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Research Article

RESEARCH ARTICLE

Size-fractionated diversity of eukaryotic microbial communities in the Eastern Tropical North Pacific oxygen minimum zone

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One sentence summary: Microbial eukaryotes distribution within the Eastern Tropical Northern Pacific water column presented evidence of niche partitioning between size fractions (mostly particle-associated versus free-living) and was structured by a chemical stratification.
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ABSTRACT

Oxygen minimum zones (OMZs) caused by water column stratification appear to expand in parts of the world's ocean, with consequences for marine biogeochemical cycles. OMZ formation is often fueled by high surface primary production, and sinking organic particles can be hotspots of interactions and activity within microbial communities. This study investigated the diversity of OMZ protist communities in two biomass size fractions (>30 and 30–1.6 μm filters) from the world's largest permanent OMZ in the Eastern Tropical North Pacific. Diversity was quantified *via* Illumina MiSeq sequencing of V4 region of 18S SSU rRNA genes in samples spanning oxygen gradients at two stations. Alveolata and Rhizaria dominated the two size fractions at both sites along the oxygen gradient. Community composition at finer taxonomic levels was partially shaped by oxygen concentration, as communities associated with versus anoxic waters shared only ~32% of operational taxonomic unit (OTU) (97% sequence identity) composition. Overall, only 9.7% of total OTUs were recovered at both stations and under all oxygen conditions sampled, implying structuring of the eukaryotic community in this area. Size-fractionated communities exhibited different taxonomical features (e.g. Syndiniales Group I in the 1.6–30 μm fraction) that could be explained by the microniches created on the surface-originated sinking particles.

Keywords: protist diversity; 18S SSU rRNA; particle-associated; water-column

INTRODUCTION

Oxygen minimum zones (OMZs) are low-oxygen oceanic regions that occur naturally in coastal and open-ocean mesopelagic waters. They are especially prominent in the northern Indian Ocean (Ulloa *et al.* 2013) and in areas of nutrient upwelling in the Eastern Tropical and subarctic Pacific (Wyrtki 1962; Stramma

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et al. 2010). Seasonal upwelling delivers cold, oxygenated and nutrient-rich waters to the surface (Stramma et al. 2010), which leads to high primary production in the euphotic layer. A portion of the particulate organic matter (POM) originating from the euphotic zone sinks into the OMZ (i.e. under the surface mixed layer (O_2 > 180 μM; 40 m), Boyd and Trull 2007; Buesseler and Boyd 2009) either directly or indirectly via attachment to decaying or living phytoplankton cells, aggregates, zooplankton feces, etc.; see Alldredge and Silver (1988) and Simon et al. (2002). Sinking POM aggregates fuel aerobic microbial respiration, contributing to a progressive loss of deep water O_2 and release of CO_2 (reviewed in Simon et al. 2002). OMZs can occur in near-surface waters down to depths greater than 1500 m due to sinking of surface primary production (Kamykowski and Zentara 1990). Depletion of dissolved oxygen can also occur seasonally in coastal and estuarine environments (e.g. Framvaren Fjord, Behnke et al. 2006; Saanich Inlet, Zaikova et al. 2010) or can be a permanent feature of certain anoxic/sulfidic basins (e.g. Cariaco Basin, Taylor et al. 2001). The three major OMZs (Eastern Tropical South Pacific, ETSP; Eastern Tropical North Pacific, ETNP; Arabian Sea) are important sites of denitrification (Codispoti et al. 2001), OMZs account for about 50% of global oceanic nitrous oxide release to the atmosphere (Keeling, Körtzinger and Gruber 2010). The ETNP (0–25°N; 75–180°W) includes the largest permanent OMZ, representing 41% of the surface area of all global ocean OMZs (~12 $\times 10^6$ km^2, Paulmier and Ruiz-Pino 2009) with an oxygen concentration <30 nM at its core (Tiano et al. 2014). Formation of the ETNP OMZ is mainly driven by a major upwelling system of the North Pacific Ocean that intensifies during the summer months (Wyrtki 1962; Kamykowski and Zentara 1990).

Suboxic and anoxic environments (<20 μM and undetectable O_2, respectively; Kamykowski and Zentara 1990; Wright, Konwar and Hallam 2012) exhibit unique prokaryotic and eukaryotic microbial communities, as shown in studies of OMZs (Stevens and Ulloa 2008; Orsi et al. 2012; Stewart, Ulloa and DeLong 2012) and anoxic basins (Lin et al. 2008; Edgcomb et al. 2011; Orsi et al. 2011). Through microbial denitrification and anaerobic ammonium oxidation processes (anammox, Paulmier and Ruiz-Pino 2009; Canfield et al. 2010; Ulloa et al. 2012), prokaryotic communities in oxygen-depleted waters impact the release of nitrogen gas from ocean waters. While the ecology of prokaryotes is starting to be understood in OMZs, a lot remains to be explored about protist communities in these environments. There is a known shift between eukaryotic communities adapted to aphotic oxic conditions, and those adapted to aphotic anoxic conditions within marine water columns showing steep oxygen gradients (Stoeck, Taylor and Epstein 2003; Edgcomb et al. 2011; Orsi et al. 2011, 2012). Symbioses between Bacteria, Archaea and Eukarya in oxygen-depleted water columns appear to be common, and may represent a means for eukaryotic protists to exploit otherwise inhospitable habitats. Based on the abundances of putative protist hosts, which frequently harbor hundreds or thousands of prokaryotic partners per host (e.g. Bernhard et al. 2000; Edgcomb et al. 2011; Orsi et al. 2012), the numerical abundance of prokaryotes in symbiosis with protists may rival that of free-living prokaryotes in some habitats, and the direct impacts of these symbionts on major nutrient biogeochemical cycles may be significant. Sinking POM constitutes a 'hotspot' for carbon and nitrogen cycling (Azam 1998; Arıstegui et al. 2009) as it harbors high concentrations of heterotrophic communities (e.g. Cho and Azam 1988; Turley and Mackie 1994). Indeed, heterotrophic protists graze on prokaryotes actively involved in remineralization of POM, and thus modify their quantity, activity and/or their physiological state (Sherr and Sherr 2002; Frias-Lopez et al. 2009; e.g. regulation of the chemoautotrophic denitrifier *Sulfurimonas* sp. by ciliophorans, Anderson et al. 2013). Protists can also impact nutrient availability by directly modifying and/or remineralizing POM (Taylor 1982; Jumars et al. 1989; Sherr and Sherr 2002). However, little is known about partitioning of eukaryotic communities, between free-living protists and those associated with sinking organic matter, along the oxygen gradient of the OMZ water column.

Only one study so far (Parris et al. 2014) has examined OMZ protist communities within multiple biomass size fractions (0.2–1.6 μm and >1.6 μm). Taking into account that most of the free-living marine protists have a size of 2.0–20 μm (for heterotrophic nanoflagellates, ciliates and dinoflagellates) we chose to examine two size fractions 1.6–30 μm and >30 μm to estimate the community diversity of protists in the free-living and particle-associated fractions, respectively. We focus on samples spanning the vertical oxygen gradient at two locations within the ETNP (continental shelf versus continental slope). This study contributes to a greater understanding of protist diversity and distribution in the ETNP, along oxygen gradients, and between size fractions.

