An Analysis of Protists in Pacific Oxygen Deficient Zones: Implications for *Prochlorococcus* and N_2 producing bacteria

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Running title: Protists in Pacific Oxygen Deficient Zones

Originality-Significance Statement

We use non-primer based methods to examine the composition and relative abundance of protists in metagenomes from the Eastern Tropical North Pacific and Eastern Tropical South Pacific Oxygen Deficient Zones. We compare this data to an oxic North Pacific station (Hawaii Ocean Time-series or HOT). It is not possible to tell from amplicon techniques if the total numbers of protists decreased in the ODZ as the results are the relative abundance of a particular 18S rDNA gene within all 18S rDNA genes. Common primers also do not amplify many important groups of protists. Our non-primer based metagenomic read placement onto phylogenetic tree method allows us to include all protists and to normalize by total reads which includes all the bacterial and archaeal genomes in its total. The goal of this work is to identify potential predators of ODZ *Prochlorococcus* and ODZ N cycling bacteria and quantify these protists in proportion to the total microbial community. We see a large drop in abundance of protists in the oxygen deficient zones and identify potential predators present there.

Abstract (200 words)

Ocean oxygen deficient zones (ODZs) host 30-50% of marine N_2 production. Cyanobacteria photosynthesizing in the ODZ create a secondary chlorophyll maximum and provide organic matter to N_2 producing bacteria. This chlorophyll maximum is thought to occur due to reduced grazing in anoxic waters. We first examine ODZ protists with long amplicon reads. We then use non-primer-based methods to examine the composition and relative abundance of protists in metagenomes from the Eastern Tropical North and South Pacific ODZs and compare these data to the oxic Hawaii Ocean Time-series in the North Pacific. We identify and quantify protists in proportion to the total microbial community. From metagenomic data, we see a large drop in abundance of fungi and protists such as choanoflagellates, radiolarians, cercozoa and ciliates in the ODZs but not in the oxic mesopelagic at HOT. Diplonemid euglenozoa were the only protists that increased in the ODZ. Dinoflagellates and foraminifera reads were also present in the ODZ though less abundant compared to oxic waters. Denitrification has been found in foraminifera but not yet in dinoflagellates. DNA techniques cannot separate dinoflagellate cells and cysts. Metagenomic analysis found taxonomic groups missed by amplicon sequencing and identified trends in abundance.

Introduction

Ocean oxygen deficient zones (ODZs), defined as water with <10 nM O_2 and without measurable sulfide, host 30-50% of marine fixed-N loss through N_2 production (DeVries *et al.*, 2013). There are two oceanic ODZs in the Pacific: the Eastern Tropical North Pacific oxygen deficient zone (ETNP ODZ) offshore from Mexico and the Eastern Tropical South Pacific oxygen deficient zone (ETSP ODZ) offshore from Peru and Chile. Populations of the cyanobacterium *Prochlorococcus* have been found photosynthesizing in the ETNP and ETSP ODZs (Goericke *et al.*, 2000; Lavin *et al.*, 2010; Garcia-Robledo *et al.*, 2017; Fuchsman *et al.*, 2019; Márquez-Artavia *et al.*, 2019) creating a secondary chlorophyll *a* maximum whenever the 1% of the deeply penetrating blue irradiance overlaps with the ODZ (Cepeda-Morales *et al.*, 2009). Production by these ODZ *Prochlorococcus* could provide up to 40% of the organic matter to the heterotrophic community in the upper ODZ (Fuchsman *et al.*, 2019). Experiments in the coastal ETSP found higher cyanobacterial growth rates than consumption rates by nanoflagellates under putatively anoxic conditions (Cuevas and Morales, 2006), and models indicate that a reduction in predation in anoxic waters could allow the creation of the secondary chlorophyll maximum (Zakem *et al.*, 2020). The theory that the secondary chlorophyll maximum is due to reduced grazing rather than increased growth rates is logical given the low growth rates of *Prochlorococcus* at these light levels (Vaulot *et al.*, 1995; Johnson *et al.*, 1999). A reduction in predation in anoxic waters also would affect bacteria and archaea mediating N cycling in the ODZ, including N_2 producers. The goal of this work is to identify potential predators of ODZ *Prochlorococcus* and ODZ N cycling bacteria and quantify them in proportion to the total microbial community.

Previous work has provided a few estimates of grazing under suboxic and anoxic conditions. At a coastal station on the Chilean coast, heterotrophic nanoflagellates preyed on cyanobacteria in waters with oxygen below the detection limit of the CTD sensors (Cuevas and Morales, 2006). Unfortunately, it is impossible to know if these waters were truly anoxic. Grazing rates per heterotrophic nanoflagellate were the same in oxic and putatively anoxic waters. However, the number of heterotrophic nanoflagellates were much reduced in the putatively anoxic waters, so the total cyanobacterial consumption rates were lower (Cuevas and Morales, 2006). Cyanobacterial growth was balanced by grazing in oxic waters, but cyanobacterial growth was 4x higher than grazing in putatively anoxic waters (Cuevas and

Morales, 2006). Additionally, in situ fluorescent labeled bacteria grazing experiments in the Eastern Tropical South Pacific found that grazing rates were twice as high in suboxic water (5 μM O_2) compared to anoxic (non-sulfidic) water at 150m at different stations, where 28% of bacteria were grazed in suboxic water and 13% of bacteria were grazed in the anoxic water (Medina *et al.*, 2017). With the same in situ technique, grazing experiments in the Mediterranean indicated similar grazing rates at 3 μM O_2 and fully oxic waters (Pachiadaki *et al.*, 2016). Thus full anoxia seems to affect predation differently than low oxygen. Altogether previous work indicates reduced but active grazing in anoxic ODZs.

The majority of studies of anaerobic protists have been in sulfidic waters and the suboxic/anoxic waters overlaying them (Zuendorf *et al.*, 2006; Behnke *et al.*, 2010; Edgcomb, Orsi, *et al.*, 2011; Orsi *et al.*, 2011, 2012; Wylezich *et al.*, 2018). Oxygen, sulfide and methane are some of the strongest variables structuring protist communities (Fenchel *et al.*, 1995; Orsi *et al.*, 2012; Pasulka *et al.*, 2016). However, oxygen deficient zones contain high concentrations of nitrate and are underlain by oxic waters. Oxygen deficient zones are defined as <10 nM oxygen due to the detection limits of the STOX sensor in the environment (Revsbech *et al.*, 2009), and thus it is difficult to determine where there is complete anoxia. Some bacteria can utilize oxygen at concentration < 10 nM (Stolper *et al.*, 2010; Tiano *et al.*, 2014; Bristow *et al.*, 2016), and it is unknown if any protists can do so. Offshore ODZs are not sulfidic and do not have influxes of sulfide or other reduced chemical species, though sulfate reduction may occur in particles (Saunders *et al.*, 2019; Raven *et al.*, 2021). ODZs on shallow coastal shelves may have sulfide fluxes from the sediments, but these are a special case not examined here (Schunck *et al.*, 2013; Callbeck *et al.*, 2018; Schlosser *et al.*, 2018). Thus, functionally anoxic ODZs are at a different redox state than sulfidic basins and are even fundamentally different from the suboxic/anoxic zones above sulfidic basins that are hugely influenced by fluxes of reduced species (manganese, ammonium, methane, reduced S) from sulfidic waters (Lam *et al.*, 2007; Fuchsman *et al.*, 2008, 2011, 2012; Kirkpatrick *et al.*, 2018). Decreased grazing rates in the ODZ are in contrast to grazing experiments at sulfide boundaries, where chemoautotrophy of reduced S cause numbers of prokaryotes and also protists and grazing to be increased (Anderson *et al.*, 2012; Pachiadaki *et al.*, 2014). Fermentation and endosymbiont methanogenesis are processes favored by protists under sulfidic conditions (Fenchel and Finlay, 1991; Boxma *et al.*, 2005; De Graaf *et al.*, 2011). It would be more energetically favorable for protists living in an ODZ to utilize nitrate in a

dissimilatory fashion than to utilize fermentative processes. Few marine dissimilatory nitrate-reducing protists have been documented (Kamp *et al.*, 2015), but some benthic foraminifera can undergo denitrification or denitrification and fermentation simultaneously (Risgaard-Petersen *et al.*, 2006; Gomaa *et al.*, 2021), some fungi and diatoms can utilize dissimilatory nitrate reduction to ammonium (Stief *et al.*, 2014; Kamp *et al.*, 2016), and some Euglenzoa have nitrate reducing endosymbionts (Edgcomb, Breglia, *et al.*, 2011).

The protists of ODZs have previously been investigated with 18S rDNA short amplicon sequencing (Parris *et al.*, 2014; Duret *et al.*, 2015; Jing *et al.*, 2015; De La Iglesia *et al.*, 2020). In the ETNP ODZ short amplicon sequencing found Acantharea, Polycystinea radiolarians, Gymnodiniales dinoflagellates, cercozoan and parasitic protists, Syndiniales and Perkinsidae, (Duret *et al.*, 2015). In the ETSP ODZ, Acantharea and Polycystinea radiolarians, dinoflagellates, Syndiniales parasites, and Euglenozoa were found (Parris *et al.*, 2014). The diversity of protists has been found to be reduced under anoxic conditions (Orsi *et al.*, 2011, 2012; Jing *et al.*, 2015). Since amplicons are analyzed using relative abundance of a particular 18S rDNA gene within all 18S rDNA genes, it is not possible to tell if the total numbers of protists decreased in the ODZ from this technique. The use of primers can bias results both because some organisms are missed due to primer mismatches and because of amplification bias where some species amplify more readily and thus look more abundant (Elbrecht and Leese, 2015). Additionally, the18S rRNA gene is not a single copy core gene (Zhu *et al.*, 2005; Galluzzi *et al.*, 2010; Kudryavtsev and Gladkikh, 2017; Gong and Marchetti, 2019), but it is the most broadly sequenced gene used to identify protists (Guillou *et al.*, 2013). Here, we start by forming OTUs with long amplicon reads (~1600 bp) of 18S rRNA gene from the ETNP and combined them with known reference sequences from the curated Protist Ribosome Database PR2 (Guillou *et al.*, 2013) to create a phylogenetic tree of protists. Then we use previously published metagenomic samples from the ETNP ODZ in April 2012 (Fuchsman *et al.*, 2017), newly sequenced metagenomic samples from the offshore ETSP ODZ in July 2013 and published metagenomes from Hawaii Ocean Time-series (HOT) (Luo *et al.*, 2020) to place metagenomic reads on this 18S rRNA gene phylogenetic tree in a non-primer biased manner. While our analysis still provides relative abundance, we normalize by total reads, which includes all the bacterial and archaeal genomes in its total. Here we identify and quantify protists found in the ODZ and consider potential predators of ODZ *Prochlorococcus* and ODZ N cycling bacteria.

