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Eukaryotic microbes, principally fungi and labyrinthulomycetes, dominate biomass on bathypelagic marine snow

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In the bathypelagic realm of the ocean, the role of marine snow as a carbon and energy source for the deep-sea biota and as a potential hotspot of microbial diversity and activity has not received adequate attention. Here, we collected bathypelagic marine snow by gentle gravity filtration of sea water onto 30 μm filters from ~1000 to 3900 m to investigate the relative distribution of eukaryotic microbes. Compared with sediment traps that select for fast-sinking particles, this method collects particles unbiased by settling velocity. While prokaryotes numerically exceeded eukaryotes on marine snow, eukaryotic microbes belonging to two very distant branches of the eukaryote tree, the fungi and the labyrinthulomycetes, dominated overall biomass. Being tolerant to cold temperature and high hydrostatic pressure, these saprotrophic organisms have the potential to significantly contribute to the degradation of organic matter in the deep sea. Our results demonstrate that the community composition on bathypelagic marine snow differs greatly from that in the ambient water leading to wide ecological niche separation between the two environments.

Introduction

Deep-sea life is greatly dependent on the particulate organic matter (POM) flux from the euphotic layer. Thus, the quantification of the POM flux into the ocean's interior and the attenuation of POM with depth in the oceanic water column have been major focal points in oceanographic research in large international research programs (for example, Martin *et al.*, 1987; Koeve and Ducklow, 2001; Buesseler and Boyd, 2009). The POM flux in the oceanic water column is typically estimated by collecting particles with sediment traps (Buesseler *et al.*, 2007; Honjo *et al.,* 2008). However, these sediment traps collect fast-sinking particles with much higher efficiency than slowly-sinking particles, while buoyant particles are not collected at all. Recently, we demonstrated that bathypelagic particles in a size range from 400 μm to several mm were more abundant than expected from the number spectrum of smaller particles (Bochdansky *et al.*, 2016). Approximately 30% of these particles did not contain apparent ballast, such as fecal pellets

or dense phytodetritus, but a large amount of transparent exopolymer particles (TEP, Alldredge *et al.*, 1993), which led us to conclude that they are either neutrally buoyant or slow sinking (Bochdansky *et al.*, 2016). Neutrally buoyant or slowly-sinking marine snow has previously been suspected to contribute significantly to the metabolism of the deep sea, providing hotspots of microbial diversity and activity (Baltar *et al.*, 2010; Bochdansky *et al.*, 2010; Burd *et al.*, 2010). Hence, it is likely that the microbial community composition and activity on bathypelagic marine snow is fundamentally different from that of the ambient water. Deep-sea studies examining differences between particle-associated and freely-suspended microbes so far have primarily been based on high-throughput sequencing of the 16S and 18S rRNA genes. For instance, bacterial, archaeal and eukaryotic microbial communities were found to be significantly different between 0.22 and 3 μm fractions in samples from the Puerto Rico trench (6000 m) (Eloe *et al.*, 2011). The examination of bathypelagic bacterioplankton communities revealed deep phylogenetic differences between particulate fractions (>0.8–20 μm) and free-living cells (0.2–0.8 μm) (Salazar *et al.*, 2015). Microbial communities of all three domains also differed between sea water samples and sediment trap (particle interceptor traps) contents (Fontanez *et al.*, 2015). In a study employing stable isotope probing, specific particle-associated clades were

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identified that specialize in the degradation of high-molecular weight compounds (Orsi *et al.*, 2016). Recently, Pernice *et al.* (2016) described a highly variable eukaryotic microbial community composition that was affected by depth, water masses, as well as the number of prokaryotes. It is thus possible that the relative amount and type of marine snow significantly contributes to this observed variability.

In the present study, we addressed the question whether the particular conditions in bathypelagic marine snow, representing oases in the nutritive desert of the bathypelagic realm, lead to altered microbial food web structures on bathypelagic marine snow as compared with the ambient water. We also hypothesized that the abundance relation between prokaryotes and eukaryotes in deep-sea marine snow more closely resembles the one in surface waters rather than the organic nutrient-deprived deep-sea ambient waters. To this end, we examined the relative distribution of eukaryotic microbes in general, as well as three specific subgroups—the kinetoplastids, fungi and labyrinthulomycetes—in bathypelagic marine snow collected on 30 μm pore-size filters. The pore size of the filters was selected to filter large volumes of water and to target amorphous marine snow. The rationale for the selection of the three subgroups was as follows. Fungi have been identified in all marine environments including the deep sea, hydrothermal vents, sea ice and in the deep biosphere below the sea floor (for example, Kutty and Philip, 2009; Le Calvez *et al.*, 2009; Edgcomb *et al.*, 2011a; Morgan-Smith *et al.*, 2013; Ciobanu *et al.*, 2014; Rédou *et al.*, 2015; Richards *et al.*, 2015; Hassett and Gradinger, 2016; Tisthammer *et al.*, 2016). While fungi are thought to have a relatively small role in pelagic environments (Newell, 1994; Kirchman, 2008), some reports have shown episodically higher numbers in the water column (Gutiérrez *et al.*, 2011). Of the labyrinthulomycetes, thraustochytrids are often associated with dead phytoplankton debris at the end of a bloom (reviewed in Raghukumar, 2002) where they can be episodically found in such high numbers that they match bacterial biomass (Damare and Raghukumar, 2008). For both groups, marine snow represents a potential nutrient-rich substrate that would suit their osmotrophic lifestyle. The third group selected for this study, the kinetoplastids, are heterotrophic flagellates that have consistently been found in deep-sea samples (e.g., Turley *et al.*, 1988; Arndt *et al.*, 2003; Morgan-Smith *et al.*, 2013). In addition, their non-parasitic forms are voracious bacterivores that are often associate with particles (Artolozaga *et al.*, 2000; Boenigk and Arndt, 2002), which makes them prime candidates to populate deep-sea marine snow.

