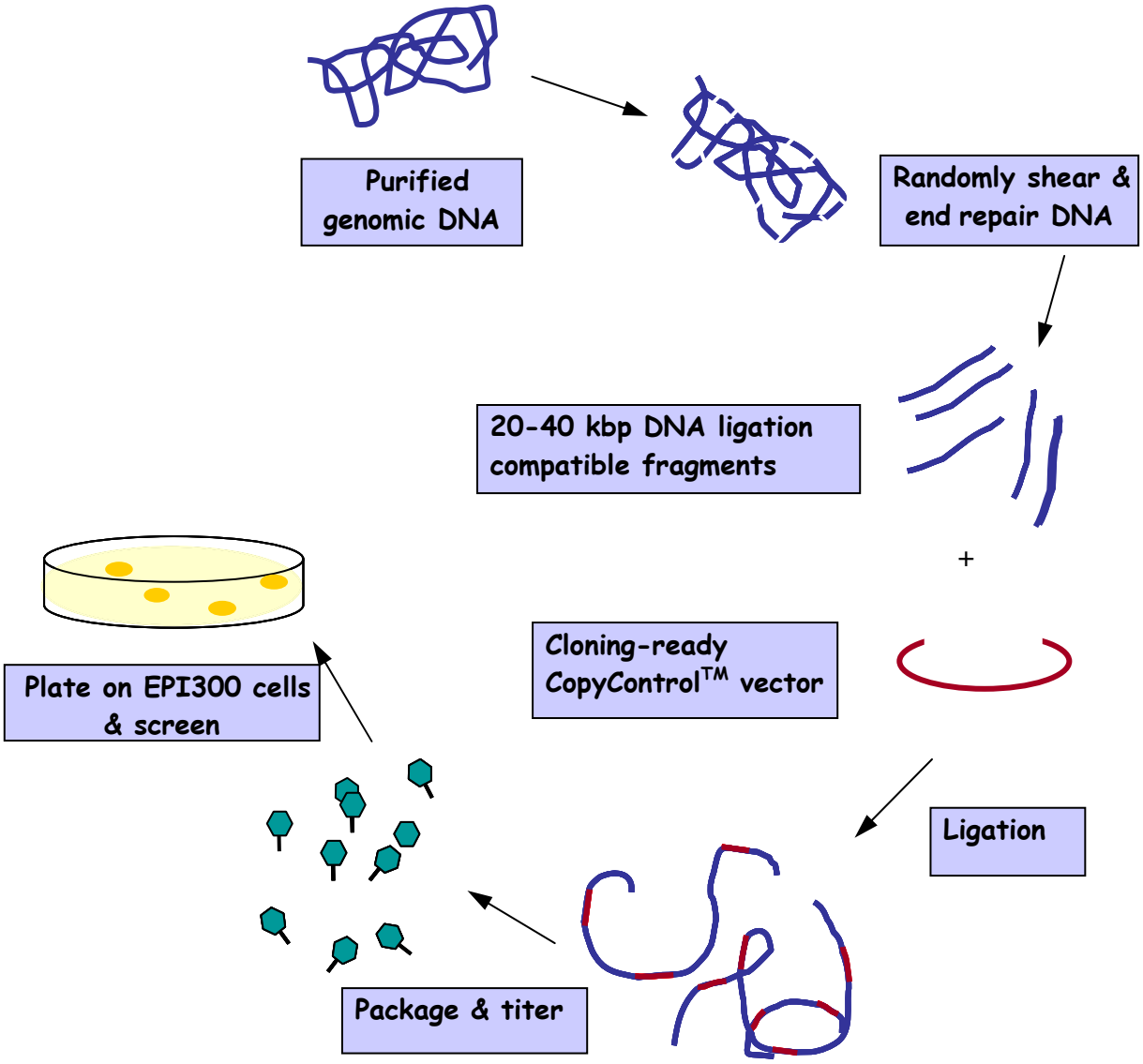


Fig. 4.5: Construction of a metagenomic library (modified according to Epicentre, Madison/USA)

5. Sequence-based screens of metagenomic libraries using a PCR-amplification approach

A sequence-based analysis of metagenomic DNA can be performed by monitoring the presence of respective key genes by PCR amplification in order to identify genes and metabolic pathways. The primers are designed based on the sequences known for the respective gene with the primers binding to conserved regions of the genes. PCR-amplification is performed using the metagenomic DNA, fosmid pools or single fosmids of the metagenomic library. The respective amplified PCR fragment is cloned (e.g. into a TA cloning vector) followed by sequence analysis of randomly chosen clones. A recent example is the identification of a gene encoding a novel cytochrome P450 monooxygenase with a robust catalytic activity in a soil metagenomic library (Kim et al. 2007). Another example is the unexpected high diversity and distribution of the *nifH*-gene, one of the functional key genes for nitrogen fixation, discovered in the surface water of the Pacific Ocean (Langlois et al. 2005b; Langlois et al. 2008). Large scale sequencing projects such as the one initiated by Craig Venter for the metagenome of the Saragossa Sea resulted in the identification of numerous novel genes and is a famous example of sequence-based metagenome analyses (Venter et al. 2004b). Recently, large-scale sequencing of complete metagenomes by massive parallel sequencing, e.g. a pyro-sequencing approach, has been performed followed by bioinformatic analyses and partial assembly of the genomes present in the habitats (Tringe et al. 2005; Green and Keller 2006).

6. Function-based screens of metagenomic libraries

Functional screens for novel genes in metagenomic libraries explore the genetic potential of a habitat by directly monitoring products or enzymatic activity of the metagenomic clones. Metagenomic libraries have been screened for various biomolecules, such as biotechnologically relevant enzymes. So far, functional screens of metagenomic libraries have identified e.g. several novel antibiotics, e.g. turbomycin A and B (Gillespie et al. 2002), aminoacylated antibiotics (Brady et al. 2002) or small antimicrobial molecules (MacNeil et al. 2001) from soil metagenomes, exoenzymes such as lipases (Henne et al. 2000) and marine chitinases (Cottrell et al. 1999) or membrane proteins (Majernik et al. 2001). In the following, the screen for cellulose degrading activity will be exemplarily described.

Screening metagenomic libraries for novel cellulose degrading enzymatic activity.

