Microbial nitrogen guilds in the oceans: from the bathypelagic to the ocean surface

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Abstract

Microbial nitrogen guilds play crucial roles in the oceanic nitrogen cycle, but their composition and importance across specific depths and conditions remain unclear. In this study, we examine the guilds participating in nitrogen transformations within the mid-latitude ocean ecosystems, from the surface down to 4000 m, using data obtained from 75 samples belonging to 11 stations in the Malaspina (MP) dataset. In particular, we used a quantitative approach to investigate the stability of nitrogen acquisition and nitrogen-redox guilds separately. Our results showed that nitrogen acquisition guilds are more stable and redundant than nitrogen-redox guilds across depths and conditions related to specific water columns. However, differential conditions such as nitrogen depletion and oxygen availability affected the two groups of guilds in different ways. These findings have significant implications for the understanding of global nutrient fluxes and the biosphere's functional diversification.

Keywords: microbial guilds, marine ecology, nitrogen cycle, microbial ecology, protein diversification

1 Introduction

Microorganisms have colonized a multitude of niches distributed over the Earth's superficial layers, including the lithosphere, the hydrosphere, and the lower layers of the atmosphere [1]. The functional repertoire found in them has diversified to non obvious extents, making the study of nutrient fluxes very hard [2–4]. Of them, water bodies represent the most important biological functional reservoirs in terms of global nutrient fluxes [5–7]. Thus, we may still be unaware of several oceanic fundamental biotic processes in the oceans. For example, anaerobic ammonia oxidation mediated by microbes (anammox) is an ecologically very relevant process ($\approx \frac{1}{2}$ of total N_2 release from the oceans is attributed to anammox [8], while oscillating between 4-79% in specific conditions [9]), but, surprisingly, it had not been uncovered until three decades ago [10].

Part of the functional variation in the oceans is explained by the fact that the Earth's water bodies do not constitute a single biome, but rather a compendium of them corresponding to variable characteristics such as salinity, temperature, access to nutrients (including irradiance), or hydrostatic pressure [11]. To functionally study microbiomes across these ever-changing environments, we propose using a suitable quantitative approach such as microbial guilds [12]. In the present contribution, microbial guilds belonging to mid-latitude oceanic biomes were analyzed, as represented by metagenomes from the Malaspina 2010 (MP) expedition [13, 14]. For this purpose, we classified the MP metagenomes according to their corresponding depth, establishing three categories: epipelagic (0-200 m depth), mesopelagic (200-1000 m depth) and bathypelagic (1000-4000 m depth). This depth criterion is typically applied to understand marine ecology because these discretized oceanic layers share many main physicochemical characteristics [15, 16]. However, geographic location is also a key factor to consider when establishing the oceanic physicochemical regions, as the Longhurst's provinces attempt to do [17]. Therefore, we considered both depth and specific locations to study how they influence the oceanic microbial functions.

In particular, we focus on the main guilds of the N cycle. The reasons for this are manifold: (i) there exist up to eight redox states in the range of possible N compounds in the ocean [18]; (ii) they consist of both organic and inorganic N species [19]; (iii) N compounds are used in a variety of ways, both as an energy source and as an essential nutrient, and can sometimes serve as an electron acceptor under suboxic conditions [20]; (iv) there is also variability in N-limitation among different areas and compounds, for example, N_2 is never limiting, whereas nitrate (NO_3-) frequently limits phytoplankton growth [18, 20]; and (v) the main functions of the N cycle and its substrates are well described [21–23]. Thus, the N cycle lies in an intermediate stage between complexity and predictability, making this an interesting candidate to describe the change in its functions in detail.

Just as we have classified different MP environments using the depth-location criterion, we'll also classify the guilds related to the N cycle according to the kind of ecological processes in which they are involved. We established the two most general categories: (1) acquisition of N compounds, and (2) redox transformation of the N species acquired from the environment.

Regarding (1), we already pointed out that there are a lot of different N compounds that microorganisms can import from oceanic waters. Within (2) we find transformation processes where imported N compounds act as electron donors or acceptors. To clarify the dynamics of N uptake and N redox transformations, and determining how they respond to depth, is something that has been tentatively explored [14, 24, 25], but it is not yet described using a quantitative ecological framework. With this clear division between biomes and guilds, their interplay in the MP metagenomes will be described.

2 Results

2.1 N/P ratios of main MP water columns

In order to look for differences between the contributions of different guilds to the N cycle, we already considered the different depth categories as environmental factors, but what geographical criteria could we apply to consistently impact N-related functions? We thought of the N/P ratio, which is an environmental factor of interest for oceanographic characterization. It provides a rough idea of how limiting is the dissolved N in an environmental context [26]. When we calculated this ratio in our samples, from the mesopelagic to the deepest, the values stabilized. We observed that the values were only slightly different from 16:1 – the Redfield ratio which is considered not to be either N or P limiting (Fig. 1). However, two stations showed markedly different profiles. The first was a water column off the coast of Mexico (corresponding to the station 120), where an Oxygen Minimum Zone (OMZ) occurs. The samples belonging to this station had a very low mesopelagic maximum (N/P = 13.86), and its values were always lower than the other samples after certain depth, as shown in Figure 1. The second was a station from the open North Atlantic Ocean (corresponding to the station 141), whose mesopelagic maximum was 18.96 and its values were always higher than the other stations after this point.

In summary, station 120 reached the mesopelagic N/P maximum faster with depth, close to $\approx$ 210m, while all the other stations showed the maximum at $>$ 400m. Station 141, on the contrary, reached the mesopelagic maximum at the mean depth, but after that point it was never a nitrogen-scarce environment (N/P $\approx$ 16). Therefore, we selected these two water columns to study further changes in N guilds, as they showed opposite departures from the general pattern found regarding N depletion.