MATERIAL AND METHODS

Sample collection

Sample collection took place in June 2013 aboard the R/V *New Horizon* during the NH1313 cruise. Two sites in the ETNP OMZ off Colima, Mexico were sampled: station 6 (18.55°N, 104.53°W) on the continental shelf, and station 10 (18.48°N, 105.12°W) off the continental slope (Fig. 1). Samples were collected from the following: the surface mixed layer (O_2 concentration >180 μM; 30 m), upper oxycline (~2 μM O_2; 85 m), and OMZ core (undetectable O_2; 100, 125, 300 m) at station 6 (Fig. 2A); the surface mixed layer (O_2 > 180 μM; 40 m), upper oxycline (~2 μM O_2; 80 m), OMZ core (undetectable O_2; 125, 300, 500, 800 m) and lower oxycline (~5 μM O_2; 1000 m) at station 10 (Fig. 2B). For each depth, microorganisms were collected onto a 30 μm pore-size nylon mesh filter (47 mm) and a 1.6 μm nominal pore-size glass fiber filter (GF/A; 47 mm) *via* in-line filtration of roughly 10 L of seawater. The larger filter was used to capture free-living microplankton and large POM-attached organisms, and the GF/A filter to capture free-living and smaller POM-attached nanoplankton. Immediately after filtration, each filter was folded and stored in a cryotube with approximately 2.0 ml of extraction buffer (40 mM EDTA, 50 mM TRIS pH 8.3, 0.73 M sucrose) and flash frozen for storage at −80°C until further processing. Two replicates were collected per depth. Oxygen concentration, temperature and salinity were measured with a CTD (conductivity, temperature, depth) equipped with a SBE43 dissolved oxygen sensor (Fig. 2). Nitrogen oxide concentrations were determined using frozen samples, which were returned to the laboratory and analyzed according to standard protocol by nitrate to nitrite reduction in hot acidic vanadium chloride for colorimetric analysis (Braman and Hendrix 1989; Fig. 2).

DNA extraction

Samples (n = 48) in extraction buffer were heated to 55°C for 30 min after thawing. Lysates were then purified using a phenol-chloroform-isoamyl alcohol (25:24:1) extraction, precipitated with 100% isopropanol and washed with 70% ethanol. The pelleted DNA was diluted in 50 μL of water and kept at −80°C until further processing.

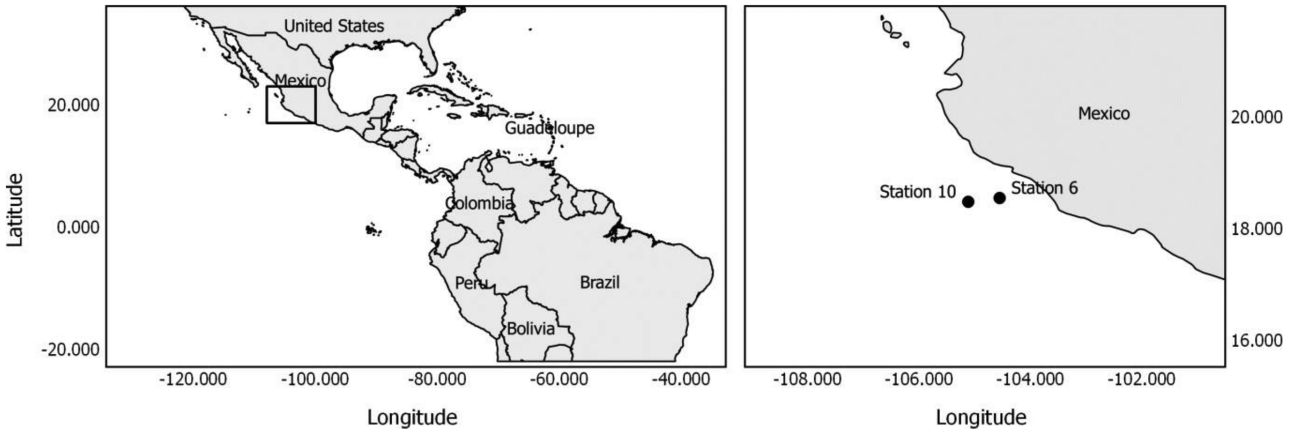


Figure 1. Sampling map. Two sites were sampled for genomics in the ETNP, off the coast of Colima (Mexico) along the oxygen gradient within depths: five depths in station 6 (maximal depth: 1500 m; 18.55°N, 104.53°W), and seven depths in station 10 (maximal depth: 2600 m; 18.48°N, 105.12°W).