Materials and methods

Sample collection
Sea water was collected during the MEDEA-2 research expedition to the North Atlantic and Arctic Basin between 22 June and 22 July 2012 on the RV *Pelagia* (Royal Netherlands Institute of Sea Research, Figure 1). At the beginning of the cruise, all Niskin bottles were filled with a 5% bleach solution overnight to remove microbes that may have grown during storage. Collection depths were chosen to target specific bathypelagic water masses (Supplementary Table S1,van Aken, 2000). Immediately after returning on deck, one 25 L Niskin bottle was removed from the rosette assembly and set aside for gentle gravity filtration. A filter cartridge was connected to the spout and a flow restrictor was put in-line after the cartridge (Supplementary Figure S1). The cartridge was loaded with a 25 mm diameter, 30 μm pore-size polycarbonate filter membrane (type PCT30025100, Sterilitech Corp., Kent, WA, USA). The flow was set to a maximum rate of 100 ml min^{-1}. Although marine snow is defined as > 500 μm in size (Alldredge and Silver, 1988), pores or meshes of that size are ineffective because these particles are too fragile to be retained. In contrast, filtration on smaller pore sizes such as 0.2 or 0.8 μm filters is limited to much smaller volumes (~100 ml and ~ 1L on 25 mm diameter filters, respectively) due to the clogging of the filter pores by TEP and finely suspended material from the ambient water. Approximately 15–21 L were filtered in this manner (see Supplementary Table S1 for volume filtered at each station), and upon inspection, all filters had a visible layer of material. While still in the cartridge, the collected material was preserved on the filter by connecting a syringe containing a 2% (final concentration) formaldehyde solution in 0.2 μm filtered deep-sea water. The filter was gently rinsed with sterile 1 × phosphate-buffered saline and ultrapure water by attaching another set of syringes to the cartridge and by gently moving the water in the
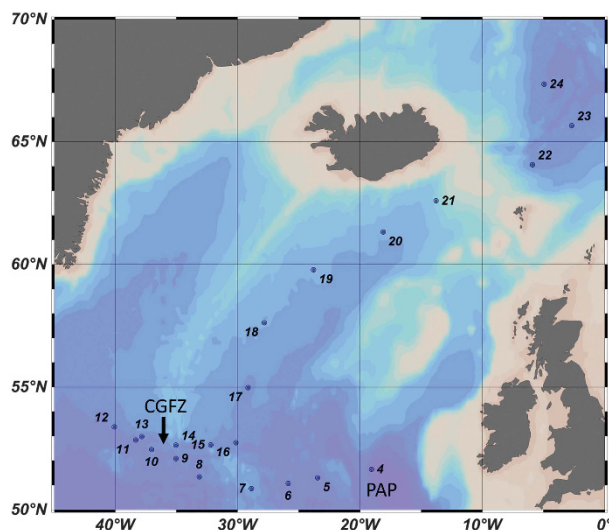

Figure 1 Map of stations at which deep-sea samples of marine snow were taken. Depths are shown in Supplementary Table 1. CGFZ, Charlie Gibbs Fracture Zone; PAP, Porcupine Abyssal Plane.

same direction as the original flow. Water samples (200–250 ml each) from the same depths and locations were preserved in 2% formaldehyde (~30 min) and subsequently filtered through 25 mm 0.2 μm pore-size polycarbonate filters (Millipore, Billerica, MA, USA) using a standard filtration manifold. Filters were also rinsed with 1× phosphate-buffered saline and ultrapure water before being stored at − 80 °C. Samples were returned to Old Dominion University on dry ice where they were stored at − 80 °C until further processing.