2.2 Global patterns of distribution of N functional markers

We determined the abundance of selected N functional markers along the 75 samples (Fig. 2) as curated normalized metagenomic counts. The taxonomic composition is shown in Fig. 2a–c in a relative way so that the composition of the less abundant markers can be appreciated. The sum of all the counts from all the samples is shown in Fig 2d–e to compare their net abundance. The most abundant genes were *amt* (marker for ammonia transport), *ilvC* (marker for branched aminoacid synthesis), *nirK* (marker for denitrification and hydroxylamine oxidation in archaea [27]), and *ureC* (marker for urea hydrolysis) regardless of depth. There was considerable

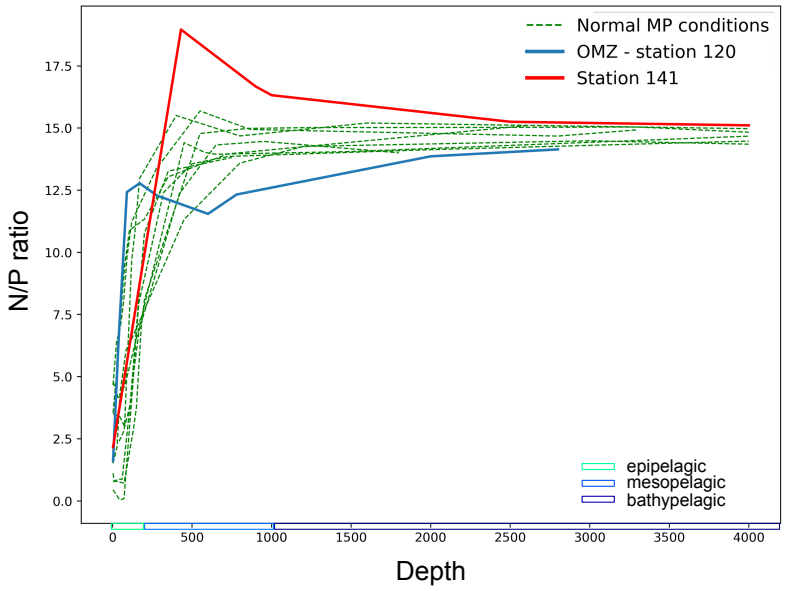

Fig. 1 Different trends in N/P ratios observed in the main MP water columns (11 stations). The bathypelagic depth makes the N/P ratio similar between markedly different stations. Of these, station 120 (Pacific, off the Mexico coast, with a reported oxygen minimum) and station 141 (North Atlantic, open ocean) have the most inconsistent profiles.

species turnover between the epipelagic and mesopelagic, but the latter and the bathypelagic were similar. There was predominance of primary producers (*Cyanobacteria*) at the surface, but it was replaced by *Nitrososphaeria* at depth. As a consequence, the *nirK* gene became more abundant than *amt*. The marker for N-fixation (*nifH*) was only found a few times with extremely few counts and, therefore, it does not appear in Figure 2d–f. However, we showed the relative proportion of the few counts in Figure 2a–c. Some functional markers were found only at some depths. For example, ammonia oxidation (*amoA*) was absent from the epipelagic but it was substantial at the other depths. Likewise, there was a maximum of *nosZ* (marker for the last step of denitrification) in the bathypelagic and *Actinobacteria* appeared at these depths, contributing moderately to several functions.

In summary (1), as expected, the most popular implementation for inorganic N uptake function in the oceans was the ammonium transporter, (2) the preferred metabolic usage of organic-N was to build branched chain amino acids regardless of depth, and (3) the main redox process involving N species in the oceans concerned *nirK* functions (denitrification / nitrification partial steps), particularly below the euphotic zone. In the next section we describe spatial and compositional trends of the main guilds.

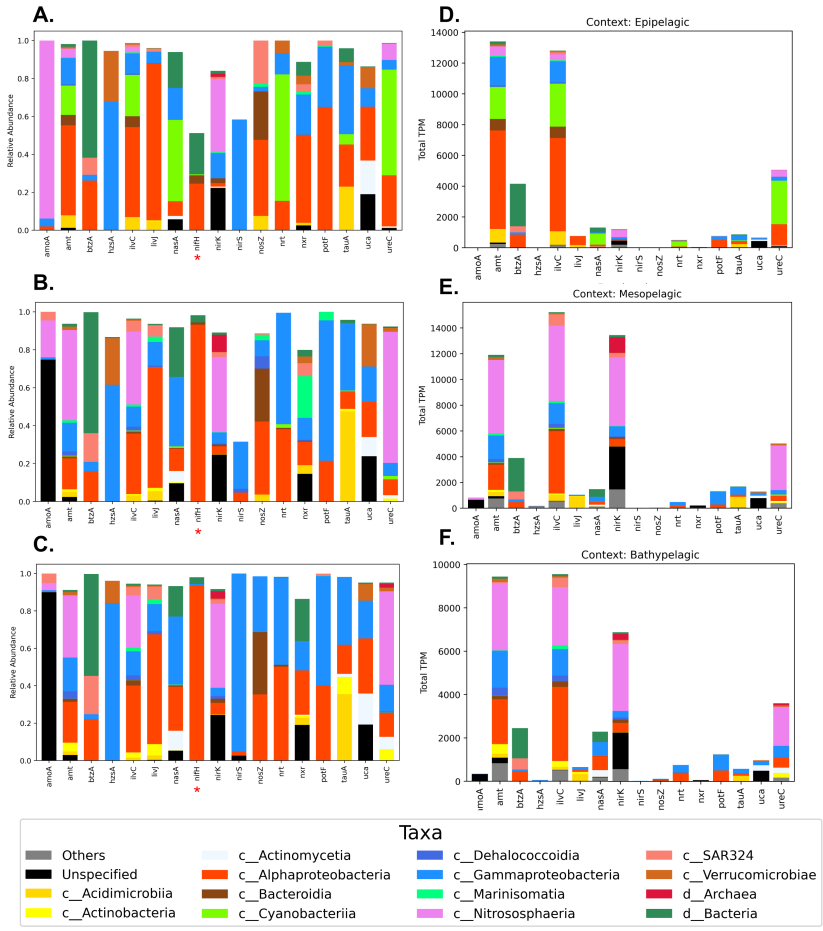

Fig. 2 Relative (a–c) and net (d–f) proportion of normalized counts (TPM) for the main N functional markers in the tropical and subtropical ocean. All the graphs shown have removed sequences not considered to carry out the assigned function. The proportion of these sequences can be seen in the relative graphs as the missing distance from the bars to unity. The most detectable changes occurred between the epipelagic (top) and mesopelagic (middle), and were minor from the latter to the bathypelagic (bottom). These changes included a threefold net increase for nitrite reduction (*nirK*) and ammonium oxidation (*amoA*) markers abundance, as well as a significant decrease in urease (*ureC*) and amino acid synthesis (*ilvC*) markers. Furthermore, these marked changes were also reflected in the taxonomic composition (presence of *Cyanobacteria* in the euphotic zone and the dominance of *Nitrososphaeria* from the mesopelagic to the bathypelagic).