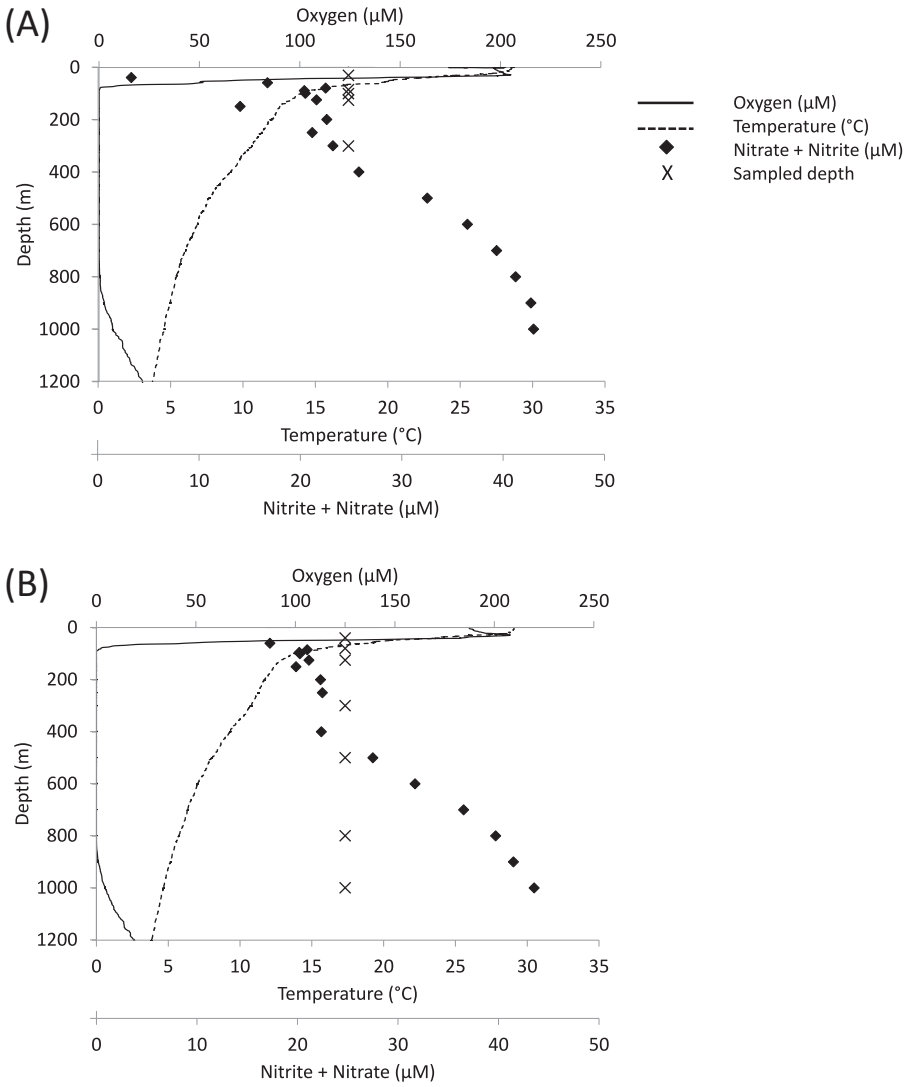


Figure 2. Vertical profiles of environmental parameters. CTD measurements of oxygen concentration (μM; split line), temperature (°C; dashed line) and nitrite + nitrate concentration (μM; dots) along depth in station 6 (**A**) and station 10 (**B**) of the ETNP during June 2013. Crosses indicate depths sampled for DNA. Two filter sizes (30 and 1.6 μm) in replicates ($n = 2$) were collected per depth (4 samples/depth).

DNA amplification and sequencing

The V4 region of 18S SSU rRNA gene was amplified by PCR using the following combination of primers: TAReuk454FWD1 (5′-CCAGCA(G/C)C(C/T)GCGGTAATTCC-3′) and TAReukREV3 (5′-ACTTTCGTTCTTGAT(C/T)(A/G)A-3′) which target most eukaryotes, with some exceptions within the excavates and the microsporidia (Stoeck *et al.* 2010). Each library was generated using a unique combination per sample of barcoded Illumina MiSeq forward and reverse primers designed according to Kozich *et al.* (2013). Each PCR mix [30 μL of MilliQ water, 50 μL of buffer (5X), 10 μL of $MgCl_2$ (25 mM), 5 μL of dNTPs (10 mM) and 0.5 μL of taq polymerase (5 u/μL)] contained 1 μL of template DNA with 1 μL of 10 μM forward and reverse primers. The following PCR conditions were used: 5 min at 95°C followed by 40 cycles of 95°C for 30 s, 55°C for 45 s, 72°C for 60 s and a final extension of 7 min at 72°C using a Vapo.protect thermocycler (Eppendorf, Germany).

Nested PCR was required to amplify the 800 (OMZ core) and 1000 m (lower oxycline) depth samples from station 10. The first PCR conditions were the following: 5 min at 95°C followed by 30 cycles of 95°C for 60 s, 55°C for 1 min, 72°C for 90 s followed by a final elongation step of 7 min at 72°C using the forward primer Euk A (5′-ATCTGGTTGATYCTGCCAG-3′) and Euk B (5′-TGATCCTTCTGCAGGTTCACCTAC-3′), amplifying the full rRNA gene (~2 kb fragments; Medlin *et al.* 1988). One microliter of the PCR product was used as a template for a second PCR using the MiSeq primers with the conditions mentioned above (Stoeck *et al.* 2010).

Success of amplification, intensity of the bands and size of the resulting PCR products were checked using gel electrophoresis (1% agarose). Successful amplicons were gel purified using the Zymoclean Gel DNA Recovery Kit (Zymo Research, USA) according to the manufacturer's instructions. The concentration of purified DNA was measured with a Qubit fluorometer (Thermo Fisher Scientific Inc., USA) according to the manufacturer's instructions. Purified amplicons were then pooled in equimolar concentration (4 nM) for sequencing on an Illumina MiSeq instrument (School of Biology, Georgia Institute of Technology) running MiSeq control software v2.4.0.4 and using a MiSeq 500 cycle v2 reagent kit. Demultiplexing and preliminary adapter trimming of the samples was carried out using the MiSeq instrument.

Bioinformatics analysis

The base quality and basic statistics of demuliplexed read pairs were visualized using FastQC v0.10.1 (Andrews; Babraham Bioinformatics). Reads were screened for Illumina adapters (AATGATACGGCGACCACCGAGATCTACAC and CAAGCAGAAGACGGCATACGAGAT) and quality trimmed using thresholds of a Phred33 score of Q25 and a minimum read length of 100 bp with TrimGalore! v0.3.3 (Krueger; Babraham Bioinformatics). Pairs containing one mate with an average quality score <25 or with a length <100 bp after trimming step were discarded. High-quality read pairs were merged using FLASH v1.2.9 (Magoč and Salzberg 2011) with the following parameters: average read length (~250 bp), fragment length (~400 ± 40 bp) and a minimum overlap of 20 bp between reads. OTU clustering was performed using QIIME v1.8 (Caporaso *et al.* 2010) with the UCLUST algorithm (Edgar 2010) with a minimum sequence similarity of 97%. The most abundant sequence of each OTU was compared against the Silva V111 Reference Set database (Quast *et al.* 2013) with the BLAST taxonomic affiliation algorithm (Buhler *et al.* 2007) on QIIME using the default settings. Non-affiliated OTUs and those affiliated with Metazoa, Archaea, Bacteria and land plants were removed from the data set. Singletons and OTUs whose representatives accounted for less than 0.002% of the overall dataset reads were not used in the subsequent analysis.

Statistical analysis

Statistical analyses were computed with R statistics software (R Development Core Team 2008). Reads abundances were normalized using the Hellinger transformation (Legendre and Legendre 2012) in order to minimize the influence of uneven sequencing effort between libraries. Abundances from replicate filters were pooled based on the average of Hellinger transformed values. A Canonical Correspondence Analysis (CCA; Gotelli and Ellison 2004) was used to elucidate relationships between eukaryotic community structure and environmental parameters, and an ANOVA was then used to statistically test the influence of these parameters on OTU distributions. Similarities between libraries were visualized with a hierarchical clustering analysis using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA; Legendre and Legendre 2012) based on Bray Curtis dissimilarity. Shared OTU composition between replicates, filter sizes, oxygen conditions and sites was quantified based on the proportion of shared OTUs.

RESULTS

DNA libraries

A total of 4 227 913 18S rRNA paired-end reads were recovered from the 48 samples analyzed. An uneven number of raw reads was recovered per library ($\mu = 88\,082 \pm 78\,745$ (se) reads/sample) and this can be explained by possible errors in estimating DNA concentration in some samples prior to pooling for sequencing. This would lead to a DNA pool that did not represent equimolar concentrations for all libraries. The effect of this disparity was minimized with the use of Hellinger transformed data. After removal of non-protist OTUs and OTUs representing <0.002% of total sequences, 2 968 747 sequences were retained in the overall dataset ($\mu = 61\,849 \pm 59\,963$ reads/sample) representing 70.2% of the original dataset, with an average length of 400 ± 40 bp. The sequences formed 2209 OTUs, and 75% of the sequences were affiliated with only 127 OTUs.