Cellulases refer to a class of enzymes that catalyze the hydrolysis of cellulose by hydrolyzing the 1,4-ß-D-glycosidic linkages. Several different kinds of cellulases are known which differ structurally and mechanistically (Ghose 1987a, 1987b). Cellulases are widely used in the pulp and paper industry for various purposes, for pharmaceutical applications (Cohen 2007), in the textile industry and are present in laundry detergents. Thus, these enzymes have a high relevance in biotechnology and novel thermal or pH-stable cellulases are continuously searched for industrial application. Metagenomic libraries can be screened for cellulose degrading activities on agar plates with a rapid and sensitive assay system using Congo Red for detection. As Congo Red shows a strong interaction with polysaccharides containing contiguous ß-(1,4)-linked D-glucopyranosyl units and a significant interaction with ß-(1,3)-

D-glucans, zones of cellulose hydrolysis around a metagenomic clone on agar plates can be visualized (Teather and Wood 1982). The following protocol exemplarily describes a plate screen for cellulose degrading enzymatic activity on plates:

- Metagenomic clones stored at -70 °C are directly transferred from the 96 well microtiter plates to the CMC agar plates with a steel stamp, followed by incubation for 24 h at 37 °C.
- The plates are flooded with an aqueous 0.2 % solution of Congo Red for 15 min.
- After pouring off the Congo Red solution the plates are further treated by flooding with 1 M NaCl for 15 min. Degradation of cellulose is indicated by a yellow zone around positive clones (see Fig. 4.6).
- The visualized zones of hydrolysis can be stabilized for at least 2 weeks by additional flooding the agar plate with 1 M HCl which changes the dye colour to blue and inhibits further enzyme activity.

In order to identify the respective open reading frame (ORF) of a confirmed fosmid conferring the desired activity, an *in vitro* transposon mutagenesis can be performed e.g. using the EZ-Tn5^{TM}<oriV/KAN-2> Insertion Kit from Epicentre (Madison/USA). Following the transposon mutagenesis clones are screened for loss of the desired activity. Fosmid DNA of clones that lost the activity are sequenced using primers hybridizing to the 5' and 3' end of the transposon reading into the flanking metagenomic regions. The obtained DNA sequences flanking the transposon are assembled in order to identify the respective ORF, which can be cloned in an expression vector to purify the protein in high amounts.

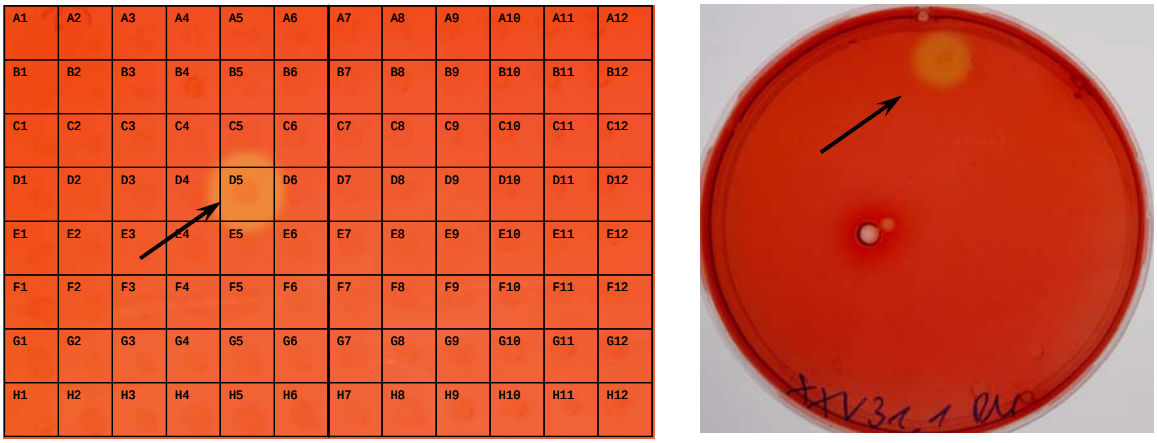


Fig. 4.6: Plate screen for cellulose degrading metagenomic clones

Notes

1. The sampling procedures have to be performed rapidly because of the changing environmental conditions.
2. The filtration should be realized as fast as possible with a supporting peristaltic pump in a cold room or for large volumes preferentially using an *in situ* pump at the respective conditions at the sampling site.
3. Based on the higher number of pores polyvinylidenfluoride filters are preferred to filter high water volumes through a single filter especially when working with small filter diameters.
4. For long time storage filters have to be frozen at -80 °C in practicable dimensions. Before liquid nitrogen treatment the filters have to be cut into convenient pieces to rule out needless freeze/thaw cycles.
5. The standard DNA extraction protocol has to be modified when the samples contain high amounts of polysaccharides and glycoproteins. In this case the sample should be treated with higher percentages of CTAB to support disintegration of samples.
6. In some cases an additional mechanical cell lyses step might be necessary as some bacteria/archaea may not be cracked with enzymatic methods.
7. Sometimes extracted metagenomic DNA shows a high degradation because of DNases present in the sample. In this case addition of EDTA to the DNA extraction buffer helps suppressing the damage of DNA. (EDTA is used for scavenging metal ions to deactivate metal-dependent enzymes).
8. The crucial step of the 16S rDNA PCR amplification is to amplify the bacterial/archaeal 16S rDNA fragments from the optimal amount of template DNA, which can differ from 1 pg to 1 µg.
9. If the extracted metagenomic DNA will be used for library construction the DNA should routinely be analyzed for degradation to decide if shearing is necessary or this step might be skipped.
10. Before preparing the End-Repair reaction the DNA concentration has to be determined precisely by measuring the absorbance at 260/280 nm, as in the following dNTPs are added and all following steps and calculations are based on this DNA quantification.
11. A size selection of 20 – 40 kb End-Repaired fragments can be performed to ensure that only large inserts are ligated into the pCC1FOS vector. In special cases the molar ratio 10:1 of fosmid vector to insert DNA can be optimized (5:1 or 7.5:1) to increase the clone number.