2.3 Description of N uptake guilds

Based on the information in figures 1 and 2, we studied separately four differential environments: OMZ with anammox off the Mexican West coast (corresponding with the blue line in Fig. 1), the open Atlantic Ocean water column (corresponding with the red line in Fig. 1), the non-OMZ near Brazil East coast water column (mean N/P conditions, but associated with an abnormal presence of *Alcanivorax* spp.), and

the average MP conditions. Then, we examined all three depths levels for each case: epipelagic, mesopelagic and bathypelagic; making a total of 12 contexts (4 x 3). We estimated the importance of functions as a product between normalized abundances and non-expected richness for the relevant N uptake functional markers (Fig. 3). This metric is known as k-values [12]. The k-values were log transformed and displayed in radial plots for clarity when identifying patterns between contexts.

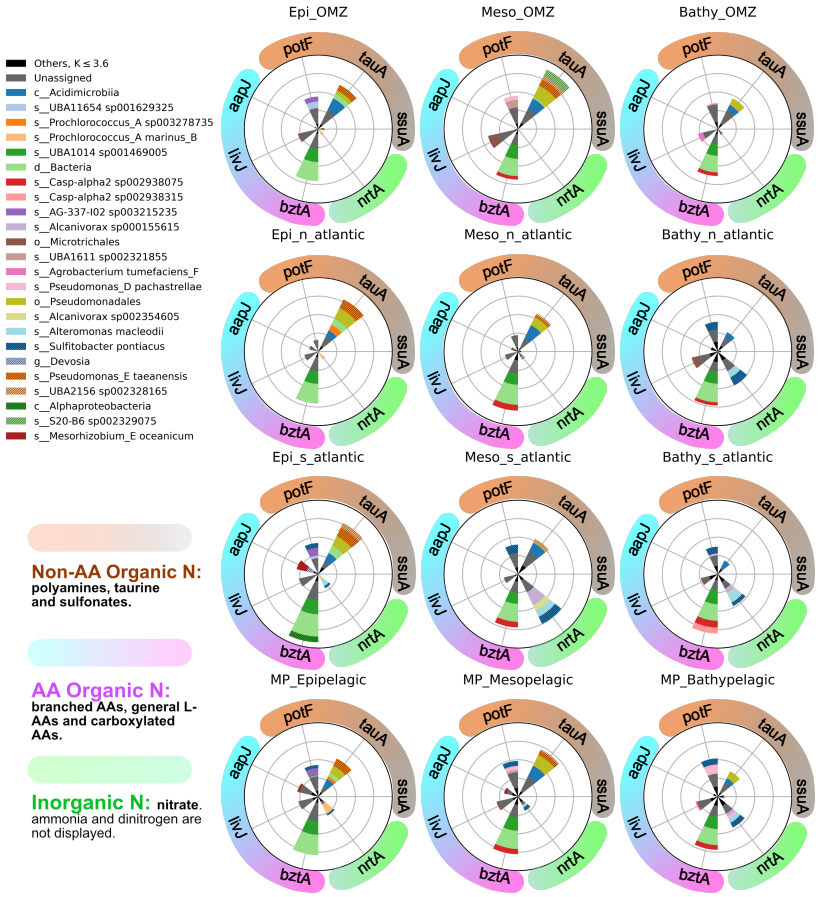

Fig. 3 N uptake guild patterns in the tropic ocean represented by log(k-value) [12]. We represented the top 25 taxonomic contributors, for the sake of clarity. The general pattern of N acquisition genes was similar in all the stations (last row, MP). Ammonia uptake was always important, as *amt* was always the most abundant gene, showing the preference of most bacterioplankton for ammonia. Therefore, we did not represented it. Next in importance were the organic nitrogen transporters for carboxylated amino-acids (*bztA*) and for taurine (*tauA*). The remaining organic-N transporters were of minor importance. The marker gene for nitrate uptake (*nrtA*) was absent from the OMZ station and was present only at the bathypelagic in the NAtl station. However, it was very important at the SAtl station, particularly at the mesopelagic.

First, we found that patterns of individual taxonomic contributors to the general N uptake guild seemed to change both among sampling stations and among depths. However, environments belonging to the same ocean layer often showed taxonomical identity. Thus, we realized that N acquisition was dominated by specific taxa in the epipelagic, regardless of the geographical location of the water column. These were mainly *Pseudomonas taenensis*, *Prochlorococcus marinus*, *Sulfitobacter pontiacus*, *Alteromonas macleodii*, and *Mesorhizobium oceanicum*. Regarding NO_3- acquisition (inferred by the presence of the substrate-binding subunit of the nitrate transporter, *nrtA*) the most important species were *Prochlorococcus marinus* and *Sulfitobacter pontiacus*, and *Alteromonas macleodii*, with some *Alcanivorax* spp. contributing in the SAtl station. Regarding organic N acquisition, the outstanding contributors were members of *Pseudomonadales* and *Acidimicrobiia*, while some belonged to the *Desulfobacteraceae* family (UBA 2156).