Pretreatment of filters with agarose and EDTA
Initial microscopic observation revealed that no microbes could be visualized using 4',6-diamidino-2-phenylindole (DAPI) despite a visible layer of material on the 30 μm pore-size filters. This was due to a thick matrix of TEP which we were able to render permeable by pretreating the filter with a 25 mM EDTA solution for 15 min (Cavaliere *et al.*, 2014). Tests were performed with shorter and longer time intervals (5 min to 2 days), with 15 min being optimal. EDTA dissolves the TEP by scavenging the cationic bridges of mainly Ca^{2+} between neighboring TEP chains (Cavaliere *et al.*, 2014; Bar-Zeev *et al.*, 2015). To minimize losses from the filter surface, we covered the filter with agarose according to the catalyzed reporter deposition fluorescence *in situ* hybridization (CARD-FISH) procedure (Teira *et al.*, 2004; Morgan-Smith *et al.*, 2013) before the EDTA treatment. Agarose forms a very porous molecular mesh that works very well to hold organisms in place but allows EDTA and probes to penetrate. Tests indicated that agarose coating did not negatively affect EDTA permeabilization.

Tyramide signal amplification CARD-FISH
CARD-FISH was performed to examine the composition of eukaryotic microbes on both the 30 μm and 0.2 μm filters. As we found a mismatch of the kinetoplastids with the universal eukaryote probe EUK516 (Bochdansky and Huang, 2010; Mukherjee *et al.*, 2015), and some diplonemids displayed a second mismatch (for example, Genbank accession #KF633466.1 and #JN542573.1), we designed a new probe (Diplo516) and used the combination of three probes (EKD: EUK516 +KIN516+Diplo516, Supplementary Table S2) to be as inclusive as possible. We evaluated this probe combination with a series of stringency tests on deep-sea samples from the same cruise (5 stations) using the protocol of Hugenholtz *et al.* (2001) and arrived at an optimal stringency of 40% formamide concentration (see probe evaluation details in the Supplementary Information). This group hybridizing with the EKD-probe combination will be referred to as 'eukaryotes'. The probe sequences and optimal formamide stringencies used in this study for kinetoplastids (KIN516), fungi (PF2)

and labyrinthulomycetes (LabY) are also given in Supplementary Table S2. After the initial agarose coating and EDTA treatment, we followed the CARD-FISH protocol using AlexaFluor 488 detailed in Morgan-Smith *et al.* (2013). Additional permeabilization as previously applied for fungi and the regular FISH protocol is not necessary when using CARD-FISH (Jobard *et al.*, 2010; Morgan-Smith *et al.*, 2013). We achieved 100% success rate of CARD-FISH in detecting fungal cells without permeabilization in tests using bread yeast and a chytrid culture. All treatments were counterstained with DAPI in an antifadent mountant (Vectashield with DAPI, Vector Laboratories, Burlingame, CA, USA). Signals were only considered positive (regarded as an eukaryotic cell) if the green hybridization signal was also co-located with a clearly identifiable DAPI-stained nucleus. Not all probes could be used on all filters, due to a shortage of filter slices after performing formamide, EDTA and agarose tests (see above). For every filter slice, prokaryotes were also enumerated using DAPI and these more numerous and easily identifiable cells served as benchmark values to normalize eukaryotic microbe numbers for each filter slice.

Enumeration and dispersion diagnostics
Two sets of counts were performed on every filter slice (30 and 0.2 μm). The first set consisted of counting both prokaryotes and eukaryotic microbes at the same time and in the same plane to obtain information on spatial heterogeneity on the filter surfaces. This was done by counting up to 150 entire microscopic fields at the highest magnification (Olympus BX51 epifluorescence microscope (Center Valley, PA, USA), U-LH100HG APO mercury burner, 100 × UPlanSApo objective lens and × 20 ocular magnification). This approach was used to test the hypothesis that organisms that landed on the 30 μm filter were not solely random intercepts on the filter membrane but due to the presence of particles. In that case, the dispersion index would be much higher than expected from a random distribution. The distribution of organisms on the 0.2 μm filter, in contrast, should have a distribution of organisms on the filter surface closer to random. In other words, the patchy arrangement of microbes on the filter surface is an indicator that particles were captured even if the exact outlines of these particles are not readily detectable. As a dispersion index, we chose the Lloyd index of patchiness (Lloyd, 1967), which has a good dynamic range at highly overdispersed (patchy) distributions (Bochdansky and Bollens, 2004). The Lloyd index of patchiness (Lp) is the ratio of the Lloyd index of mean crowding ($\dot{m}$) divided by the mean (m), or

$$Lp = \frac{\dot{m}}{m} = \left[m + \left(\frac{\sigma^2}{m} - 1 \right) \right] m^{-1}$$

where σ^2 is the variance (Lloyd, 1967). An Lp of 1 means that microbes are randomly distributed on the filter, above 1 they are overdispersed.

A second set of counts was performed at lower (×10 ocular) magnification to enumerate all protists found on the entire filter slice (up to 300 entire microscopic fields). This improved the enumeration of less represented groups. Length and average width of a subsample of fungi and the labyrinthulomycetes were measured (this included single cells, thalli and sporangia, but did not include any hyphen that permeated the particle matrix, Jobard *et al.*, 2010). Volume calculations were based on prolate spheroid geometry ($\pi/6 \times$ width$^2 \times$ length) for eukaryotic microbes (Pernice *et al.*, 2015), and on the equation ($\pi/4 \times$ width$^2 \times$ (length – (width/3)) for cocci and bacilli (Bratbak, 1985). Estimates of eukaryotic microbial biomass are minimum estimates of total biomass especially for organisms that may have significant biomass allocated in hyphen structures. Non-parametric tests were used *in lieu* of Student's *t*-tests because residuals were not normally distributed, or sample size was too small to test for normality of the residuals.