Discussion

The overall goal of this study was to identify microbes involved in key processes of the marine N-cycle and to assess their contribution to the respective processes under changing O_2 conditions in the ocean. We were particularly interested in N_2-fixation and the biological production of the greenhouse gas N_2O. Ultimately, our results were supposed to lead towards predicting future changes of the strongly O_2-sensitive biological N-cycle processes with regard to the expansion of oceanic OMZs and their ongoing deoxygenation.
In the following, the role of OMZs of different intensities as hotspots for N-turnover processes will be discussed; trends and open questions with regard to the marine N-cycle and the potential of meta-omics for marine studies will be assessed.

The N-cycle

N_2-fixation and the fixed N deficit in the ocean:
According to current estimates, a large deficit in oceanic fixed N resulting from a total N-loss term exceeding the N-gain is suggested. As an overestimation of the N-loss term appears unlikely (Codispoti 2007), the apparent deficit in fixed N (~200 Tg N a^{-1}) is ascribed to an underestimation of N_2-fixation as the major source for oceanic fixed N. Three major reasons for an underestimation of the fixed N_2 term were identified during our studies, of which the first is a methodological underestimation of N_2-fixation rates resulting from using the classical $^{15}N_2$-tracer technique (Montoya et al. 1996). The development of a novel method (Mohr et al. 2010) demonstrated a non-linear underestimation of N_2-fixation in the environment of up to 6-fold compared to the classical method (Grosskopf *et al.*, submitted).
We further demonstrated that the classical view of N_2-fixation taking place mainly in nutrient-depleted surface waters and to only a minor extent in deeper waters has to be reassessed. The detection of eight novel *nifH* clusters in the ETSP and two novel *nifH* clusters in the ETNA (Joshi and Löscher, in prep.), their presence along with active N_2-fixation and at least occasional expression in those areas indicated an important role of those clusters, particularly, under extremely anoxic conditions as present in the OMZ off Peru. Those diazotrophs consisting at least partially of heterotrophic clades, as hypothesized from glucose fertilization experiments, might contribute essentially to the oceanic N-input. With regard to the ongoing O_2 decrease in OMZs along with eutrophication (Codispoti et al. 2001; Capone 2008; Stramma et al. 2008; Stramma et al. 2010), those clusters, preferably present in OMZ waters, might be dominating the oceanic diazotrophic community in the future. In addition, we detected highest N_2-fixation comparable to those from waters dominated by major *Trichodesmium* blooms connected to an anoxic sulphidic event (Chapter 1). This temporally limited significant enhancement of N_2-fixation might result from a variety of factors present during this event, e.g. the

increased availability of trace metals, such as dissolved iron, predominantly present as Fe^{2+} during this event (Schlosser and Croot, unpublished). Enhanced N-loss processes, as detected at this transient event, as well, might have additionally triggered N_2-fixation by rising P*. Assuming such events will occur more frequently in the futures possibly as a result of large plankton blooms as a consequence of ongoing eutrophication and their subsequent degradation; we speculate that this term might substantially impact on the marine N-budget. Thus, it is crucial to understand and explore the genetic and metabolic potential present in the microbial community to assess the ability to adapt to those rapidly changing conditions and thus to the future trend of expanding OMZs.

OMZs were previously proposed potential niches for diazotrophs and to promote N_2-fixation, as those waters are rather depleted in biological available N (Deutsch et al. 2007) as a result of massive N-loss (Kuypers et al. 2005; Deutsch et al. 2007). Hence it has been suggested, that high P* values as present in upwelled N-depleted waters connected to OMZs trigger N_2-fixation, normalizing the N-deficit towards the open ocean as previously predicted by biogeochemical models (Deutsch et al. 2001; Deutsch et al. 2007). An even closer spatial link between N-loss and N_2-fixation in OMZ waters has been suggested by our studies (Chapter 1). The co-occurrence of one novel cluster (P1) with key functional genes and transcripts of nitrification, denitrification and anammox as well as the demonstrated activity of those processes (mainly nitrification and anammox, detected by isotope pairing studies, Kalvelage *et al.*, unpublished) support a co-occurrence of N-loss and N-input in the OMZ off Peru. Hence, we propose that the deficit in bio-available N resulting from N-loss processes triggers N_2-fixation directly. However, we can not directly exclude that N-loss and N_2-fixation are triggered by a similar unknown parameter. The overall presence of P* in the ETSP (Deutsch et al. 2007) still points towards the fact that N_2-fixation is not able to balance the N-loss in that area. Thus, we argue, that the pronounced presence of P*results to major part from enhanced P regeneration in the sediments and shelf bottom waters favouring substantially the release of reactive P to the water column (Ingall and Jahnke 1994). Consequently, the present P* would not exclusively result from N-loss. OMZs with high P* might therefore provide an ideal niche for diazotrophs.

Taking the methodological underestimation (Mohr et al. 2010) along with the suggested major contribution of deep heterotrophic N_2-fixation and with transient events in rapidly changing systems into account, oceanic N_2-fixation needs to be revised upwards. Further studies need to focus on reassessing the in situ N_2-fixation using the novel method and moreover the distribution of the *nifH* gene and the diazotrophs in general to estimate the potential of the diazotrophic community to react to changes in dissolved O_2. Moreover, the role of OMZs as hotspots for N-turnover as suggested in Chapter 1 and thus as potential niches for diazotrophs has to be analyzed in depth.

OMZs as hotspots of N_2O production:

Besides the importance for oceanic N_2-fixation, OMZs were also proposed important areas for N_2O production. Favoured by low O_2 conditions as present in O_2 deficient waters of the OMZs (Naqvi et al., 2010), the biological formation of N_2O is predicted to strongly react to even minor changes

(Codispoti 2010). However, current estimates ascribe 25-50% of oceanic N_2O production to OMZs (Suntharalingam et al. 2000); this term might severely increase with ongoing deoxygenation of OMZs and their expansion.

The paradigm shift of N_2O production by mainly ammonia-oxidizing archaea (AOA) rather than by ammonia-oxidizing bacteria (AOB) as previously proposed (Wuchter et al. 2006; Church et al. 2009; Santoro et al. 2010; Santoro et al. 2011) and verified by our studies using a pure culture of *Nitrosopumilus maritimus*, demonstrated the rather poor understanding of major pathways of the marine N-cycle. Although the abundant presence of AOA in the water column of the ETNA and ETSP along with maxima in N_2O has already been observed (Church et al. 2009; Santoro et al. 2010), our onboard incubation experiments using an archaea-specific inhibitor were the first direct evidence for archaeal N_2O production in the ocean (Fig. 3.5, Chapter 2).