Environmental parameters and protist community structuring

The OMZ was situated between 80 and 900 m (80–800 m and 90–900 m for station 6 and 10, respectively; Fig. 2A and B). Both stations showed a stratified water column with a marked thermocline situated at 80 and 90 m for station 6 and 10, respectively and extending to ~100 m (Fig. 2A and B). Salinity did not exhibit a strong depth gradient at either station and did not significantly impact community distribution. CCA explained 11.2% of the OTU distribution (5.8 and 5.4% for axis 1 and 2, respectively; Fig. S1A, Supporting Information). All the factors tested in the CCA (i.e. station, filter pore size, depth, oxygen concentration and temperature) were significant determinants of community composition and OTU distribution (P values < 0.01; Table S1B, Supporting Information). Nitrite and nitrate concentrations could not be tested in multivariate analyses, as concentrations were not available for all samples, and the statistical packages do not allow missing values for any analyzed sample. However,

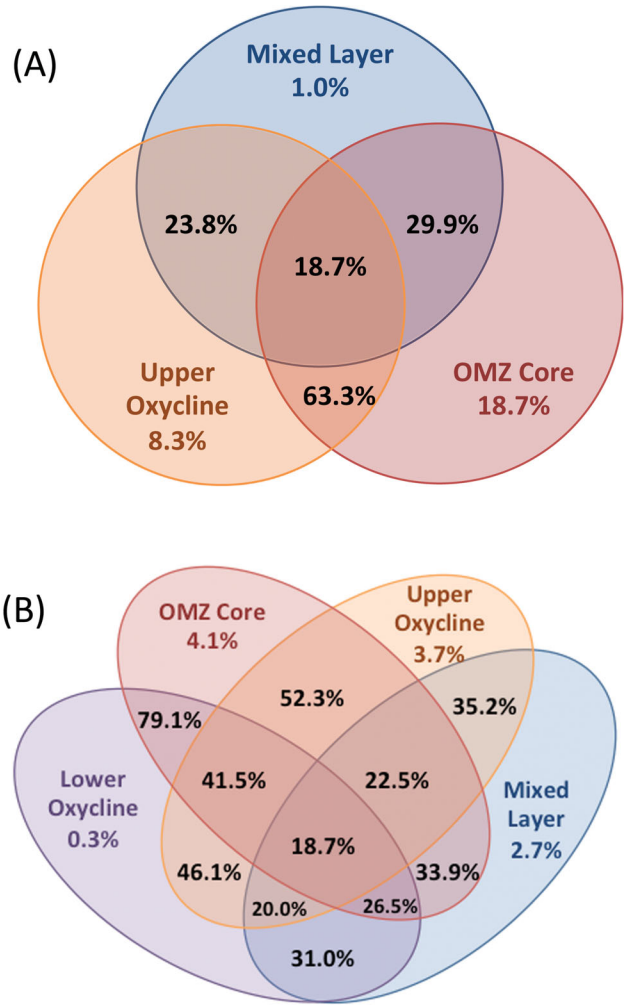


Figure 3. Protist community structure within oxygen gradient. Venn diagrams of shared diversity between oxygen conditions in station 6 (**A**) and station 10 (**B**) of the ETNP during June 2013. Percentages of unique OTUs per oxygen condition are relative to the number of total OTUs within each station ($n = 1847$ and 2196 OTUs for station 6 and station 10, respectively). Percentages of shared OTUs are relative to the total number of OTUs present in the compared oxygen conditions. The color indicates the oxygen condition [blue corresponds to the mixed layer (>180 μM O_2), orange corresponds to the upper oxycline (~2 μM), red corresponds to the OMZ core (~0 μM) and purple corresponds to the lower oxycline (~5 μM)].

nitrite and nitrate concentration variables were tested using the same methodology by substituting a missing value with an interpolated value when possible (i.e. if the 300 m value was missing, the mean of the 200 + 400 m values was used). In both of these scenarios, nitrogen oxide concentrations did not show significant effects on the community taxon composition variability.

A total of 83% OTUs was shared between both stations with equivalent shared proportions between the larger and the smaller size fractions (75.1 and 76.0%, respectively; data not shown). However, certain OTUs were differentially detected in the two size fractions at both stations (e.g. *Duboscquella* sp., Syndiniales, were present in the larger size fraction of station 6 and the smaller size fraction of station 10). At both stations, 18.7% of the total OTUs were detected under all oxygen conditions sampled within a station (Fig. 3) while 215 OTUs (i.e. 9.7% of the total 2209 OTUs) were represented at both stations under all oxygen conditions.

Diversity

OTUs representing the diverse higher taxonomical groups of Alveolata and Rhizaria (62.4 ± 20.4% and 30.0 ± 18.3%, respectively; Figs S2 and S3, Supporting Information) dominated total read abundances in most of the samples from both stations. Due to the potential for differential 18S rRNA operon copy numbers per genome (i.e. dinoflagellates are known to possess large and variable numbers of copies; e.g. 100 copies in *Pfiesteria piscicida*, Saito *et al.* 2002) and biases introduced during PCR (e.g. differential primer binding), relative OTU abundances may not accurately reflect relative cell abundances. Abundance data should be interpreted carefully and confirmed with complementary methods such as fluorescence *in situ* hybridization (FISH) counts.

Of the total 2209 OTUs, 58.0% were shared among size fractions (data not shown). 58.6 and 61.0% of the OTUs affiliated with Alveolata and Rhizaria, respectively, were shared between both size fractions (data not shown). However, one replicate sample from the >30μm fraction at 100 m (OMZ core) of station 6 displayed different traits (Fig. S2, Supporting Information). In the sample from 100 m, Archaeplastida represented 66.1% of total reads, almost all of which were affiliated to *Dunaliella* sp. We interpret the taxonomic assignment of this result with caution. PCR controls did not suggest any contamination, so the presence of sequences affiliated to *Dunaliella* sp. in the >30 μm fraction of this one sample suggests entrainment of intact DNA of this photosynthetic species or an undescribed close relative within a sinking large particle. Stramenopiles were mainly represented in the larger size fraction at both stations (Figs S2 and S3, Supporting Information).

At finer taxonomic levels, the orders and classes within Alveolata and Rhizaria differed in relative abundances among oxygen conditions and size fractions (Figs 4 and 5). Gymnodyniales (38.1 ± 11.8%; Fig. 4) were the dominant Alveolates in the larger size fraction at both stations, except at 800 (OMZ core) and 1000 m (lower oxycline) at station 10 which contained a higher proportion of Syndiniales (Fig. 4B). However, some patterns were station specific, such as the presence of Gonyaulacales in the mixed layer of the column above the continental shelf (Fig. 4A). Syndiniales mainly represented Alveolata in the smaller size fraction (44.4 ± 17.9%; Fig. 4). Gymnodiniales also represented an important proportion of alveolate richness in all samples (30.0 ± 15.9%; Fig. 4).

Orders affiliated with Rhizaria exhibited more complex patterns than those of Alveolates (Fig. 5). Both the >30 μm and 1.6–30 μm size fractions showed an increased proportion of uncultured Granofilosea with depth (Fig. 5). Phaeogromida represented 44.6% of Rhizaria sequences in the larger size fractions in the surface mixed layer of both stations (Fig. 5A and B). Collodaria represented 27.5 ± 19.5% of OTUs in the upper oxycline and 100–125 m (OMZ core) at both stations (Fig. 5A and B).