Results

Prokaryote counts on 30 μm filters were highly variable among different microscopic fields, and were significantly more patchy than those observed on 0.2 μm filters. The Lp was on average 5.22 (s.d. = 4.33, $n = 61$) on the 30 μm filters, and on average 1.39 (s.d. = 0.27, $n = 59$) on 0.2 μm filters with the means being significantly different from each other (Wilcoxon signed-rank test, $n = 58$, z-score = 6.62, $P < 0.0001$). This means that while prokaryotes were only 1.4 times more overdispersed than expected from a random distribution on 0.2 μm filters, prokaryotes were 5 times more overdispersed on 30 μm filters. The standard deviation of prokaryote numbers for individual fields was also much higher on the 30 μm filters (83% of mean) than on the 0.2 μm filters (19% of the mean). This demonstrates that microbes that landed on the 30 μm filters were not random intercepts with the filter surface but that parts of aggregates were responsible for the patchy distribution. Figure 2 shows an example of a large particle with embedded microbes under DAPI as found on a 30 μm filter; particle boundaries were not always as clear as in that particular case however. This image also demonstrates the typically large amount of transparent exopolymer matrix that holds the particle and microbes together (Figure 2).

Examples of eukaryotic microbes with positive hybridization signals from CARD-FISH are displayed in Figure 3. The ratios between eukaryotic and prokaryotic microbes (normalized eukaryote numbers, NEN) are shown in Figure 4. The differences in NEN between the 0.2 μm and the 30 μm filters were highly significant for the eukaryotes (Wilcoxon
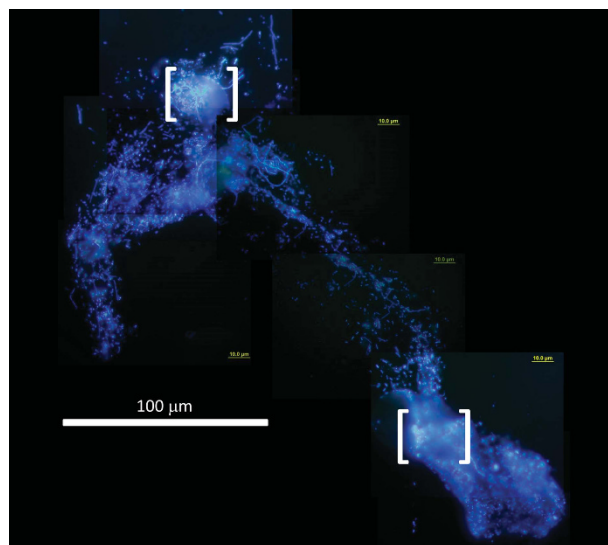

Figure 2 Epifluorescence image mosaic of a bathypelagic particle from 3390 m collected at the Charlie Gibbs Fracture Zone and retained by a 30 μm pore-size filter, pretreated with 25 mM EDTA, and stained with DAPI. Note: hazy areas [brackets] are not large objects but represent backscatter from the edges of the 30 μm filter pores.

signed-rank test, z-score = 3.92, $P = 4.5 \times 10^{-5}$), the labyrinthulomycetes (Wilcoxon signed-rank test, z-score = 3.46, $P = 2.7 \times 10^{-4}$) and the fungi (Wilcoxon signed-rank test, z-score = 3.48, $P = 2.5 \times 10^{-4}$). Fewer samples were available for kinetoplastids but the difference was still significant between NEN on the 0.2 and the 30 μm filters (Mann–Whitney *U*-test, $U = 16$, $P = 0.0286$). Fungi and labyrinthulomycetes accounted for ~ 1/5 each of all eukaryotic microbes on particles (30 μm filters) but for a much lesser extent on the 0.2 μm filter samples (Figure 4). The relative enrichment ratios on particles (that is, the enrichment of eukaryotic microbes in excess of the enrichment of prokaryotes, calculated as the ratio between NEN on the 30 μm filters and NEN on the 0.2 μm filters) were 78, 25, 42 and 141 times that of prokaryotes for total eukaryotes, kinetoplastids, labyrinthulomycetes and fungi, respectively (Figure 4).

The volumes of fungi and labyrinthulomycetes were on average 115 μm^3 (s.d. = 203, $n = 16$) and 106 μm^3 (s.d. = 125, $n = 22$), respectively, and thus much larger than those of prokaryotes (cocci: 0.061 μm^3, s.d. = 0.073, $n = 90$; bacilli: 0.079 μm^3, s.d. = 0.089, $n = 88$) in the same samples. For fungi and labyrinthulomycetes, the volumes were measured for each occurrence, which may include more than one cell per occurrence (for example, in a sporangium or thallus).