The pronounced inverse correlation of O_2 concentrations and N_2O formation (Fig. 3.6) determined in the presented experiments using pure cultures demonstrates the strong sensitivity of N_2O formation to changes in dissolved O_2, possibly explaining the high amounts of N_2O present in OMZs. Although further studies are required to identify the O_2 threshold for N_2O production by AOA, Kalvelage *et al.* (Kalvelage *et al.*, unpublished) showed by rate measurements that ammonia oxidation is possible at O_2 concentrations as low as ~1 µM O_2, which is considerably lower than previously expected. Assuming an ongoing inverse correlation of N_2O production via nitrification at those low O_2 conditions and moreover additional N_2O formation via denitrification, this would possibly explain the maxima in N_2O detected in and close to OMZs.

In particular, massive N_2O concentrations have been detected in the OMZs off Peru (~370 nM Löscher, unpublished) and West India (Naqvi et al. 2000) (Fig. 5.1). While Naqvi *et al.* (Naqvi et al. 2000) ascribed large parts of the N_2O production at hypoxic conditions to denitrification; we propose a combination of denitrification and nitrification in the OMZ off Peru resulting in the present high N_2O concentrations. Regarding the gene abundance and activity of the archaeal *amoA* gene, used as the functional marker for archaeal ammonia oxidation in this investigation, a contribution of this process to N_2O production is highly likely. Nevertheless, a ΔN_2O/AOU correlation has not been detected, (Loescher et al. 2011; Ryabenko et al. 2011) and delta ^{15}N values strongly point towards additional denitrification activity (Ryabenko et al. 2011). Possibly depending on the time point with regard to deoxygenation of the water column and later on re-oxygenation, one of those processes might dominate the overall production. While Naqvi *et al.* and Farias *et al.* (Naqvi et al. 2000; Farías et al. 2007) proposed high N_2O production due to denitrification during deoxygenation; we propose additional production of N_2O due to beginning nitrification when the water recovers from anoxia. Pulse experiments using H_2S demonstrated, that nitrification is significantly enhanced during re-oxygenation after the sulphide pulse (Erguder et al. 2008), those findings in line with observations from the Baltic Sea (Schweiger et al. 2007), where nitrification was found to be significantly enhanced during re-oxygenation, strongly point towards an important role of ammonia oxidation for N_2O production at rapidly changing conditions.

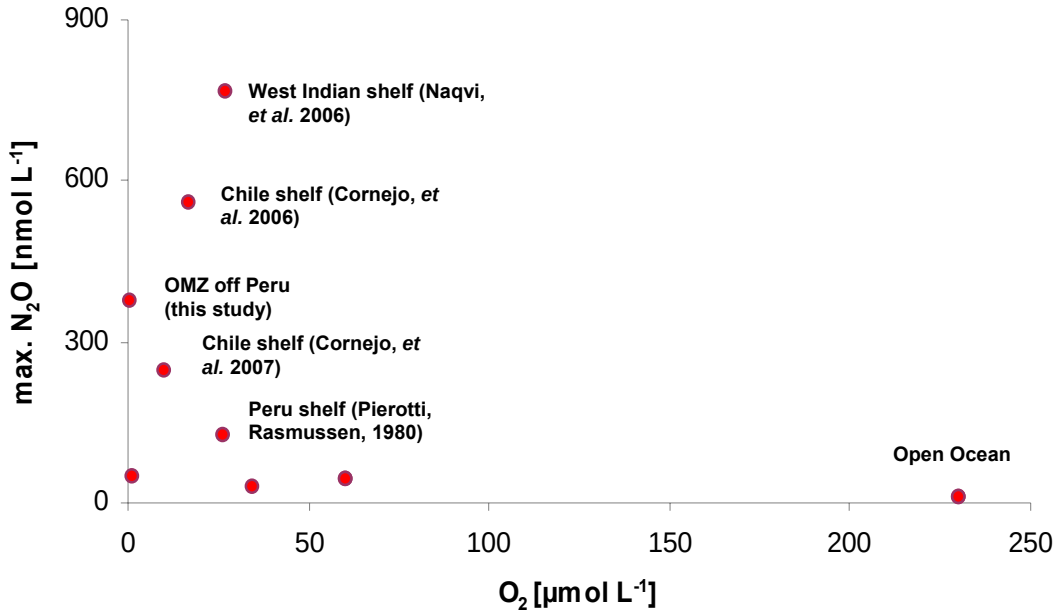


Fig. 5.1: Maximum N_2O concentrations vs. associated O_2 concentrations in coastal upwelling regions. For comparison a typical N_2O surface concentration in the tropical open ocean is shown as well. Data sources: W. India and Oman – W. Naqvi, pers. comm.; Open ocean off Mauritania – A. Kock and H.W. Bange, unpublished; Chile – Cornejo *et al.* (2006); Peru – C.R. Löscher and H.W. Bange, unpublished.

These findings demonstrate that under low O_2 conditions, as present in OMZs, N_2O production in those environments is highly complex and might rapidly change over short time periods.
To improve the understanding of the oceanic distribution of N_2O and the physiology and metabolism of the major production processes it is crucial to apply novel developed molecular techniques, which allow gaining insight into those complex processes responsible for N_2O production.

In summary, the classical view of N-turnover processes, of the habitats of the contributing microorganisms and their O_2 tolerance has to be reassessed, we propose an extension of the diazotrophic habitat to low O_2, high NO_3^- habitats, further, we suggest, that nitrification can occur along with anammox and denitrification (Fig. 5.2), and that N_2O is mainly produced by AOA and not AOB at suboxic to hypoxic conditions.