144

145 **Methods**

146 *Long read amplicon sequencing*

147 Bulk water samples were collected for long read amplicon sequencing from ETNP station
148 161 (15.9°N -107.9°W) (Figure S1) at 12 depths (75m-300m) in April 2012 aboard the R/V
149 *Thompson* TN278 using 10 L Niskin bottles on a CTD-rosette. A Seabird SBE-43 dissolved
150 oxygen sensor and a WETLabs ECO Chlorophyll Fluorometer and a STOX sensor (Revsbech *et*
151 *al.*, 2009) were attached to the rosette. Two liters of Niskin water were vacuum filtered onto a
152 0.2 µm SUPOR filter. DNA was extracted from filters using freeze thaw followed by incubation
153 with lysozyme and proteinase K and phenol/chloroform extraction as in Fuchsman et al (2017).
154 Nutrients and N_2 gas measurements for this station and cast are previously published in
155 (Fuchsman *et al.*, 2018). Hydrographic and nutrient data from this cruise are deposited at
156 http://data.nodc.noaa.gov/accession/0109846.

157 Extracted DNA was sent to the company Mr. DNA (www.mrdnalab.com, Shallowater
158 TX, USA) where long reads amplicon sequences were amplified using 18S rDNA primers
159 designed for anoxic protists (EK-82F (GAAACTGCG AATGGCTC) combined with 25F (5′-
160 CATATGCTTGTCTCAAAGATTAAGCCA-3′) and 18S-1630Rev (5′-CGA CGG GCG GTG
161 TGT ACA A-3′)) and PCR methodology from (Wylezich and Jürgens, 2011) except that
162 HotStarTaq Plus Master Mix (Qiagen, USA) was used. PCR products were purified using
163 Ampure PB beads (Pacific Biosciences). SMRTbell libraries (Pacific Biosciences) were prepared
164 following manufacturer's guidelines and sequenced using a Pac Bio Sequel. After completion of
165 initial DNA sequencing, each library underwent Circular Consensus sequencing using PacBio's
166 CCS algorithm where the forward and reverse reads from each template were aligned to identify
167 stochastic errors. Five thousand amplicons were sequenced per sample. Amplicon sequences
168 were then processed using the Mr. DNA analysis pipeline. In this pipeline, barcodes were
169 trimmed and sequences with ambiguous bases or chimeras were removed, then operational
170 taxonomic units (OTUs) were created by clustering at 97% similarity. OTUs were identified
171 using BLASTn against the PR2 database (Guillou *et al.*, 2013). We note that several OTUs were
172 not 18S rRNA. After quality control, 332 OTUs contained >10 amplicons. These OTUs
173 represent 14,492 individual amplicons (Table S2). These OTU consensus sequences are
174 submitted to NCBI Genbank Accession numbers MW695537-MW695844.

175

176 *Extraction of DNA for metagenomic sequencing*

177 Bulk water samples were collected from ETSP Station 9 cast 32 (13ºS and 82.2ºW) at 16
178 depths (80-400m) and ETSP Station 17 cast 40 (16.7ºS and 79ºW) at 14 depths (110m-1000m) in
179 July 2013 (Figure S1) aboard the R/V *Nathaniel B. Palmer* using 12 L Niskin bottles on a CTD-
180 rosette. Water was sampled with a focus on the oxycline (hypoxic waters above the ODZ) and
181 the ODZ and no fully oxygenated samples were obtained. A Seabird SBE-43 dissolved oxygen
182 sensor and a WETLabs ECO Chlorophyll Fluorometer and a STOX sensor (Revsbech *et al.*,
183 2009) were attached to the rosette. Four liters of Niskin water were filtered onto a 0.2 µm
184 SUPOR filter using a peristaltic pump. DNA was extracted from filters using freeze thaw
185 followed by incubation with lysozyme and proteinase K and phenol/chloroform extraction as in
186 Fuchsman et al (2017). Libraries were created and run on a 300 cycle NovaSeq S1 flowcell at the
187 Northwest Genomics Center (Seattle, WA). Nutrients and N_2 gas measurements for this station
188 are previously published in (Peters *et al.*, 2018). Hydrographic and nutrient data from this cruise
189 are deposited at http://data.nodc.noaa.gov/accession/0128141. The Bioproject containing this
190 data is PRJNA704804 and individual accession numbers can be seen in Table S1 along with
191 oxygen, nitrite and nitrate concentrations.

192 Previously published ETNP metagenomes from bulk water samples (2-4 L) were
193 collected onto 0.2 µm SUPOR in April 2012 at Station 136 (17.04 ºN, 106.54 ºW) on R/V
194 *Thompson* TN278 as indicated in (Fuchsman *et al.*, 2017). Nutrients and N_2 gas measurements
195 for this station and cast are previously published in (Fuchsman *et al.*, 2017, 2018). These
196 sequences can be found at Bioproject PRJN350692. Previously published HOT metagenomes
197 were sampled at (22º45'N and 158ºW) from bulk seawater (2-4 L) onto 0.2 µm SUPOR at 12
198 depths (5-500m) in May, August, and November 2015 (Luo *et al.*, 2020) and can be found at
199 Bioproject PRJNA352737. Nutrient and CTD measurements for these cruises can be found in
200 (Luo *et al.*, 2020).

201

202 *Phylogenetic trees and metagenomic read placement*

203 A representative of each family in the Protist Ribosomal Database PR2 version 4.14.0
204 (Guillou *et al.*, 2013) were obtained and combined with the consensus long read amplicons for
205 each 18S rDNA OTUs with ≥10 amplicons from the ETNP St 161 and their close relatives

(Figure 3, Figure S4A). These combined full length sequences were aligned using MUSCLE (Edgar, 2004) in order to construct a nucleotide maximum likelihood phylogenetic tree with bootstrap analysis (n=100) using the General Time Reversible (GTR) nucleotide substitution model under a Gamma rate heterogeneity model with RaxML-ng (Kozlov *et al.*, 2019). Groups within the phylogenetic trees were then labeled based on the Order of the references within that group. We note that to really understand the evolution of eukaryotic protists, concatenation of multiple genes should be used. However, this 18S rRNA gene tree produced coherent phylogenetic groups which could be used for read placement. Foraminifera did not align well with the other protists, so we made a separate foraminifera tree (Figures S4B) using all the foraminifera sequences in PR2 (Guillou *et al.*, 2013).

The sequences making up the tree were then BLASTed (blastn) very broadly (e-value=10^-5) against an ETNP, ETSP, and HOT metagenomic read databases. The short reads were then aligned to the reference tree using PaPaRa Parsimony-based Phylogeny-Aware Read Alignment program 2.0 (Berger and Stamatakis, 2011). Non-overlapping paired end reads were then combined into one aligned sequence and placed on the tree by EPA-ng (Barbera *et al.*, 2019). Placed reads have a pendant length indicating the similarity between a query read and the location it places on the tree. Previously it was determined that reads with a pendant length greater than 2 were different from the gene under examination (Fuchsman *et al.*, 2017). Reads that placed with a pendant length greater than 2 were removed (4 reads total for the ETNP, 2 reads for the ETSP, and 1 read for the HOT samples). The remaining reads were enumerated for each taxonomic group using the assign subcommand of gappa (Czech *et al.*, 2020). Taxonomic read counts were normalized using the method previously described (Fuchsman *et al.*, 2019) where normalization factors for each sample were determined by dividing the number of good quality reads in a sample by the 100 m ETNP sample. The read counts were multiplied by the sample normalization factor, divided by the length of gene (1700 bp for 18S rRNA), and then multiplied by 100 in order to make visualization easier. We list both the original numbers of reads placed and the normalized reads for each taxonomic group in Table S3.

We note that we attempted to use this analysis with BioGeotraces metagenomic data (Biller *et al.*, 2018). However, not enough protist reads were present. We believe this could be because BioGeotraces metagenomes were created from 200 mL of water (Biller *et al.*, 2018), so not enough protists were sampled.

The 18S rRNA gene is not a single copy core gene. Single cell eukaryotes are known to often have multiple copies of the 18S rRNA gene (Zhu *et al.*, 2005; Galluzzi *et al.*, 2010; Kudryavtsev and Gladkikh, 2017; Gong and Marchetti, 2019). However, it is the gene with the most comprehensive protist dataset (Guillou *et al.*, 2013). Additionally, some protists, such as dinoflagellates, can have multiple copies of their entire genome (Lin, 2011). We assume in this paper that copy number within a group is fairly consistent with depth though this is more likely to be true in more narrowly-defined taxonomic groups. However, the changes between oxic and anoxic water are so large that 18S rRNA gene copy number, though important to keep in mind, does not determine our big picture conclusions.

The phylogenetic trees used to obtain hydrazine oxidoreductase (*hzo*) to represent anammox bacteria and nitrous oxide reductase (*nosZ*) to represent denitrifying bacteria abundances in the ETSP were identical to previously published trees for the ETNP (Fuchsman *et al.*, 2017). Phylogenetic read placement was used for *hzo* and *nosZ* as described above. ETNP placement results for these genes were previously successfully compared to ETNP rate and lipid abundance data (Fuchsman *et al.*, 2017).