Replication

Replicates of the smaller size fraction from station 6 (30, 85, 100, 125 m; surface mixed layer down into the OMZ core) and station 10 (80, 125, 300, 500 m; surface mixed layer down into the OMZ core) clustered in pairs in the UPGMA tree based on a Bray Curtis threshold of 75% similarity. None of the larger size fraction replicates shared 75% or more similarity (Fig. 6).

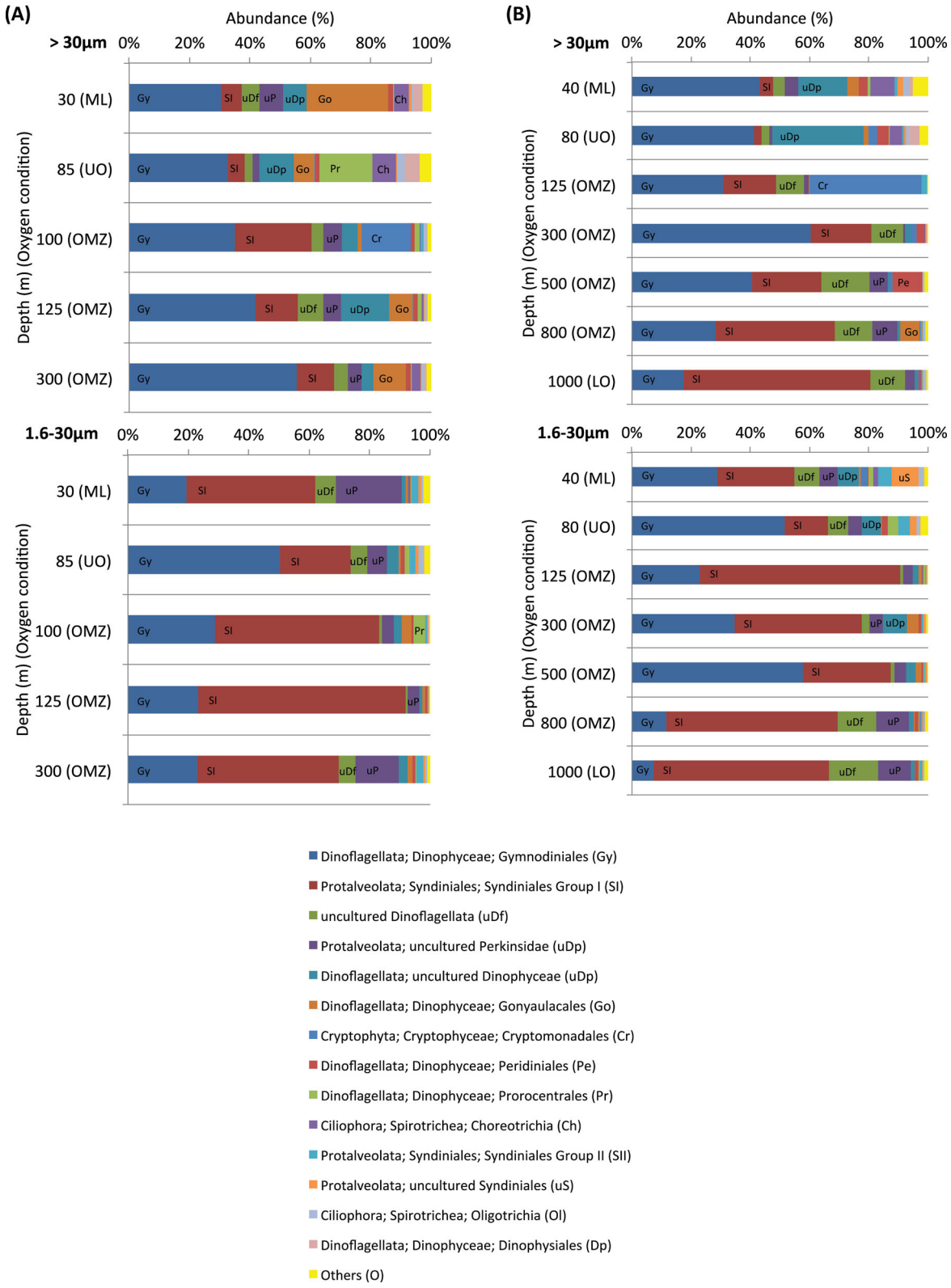


Figure 4. Community alveolates diversity within depth. Diversity within Alveolata from station 6 (**A**) and station 10 (**B**) of the ETNP within depth for both size fractions (>30 μm and 1.6–30 μm). Replicates were pooled and the average of their Hellinger transformed abundances was used. Oxygen conditions are indicated next to depth (ML: mixed layer, UO: upper oxycline, OMZ: OMZ core, LO: lower oxycline). Taxa represented by less than 0.5% on average per sample were pooled and represented as 'others'. Abundance of these specific taxa can be found in Fig. S3 (Supporting Information).