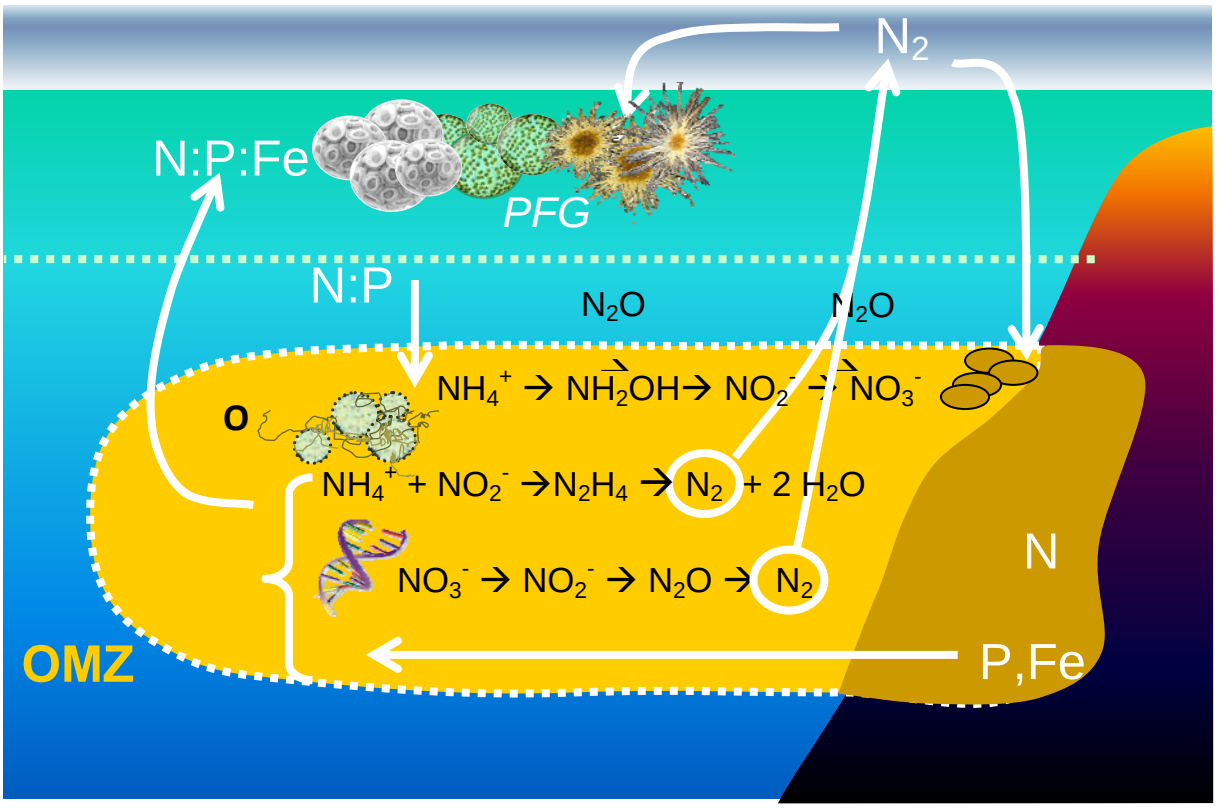


Fig. 5.2: Model of N-cycle processes in and near OMZs: A co-occurrence of nitrification, denitrification and anammox in OMZ waters and in the oxycline is proposed.

Meta-omics

Applying metagenomics and metatranscriptomics on marine samples

Although microorganisms are driving the majority of biogeochemical cycles, thus shaping the environment of the earth and its oceans, their diversity and metabolic potential is understood to only a minor extent (Amann et al. 1995; Venter et al. 2004; DeLong and Karl 2005; DeLong 2009). The development of culture-independent approaches, e.g. large insert clone libraries or whole (meta-) genome sequencing techniques led to surprising novel insights in the microbial potential in the ocean over the last few years, e.g. the identification of proteorhodopsin (Beja et al. 2000; Beja et al. 2001) and the presence of the *amoA* gene in archaea (Schleper et al. 2005). A substantial progress in the field of marine meta-omics was initiated by the results obtained from the global ocean survey (Venter et al. 2004). This large-scale investigation demonstrated the wealth of diversity in marine microbial communities and functional genes in the ocean. In addition to classical Sanger-sequencing, which can exclusively reveal the diversity in previously selected target genes, the pyrosequencing approach has the potential to detect whole (meta-) genomes and thus genes of unknown functions and varying molecular structures. Moreover, it allows a substantially deeper insight into the community structure present in environmental samples as suggested by the preliminary evaluation of our studies (Fig. 5.3).

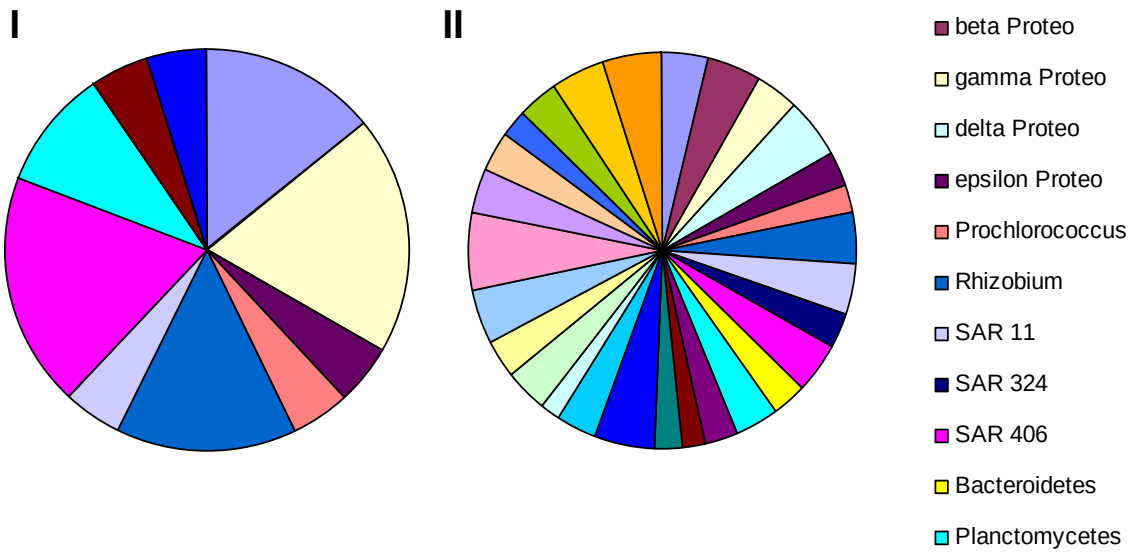


Fig. 5.3: 16S rDNA Sanger sequencing (I) compared to Pyrosequencing (II): Only major groups (which are mentioned in the legend) were detected by classical Sanger sequencing, a broader diversity was recovered from the Pyrosequencing dataset (samples from M77/3, #3, 50 m).