Results and Discussion

We examined the protist community in three systems: the Eastern Tropical North Pacific oxygen deficient zone (ETNP ODZ), the Eastern Tropical South Pacific oxygen deficient zone (ETSP ODZ), and Hawaii Ocean Time-series (HOT), a long-term monitoring station representing the oxic North Pacific Ocean. For ETNP and ETSP stations, a STOX sensor was used to verify the functional anoxia of the Oxygen Deficient Zone (Tiano *et al.*, 2014; Garcia-Robledo and Revsbech, unpublished data). In the ETNP, station 161, used for long-read amplicons, and station 136, used for metagenomics, were 160 km apart (Figure S1), had similar hydrographic profiles, with the ODZ starting ~100m and remaining functionally anoxic until 750m, and both stations had a large secondary chlorophyll maximum in the upper ODZ (Figure S2). At ETNP station 136, the cyanobacteria *Prochlorococcus* was the dominant photosynthesizer in the ODZ (Fuchsman *et al.*, 2019) and oxygen production by this cyanobacteria in the ODZ was inferred from transient measurable oxygen concentrations at the secondary chlorophyll maximum (Tiano *et al.*, 2014). Nitrite had a maximum of 4.5 μM from 140m-160m (Figure 1B; Table S1). Anammox and denitrifying N_2 producing bacteria had depth

profiles offset from each other (Fuchsman *et al.*, 2017) with anammox bacteria, as measured by phylogenetic read placement of hydrazine oxidoreductase (*hzo*), having a maximum at 160m and denitrifiers, as measured by phylogenetic read placement of nitrous oxide reductase (*nosZ*), having a maximum from 100-120m (Figure 1B). Depth profiles for all the genes in the dissimilatory N cycle in the ETNP and their various phylotypes are found in (Fuchsman *et al.*, 2017). ETSP station 9 contained a small secondary chlorophyll maximum (Figure 1C). The ODZ in the ETSP was less thick, corresponding to 108m to 350m (Figure 1C). Thus, in the ETSP station 9, our metagenomic samples spanned the entire ODZ. Nitrite had two maxima in the ETSP St 9 ODZ corresponding to 130m (8 µM) and 260m (6 µM) (Figure 1D; Table S1; (Peters *et al.*, 2018)). Normalized reads for anammox bacteria (*hzo*) had a maximum from 160m-260m and denitrifiers (*nosZ*) had a maximum from 110m-150m (Figure 1D). For ETSP St 17, the ODZ spanned from 140m to 350m and no secondary chlorophyll maximum was found (Figure 1E). Nitrite had a single maximum of 4 µM at 210m (Figure 1F; Table S1). Normalized reads for anammox bacteria were only found in the ODZ, but denitrifiers were found at depths with <30 µM oxygen (Figure 1F). In all three ODZs, nitrate concentrations were >10 µM (Table S1). The HOT station has a single chlorophyll maximum at 150m in May 2015, 125m in August 2015 and at 100m in November 2015 and oxygen remained >100 µM in the top 500m (Figure S3). The HOT station serves as our oxic comparison. With the goal of identifying grazers in oxygen deficient waters, we examined the protist community in offshore ETNP, offshore ETSP and oxic HOT station.

ETNP Long amplicon reads

Long read amplicons were sequenced from samples from ETNP St 161 in 2012. The top of the ODZ at this station was 100 m and the secondary chlorophyll max ranged from 95-120m (Figure S2). The number amplicons of each OTU sequence found at each depth can be found in Table S2. To reduce the possibility of including sequences with errors, we only examine OTUs with >10 total amplicons and thus we are missing rare taxa. Long read amplicons indicated more Polycistinea radiolarians and sister clade Acantherea in the hypoxic oxycline and the upper ODZ than in the deep ODZ (Figure 2). Large (up to 250 µm) mixotrophic protists such as Polycystinea radiolarians (Spumellarida and Nassalarida), known for their silica shells, and Acantharea, their

sister phyla with strontium shells can have photosynthetic endosymbionts that range from dinoflagellates to prymnesiophyte algae, to cyanobacteria (Michaels, 1991). Another dominant group of amplicons in both oxic and anoxic waters in the system was the Gymnodiniales order of dinoflagellate. This dinoflagellate group was equally important at all depths including oxic waters (Table S2). In both oxic waters and at depths in the ODZ below the secondary chlorophyll maximum, amplicons for parasitic Syndiniales and Eugregarinorida Apicomplexa were abundant (Figure 2). We remind the reader that these abundances are relative to total protist amplicons, so if total protists are reduced in the deep ODZ, as we might expect, the relative abundance of groups of protists may increase without their actual numbers increasing. All these groups have been previously found in the ETNP using short read amplicons (Duret *et al.*, 2015). We further use representatives of these long-read amplicons as reference sequences in a 18S rRNA gene phylogenetic tree (Figure 2 and S4).

Depth profiles of protists from placement of metagenomic reads

Metagenomic reads from ETNP station 136 in the and ETSP stations 9 and 17 were placed on the 18S rRNA gene phylogenetic tree, and normalized based on sequencing depth, which quantified them in proportion to the total microbial community. Fungi and the vast majority of protists, including algae Chlorophyta, Cryptophyta, Haptophyta and Stramenopiles, and heterotrophs chaonoflagellates, telonemia, radiolarians, and Acantharea had a maximum in normalized read abundance under oxygenated conditions, which then decreased sharply in anoxic waters (Figures 3 and 4 and Table S3). We remind the reader that 18S rRNA is not a single copy core gene (Zhu *et al.*, 2005; Galluzzi *et al.*, 2010; Kudryavtsev and Gladkikh, 2017; Gong and Marchetti, 2019). However large changes in the abundance of normalized reads likely reflect changes in abundance in any given taxa. Fungi in ETNP metagenomes have been examined in detail elsewhere (Peng and Valentine, 2021). Amoebozoa, ciliates, and cercozoan were still found at low levels in the upper ETNP ODZ, coincident with the secondary chlorophyll maximum, where oxygen is produced by photosynthesis (Tiano *et al.*, 2014; Garcia-Robledo *et al.*, 2017) but were nearly absent from ETSP ODZ samples. This sharp decrease in protist normalized reads in the mesopelagic is not seen at HOT, where oxygen was >100 μM (Figure 6, S3 and S5 and Table S3). In fact, in the ETSP, abundance of chaonoflagellates, fungi, and ciliate

reads increased again below the ODZ (Figure 5). We will focus below on those organisms who were present in the ODZ.

From metagenomic reads, dinoflagellates appear to be reduced but still present in the ODZ (Figures 4 and 5 and Table S3). However, as dinoflagellates can have a very large range of copy numbers for 18S rRNA genes (Galluzzi *et al.*, 2010), it is particularly difficult to interpret these results. The Dinophycea part of our phylogenetic tree contains many dinoflagellate orders without clear separation (Figure S4). Dinoflagellates in the ODZ are dominated by Gymnodiniales in our long read amplicon data (Table S2) which is consistent with previous amplicon data (Parris *et al.*, 2014; Duret *et al.*, 2015; Jing *et al.*, 2015). However, Gymnodiniales dinoflagellate amplicons also dominate in oxic waters above the ODZ (Table S2). Our techniques cannot differentiate between live, dead or resting cells. Gymnodiniales dinoflagellates can form cysts to survive low oxygen conditions and it has been suggested that these cysts would explain their presence in the ODZ (Morquecho and Lechuga-Devéze, 2003; Jing *et al.*, 2015; More *et al.*, 2018). Dinoflagellates cysts have been documented sinking into anoxic waters (Bringué *et al.*, 2018). No dinoflagellates have been found to utilize dissimilatory nitrate reduction (Kamp *et al.*, 2015), which would be the most energetic pathway to function in the ODZ. However, that does not mean that no dinoflagellates can do so.

From metagenomic reads, Syndiniales parasites are also present in the ETNP and ETSP ODZs (Figures 4 and 5 and Table S3). However, their read abundance also decreased in the ODZ and increases again below the ODZ (Figures 4 and 5). This decrease in reads was not seen in oxic waters at HOT (Figure 6). These parasites are common in previously published amplicons in low oxygen, anoxic and sulfidic waters (Guillou *et al.*, 2008; Parris *et al.*, 2014; Duret *et al.*, 2015; Torres-Beltrán *et al.*, 2018) as well as in fully oxic waters at HOT (Ollison *et al.*, 2021). We also find low levels of Icthyospora and Apicomplexa parasites with our metagenomic data (Figures 4-6, S5). All these parasites prey on both multicellular and single-cell eukaryotes (Jephcott *et al.*, 2016; Zamora-Terol *et al.*, 2020), not on cyanobacteria or other bacteria.

Foraminifera are present in ETNP and ETSP ODZs, but are reduced compared to oxic waters (Figures 4 and 5 and Table S3). Previous microscopic work on calciferous foraminifera identified Globigerinacea in the ETNP ODZ (Davis *et al.*, 2021). We also find Globigerinacea in the ETNP ODZ and it represents the majority of the class Globothalamea (calciferous

foraminifera) there (Figure 4). Similar to our data, microscopic counts indicated that calciferous foraminifera decreased greatly in the ETNP ODZ (Davis *et al.*, 2021). However, we also find soft walled Monothalamid foraminifera. These Monothalamid foraminifera have been previously studied at the oxic/anoxic transition in the Black Sea (Sergeeva *et al.*, 2019), but have not previously been examined in ODZs. In the Black Sea redox transition zone, Monothalamid forams were more abundant that calciferous foraminifera (Sergeeva *et al.*, 2019), which is similar to what we see here in the ODZs (Figure 4 and S6). Some benthic foraminifera can undergo denitrification (Risgaard-Petersen *et al.*, 2006; Høgslund *et al.*, 2008). It is assumed that foraminifera in ODZs also undergo denitrification (Davis *et al.*, 2021).

Euglenozoa are the only protists that increased in read abundance in the ODZ (Figures 4, 5), but also increased in read abundance at depth in the oxic mesopelagic at HOT (Figure 6, S5) and below the ETSP ODZ (Figure 5). The Diplonemid subgroup was the dominant euglenozoan (Figures 4, 6, S7). Marine Diplonemids are understudied heterotrophic flagellates that are common in the mesopelagic ocean (Flegontova *et al.*, 2016) and in deep sea sediments (Schoenle *et al.*, 2021). However, their ecological function, as bacterivores or parasites, is unknown (Flegontova *et al.*, 2016). Many better studied Euglenozoa appear to harbor bacterial endosymbionts. A sulfidic Euglenozoa has been shown to have nitrate reducing (S-oxidizing) bacterial endosymbionts (Edgcomb, Breglia, *et al.*, 2011) and other Euglenozoa have been found to have unidentified bacterial endosymbionts in low oxygen water (Simpson *et al.*, 1997; Bernhard *et al.*, 2000; Buck *et al.*, 2000; Leander and Farmer, 2000). Endosymbiotic bacteria are certainly an evolutionary adaption of protists to sulfidic waters (Bernhard *et al.*, 2000), and could also be an adaption to anoxic nitrate-containing waters. Further microscopic work is needed to identify if Euglenozoa in the ODZ have endosymbionts. Euglenozoa are potential predators functioning in the ODZ.