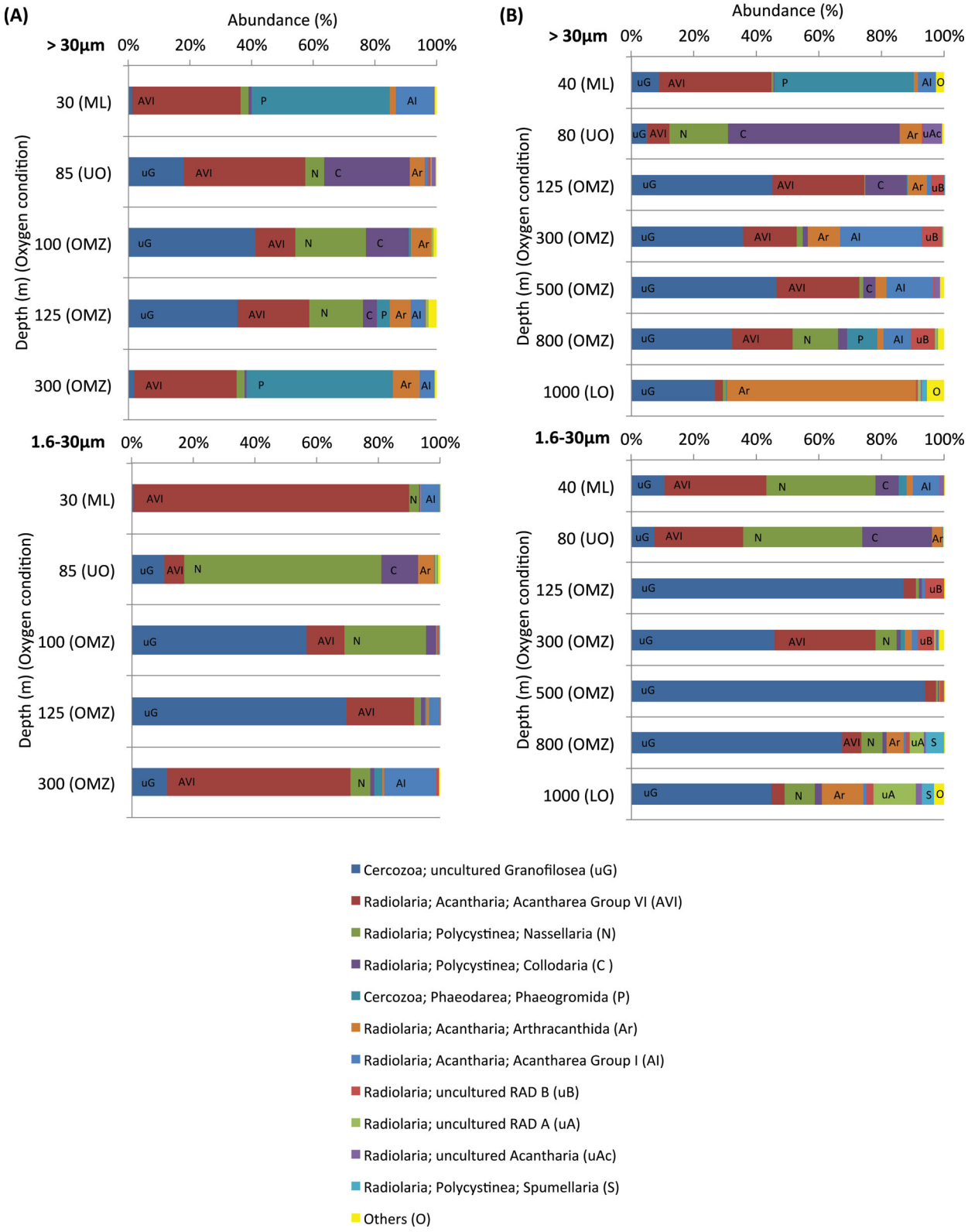
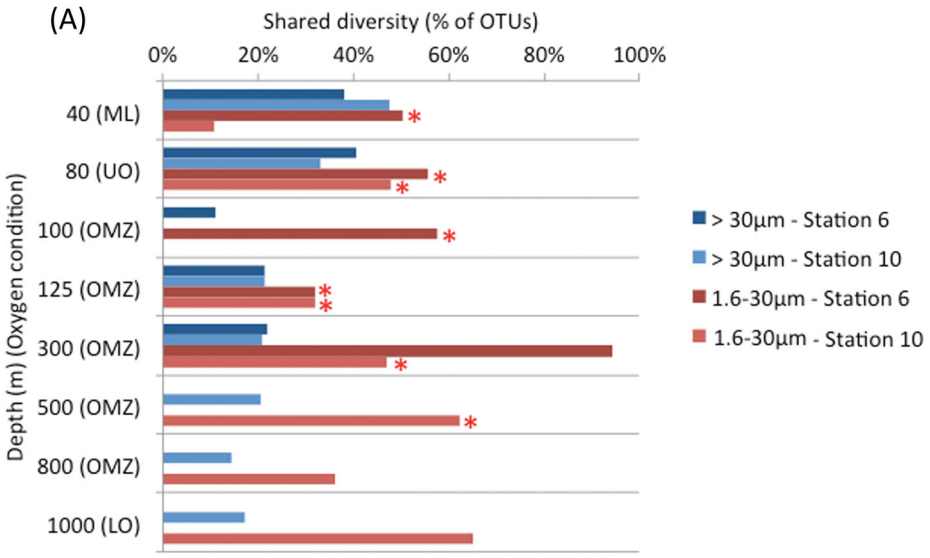


Figure 5. Community rhizarian diversity within depth. Diversity within Rhizaria from station 6 (**A**) and station 10 (**B**) of the ETNP within depth for both size fractions (>30 μm and 1.6–30 μm). Replicates were pooled and the average of their Hellinger transformed abundances was used. Oxygen conditions are indicated next to depth (ML: mixed layer, UO: upper oxycline, OMZ: OMZ core, LO: lower oxycline). Taxa represented by less than 0.5% on average per sample were pooled and represented as 'others'. Abundance of these specific taxa can be found in Table S4 (Supporting Information).



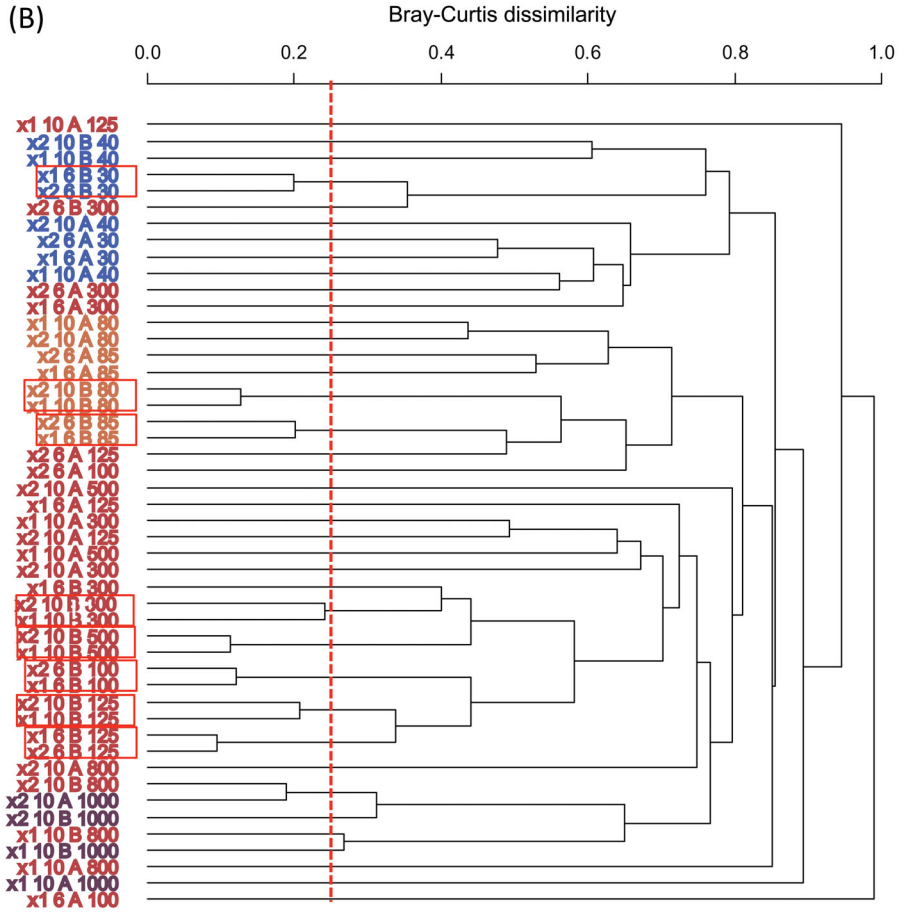


Figure 6. Shared diversity between replicates and similarity clusters. (**A**) Proportion of shared OTUs between replicates of the >30 μm fraction (blue) and the 1.6–30 μm fraction (red) in station 6 and station 10 of the ETNP within depth. Red asterisks show replicates clustered in pairs at a 75% threshold similarity level. Oxygen conditions are indicated next to depth (ML: mixed layer, UO: upper oxycline, OMZ: OMZ core, LO: lower oxycline). (**B**) UPGMA hierarchical tree-based Bray Curtis dissimilarities between libraries from station 6 and station 10. Red frame show libraries clustered together at a 75% threshold similarity level. The color indicates the oxygen condition [blue corresponds to the mixed layer (>180 μM O_2), orange corresponds to the upper oxycline (~2 μM), red corresponds to the OMZ core (~0 μM) and purple corresponds to the lower oxycline (~5 μM)]. The sample names follow this logic: x1/2 (corresponds to the replicate number), A/B (corresponds to the filter size: A – 30 μm and B – 1.6 μm), depth in m.