With depth, *Nitrososphaeria* dominated the inorganic N uptake via ammonia import, as shown in Figure 2. However, regarding the import other N compounds, this group was not found to be relevant. In the mesopelagic, the general N uptake guild pattern (bottom row of Fig. 3) was remarkably similar to the epipelagic, slightly increasing importance over non-AA uptake, but decreasing importance considering the rest. However, in specific water columns, we found several differences, which can be related to specific conditions. Most differences occurred regarding NO_3- import, where there was absence in mesopelagic OMZ, while in the higher N/P water column it was inferior but similar to mean conditions. Finally, in the SAtl station, we found a remarkable increase in *nrtA* relevance, mainly due to the *Alcanivorax* sp002354605 and sp000155615 abnormal presence. There are other differences in the acquisition of taurine, polyamines and AAs, but these are of lesser importance. In general, *Sulfitobacter pontiacus* was found to be a key contributor in terms of nitrate and putrescine acquisition from the mesopelagic onwards, although never in the OMZ water column nor in the NAtl mesopelagic.

As for the bathypelagic, we found that patterns were more similar between stations, but it was the most different pattern in terms of depth. However, top N importers were less diverse. NO_3- acquisition increased in all cases except for SAtl station, while general AA acquisition was partially replaced by branched AA acquisition. It was also the only depth at which the import function of sulphonated compounds was relevant (*ssuA*; taurine is a shared substrate between Ssu and Tau ABC transporters), which can serve as a source of N and S. However, regarding Non AA organic N assimilation, putrescine uptake was of higher importance at this layer.

2.4 Description of N-redox transformation guilds

Using the same environmental classification, we studied N-redox functional relationships between species (Fig. 4). In this case changes were very marked between depths and between stations. In general, the two Atlantic stations behaved similarly to the ocean average (bottom of Fig. 4), but with lower species and function richness.

In general, ammonia oxidation was relevant in the mesopelagic, mainly performed by *Nitrosopelagicus brevis* and related archaea. Denitrification first step (*narG*) was relevant in all stations. However, both denitrification and nitrification became more

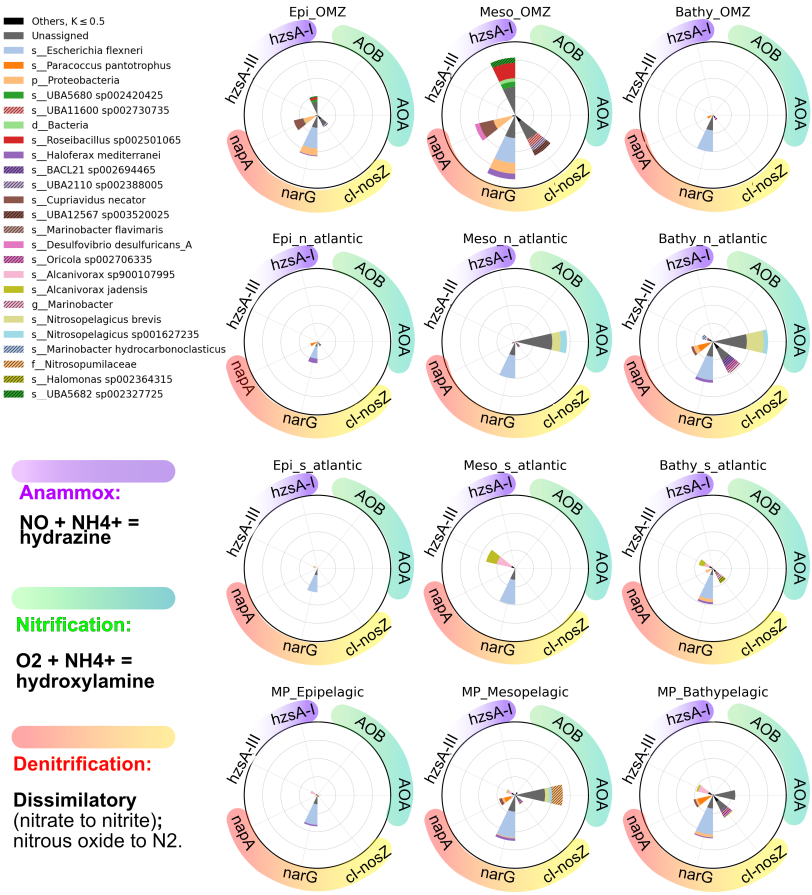

Fig. 4 The N redox guild patterns in the tropic ocean. Unlike with the nitrogen acquisition genes, those involved in redox processes varied substantially from one station to another. Annamox (reflected by *hzsA*-I), as expected, was important only in the OMZ station in the mesopelagic. Denitrification followed the same pattern as in the global ocean (last row) with *narG* as the most important gene and increasing with depth, being similar between the mesopelagic and the bathypelagic. The exception was the OMZ station, where the three examined genes were more important at the surface and the mesopelagic. Nitrification was surprisingly absent from both the OMZ and the SAtl station.

important with depth, and were comparatively weak in the epipelagic. This fitted well with our environmental data, where the epipelagic always represented a N scarcity scenario. However, station 120 (OMZ) showed a different pattern. Anammox (*hzsA-I*) was only present under OMZ conditions, where denitrification markers were also more abundant, with strong presence of *nosZ*, the last step.

In the SAtl water column, we found a very surprising change: there was no ammonium oxidation in the mesopelagic, but instead we reported a type of *hzsA* (a marker of hydrazine synthesis, main step in anammox, which requires the product of *nirS* (NO) and ammonia, as well as very low concentrations of O_2) that will only be relevant in

Table 1 Generalized Linear Model Regression of *delta* values for protein diversification.