Discussion

Methodological considerations
The use of 30 μm pore-size filters in combination with gentle gravity filtration was a suitable
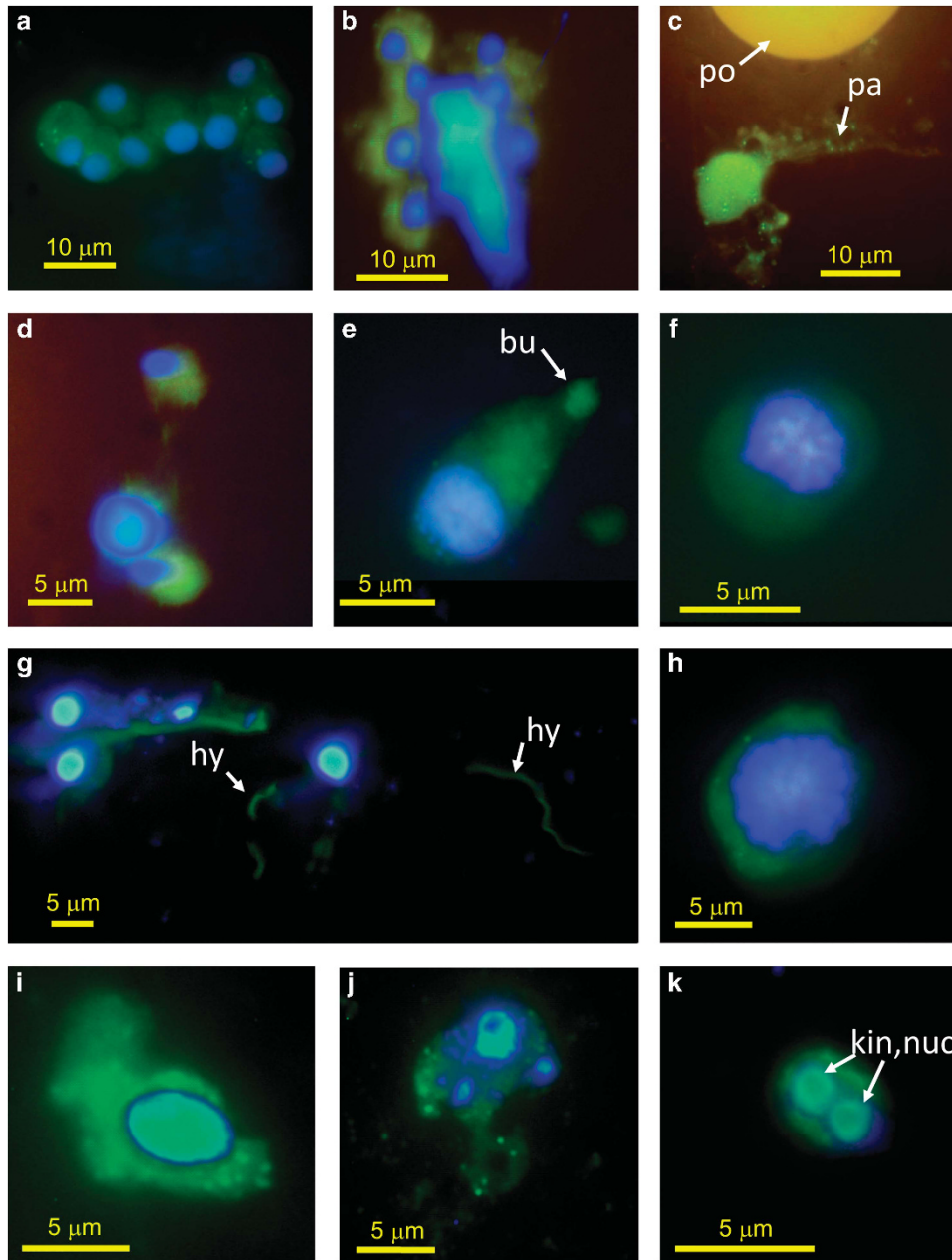
Figure 3 Examples of protists hybridized with four different probes. Images are overlays of the CARD-FISH probes (green) and DAPI counterstain (blue). (**a–d**) Organisms hybridizing with the LabY probe for labyrinthulomycetes; (**c**) green channel only, po = filter pore, pa = particle fragment. (**e–h**) Organisms hybridizing with the broad fungal probe PF2. (**e**) bu = cell shape is similar to a yeast cell forming a bud; (**g**) hy = structures that appear to be fungal hyphae similar to those shown in Jobard *et al.* (2010). As only ribosomes embedded in cytoplasma (and not chitin cell walls) light up in CARD-FISH, hyphae may appear thinner than when chitin stains are used. (**i** and **j**) Organisms hybridizing with the universal eukaryote probe combination to include kinetoplastids and diplonemids (probe: EKD). (**k**) Kinetoplastid showing the kinetoplast (kin), a large mitochondrion and the nucleus (nuc) side by side (probe: KIN516).