Although, the results obtained from those studies are impressing, the bottleneck to overcome nowadays is the bioinformatic evaluation of the large amount of obtained metagenomes and –transcriptomes, particularly with regard to the definition of quality standards, comparability and database management.

The meta-omic results obtained from this projects' dataset (Schunck *et al*., unpublished) demonstrate the need for improving the bioinformatics tools used in meta-omics. In this dataset, an average relative amount of ~ 60 % of sequences present in the obtained libraries was denoted genes of unknown function. This amount of e.g. hypothetical proteins, as also detected in comparable proportions in other datasets (Tyson et al. 2004; Venter et al. 2004; Tringe et al. 2005) sustains a vast unexploited source for novel enzyme driven pathways which might possibly be unravelled by combining gene patterns to environmental parameters and present biogeochemical processes (Bohnebeck et al. 2008).

With regard to high resolution gene profiling, significant progress was made during this project by developing highly sensitive qPCR detection systems (gene- and cluster-specific) for all key genes known to catalyze reactions in the marine N-cycle. Particularly with regard to the *nifH* gene, a need for cluster specific gene quantification has been demonstrated, as this enables to ascribe certain ecotypes to biogeochemical patterns. Thus, a deeper understanding of the cluster specific distribution is crucial to assess future changes in the community composition of diazotrophs (in terms of *nifH*) and thus in marine N_2-fixation.

The development of a (70mers) DNA-microarray during this study enables an additional fast and highly standardized initial screen of large amounts of samples for genes involved in the N- cycle, followed by more quantitative methods, e. g. RT-qPCR. The developed microarray represents a set of

key functional genes involved in the N-cycle. It contains 154 different oligomers (Fig. 5.4, 104 oligomers were deducted from sequences derived from samples from the water column of the ETNA and ETSP, a set of 40 oligomers was deducted from genes involved in the N-cycle obtained from the NCBI database to cover a broader diversity on the microarray and to include cultivated model organisms). Initial evaluation of the microarray showed a very high consistence with qPCR datasets for an exemplary sample (Fig. 5.4).

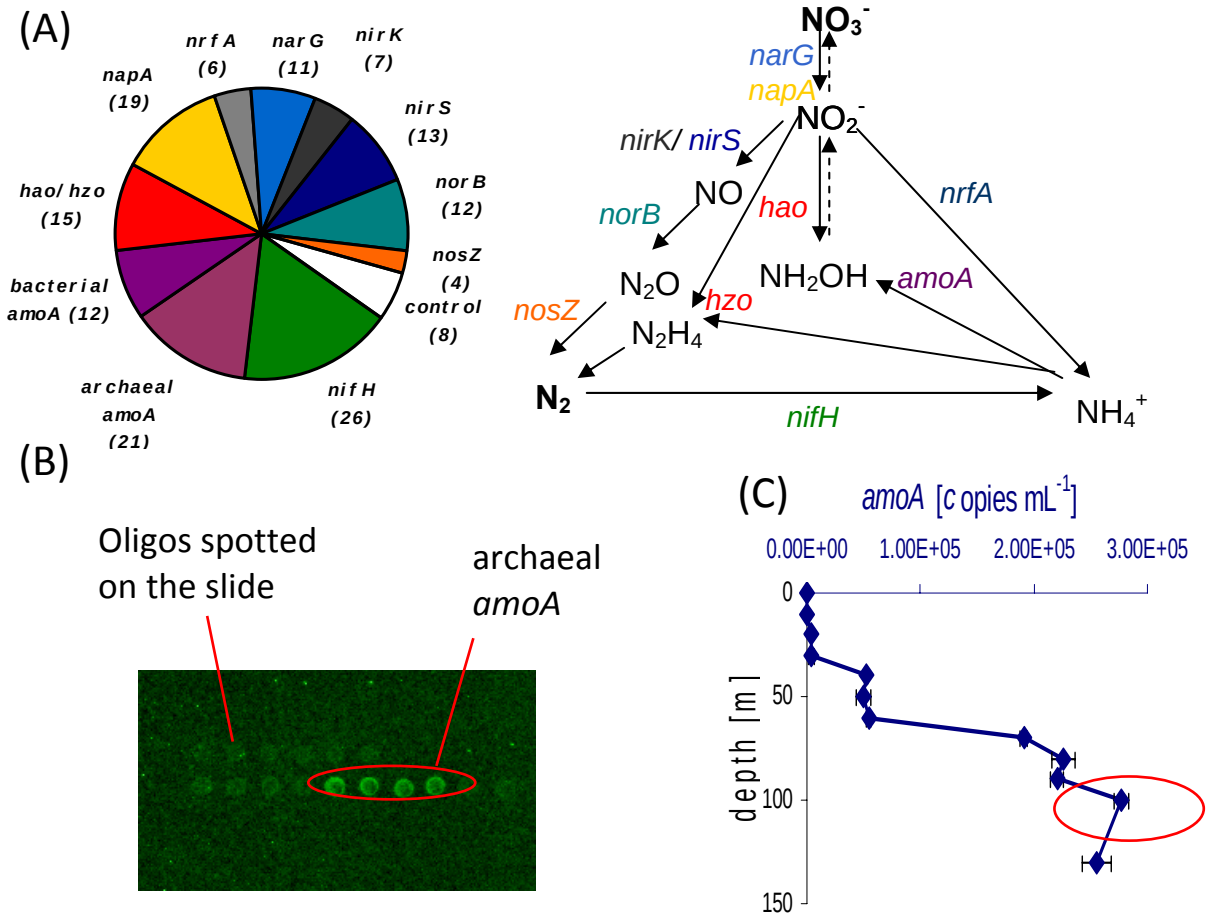


Fig. 5.4: (A) A 70mer microarray containing a broad set of key genes involved in the N-cycle was constructed; (B) a first test run using DNA from the Pacific showed results consistent with the qPCR quantification (C)

Although the detection systems developed during this study appear to reveal realistic distribution patterns with regard to gene abundance, the detection of gene expression is still not satisfactory. A large discrepancy in gene abundance and gene expression has been detected for all measured genes; variations ranged from 1-2 orders of magnitude in case of *amoA* (Chapters 1, 2) to not even detectable expression in case of *nifH* (Chapter 1). In both cases, the activity of the respective process was demonstrated by rate measurements or inhibitor experiments; hence the observed difference is proposed to result from a methodological problem. Therefore, we hypothesize, that the low detected transcript numbers result from rapid RNA degradation in environmental samples from OMZs, where

RNAs might degrade when getting oxygenated. Similar observations have been made in *Prochlorococcus*, where some RNAs encoding for O_2-sensitive enzymes degraded very rapidly ($T_{1/2}$ = 2.4 min). Preliminary results of experiments using pure cultures of *Methanosarcina mazei* Gö1, *Klebsiella pneumoniae* M5A1 and *Azotobacter vinelandii* (DSMZ 332) indicate that the difference in gene copies and transcripts results from an O_2-dependent RNA degradation, further investigations are urgently needed, in order to optimize the sampling method Chapter 1, supplemental material).