Context	$\text{coef}_{N_{uptake}}$	$\text{std. err}_{N_{uptake}}$	$P > \lvert z \rvert_{N_{uptake}}$	$\text{coef}_{N_{redox}}$	$\text{std. err}_{N_{redox}}$	$P > \lvert z \rvert_{N_{redox}}$
Meso	0.045	0.056	0.417	0.33	0.117	0.005 ***
OMZ	-0.027	0.043	0.532	-0.203	0.075	0.007 ***

aerobic environments (*hzsA*-III). We propose that this type of protein sequences corresponds to a neofunctionalization [28] of the *hzsA* gene (Fig. S1), since (1) anammox function is only possible under low O_2 tension (upper limit explored to be 20 μmol/L [29]) and here it was found at a concentration of 191.52 μmol/L; (2) hydrazine synthase activity is thought to be bound to the anammoxosome, an organelle not present in *Alcanivorax* [30]; and (3) this type of protein sequence has never been found where ideal conditions for anammox occur in our dataset (0.89 μmol/L O_2, in samples from the OMZ water column); instead, the orthodox *hzsA*-I associated with *Planctomycetota* was found with a remarkable relevance.

We also concluded that, in contrast to N uptake guilds, N-redox guilds seemed to be more taxonomically linked, as we never identified different markers implemented in the same taxa among the top contributors.

2.5 Diversification of N cycle proteins

Functions can be carried out by a single protein sequence or by a multitude of equivalent sequence variants. To study how protein diversification increased or decreased depending on the environment, we calculated a ratio between observed and expected unique sequence richness, *delta* (Methods). Then, we compared *delta* values of the previously defined functional categories: proteins that serve for the acquisition of different N compounds and proteins that transform these compounds by redox processes.

Using a generalized linear model, we found that the depth of individual samples did not explain significant *delta* variance in either protein type. However, in general, we found a highly significant tendency for N redox-related proteins to diversify above the expected in the tropical and subtropical ocean mesopelagic (Table 1). In addition, we tested whether or not the OMZ present in station 120 had an effect regarding protein diversification. We found that N redox protein diversification was significantly lower at this station. This correspondence between *delta* variation and individual stations was only found in this water column, regardless of the protein function type.

3 Discussion

3.1 Are functional compartiments in the oceans taxonomically linked?

Recent research has improved our understanding of marine microbial functions and their role in biogeochemical cycling, as well as helped us to identify the main players driving these processes [31–33]. First of all, our results yielded novel data on the oceanic N guilds taxonomic composition, which remained, to some extent, compartmentalized throughout the water column (Fig. 2). However, taxonomic stratification is not a new concept in marine microbial ecology [34]. For example, we already knew of

specific *Prochlorococcus* populations linked to the bottom of the photic zone, or some *Crenarachaeota* to be important regarding ammonia oxidation in sub-mesopelagic depths [35]. Likewise, the dynamics of particles – the so-called *marine snow* – are of great importance (whether they sink, whether they are dissolved, whether they are large and taxonomically diverse [36, 37]). In addition, the internal characteristics of these particles are also critical to support metabolisms and species across depths [38, 39].

Surprisingly, we found that top contributors from N guilds retained more identity with depth than with specific conditions (Figs. 3 & 4). We interpreted that N guilds were more dependent on the resource availability and environmental constraints than on pure depth.

3.2 How N scarcity affected the oceanic N guilds

Depth and N availability are closely related in the ocean. However, this relationship is variable with geographic location, as we saw in Figure 1. This is why we aimed our efforts to determine N/P unusual ratios for our working MP samples. Once we identified the two water columns with the most distant distributions, we analyzed both of them separately. The results were already shown and discussed above, but further conclusions can be inferred regarding N shortage or oversupply. In summary, what we observed was that most N-redox guilds were highly dependent of depth, especially compared to the N uptake guilds. In addition, certain specific features of the water column also altered the N-redox guilds more than the N uptake guilds. However, now we will focus on discussing the feasible causes of these differential perturbations regarding only N depletion.

The lower values of N/P correspond to the OMZ water column, as shown in Figure 1. Typically, the scarcity of a resource constrains the type and amount of biological processes that depend on it [40, 41]. Interestingly enough, our results are telling us the opposite, at least within guilds for redox transformation of the available N species (Fig. 4): the importance and variety of processes and their contributors exponentially increase when N (the limiting resource) is scarcer compared to all the other water columns in the mesopelagic. However, one straightforward explanation for the fact that N transformation processes multiply as N depletion increases is precisely that, in the absence of oxygen as the ultimate electron acceptor, N would be used profusely (and by a lot of different functions) to obtain reducing power. Surprisingly, the richness of unique protein sequences for the different N redox transformations in the OMZ was lower than expected for their abundance when compared to the rest of the oceanic environments (Table 1). This latter result is not explained by a poor taxonomic diversity, as OMZs are considered well diverse environments [42].

We interpreted both results together. We conjecture that the diversification of proteins exploiting a resource is a process somehow leveraged by the scarcity – and by the need – of it. This effect would emerge, of course, from the implied eco-evolutionary mechanisms (whether known or unknown) [43, 44], and should remain, at least partially, independent from taxonomic variation.

Finally, it can be categorically stated that the lowest N/P mesopelagic minimum in our dataset (13.86) was not limiting for N transforming guilds, in fact, was a potentiator of them. In addition, N uptake guilds were limited by the fact that the availability of the different N species was biased (highly oxidated N states like NO_3- cannot exist in highly reductive environments). Therefore, nitrate acquisition was not relevant in the OMZ, while reduced forms of N were preferred (taurine, carboxylated amino acids, and ammonia).

On the other hand, we explored the higher N/P ratios in our dataset, corresponding with a NAtl water column (Fig. 1). We reported a slight decrease in denitrification in the mesopelagic compared to the mean oceanic conditions, but an increase in the bathypelagic. The most remarkable thing is that the first step in nitrification is still very important, even more than in the mesopelagic. Ammonia oxidation is usually inhibited in euphotic zones [45] and remains very prevalent in the mesopelagic [46]. In this specific case and with our approach, denitrification and nitrification guilds were quantified at its maximum in the bathypelagic, with a strong correspondence with the higher N/P profile of these samples from station 141.

3.3 Stability of N guilds along the water column

With the introduction of a quantitative approach for guilds, we now have a better chance to investigate whether microbial resource-related functions are stable or not in changing environments. However, despite this improvement, the windows we have for observation are still not sufficient to fully and/or accurately determine the stability of guilds in all aspects. Our ability to determine the stability of N guilds depended on three factors: (1) on the reliability of our methods for functional classification of retrieved metagenomic sequences, (2) on how we measure the ecological contribution of their members, and (3) on the proper discrimination of the guilds. These factors have been discussed at length in Rivas-Santisteban *et al.* [12], and we therefore followed the methodological guidelines established in this previous study.