DISCUSSION

Environmental conditions and community structure

This study investigated the size-fractionated composition of microbial eukaryotic communities in the ETNP OMZ. OMZs harbor a wide diversity of microbial eukaryotic communities showing a non-random partitioning of their members along the oxygen gradient and size fractions (Edgcomb *et al.* 2011; Orsi *et al.* 2011, 2012; Ulloa *et al.* 2012; Parris *et al.* 2014). Structuring of eukaryotic communities within the ENTP OMZ is evidenced by the observation that only 9.7% of the total 2209 OTUs recovered were present in every oxygen condition sampled at both stations. These cosmopolitan OTUs, affiliated predominantly with the taxonomic classes Dinophyceae and Polycistinea, as well as the order Syndiniales, nonetheless showed high variability in abundance (based on rRNA signatures) along the oxygen gradient of the OMZ (Figs 4 and 5). Indeed, the most abundant of these OTUs, a member of Gymnodiniales, was detected at 100–125 m (the OMZ core) at both stations, which is consistent with previous reports for its closest relatives in oxygen-depleted habitats (Edgcomb *et al.* 2011; Orsi *et al.* 2011, 2012). Parasitic group I Syndiniales often accounts for a large majority of sequences in anoxic and suboxic water column samples (Guillou *et al.* 2008). This was also observed in this study (Fig. 4). In contrast, OTUs affiliating with group II Syndiniales were primarily found in the surface mixed layer samples.

Radiolarians from Acantharea (Group I, Group VI, Arthracantida; Fig. 5) were well represented in this ETNP data set and were detected at every depth regardless of oxygen concentration, as reported previously in the ETSP (Parris et al. 2014). This group has been reported to include mixotrophic members, which can produce blooms in surface waters, and are known to host a high number of eukaryotic symbionts (Gilg *et al.* 2010). These radiolarians are thought to contribute significantly to vertical fluxes of carbon from surface waters due to their large size and their silicate tests that create ballast (Michaels *et al.* 1995). Nonetheless, radiolarian abundance appeared to vary substantially between depths, as shown for example in the smaller size fraction of samples from station 6 (Fig. 5A). OTUs affiliated with Acantharea comprised 96.0% of total OTUs detected in the upper mixed layer and 76.7% at 300 m depth (the OMZ core) while representing as few as 12.3% at 100 m (top of the OMZ core) at this station. Their uneven abundance along depth is contradictory with the classical vertical flux usually observed for these radiolarians, i.e. an increase in abundance along depth. While estimations of abundances *in situ* based on relative abundances of rRNA sequences must be interpreted with caution, this may simply reflect periodic pulses of sinking radiolarians coincident with separate bloom events. This hypothesis requires further testing in the future.

Oxygen concentration within the ETNP in June 2013 exhibited a typical profile of an upwelling zone (Fernández-Álamo and Färber-Lorda 2006; Stramma *et al.* 2008), with an OMZ core spanning from 80–900 m (Fig. 2), while circulation of deep oxygenated waters from the Western Pacific along the Equator leads to a deep suboxic to oxic water layer under the Tropical Pacific OMZ (>1000 m; Fiedler and Talley 2006). Not surprisingly, CCA and ANOVA analyses indicated that O_2, along with all the parameters tested, affected eukaryotic community composition (P value < 0.01; Fig. S1, Supporting Information). However, the two principal axes of the CCA only explained 11.2% of the community variability, highlighting the fact that other factors (abiotic and/or biotic) are also influencing the distribution of OTUs in this complex ecosystem. Chemical factors, such as sulfide and methane concentrations, have been shown to be significant drivers of microbial eukaryotes in anoxic marine environments (e.g. Behnke *et al.* 2006; Orsi *et al.* 2012). Sulfide in water samples collected for this study was not measured. While the smell of sulfide was not detected, and sulfide is not thought to typically accumulate in large Pacific OMZs since it is likely turned over rapidly (Canfield *et al.* 2010), its presence and influence cannot be ruled out. Aside from the complex environmental chemistry of OMZs, the availability of prokaryotic prey may also affect protist distributions. Indeed, prokaryote abundance and community structure vary significantly along OMZ redox gradients (Lin *et al.* 2008, Wright, Konwar and Hallam 2012) as well as between size fractions (Ganesh *et al.* 2014). Phagotrophic protist communities can be shaped by distributions of their prey (Fenchel 2002; Sherr and Sherr 2002; Anderson et al. 2013). Protists can also be associated with specific prokaryotic symbionts (Bernhard *et al.* 2000; Edgcomb *et al.* 2011; Orsi *et al.* 2012). Consequently, symbiont-bearing protists may be restricted to waters with the chemical conditions that are optimal for their partners. FISH observations and examination of prokaryotic community distribution are necessary to test this hypothesis.

The relatively low proportion of shared OTUs between the mixed layer versus the OMZ core located off the continental shelf (i.e. station 6) and on the continental slope (i.e. station 10); 29.9 and 33.9% shared OTUs, respectively, (Fig. 3) supports the notion of habitat specialization within the eukaryotic community along the ETNP water column. These patterns suggest a degree of niche specialization, likely influenced not only by oxygen concentration, but currents and other related chemical/biological factors as well as proximity to coastal influences.

Dissolved oxygen concentration impacts protists directly by selecting for physiological adaptations to anoxia, or indirectly through creation of specific biogeochemical niches (Fenchel 2002). A total of 18.7% of the OTUs recovered in the OMZ core at station 6 were unique to this environment (Fig. 3A). The unique species found in oxygen-depleted habitats are most likely lineages that have adapted their physiology to the specific conditions present within these environments, and in some cases, may represent primitive lineages that have inhabited anoxic habitats throughout their evolutionary history. The lower proportion of unique OTUs in the OMZ core at station 10 (4.1%; Fig. 3B) may be explained by a strong pulse of sinking particles at this location from the upper oxycline and the mixed layer, containing residual DNA of species adapted to oxic conditions. Alternatively, some fraction of these OTUs may share similar physiologies and/or may be capable of adapting to different oxygen conditions. Nonetheless, detection of photosynthetic cryptophytes in the larger size fraction of samples from station 10 on the continental slope in the surface mixed layer and in the upper oxycline (Fig. 4) likely reflects the sinking of a previous local bloom event triggered by water stratification in this area. The lower oxycline layer shared a relatively high percentage of OTUs with the OMZ core, upper oxycline and surface mixed layer (79.1, 46.1 and 31.0%, respectively), and had as few as 0.4% unique OTUs that were not detected in any other oxygen condition (Fig. 3B). All together, this suggests vertical connectivity of the microbial community *via* sinking particles. It has been estimated that 10–15% of the surface primary production sinks out of the euphotic zone and transits through the water column in the ETNP (Karl and Knauer 1984). POM-associated eukaryotes likely impact carbon cycling by contributing directly to mechanical and/or chemical modification of the POM (Simon *et al.* 2002), and by grazing on prokaryotes and/or other smaller protists attached to sinking particles (Kiørboe *et al.* 2003). Feeding activities

also contribute to the process of disaggregation, thus modifying the composition of the aggregates as well as increasing the surfaces accessible to other active remineralizers. In the surface mixed layer mixotrophic protists further contribute to carbon cycling by combining autotrophy with grazing directly on particles (Fenchel 2002).