compromise to sample marine snow aggregates from large volumes of water, because the large filter pores let single organisms pass, but retained some marine snow. However, these aggregates are too fragile to be collected quantitatively with this method as they can easily fragment even under the gentlest collection and fixation protocols on filters. A better method is to visually locate these particles in the water column and then collect them selectively with suction samplers or syringes, being careful to exclude ambient water. This type of collection is either carried out by divers (for example, Bochdansky and Herndl, 1992; Silver, 2015) or by submersibles (Silver and Alldredge, 1981; Alldredge and Youngbluth, 1985). However, the bathypelagic ocean is beyond the reach of divers, and submersibles are prohibitively expensive to deploy over large ocean regions as encompassed in this study. In addition,
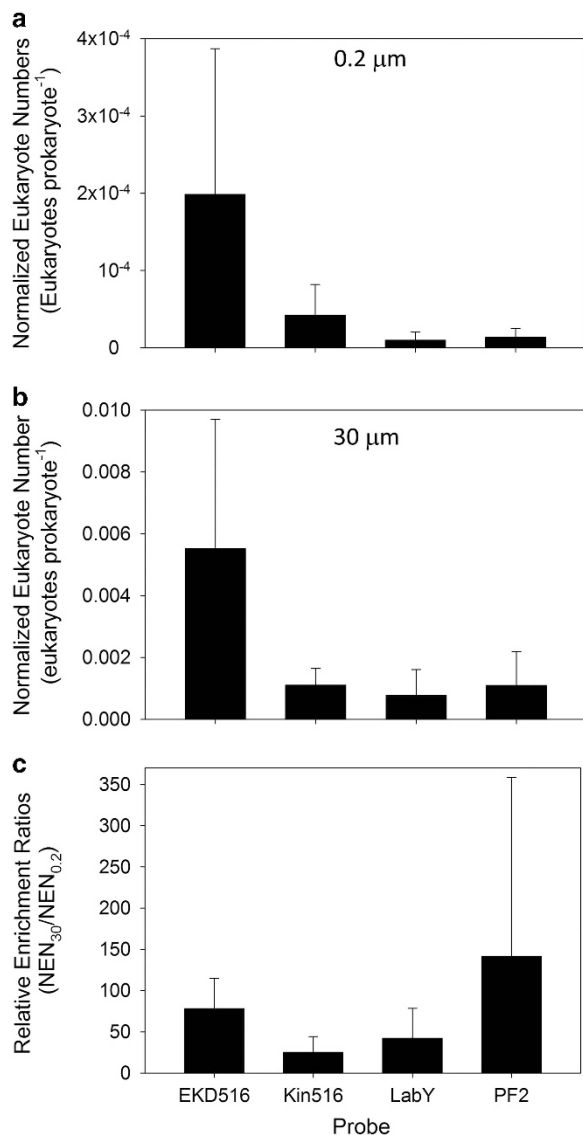
Figure 4 NEN (number of eukaryotic microbes divided by number of prokaryotes) on 0.2 μm pore-size (**a**) and on 30 μm pore-size (**b**) filters. The ratio between NEN_{30} and $NEN_{0.2}$ provides the relative enrichment ratios on marine snow (**c**). This ratio is an indicator for how much more eukaryotes are enriched in particles over the already highly enriched prokaryotes. It demonstrates that the contrast between the particles and ambient water environments are much larger for eukaryote than for prokaryotic numbers. Error bars are standard deviations.

marine snow less than a few millimeters is very difficult to collect from a submersible (Silver and Alldredge, 1981; Alldredge and Youngbluth, 1985) so that a large portion of relevant particles ($>400\,\mu m$) is unaccounted for (Bochdansky et al., 2016). Although we concede some inefficiency in our sampling protocol, we are also confident that marine snow was retained by our method. The organisms on the 30 μm pore-size filters were much more overdispersed than those on the 0.2 μm filters, rejecting the notion that these organisms were just randomly captured by direct interception with

the filter surface. In addition, a thick layer of TEP (the invisible matrix that typically holds marine snow together) was present on all 30 μm pore-size filters—so much so that EDTA had to be used to allow probes and nucleic acid stains to reach the embedded microorganisms. Normalizing all counts to the more abundant prokaryotes leads to relative enrichment ratios of eukaryotic microbes in an unbiased manner and also circumvents the problem of uneven material collection on different parts of the filters.

Kinetoplastids

Non-parasitic species of this group of flagellates are known to feed well on bacteria attached to particles (Artolozaga et al., 2000; Boenigk and Arndt, 2002) and in biofilms (Chavez-Dozal et al., 2013). Members of this group (*Bodo* sp., *Neobodo designis*) do not feed well on freely-suspended cells but prefer to feed on attached prokaryotes (Caron, 1987; Bochdansky and Clouse, 2015). The ratio of eukaryotic microbes to prokaryotes on particles in this study is very similar to those found in surface environments (for example, Gasol et al., 1997) where bacterivores together with viruses have an important role in controlling prokaryotic populations (for example, Fuhrman and Noble, 1995). Thus, it is likely that bacterivores have the same capacity to control prokaryotes on marine snow as in surface waters. A recent study demonstrated that kinetoplastids are also very abundant in hypolimnetic systems where they can represent up to 44% of total flagellates (Mukherjee et al., 2015). It would thus be very interesting to further study their association with particles in freshwater systems.