Even though the applied techniques might be biased to a certain degree, they allow to specifically detect for key genes of N-cycle pathways, thus helping to understand the diversity and distribution of genes and organisms involved in the N-cycle, respectively. Nevertheless, a most realistic assessment of the ongoing processes of the oceanic N-cycle leading to valuable future predictions can only be achieved by a combination of molecular tools with rate measurements and the determination of the distribution of N compounds in the oceans' water body.

Future development of the marine N-cycle under changing O_2 conditions

OMZs of different intensity: ETNA and ETSP

The pronounced difference between the two investigated areas (ETNA and ETSP), with regard to the N-cycle raised the question on the origin of those variations. As basic features of the water mass, the age and thus the O_2 and nutrient content combined with the depth of the shelf and the overall volume are proposed important factors, with large shelf areas, and relatively low water depths leading to deoxygenation via a close spatial coupling of plankton blooms and the recycling and respiration of sinking organic matter. Independent of the age of the water mass and the expansion of the shelf, the difference in expression of the OMZs in the ETNA and the ETSP another fact might be of importance: Both systems show a rather comparable primary production, thus, comparable amounts of organic matter (OM) are assumed sinking down to the OMZ. Nevertheless, it appears, that a difference in respiration in the OMZ is present thus resulting in a variation in O_2 consumption and consequently in deoxygenation. Possibly, the residence time of OM in the OMZ of the ETNA is influenced by the input of Saharan dust particles (in contrast to the ETSP, where dust input is rather low), increasing the sinking speed of OM and enhancing the export from the OMZ, thus shortening the remineralization time. Although, information on particle sinking speeds in the ETSP and ETNA is rather sparse, it might be one factor shaping OMZs of different intensity (Fig. 5.5).

Besides these general differences, the occurrence of the transient H_2S event demonstrated that such extreme situations act as temporally limited hotspots of N-turnover, reflected by highest N_2-fixation rates and significantly enhanced N_2O production. Although data on the frequency of the occurrence of those events is rather sparse, eutrophication along with ongoing water deoxygenation might provoke them.

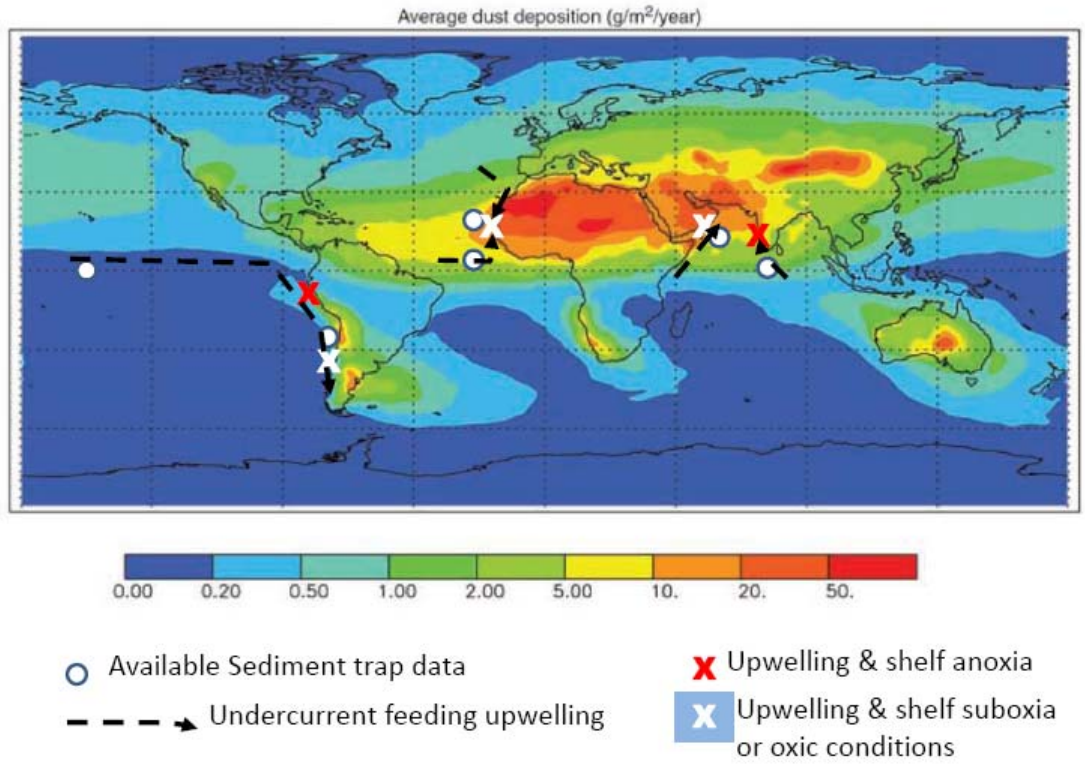


Fig. 5.5: Particle sinking speeds might influence the formation and future development of OMZs (Bange, unpublished)**.** The Saharan dust input in the ETNA might decrease residence times of organic matter in the OMZ off Mauritania, decreasing respiration times and ultimately resulting in lower deoxygenation.

The N-cycle in the ocean: future trends and open questions

Several striking results regarding the marine N-cycle in and close to OMZs were obtained over the recent years, e.g. by identifying the importance of anammox, particularly in OMZ waters (Dalsgaard et al. 2003; Kuypers et al. 2003), the leading role of archaeal for oceanic ammonia-oxidation (Wuchter et al. 2006) and the unexpected higher diversity of diazotrophs, also particularly important in OMZs (Fernandez et al. 2011; Hamersley et al. 2011). It has already been speculated, that it will soon be time to rewrite the textbooks on the marine N-cycle (Deutsch et al. 2007). However, there are several topics and open questions which have to be addressed, before.