Amoebozoa are understudied due to the fact that they are destroyed by commonly used field collection techniques and are difficult to visualize microscopically once collected (Juhl and Anderson, 2014). Additionally, common primers for amplicon sequencing do not amplify Amoebozoa (Juhl and Anderson, 2014; Parris *et al.*, 2014; Duret *et al.*, 2015). Nevertheless, planktonic naked amoebas have been found to be important bacterivores in other aquatic systems (Murzov and Caron, 1996; Rogerson *et al.*, 2003; Lesen *et al.*, 2010). Planktonic amoebas are associated with particles (Caron *et al.*, 1982; Rogerson *et al.*, 2003; Anderson, 2011) probably

because the amoebas cannot feed properly in their suspended form (Pickup *et al.*, 2007). Thus amoebas are unlikely and potentially unable to feed on free-living bacteria (Pickup *et al.*, 2007). The Amoebozoa found in the ODZ are likely entering the ODZ on sinking particles. Amoebozoa, ciliates, and Cercozoa have almost identical normalized read profiles from metagenomic data (Figures 4 and 5). Ciliates and Cercozoa reads were abundant on deep sinking particles at HOT (Boeuf *et al.*, 2019). We hypothesize that all three of these groups are falling into the ODZ on particles and thus may not be active.

Comparison between amplicons and metagenomic reads

The use of phylogenetic placement of metagenomic reads has added to our understanding of marine protists. Amplicon sequencing has indicated that the diversity of protists is reduced under anoxic conditions (Orsi *et al.*, 2011, 2012; Jing *et al.*, 2015). Altogether, the read placement method indicated a general decrease in abundance of protists in ODZ, which could not be seen by traditional amplicon methods. Placement of metagenomic reads avoided primer bias and thus allowed us to examine groups missed from the long read amplicons, such as chaonoflagellates, marine fungi, Amoebozoa, Euglenozoa, and foraminifera. All these groups appear to be important in the offshore ETNP (Figure 4). Additionally, Acantherea and radiolarians did not seem to be abundant in the ODZ by metagenomic methods, indicating that primers may overly amplify some members of these groups. However, the primer set used here for long amplicons is not typically used by the scientific community. Instead short read amplicon primer sets have been developed. Short amplicons from the V4 18S rRNA region were recently published from HOT in August 2015 (Ollison *et al.*, 2021). We examine metagenomes from HOT in August 2015 and thus can directly compare (Figure 6). Profiles of protists amplified by the V4 primers and detected by metagenomic read placement are qualitatively similar for some groups. For example, the Stramenopile orders match between V4 amplicons and in the metagenomic profiles (Figure 6, S8) though our long read amplicons do a worse job for Stramenopile groups (Figure 2, 4). However, the short read amplicons miss Amoebozoa, Euglenozoa, and foraminifera (Ollison *et al.*, 2021), all important members of the protist community (Figure 6). While Euglenozoa and foraminifera feature prominently in all our ODZ datasets, they are missed or undercounted from published ODZ amplicon data (Parris *et al.*, 2014; Duret *et al.*, 2015; Jing *et al.*, 2015; De La Iglesia *et al.*, 2020). Both long read and short

read amplicon methods appear to under count Polycystinea radiolarians (Figure 2, 4, 6) (Ollison *et al.*, 2021). Upon closer examination the amplicon methods appear to specifically under count Spumellarida, which is the most abundant radiolarians in these systems (Figure 4, 6, S9, Table S2, (Ollison *et al.*, 2021)).

Ciliates

Ciliates are phagotrophic protists that are covered with cilia that allow them to move and to eat and that choose their prey selectively by size with the prey size range varying between ciliates (Fenchel, 1980). Though also present under oxic conditions, ciliates are a dominant protist taxon under sulfidic conditions (Orsi *et al.*, 2012) with the type of ciliate varying greatly between oxic and sulfidic conditions (Forster *et al.*, 2012). In sulfidic waters, instead of having mitochondria which create ATP utilizing oxygen, ciliates often have degenerative mitochondria such as hydrogenosomes, anaerobic organelles which use protons as an electron acceptor and produce hydrogen via fermentation and ATP from substrate level phosphorylation (Boxma *et al.*, 2005; De Graaf *et al.*, 2011). Anaerobic ciliates also can have methanogenic archaea endosymbionts that utilize the H_2 created by the hydrogenosome to produce methane and biomass and these methanogens in turn provide organic matter to the host (Fenchel and Finlay, 1991). Other anoxic (sulfidic) protists have similar organelles to hydrogenosomes (De Graaf *et al.*, 2011). However, fermentation and methanogenesis are processes favored under sulfidic or equally reducing conditions, not in nitrate replete waters.

Ciliates have not been found to be abundant in ODZs by amplicon sequencing (Parris *et al.*, 2014; Duret *et al.*, 2015; De La Iglesia *et al.*, 2020; Figure 2), and were not found to be abundant here by non-primer biased methods (Figures 4, 5). ODZs contain high concentrations of nitrate (Table S1; see (Fuchsman *et al.*, 2018; Peters *et al.*, 2018)) and are not sulfidic. Thus, functionally anoxic ODZs are at a different redox state than sulfidic basins. It would be more energetically favorable for protists living in an ODZ to utilize nitrate in a dissimilatory fashion than to utilize fermentative processes. In support of this, dissimilatory nitrate reduction is the dominant metabolism among the bacterial community in ODZs (Lam *et al.*, 2009; Fuchsman *et al.*, 2017). However, only one freshwater ciliate, *Loxodes*, has been found to utilize dissimilatory nitrate reduction (Finlay *et al.*, 1983). Thus, we hypothesize that the low numbers of ciliates in the ODZ has to do with the adaption of most anoxic ciliates to more reduced conditions.

Predation on ODZ Prochlorococcus and N_2 producing bacteria

A secondary chlorophyll maximum in the ODZ that is dominated by Low Light V *Prochlorococcus* is found at many stations in the ETNP and some stations in the ETSP (Lavin *et al.*, 2010; Garcia-Robledo *et al.*, 2017; Fuchsman *et al.*, 2019). Models indicate that a large reduction of predation in anoxic waters allows the creation of a secondary chlorophyll maximum in ODZs assuming nutrient availability (Zakem *et al.*, 2020) despite the low growth rates of *Prochlorococcus* at these depths and light levels (Vaulot *et al.*, 1995; Johnson *et al.*, 1999). The data available support this theory. In the coastal ETSP, cyanobacterial growth was 4x higher than grazing by heterotrophic nanoflagellates in putatively anoxic waters (Cuevas and Morales, 2006) and in general grazing was reduced from suboxic to anoxic conditions (Medina *et al.*, 2017). Reduced grazing could also allow N cycling bacteria in the ODZ to have higher abundance and perhaps higher N transformation rates. We saw that normalized reads for a large number of bacterivore predators, such as chaonoflagellates, radiolarians, ciliates are much reduced in anoxic waters (Figures 4 and 5). According to our phylogenetic read placement analysis, groups known to include bacterivore predators that were found in the ODZ include dinoflagellates, Euglenozoa, and foraminifera. Each of these potential predators has unknowns associated with them. Dinoflagellates can have thousands of copies of the 18S rRNA gene, thus enumerating them with 18S rRNA is not possible (Galluzzi *et al.*, 2010). Dinoflagellates also can form resting cysts under low oxygen conditions that could sink into anoxic water (Jing *et al.*, 2015; Bringué *et al.*, 2018; More *et al.*, 2018). Thus, the numbers of active dinoflagellates in the ODZ cannot be determined by these methods. Euglenzoa of the diplonemid group remain likely bacterivore predators in the ODZ. Other Euglenzoa have even been shown to have nitrate reducing endosymbionts (Edgcomb, Breglia, *et al.*, 2011). However, Flegontova et al (2016) point out that we do not know if marine Diplonemids are parasites or bacterivores. Foraminifera likely can use denitrification to prey on bacteria in the ODZ (Davis *et al.*, 2021). Thus, the potential predators of ODZ bacteria offshore have been identified but questions remain.

An alternative source of death for the ODZ *Prochlorococcus* and N_2 producing bacteria is viruses. Abundant cyanophage have been found in cellular and particulate fractions in the ETNP ODZ (Fuchsman *et al.*, 2019, 2021). Additionally, viruses infecting N cycling bacteria in the ODZ have also been identified in the ETSP ODZ (Gazitúa *et al.*, 2021). Grazing causes the organic carbon to move up the food chain, while viral lysis makes the cell's organic carbon

available to heterotrophic bacteria (Fuhrman, 1999). As *Prochlorococcus* is an important source of organic C to the upper ODZ (Fuchsman *et al.*, 2019), its mode of death can have a profound effect on biogeochemical cycling. To further understand if dinoflagellates, foraminifera and diplonemid euglenozoa are preying on ODZ *Prochlorococcus* and N cycling bacteria in the ODZ, we believe that visual examination by microscopy and grazing studies that quantify ingested prey are necessary.

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Figure Captions

Figure 1. Setting the scene. A) Oxygen and chlorophyll a profiles for ETNP St 136 in April 2012. B) ETNP St 136 nitrite concentrations and normalized metagenomics reads for genes for denitrification (nitrous oxide reductase; *nosZ*) and anammox (hydrazine oxidoreductase; *hzo*). C) Oxygen and chlorophyll a profiles for ETSP St 9 in July 2013. D) ETSP St 9 nitrite concentrations and normalized metagenomics reads for genes for denitrification and anammox. E) Oxygen and chlorophyll a profiles for ETSP St 17 in July 2013, and F) ETSP St 17 nitrite concentrations and genes for denitrification and anammox. Dashed lined indicate the top and bottom of the ODZ.