Information on activities of protists attached to POM would aid in understand carbon cycling in this dynamic part of the global ocean. Further investigations, based on proxies reflecting activity such as mRNA, are required to decipher which community members are active members of the OMZ community versus transients on particles.

Variation among size fractions

The mean number of shared OTUs between size fractions at every depth was relatively high (39.2 ± 12.9%; Fig. 6). Operationally, distinguishing between particle-associated and free-living protists is challenging because of cell size distributions, complex life cycles and possible artifacts of filtration (discussed below). This may explain some of the overlapping reported OTU composition between the free-living and the particle-associated communities. Additionally, the high number of total shared OTUs between size fractions (58%) may be explained partly by the methodology used for sample collection. Filtration pressures were not consistently controlled. Filter clogging during filtration could cause elevated pressure gradients across the filter, potentially leading to lysis of large cells and disruption of particles, as well as retention of smaller size organisms on the larger pore size filter. Lysis of pressure-sensitive protists, potentially with passage of large protist nuclei through the 30 μm mesh and retention on the smaller pore size filter (Not *et al.* 2007) would result in an overestimation of OTU composition shared between the two size fractions. The effects of filter pressure regulation on the separation of different size fractions could be assessed in the future with FISH experiments. Furthermore, the existence of protist extracellular DNA (e.g. Radiolaria; Not *et al.* 2009) present in the environment could also confound interpretation of richness within the two size fractions if this DNA is retained on the clogged filters. Together, these factors could explain the recovery of large protists, such as acantherids (50–5000 μm) in the smaller size fraction. Data interpretation here must therefore be focused on observed differences rather than on similarities in taxonomic composition between the two size fractions.

Size partitioning has been demonstrated in eukaryotic communities from other sites, sampled using varying filter pore sizes (<0.8 and 0.8–3 μm, Not *et al.* 2009; 0.45–2 μm and > 2 μm, Not *et al.* 2007; 0.2–3 μm and 3–10 μm, Sauvadet, Gobet and Guillou 2010; 0.2–1.6 μm, >1.6 μm, Parris *et al.* 2014). In these studies, the larger size fractions were dominated by dinoflagellates (Alveolata) and radiolarians (Rhizaria). In the present study, the large free-living predators Gymnodiniales dominated the >30 μm size fraction (Fig. 5 and Figs S2 and S3, Supporting Information). The 1.6–30 μm size fraction was dominated by Syndiniales Group I; these groups were also present, at lower levels, in the larger size fraction (Fig. 5 and Figs S2 and S3, Supporting Information). Described Syndiniales are parasitoids (i.e. the death of their host is necessary for them to complete their life cycle) known to prey on a wide range of species (Guillou *et al.* 2008), including copepods (Skovgaard *et al.* 2005). Syndiniales produce small flagellated cells, i.e., dinospores (from 1–12 μm) involved in infection transmission to new hosts (Sauvadet, Gobet and Guillou 2010). The detection of Syndiniales in the large size fraction most likely corresponds to infected host organisms affiliating with diverse Dinophyceae and Rhizaria (Guillou *et al.* 2008). Likewise, Syndiniales Group I sequences found in the smaller size fraction may originate from infective dinospores dispersed in the water. Alternatively, they may correspond to Syndiniales Group I targeting a smaller host. As parasitoids, Syndiniales could have a large impact on the proportion of POM and dissolved organic matter available in the water column through host lysis as well as through modification of host distribution in the water column. Further investigations are needed to understand the role of parasites in marine elemental cycles. The high proportion of Phaeosphaeria recovered in the large size fraction in the mixed layer of the continental slope station (Fig. 5B) could correspond to a bloom of these members of Polycistinea. The order Collodaria includes radiolarians known to play a role in carbon fixation in the oligotrophic ocean, and are the only radiolarians exhibiting a colonial lifestyle (Ishitani *et al.* 2012). Their sizes and lifestyle explain why these radiolarians were recovered in the larger size fraction of both stations (Fig. 5).

Ciliates are often prominent members of low-oxygen water columns (see review by Edgcomb and Pachiadaki 2014). While ciliate signatures did not dominate our libraries, examination of taxonomic affiliation within ciliate OTUs shows that OTUs assigned to the oligotrichs Pleuronema and Strombidium were the most common oligotrichs detected in samples from the OMZ cores (the 100 m sample at Station 6 and the 125 and 500 m samples at station 10), and were not detected in the oxic mixed layer. Both taxa have been described from suboxic and sulfidic waters of the Black Sea (Wylezich and Jürgens 2011). A similar pattern was observed for other unaffiliated oligotrich OTUs.

Phylogenetic differences of the eukaryotic communities existing between the two size fractions could be explained by microniches created by the sinking POM generated in surface waters by primary producers. Mobile protists, such as nanoflagellates, dinoflagellates and ciliates, have the ability to feed on particle-associated bacteria and then detach (Kiørboe *et al.* 2003). Suspended or sessile protist feeders can also use sinking organic matter aggregates as a ballast to gain access to deeper water layers (ChristensenDalsgaard and Fenchel 2003) rather than feeding directly on the aggregates. Sinking particles may therefore carry protists to depths where they may not otherwise inhabit.

Replication

Fenchel and Finlay (2004) hypothesized that smaller protists are more likely to have a wider and more homogeneous dispersal. Consistent with this, at both stations, the 1.6–30 μm fraction replicates were more similar to one another in terms of composition (49.2 ± 20.9% of shared OTUs; Fig. 6A), compared to replicates in the larger size fraction (25.6 ± 11.4% of shared OTUs; Fig. 6A) at both stations. This may produce a more complete diversity picture for smaller organisms when analyzing the same amount of seawater, as the larger fraction may be less homogeneous. Differences observed between replicates suggest either that ETNP waters are very heterogeneous or that filtration pressures were unequal between replicates. Alternatively, differences in community evenness between the size fractions may affect the potential for replicate sequencing to consistently recover the same OTUs.

CONCLUSION

This study showed that microbial eukaryote communities of the ETNP OZM water column are not only shaped by oxygen concentration, but also by other factors not monitored here.

Further descriptive studies have to be carried out in order to better understand factors structuring protist communities of this area (e.g. sulfide or methane concentrations, prokaryotic prey distribution). It would be valuable to collect more information about temporal variations of environmental parameters in this area, in order to understand upwelling currents and their impacts on microbial community dynamics. This study also highlights the importance of maintaining the lowest possible pressure drop across filtration membranes in samples in order to separate size fractions more accurately. Despite this, differences in richness and abundances observed between size fractions suggest that, at least partially, taxonomically different protists are associated with POM compared to the free-living communities. Metatranscriptomics studies could provide an understanding of whether particle-attached protist communities are active, as well as identify the nature of their activities.

SUPPLEMENTARY DATA

Supplementary data is available at FEMSEC online.

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