Fungi

In our analysis, approximately one fungal cell occurs for every 1000 prokaryotes on marine snow making their biomass approximately at par with that of prokaryotes (Table 1). This raises the intriguing question on how important fungi are for the degradation of organic material in the deep sea. Many species of fungi are known to be saprotrophic, and their osmotrophic food acquisition is suitable for substrates they can attach to and on which their extracellular enzymes are not lost to the environment by diffusion (Richards et al., 2015). Fungi are often better than bacteria at breaking down recalcitrant organic material (Clipson et al., 2006). There is evidence from isotopic analyses that particles absorb some of the old refractory dissolved organic carbon in the deep sea (Hwang et al., 2006) and it is possible that this recalcitrant material can be utilized by fungi. In this context, it is important to keep in mind that bacteria and fungi are not always in competition with each other but that both may contribute to the degradation and benefit from each other's breakdown products in a mutualistic rather

Table 1 Biomass (carbon) ratios of labyrinthulomycetes (LabY) and fungi (PF2) to prokaryotes

Group	Average volume V&P (µm³)	Carbon range N&ST (pg)	Carbon range M&L (pg)	Carbon V&P (pg)	Biomass ratio N&ST	Biomass ratio M&L	Biomass ratio V&P
LabY	106	5.83–23.90	7.42–33.39	9.48	0.23–0.95	0.29–1.29	0.37
PF2	115	6.33–25.95	8.05–36.23	10.13	0.35–1.42	0.44–1.98	0.53

Calculations were based on 0.1–0.41 g cm^{-3} dry weight to wet volume (van Veen and Paul, 1979), and 0.2–0.9 g dry weight cm^{-3} wet volume (Newell and Statzell-Tallman, 1982). Note: 1 g cm^{-3} = 1 pg µm^{-3}. Dry weight conversion to carbon was 55% for *Cryptococcus albidus* (shake culture only) (van Veen and Paul, 1979), and 35% (Newell and Statzell-Tallman, 1982). Carbon values were also obtained from volume using the equation C (pg) = 0.216 × volume$^{0.811}$ (µm³) for non-diatom protists (Menden-Deuer and Lessard, 2000). The same equations were applied to both groups of organisms. For bacteria, 20 fg per cell was assumed although this may be an overestimation of bacterial carbon in the deep sea (Ducklow and Carlson, 1992; Fukuda *et al.* 1998). Biomass ratio = C of eukaryotic microbes/C of prokaryotes (V&P: van Veen and Paul (1979); N&ST: Newell and Statzell-Tallman (1982); M&L: Menden-Deuer and Lessard (2000)).

than antagonistic manner (Frey-Klett *et al.*, 2011). Deep-sea POM has a high C:N ratio (Baltar *et al.*, 2010). As fungi have a higher C:N ratio than bacteria (~10 vs 5), using substrates with high C:N ratios could give them a stoichiometric advantage over bacteria (Wang *et al.*, 2012), although this view is not universally accepted (de Vries *et al.*, 2006). Although fungi may contribute to the degradation of particles in the water column, there is also the intriguing possibility that their hyphen stabilize marine snow as they do with soil particles (Chenu and Stotzky, 2002). Fungal hyphae permeating the particle matrix was shown in Jobard *et al.* (2010) for 'lake snow'.

Fungi in deep-sea sediments and the deep biosphere dominate among active eukaryotes (Takishita *et al.*, 2006; Edgcomb *et al.*, 2011a) where they also dominate active eukaryotic gene expression in metatranscriptomes (Orsi *et al.*, 2013). However, there is considerable genetic separation between benthic and pelagic fungal groups, implying that pelagic fungi are not merely resuspended benthic fungi (Tisthammer *et al.*, 2016). In fact, pelagic fungal abundance and diversity are influenced by environmental factors such as freshwater input, temperature and increases in POM (Taylor and Cunliffe, 2016). Specifically, chytrid fungi that are both parasites and decomposers of phytoplankton increase in numbers during diatom blooms (Taylor and Cunliffe, 2016). Other pelagic environments where fungi are found in large numbers are on *Trichodesmium* sp. colonies (Sheridan *et al.*, 2002) and in the surface waters of upwelling regions where fungi episodically exceed prokaryote biomass (Gutiérrez *et al.*, 2011). Recently, a survey of bathypelagic eukaryotic microbes concluded that Basidiomycota (a major group of fungi) are among the four most dominant groups (Pernice *et al.*, 2016). That study did not distinguish between marine snow and ambient water but screened their samples through a 20 µm mesh. While screening will prevent most metazoans from entering the sample, some marine snow will fragment and associated organisms will be mixed into the sample. Given our result of an extreme enrichment of fungi on marine snow, it is very likely that fungi in these samples came primarily from marine snow. The variability in taxonomic composition of eukaryotic microbes was high in that study with much of the variance explained by water mass (Pernice *et al.*, 2016). As the amount of deep-sea marine snow changes dramatically by water mass and location (Bochdansky *et al.*, 2010, 2016), some of the high variability in microbial eukaryotes is very likely due to the relative abundance and type of marine snow in these layers.