Figure 2. 18S rRNA long read amplicons from ETNP St 161. Depths are labeled by Hypoxic (<60 µM O_2) or ODZ

Figure 3. Protist 18S rRNA bootstrapped phylogenetic tree. Colored bars indicate the labeled group. Stars indicate ETNP long read amplicon OTUs. Bootstrap strength are indicated by branch color where red = >75. An expanded version of the tree can be seen in Supplemental Figure 4.

Figure 4. 18S rRNA normalized metagenomic read depth profiles from ETNP St 136 for A) protists found in oxic waters, B) protists found in oxic and anoxic waters, C) algae, and D) protist parasites. Depth profiles at the level of order for E) Radiolarians, F) Euglenozoa, G) Stramenopiles, and H) Foraminifera. Globigerinacea is an order in the class Globothalamea and thus is a subset of Globothalamea. The dashed line indicates the top of the ODZ. Radiolarians and Acantharea have a separate horizontal axis in panel A, and Spumellarida have a separate horizontal axis in panel E.

Figure 5. 18S rRNA normalized metagenomic read depth profiles from ETSP stations 17 (A-D) and 9 (E-H) for A&E) protists found in oxic waters, B&F) protists found in oxic and anoxic waters, C&G) algae, and D&H) protist parasites. The dashed line indicates the top and bottom of the ODZ. Radiolarians and Acantharea have a separate horizontal axis in panels A and E.

Figure 6. 18S rRNA normalized metagenomic read depth profiles from HOT 275 in August 2015 for A) protists found in oxic waters, B) protists found in oxic and anoxic waters, C) algae, and D)

protist parasites. Depth profiles at the level of order for E) Radiolarians, F) Euglenozoa, G) Stramenopiles, and H) Foraminifera. Globigerinacea is an order in the class Globothalamea and thus is a subset of Globothalamea. Radiolarians and Acantharea have a separate horizontal axis in panel A.

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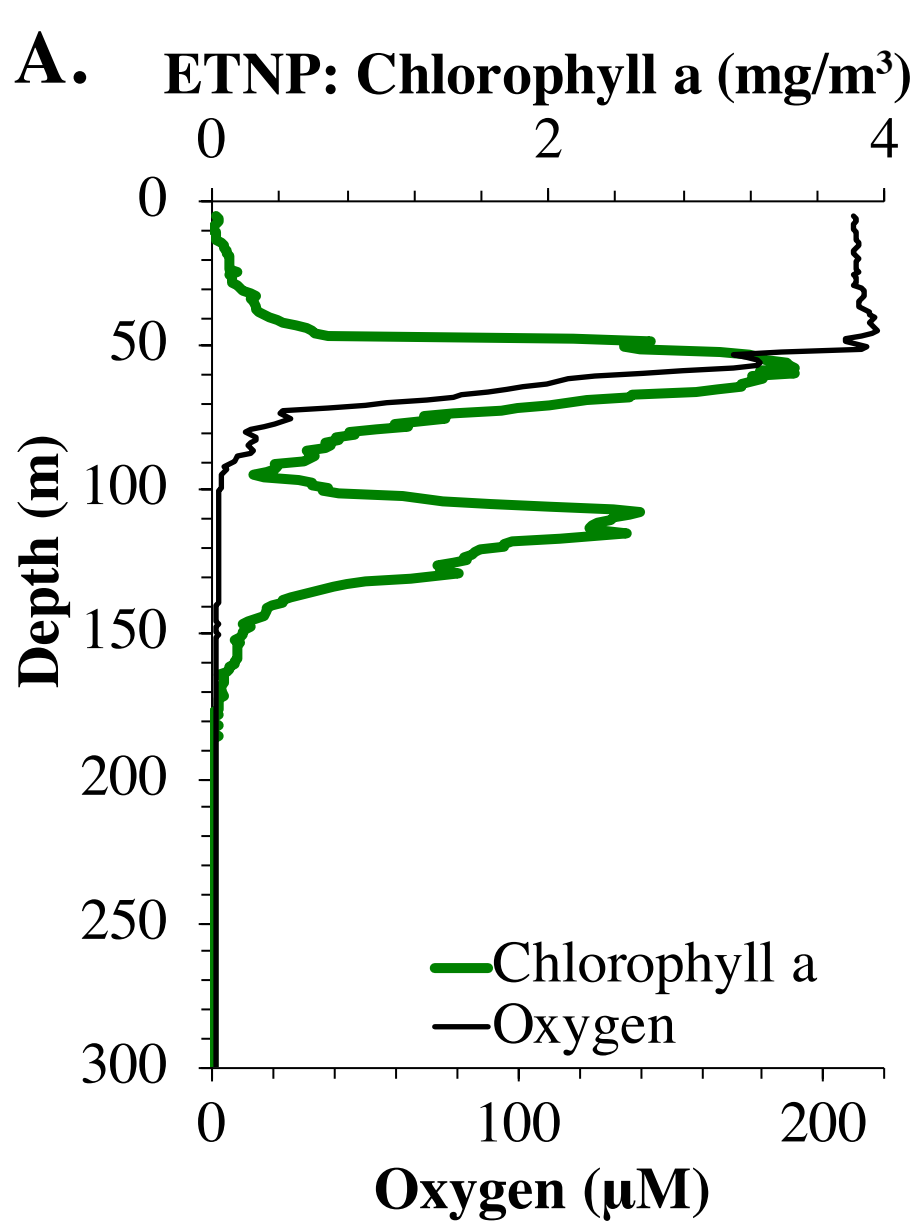

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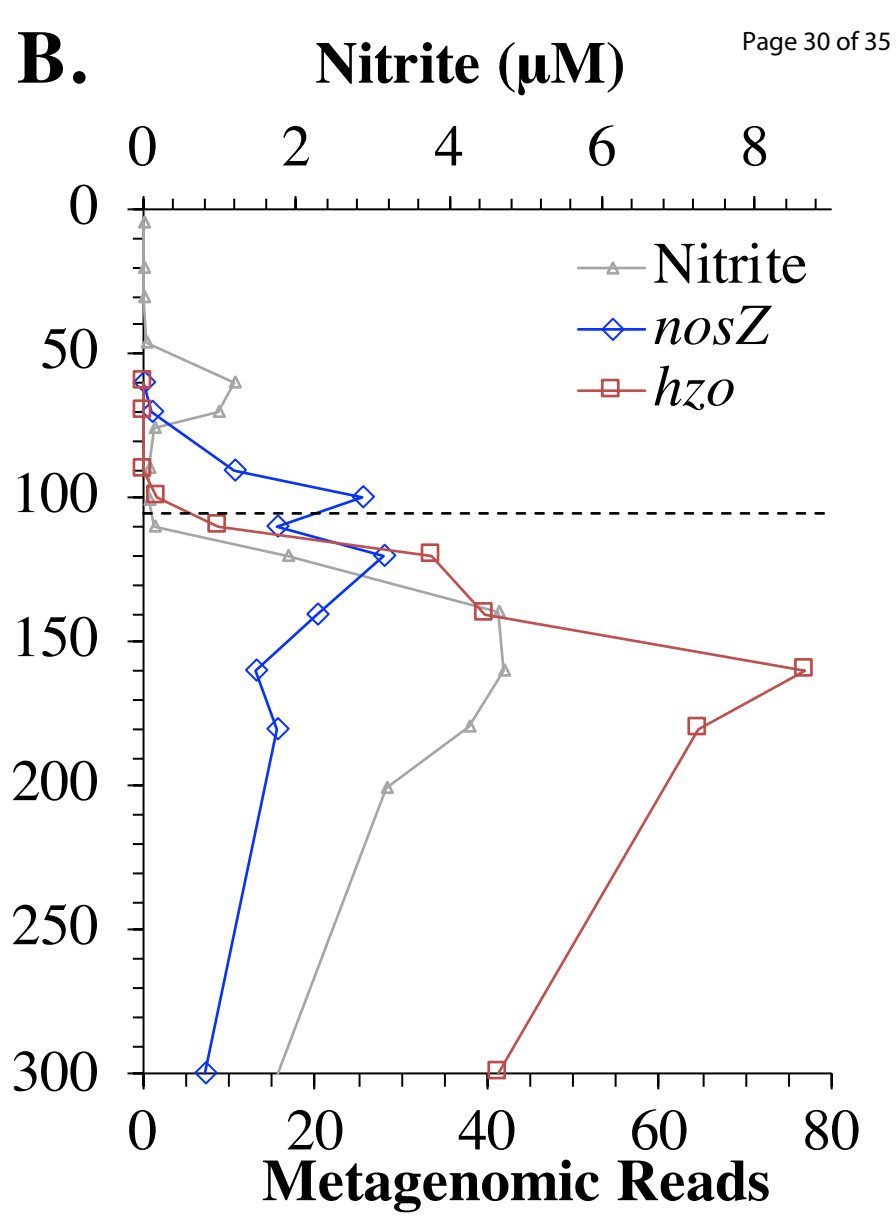