Some lacks of knowledge might be addressed more directly, such as the potential role of archaea for other pathways than nitrification in the marine N-cycle. Cabello *et al.* (Cabello et al. 2004) discussed the overall potential of archaea to denitrify in low oxygen environments; however, direct evidence for an archaeal contribution to denitrification has yet not been demonstrated in the ocean. The presence of several clusters of crenarchaeota and euryarchaeota (among those e.g. methanogens known to fix N_2 and partially containing several reductase for denitrification) in metagenomes from the OMZ off Peru (Löcher, unpublished) might point towards role of those organisms in denitrification of N_2-fixation. An additional goal would be to identify a key functional marker gene or a similar molecular tool for the oxidation of nitrite to nitrate in marine systems, which is currently lacking. Moreover, it is crucial to unravel the exact metabolism and the full enzymatic pathway which is used in archaeal ammonia

oxidation; the step of N_2O formation in those organisms has to be identified and the missing intermediate product, which corresponds to hydroxylamine in AOB, must be determined.

The possibility of N_2-fixation in OMZ waters as demonstrated by our studies opened a door for consecutive questions including a reassessment of N_2-fixation rates at depth, environmental controls on N_2-fixation and finally a re-estimation of the marine N-budget, accordingly. Nevertheless, also waters beyond OMZs (as a cut-off with regard to depth could not be determined in our studies; i. e. P8 was still present at > 4000 m depth in the ETSP), sediment N_2-fixation and the rarely explored possibility of N_2-fixation in cold waters have to be considered, when re-calculating the oceanic N-budget.

In a global context with regard to future climate change, it is crucial to determine and understand the effects of global warming, ocean deoxygenation and ocean acidification and their co-occurrence on the marine N-cycle. Direct impacts of temperature on e.g. on the distribution of diazotrophs have previously been described (Langlois et al. 2008; Stal 2009); however, information of temperature dependency of other N-cycle processes is sparse. Further, an impact of changes in pH has been demonstrated on major clades of diazotrophs. However, previous studies focussed mainly on cyanobacterial diazotrophs (Hutchins et al. 2007; Levitan et al. 2007; Ramos et al. 2007; Fu et al. 2008) and not on heterotrophic diazotrophs in OMZs. Although a decrease in nitrification by ocean acidification has been demonstrated (Beman et al. 2011), we speculate, that this would not necessarily impact on the production of the by-product N_2O, which is consequently attractive to explore. Overall, a direct effect of oceanic deoxygenation on N_2-fixation and N-loss has been expected and moreover been demonstrated in our studies, as all involved processes are strongly sensitive towards changes in dissolved O_2. Thus, the expansion of OMZs will affect the N-cycle in the future, and thus it will impact on the global ocean, with fundamental impacts on marine ecosystems and marine resources.

Outlook

Following up the unexpected findings of this study several questions rose with regard to the marine N-cycle. First, the presence of novel diazotrophic clusters and the application of a novel method allowing more accurate N_2-fixation rate measurements (Mohr et al. 2010) demonstrated the urgent need for reassessing the marine N-budget. The surprisingly broad diversity of diazotrophs recovered from the diversity in *nifH* genes and the fact that some of them are likely heterotrophs as deducted from incubation experiments further demonstrated the need to recover partial or complete genomes of those organisms in order to assess their complete metabolic potential. This issue will be addressed, now, by high throughput sequencing of fosmids, carrying the *nifH* gene, and enrichments or isolates, gained from seawater samples. In addition to unravel unknown genomes of diazotrophs, fosmids carrying the *amoA*, the *nirS* and the *hzo* genes are present in already existing metagenomic libraries and are attractive being analyzed by high through put DNA sequencing, as well.

An initial fast gene presence and expression screening will be performed on samples from depth profile by using the microarray designed and constructed during this study. Moreover, the existing TaqMan probes and detection system will be used for high resolution screening of functional genes, novel probes might be designed to detect potentially existing additional clusters.

Over the next years, the functional diversity of microorganisms present in and near to OMZs off Peru and Mauritania will be assessed, in detail. Key microorganisms capable of nitrification, DNRA, anammox, denitrification, N_2O production and N_2-fixation in those areas will be identified; their distribution will be detected and quantified in high resolution along vertical and horizontal O_2 gradients. Here, our particular interest will be set on gene distribution and activity patterns including all novel *nifH* and *amoA* clusters.

The possibility to measure intermediate short lived N compounds, now, such as hydroxylamine (NH_2OH), hydrazine (N_2H_4) and nitric oxide (NO) will be used to identify zones of ongoing N regeneration, N-loss and N_2O production and might further lead to a deeper insight in missing links in the marine N-cycle.

The observed difficulty of RNA degradation will be addressed by developing a filtration system which allows pre-filtration leading to shorter filtration periods and which uses a cover gas atmosphere to protect O_2-sensitive RNAs from O_2-dependent degradation.

Novel mRNA-based techniques developed by Schunck *et al* (unpublished) will be applied on samples along vertical O_2-gradients and on samples from O_2-manipulation incubation experiments in combination with rate measurements using stable isotope tracers.

To address those goals, we will participate in 4 cruises to the eastern tropical South Pacific OMZ and in TENATSO (tropical eastern North Atlantic Time Series Observation, near Cape Verde) sampling.

The TENATSO long term study will allow to monitor changing conditions over a time period of several years, as it has been monitored by us since 2007, whereas the consecutive cruises to the Peruvian OMZ will allow monitoring this system in depth.

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Erklärung

Ich versichere hiermit, dass ich die vorliegende Dissertation nach den Regeln guter wissenschaftlicher Praxis selbst verfasst habe und ausschließlich die angegebenen Hilfsmittel benutzt habe. Ich habe dabei keine Hilfe außer der wissenschaftlichen Beratung durch meine Betreuerin Prof. Ruth Schmitz-Streit in Anspruch genommen. Darüber hinaus erkläre ich, dass ich noch keinen Promotionsversuch unternommen habe.

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Kiel, den

Carolin Löscher