Considering the diversification of the specific protein effectors that perform a function to measure the stability of that function seems appropriate. This is motivated by the argument that the greater the number of functionally redundant unique sequences for a given env_i, the more sequences will still be able to act within their physiological response when a env_j replaces the former environment. Therefore, effector diversification is congruent with functional stability [47].

Bearing in mind the above, our results indicate that the N acquisition guilds were far more stable and redundant than the N-redox guilds across depths and conditions related to specific water columns (Figs. 3 & 4). Elaborating further, we considered the probability distributions of the diversification coefficients, *delta*, across the twelve contexts analyzed (Fig. S2). It seemed that the differential conditions – N depletion and O_2 availability – affected the two group of guilds distinctively. The N uptake guilds were systematically narrower in their probability distributions than the N redox guilds. However, if only the average oceanic conditions were considered (all MP samples, bottom row of Fig. S2), these were more similar, although they still followed a trend with markedly less variability. When comparing these with the probability distributions considering only the normalized abundance values, we did not find a strict

correspondence, especially in the case of N uptake guilds (Fig. S3). In general, these latter distributions appeared more stable (i.e. overlapped) with depth-variation than the corresponding distributions of k-values not in the average conditions, but in the specific contexts.

A reasonable interpretation for these results is that the processes of protein *overdiversification* and *underdiversification* are, despite their obscure foundations, modulators of the stability of molecular functions, which operate in response to changing environments, as we have seen in Table 1. However, these processes did not always operate in the same way, and the *delta* values of the specific environments shifted significantly more from the expected diversification when the proteins are related to N redox functions, in contrast to N uptake functions, where the mean barely moves from the expected (Fig. S4). This provided yet another argument in favour of considering N acquisition guilds to be more stable with depth and with specific contexts than redox N guilds.

4 Conclusions

We have been able to describe novel functional dynamics and key players in the N cycle as a function of depth and some specific water columns with respect to their unique characteristics for the Malaspina dataset. In addition to the above, the guild quantification has allowed us to outline the following important effects:

1) N redox guilds showed greater functional variability with depth and with geographic location than N uptake guilds. In contrast, taxonomic composition of N uptake guilds was more diverse.

2) The importance of denitrification/nitrification in the bathypelagic was strongly dependent on the availability of N.

3) The over/under diversification of proteins responds to very specific environmental variables, those that relax or exacerbate the constraining selection over the effectors of the function that the proteins compromise. A clarifying example is that the proteins with redox activity are significantly less diverse than expected for each taxon in samples from the OMZ (the largest reducing environment in the open ocean). However, this is not the case for proteins that carry out N uptake functions. Thus, we conclude that diversification is not contingent to the species: it is linked to the specific relationship between environment and function.

4) As we discussed, some types of anammox marker proteins (related to hydrazine synthesis) might not have a relevant role in OMZs. We called them *hzsA*-III (corresponding to oxygenic conditions with an increased presence of *Alcanivorax* spp. in the SAtl station), in contrast to *Planctomyces*-like *hzsA*-I proteins (corresponding to anoxygenic conditions).

5 Methods

5.1 Sampling and environmental data collection

During the Malaspina 2010 expedition, conducted from 2010 to 2011, genetic and environmental data were gathered from 37 sampling stations spanning various oceanic

regions, including the tropical and subtropical zones of the Atlantic, Indian, and Pacific Oceans, at depths ranging from 3 to 4000 meters. The majority of samples originated from the Atlantic, Pacific, and notably the Indian Ocean, where many parameters were documented for the first time during the expedition. Sampling methods and protocols are outlined in [13]. Given the tropical and subtropical nature of the samples, seasonal climate variations are expected to have minimal impact. Metadata encompassed date, latitude, longitude, depth, temperature, filter size, conductivity, oxygen levels, fluorescence, Photosynthetic Active Radiation (PAR), Direct Photosynthetic Active Radiation (SPAR), turbidity, and salinity.

5.2 Sequencing and processing

All the samples from Malaspina (116) were filtered in two size fractions: small (0.2 μm) and large (3 μm) using 142 mm polycarbonate membrane filters. The small fraction comprised free-living bacteria, archaea, and picoeukaryotes, while the large fraction contained larger eukaryotes and particle-attached microorganisms. This study focused solely on samples from the small fraction. DNA sequencing was conducted using Illumina MiSeq with a 2 x 250 bp reads configuration, yielding a total read count ranging from 319 to 508 million reads per sample. Initial read processing involved contaminant scanning (e.g., Illumina adapter sequences) and quality-based cleaning using FastQC [48], FastX-Toolkit [49], and SolexaQA [50] software. Trimmed reads falling below 45 nucleotides and quality score 33 were subsequently discarded. Functional annotation was done by homology search with DIAMOND v.0.9.13 [51] against the NCBI database for taxonomic assignment and KO (KEGG Orthology) and COG databases for automatic functional assignment.

All relevant steps were conducted with SqueezeMeta v.1.5.1 [52], including comprehensive analysis steps, multi-metagenome support for coassembling related metagenomes and retrieving individual genomes through binning procedures.

5.3 Functional delineation of metagenomic queries

Functional annotation of genetic markers and filtration of false positives were conducted as described in Rivas-Santisteban et al. [12].

5.4 Assessment of protein diversification

We assessed the expected sequence richness by fitting

$$d_{exp,s} = c_s A_s^{\gamma_s} \tag{1}$$

where s were sequences considered for a taxon, implementation and environment, abundance was measured in TPM, richness was the natural number of unique sequences, and where c_s and γ_s were constants. Thus, *delta* was calculated as a coefficient between $d_{obs,s}$ and $d_{exp,s}$. Kernel density estimates were obtained with custom software. More details can be found in Figure S5.