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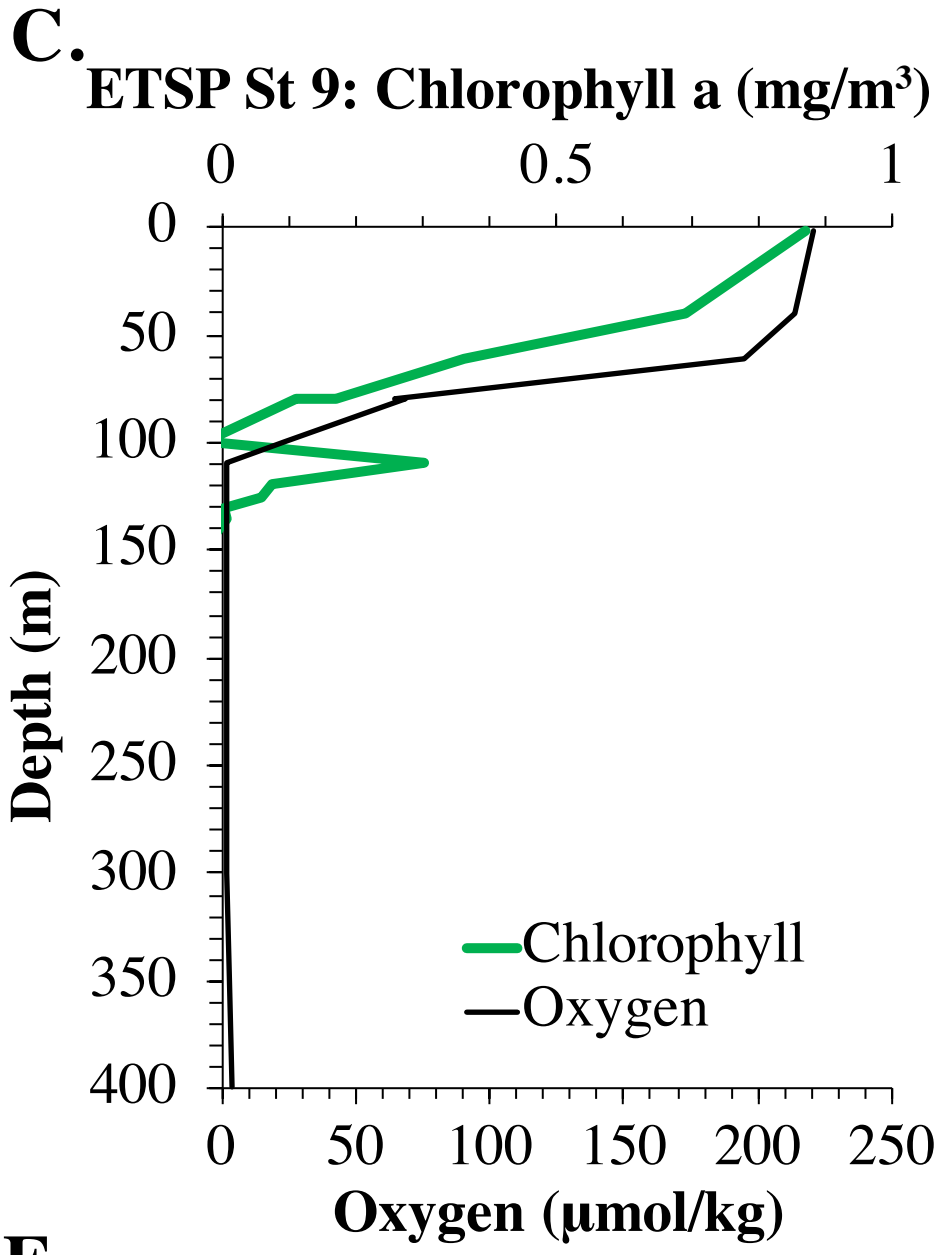

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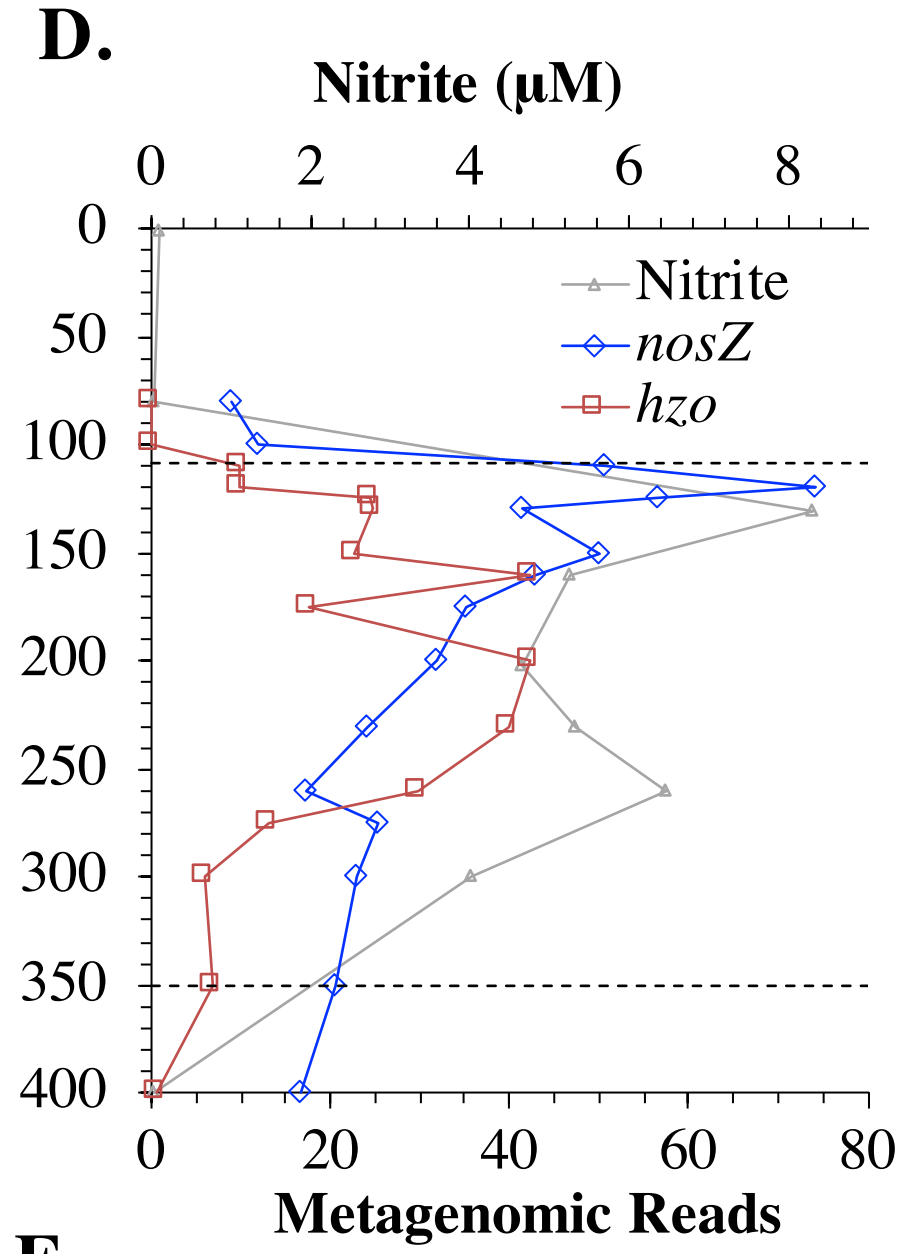

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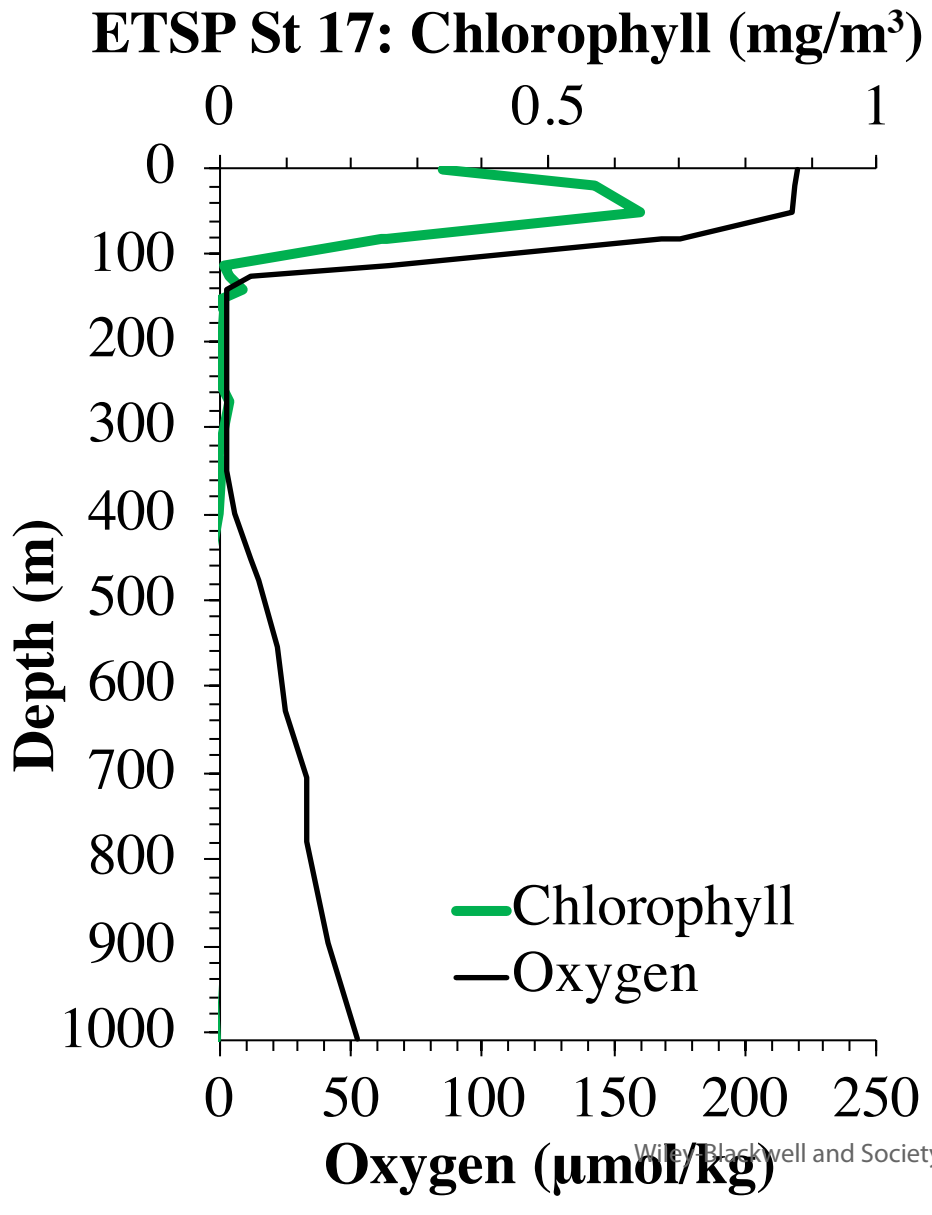

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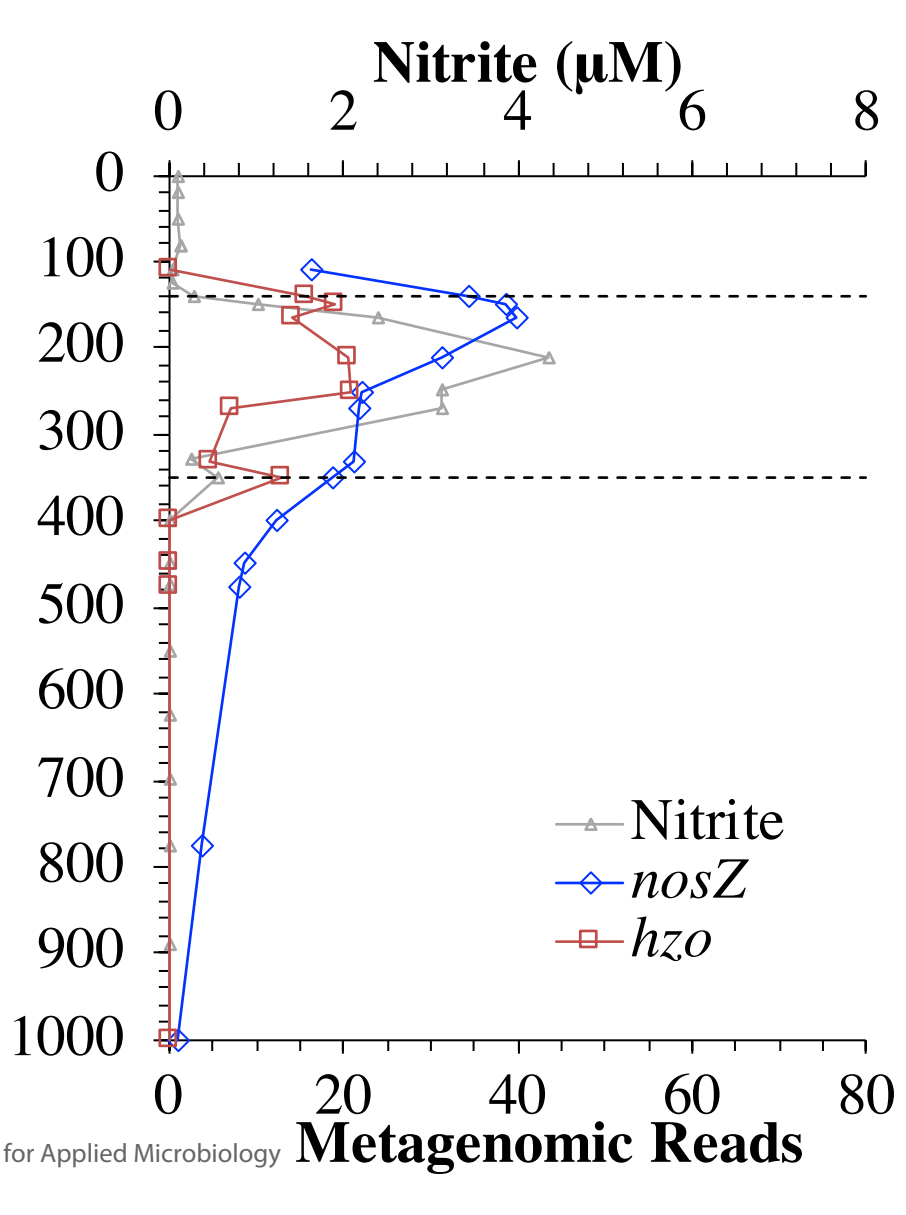