Supplementary information. More information can be found in the attached supplementary file.

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Declarations

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- Consent to participate
- Consent for publication
  The authors consent to publication.
- Availability of data and materials
  Request for data and materials should be addressed to J.R.S.
- Code availability
  Code for guild display is available in https://github.com/pyubero/microguilds. Custom code for diversification kernel density estimations on request.
- Authors' contributions
  J.R.S.: Conceptualization, Methodology, Validation, Writing - Original Draft, Investigation, Data curation, Visualization, Software, Formal analysis; N.F.G.: Methodology, Software, Writing - Review & Editing; R.L.P.: Methodology, Software, Writing - Review & Editing; J.T. and C.P.A.: Conceptualization, Writing - Review & Editing, Project administration, Supervision, Funding acquisition.

References

[1] Vernadsky, V.I.: The Biosphere, pp. 121–125. Springer, Berlin (1998)

[2] Schuetz, R., Zamboni, N., Zampieri, M., Heinemann, M., Sauer, U.: Multidimensional optimality of microbial metabolism. Science **336**(6081), 601–604 (2012)

[3] Fuhrman, J.A., Cram, J.A., Needham, D.M.: Marine microbial community dynamics and their ecological interpretation. Nature Reviews Microbiology **13**(3), 133–146 (2015)

[4] Zhou, Z., Tran, P.Q., Kieft, K., Anantharaman, K.: Genome diversification in globally distributed novel marine proteobacteria is linked to environmental adaptation. The ISME Journal **14**(8), 2060–2077 (2020)

[5] Falkowski, P.G.: The role of phytoplankton photosynthesis in global biogeochemical cycles. Photosynthesis research **39**, 235–258 (1994)

[6] Arrigo, K.R.: Marine microorganisms and global nutrient cycles. Nature **437**(7057), 349–355 (2005)

[7] Matear, R.J., Wang, Y.-P., Lenton, A.: Land and ocean nutrient and carbon cycle interactions. Current Opinion in Environmental Sustainability **2**(4), 258–263 (2010)

[8] Crowe, S.A., Treusch, A.H., Forth, M., Li, J., Magen, C., Canfield, D.E., Thamdrup, B., Katsev, S.: Novel anammox bacteria and nitrogen loss from lake superior. Scientific reports **7**(1), 13757 (2017)

[9] Engström, P., Dalsgaard, T., Hulth, S., Aller, R.C.: Anaerobic ammonium oxidation by nitrite (anammox): implications for n2 production in coastal marine sediments. Geochimica et Cosmochimica Acta **69**(8), 2057–2065 (2005)

[10] Kuenen, J.G.: Anammox bacteria: from discovery to application. Nature Reviews Microbiology **6**(4), 320–326 (2008)

[11] Fay, A., McKinley, G.: Global open-ocean biomes: mean and temporal variability. Earth System Science Data **6**(2), 273–284 (2014)

[12] Rivas-Santisteban, J., Yubero, P., Robaina-Estevez, S., Gonzalez, J.M., Tamames, J., Pedros-Alio, C.: Quantifying microbial guilds. bioRxiv (2023) https://doi.org/10.1101/2023.07.23.550202

[13] Duarte, C.M.: Seafaring in the 21st century: the malaspina 2010 circumnavigation expedition (2015)

[14] Acinas, S.G., Sánchez, P., Salazar, G., Cornejo-Castillo, F.M., Sebastián, M., Logares, R., Royo-Llonch, M., Paoli, L., Sunagawa, S., Hingamp, P., *et al.*: Deep ocean metagenomes provide insight into the metabolic architecture of bathypelagic microbial communities. Communications Biology **4**(1), 604 (2021)

[15] Karner, M.B., DeLong, E.F., Karl, D.M.: Archaeal dominance in the mesopelagic zone of the pacific ocean. Nature **409**(6819), 507–510 (2001)

[16] Kampouris, I.D., Gründger, F., Christensen, J.H., Greer, C.W., Kjeldsen, K.U., Boone, W., Meire, L., Rysgaard, S., Vergeynst, L.: Long-term patterns of hydrocarbon biodegradation and bacterial community composition in epipelagic and mesopelagic zones of an arctic fjord. Journal of hazardous materials **446**, 130656 (2023)

[17] Longhurst, A.R.: Ecological Geography of the Sea. Elsevier, ??? (2010)

[18] Capone, D.G.: The marine nitrogen cycle. Microbe Magazine **3**(4), 186–192

(2008)

[19] Sharp, J.H.: The distributions of inorganic nitrogen and dissolved and particulate organic nitrogen in the sea. Nitrogen in the marine environment, 1–35 (1983)

[20] Kirchman, D.L., Gasol, J.M.: Microbial Ecology of the Oceans, pp. 361–364. John Wiley & Sons, ??? (2018)

[21] Ward, B.B.: Molecular approaches to marine microbial ecology and the marine nitrogen cycle. Annu. Rev. Earth Planet. Sci. **33**, 301–333 (2005)

[22] Casciotti, K.L.: Nitrite isotopes as tracers of marine n cycle processes. Philosophical Transactions of the Royal Society A: Mathematical, Physical and Engineering Sciences **374**(2081), 20150295 (2016)

[23] Hutchins, D.A., Capone, D.G.: The marine nitrogen cycle: new developments and global change. Nature Reviews Microbiology **20**(7), 401–414 (2022)

[24] Bronk, D.A., Glibert, P.M., Ward, B.B.: Nitrogen uptake, dissolved organic nitrogen release, and new production. Science **265**(5180), 1843–1846 (1994)

[25] Kuypers, M.M., Marchant, H.K., Kartal, B.: The microbial nitrogen-cycling network. Nature Reviews Microbiology **16**(5), 263–276 (2018)

[26] Weber, T.S., Deutsch, C.: Ocean nutrient ratios governed by plankton biogeography. Nature **467**(7315), 550–554 (2010)