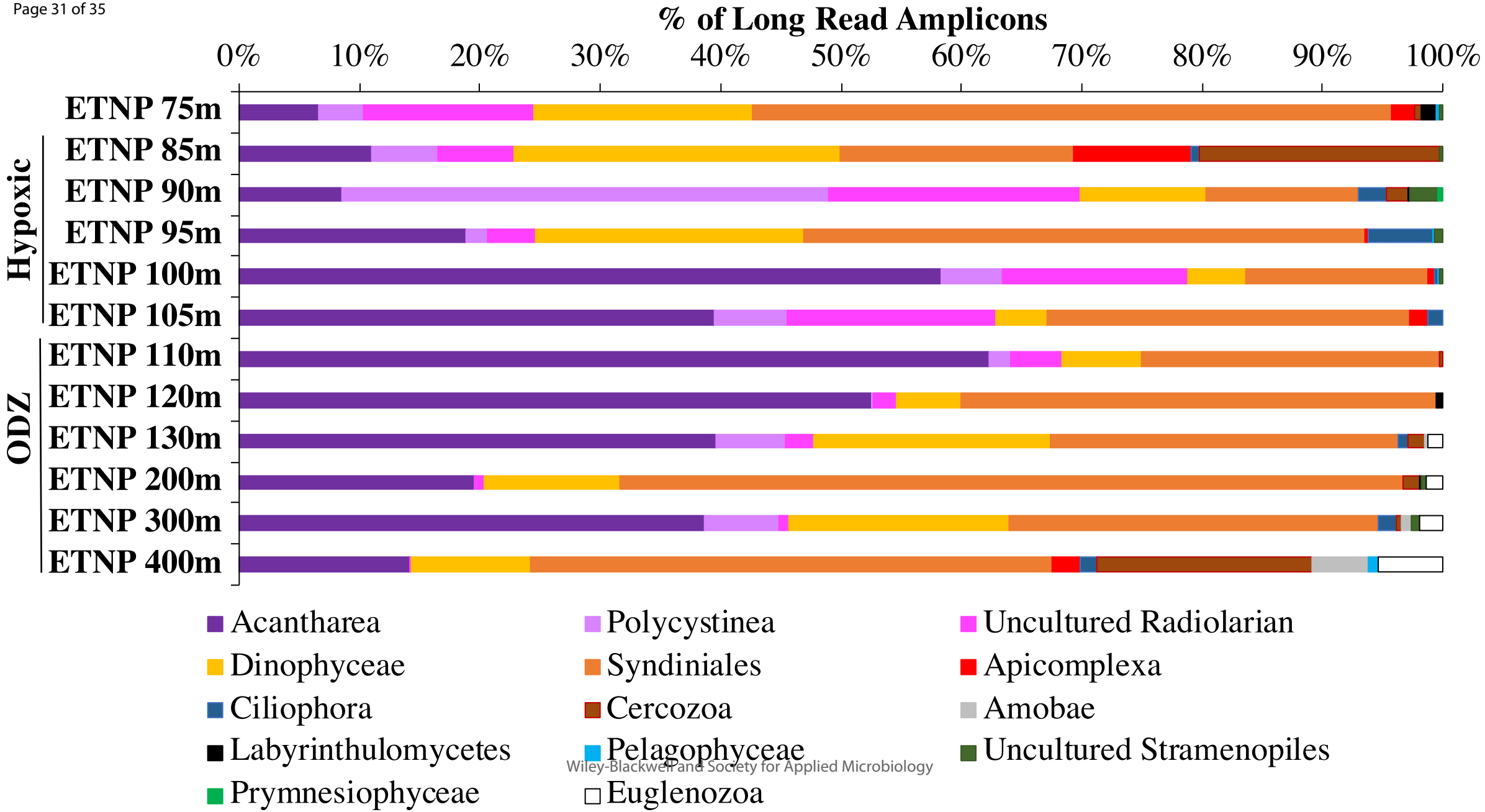

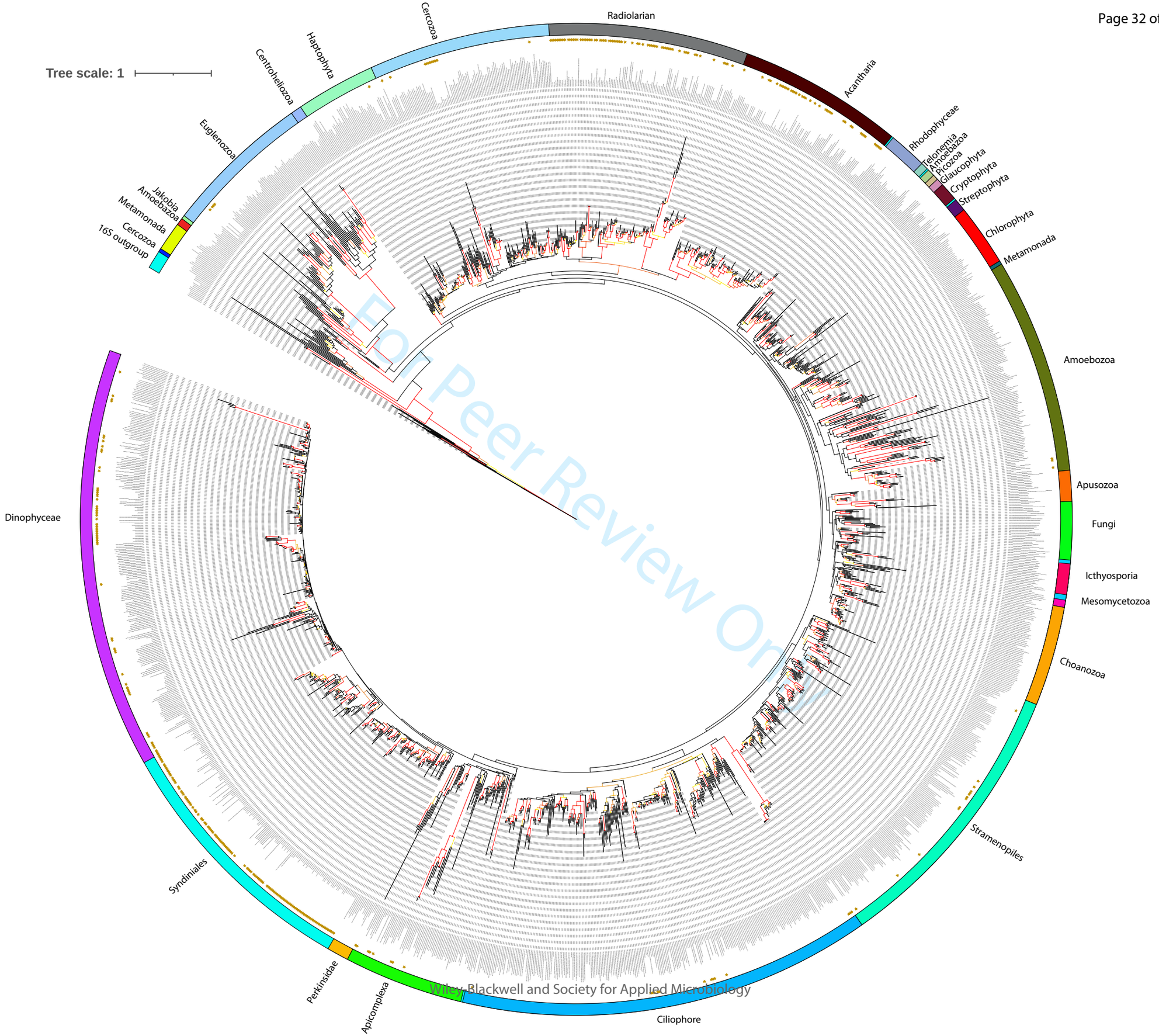

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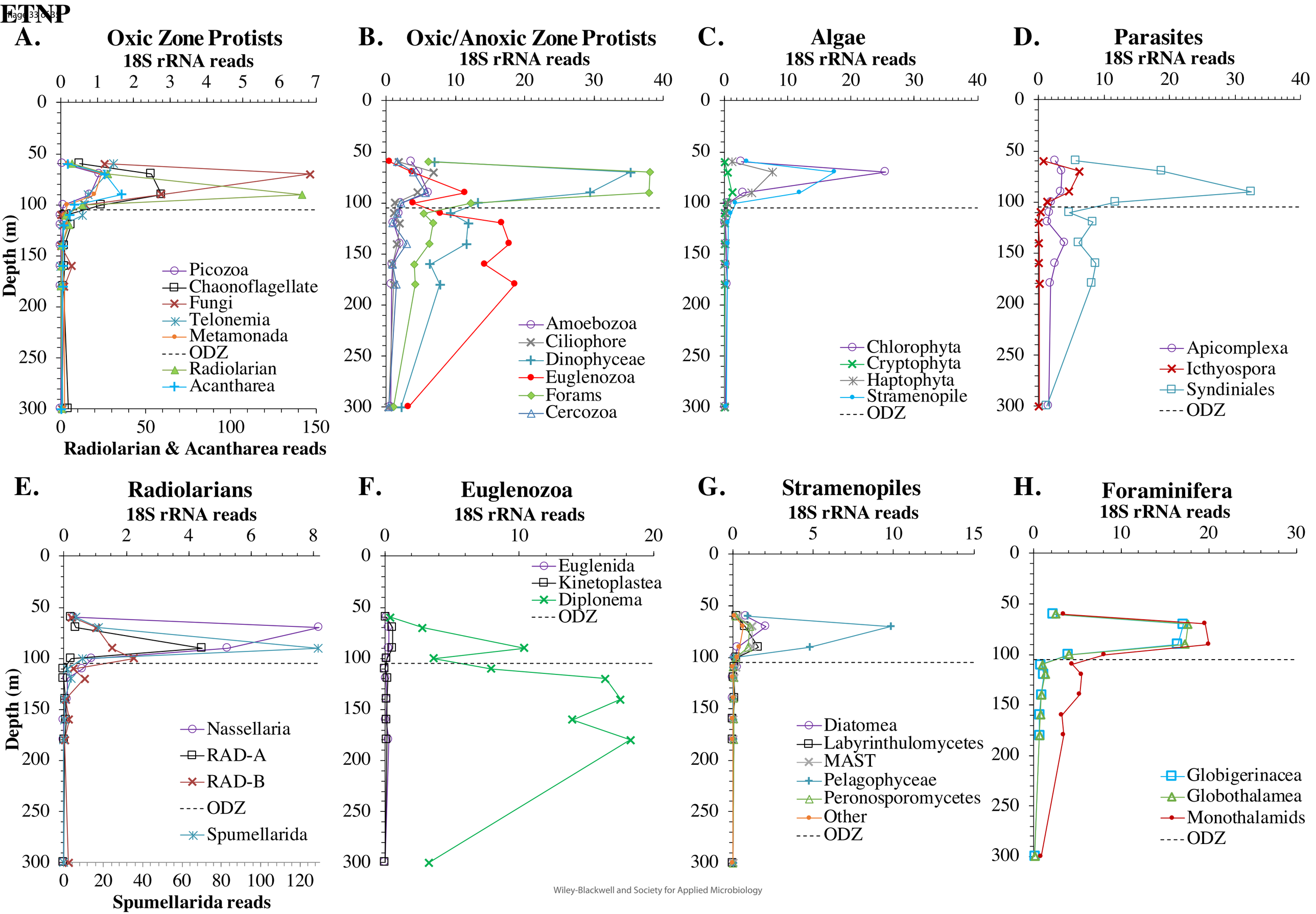

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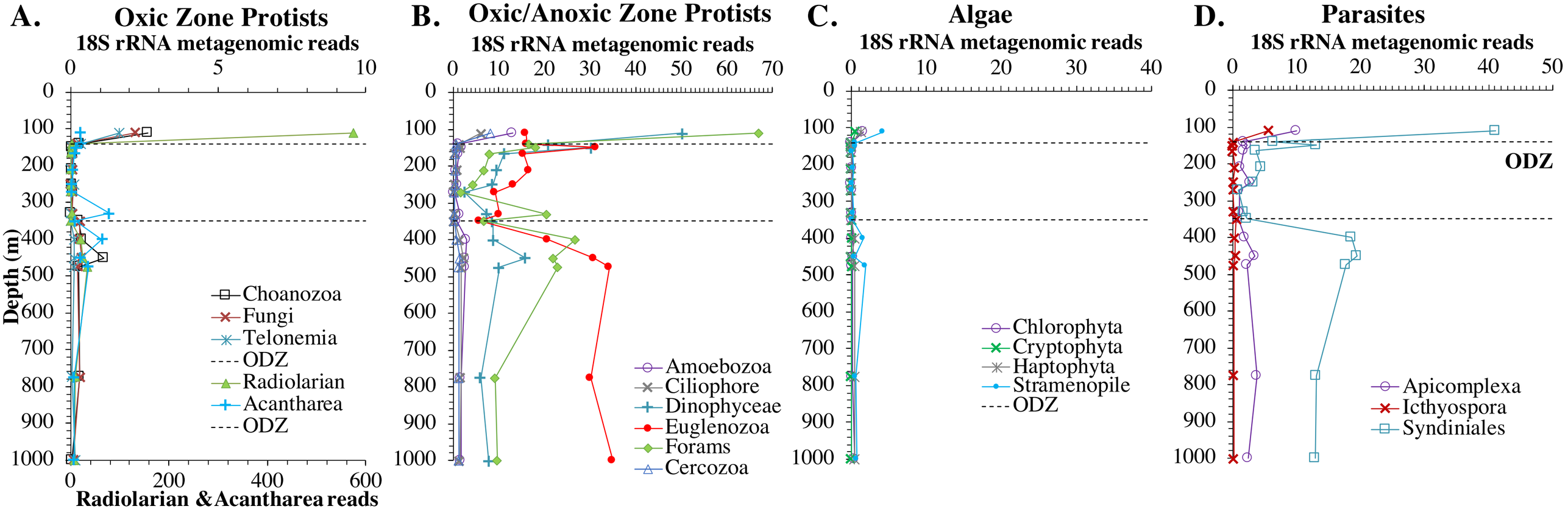

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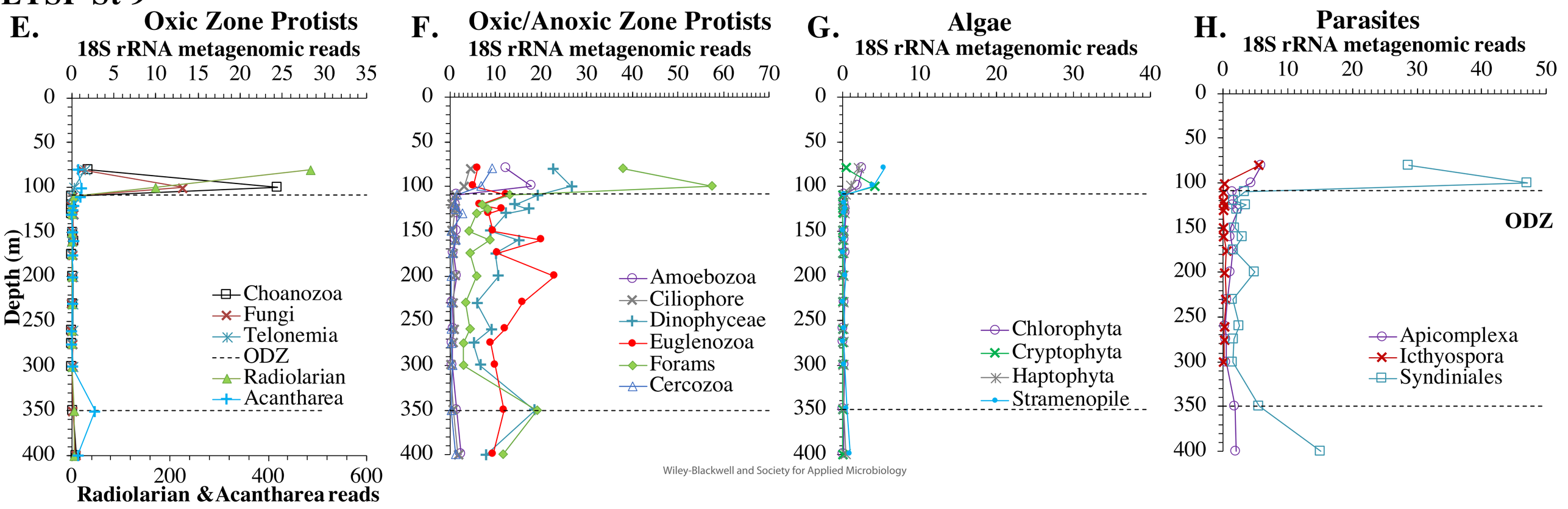

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