[27] Kobayashi, S., Hira, D., Yoshida, K., Toyofuku, M., Shida, Y., Ogasawara, W., Yamaguchi, T., Araki, N., Oshiki, M.: Nitric oxide production from nitrite reduction and hydroxylamine oxidation by copper-containing dissimilatory nitrite reductase (nirk) from the aerobic ammonia-oxidizing archaeon, nitrososphaera viennensis. Microbes and environments **33**(4), 428–434 (2018)

[28] Deng, C., Cheng, C.-H.C., Ye, H., He, X., Chen, L.: Evolution of an antifreeze protein by neofunctionalization under escape from adaptive conflict. Proceedings of the National Academy of Sciences **107**(50), 21593–21598 (2010)

[29] Kalvelage, T., Jensen, M.M., Contreras, S., Revsbech, N.P., Lam, P., Günter, M., LaRoche, J., Lavik, G., Kuypers, M.M.: Oxygen sensitivity of anammox and coupled n-cycle processes in oxygen minimum zones. PloS one **6**(12), 29299 (2011)

[30] Van Teeseling, M.C., Neumann, S., Van Niftrik, L.: The anammoxosome organelle is crucial for the energy metabolism of anaerobic ammonium oxidizing bacteria. Microbial Physiology **23**(1-2), 104–117 (2013)

[31] Arístegui, J., Gasol, J.M., Duarte, C.M., Herndld, G.J.: Microbial oceanography of the dark ocean's pelagic realm. Limnology and Oceanography **54**(5), 1501–1529 (2009)

[32] Bowler, C., Karl, D.M., Colwell, R.R.: Microbial oceanography in a sea of opportunity. Nature **459**(7244), 180–184 (2009)

[33] Grossart, H.-P., Massana, R., McMahon, K.D., Walsh, D.A.: Linking metagenomics to aquatic microbial ecology and biogeochemical cycles. Limnology and Oceanography **65**, 2–20 (2020)

[34] Bouman, H.A., Ulloa, O., Barlow, R., Li, W.K., Platt, T., Zwirglmaier, K., Scanlan, D.J., Sathyendranath, S.: Water-column stratification governs the community structure of subtropical marine picophytoplankton. Environmental Microbiology Reports **3**(4), 473–482 (2011)

[35] Shi, Y., Tyson, G.W., Eppley, J.M., DeLong, E.F.: Integrated metatranscriptomic and metagenomic analyses of stratified microbial assemblages in the open ocean. The ISME journal **5**(6), 999–1013 (2011)

[36] Alcolombri, U., Peaudecerf, F.J., Fernandez, V.I., Behrendt, L., Lee, K.S., Stocker, R.: Sinking enhances the degradation of organic particles by marine bacteria. Nature Geoscience **14**(10), 775–780 (2021)

[37] Cael, B., Cavan, E.L., Britten, G.L.: Reconciling the size-dependence of marine particle sinking speed. Geophysical Research Letters **48**(5), 2020–091771 (2021)

[38] Ciccarese, D., Tantawi, O., Zhang, I.H., Plata, D., Babbin, A.R.: Microscale dynamics promote segregated denitrification in diatom aggregates sinking slowly in bulk oxygenated seawater. Communications Earth & Environment **4**(1), 275 (2023)

[39] Zehr, J.P., Capone, D.G.: Unsolved mysteries in marine nitrogen fixation. Trends in Microbiology (2023)

[40] Cullen, J.J.: Hypotheses to explain high-nutrient conditions in the open sea. Limnology and Oceanography **36**(8), 1578–1599 (1991)

[41] Moore, C., Mills, M., Arrigo, K., Berman-Frank, I., Bopp, L., Boyd, P., Galbraith, E., Geider, R., Guieu, C., Jaccard, S., *et al.*: Processes and patterns of oceanic nutrient limitation. Nature geoscience **6**(9), 701–710 (2013)

[42] Medina-Silva, R., Oliveira, R.R., Pivel, M.A., Borges, L.G., Simão, T.L., Pereira, L.M., Trindade, F.J., Augustin, A.H., Valdez, F.P., Eizirik, E., *et al.*: Microbial diversity from chlorophyll maximum, oxygen minimum and bottom zones in the southwestern atlantic ocean. Journal of Marine Systems **178**, 52–61 (2018)

[43] Hurst, L.D.: The ka/ks ratio: diagnosing the form of sequence evolution. TRENDS in Genetics **18**(9), 486–487 (2002)

[44] Kreitman, M.: The neutral theory is dead. long live the neutral theory. Bioessays

18(8), 678–683 (1996)

[45] Merbt, S.N., Stahl, D.A., Casamayor, E.O., Martí, E., Nicol, G.W., Prosser, J.I.: Differential photoinhibition of bacterial and archaeal ammonia oxidation. FEMS microbiology letters **327**(1), 41–46 (2012)

[46] Newell, S.E., Babbin, A.R., Jayakumar, A., Ward, B.B.: Ammonia oxidation rates and nitrification in the arabian sea. Global Biogeochemical Cycles **25**(4) (2011)

[47] Margalef, R.: Diversity, stability and maturity in natural ecosystems. In: Unifying Concepts in Ecology: Report of the Plenary Sessions of the First International Congress of Ecology, The Hague, the Netherlands, September 8–14, 1974, pp. 151–160 (1975). Springer

[48] Andrews, S.: Fastqc version 0.11. 5. A Quality Control Tool for High Throughput Sequence Data (2016)

[49] Gordon, A., Hannon, G.: FASTX-Toolkit. FASTQ/A short-reads pre-processing tools (version 0.0. 13) (2010)

[50] Cox, M.P., Peterson, D.A., Biggs, P.J.: Solexaqa: At-a-glance quality assessment of illumina second-generation sequencing data. BMC bioinformatics **11**(1), 1–6 (2010)

[51] Buchfink, B., Xie, C., Huson, D.H.: Fast and sensitive protein alignment using diamond. Nature methods **12**(1), 59–60 (2015)

[52] Tamames, J., Puente-Sánchez, F.: Squeezemeta, a highly portable, fully automatic metagenomic analysis pipeline. Frontiers in microbiology **9**, 3349 (2019)