Labyrinthulomycetes
This group is even less studied in marine pelagic environments than fungi. Methods previously used in pelagic environments were either most probable number estimates using pollen as bait or growth substrate or the Acriflavine direct detection method based on sulfated polysaccharides in their cell walls (Raghukumar, 2002). The first time CARD-FISH used to systematically evaluate labyrinthulomycetes in the deep-sea water column was Morgan-Smith *et al.* (2013) using the LabY probe (Stokes *et al.*, 2002). Cells of this group have been reported to occur at higher numbers than fungi in the water column (Raghukumar, 2002). In our analysis, labyrinthulomycetes were approximately equally abundant as fungi on the 0.2 µm filters and less enriched than fungi on marine snow (Figure 4). Of the labyrinthulomycetes, the thraustochytrids are the most dominant group in pelagic marine systems, and they are often associated with phytodetritus but are interestingly suppressed during intense phytoplankton blooms (Raghukumar, 2002). Thraustochytrids and their 18S rRNA sequences have been found to be abundant in the bathypelagic environment down to 4000 m (Gaertner, 1982; López-García *et al.*, 2001). Thraustochytrids are extremely well adapted to cold temperatures and high pressures (Bahnweg and Sparrow, 1974; Riemann and Schaumann, 1993; Ragunkumar and Ragunkumar, 1999), and have even been found in dense clusters in Antarctic sediments (Riemann and Schrage, 1983). They have been suggested to be important agents in the degradation of organic material on deep-sea particles (Raghukumar, 2002) as they are an important group

of decomposers of coastal leaf litter and phytoplankton in sediments (Bongiorni, 2012). Because many are also parasitic or symbiotic (Damare and Raghukumar, 2010), thraustochytrids' ecological role cannot be inferred from the data at hand. However, their large biomass contribution to microbes associated with particles suggests a substantial role in deep-sea biogeochemical processes.

Biomass calculations
Carbon ratios of fungi and labyrinthulomycetes to prokaryotes are shown in Table 1. We used various conversion factors; however, the individual biomass estimates are very close to each other (Table 1). The carbon estimates for labyrinthulomycetes in this study are close to those previously determined for thraustochytrids (Kimura *et al.*, 1999). Previous estimates of the contribution of thraustochytrids to total decomposer biomass (bacteria+protists) ranged from 3.5 to 29% (Kimura *et al.*, 1999). In another study, the biomass of thraustochytrids was ~ 10% to ~ 100% of that of bacterial biomass (Damare and Raghukumar, 2008). Our calculations suggest that labyrinthulomycete biomass is approximately equal to that of prokaryote biomass on bathypelagic marine snow. If we combine the biomasses of fungi and labyrinthulomycetes, then eukaryotic saprotrophs exceed prokaryotes in terms of biomass, even when we exclude all other eukaryotic microbes that are present on particles. The biomass distribution of eukaryotic microbes on deep-sea marine snow is therefore much more similar to the biomass distribution in typical surface waters where eukaryotic microbes (including primary producers) exceed prokaryotic biomass (for example, Gasol *et al.*, 1997; Church, 2008). While some misclassification of taxa by means of CARD-FISH is inevitable due to errors when there is not a perfect sequence match, it is important to emphasize that the distinction between prokaryotes and eukaryotic microbes is very robust, as only those organisms were considered eukaryotic microbes that had both a cell body hybridizing with the probes and a clearly visible nucleus inside that (Figure 3).

Colonization of particles by eukaryotic microbes
Laboratory-made marine snow in rolling tanks is quickly colonized by heterotrophic protists such as flagellates, amoeba and ciliates (Artolozaga *et al.*, 1997) for which enrichment factors range from 5 to ~ 10 000 (Silver *et al.*, 1978; Caron *et al.*, 1982; Artolozaga *et al.*, 1997). The species composition on particles is more closely related to that found in benthic habitats than in pelagic habitats (Artolozaga *et al.*, 1997). For instance, pelagic amoeba are typically found associated with aggregates (Arndt, 1993; Rogerson *et al.*, 2003), and some amoeboid-type shapes were indeed present on the bathypelagic particles examined here (Figures 3i and j).

The conclusion that marine snow is a unique microenvironment for eukaryotic microbes also resonates with earlier findings of highly enriched metazoan plankton, some of which are almost exclusively found on marine snow such as acoel turbellarians and nectochaeta larvae of polychaetes (Bochdansky and Herndl, 1992). All these indicate that marine snow provides a benthos-like yet unique habitat in the water column.

Comparison between CARD-FISH and amplicon sequencing
There are similarities and discrepancies in the relative abundance of eukaryotic microbes in the deep sea between methods when comparing amplicon sequencing and direct cell counts. As shown above, the high number of Basidiomycota in deep-sea samples (Pernice *et al.*, 2016) is supported by our findings of a large number of fungi. Direct counts and 16S/18S diversity approaches also agree in the relative abundance of diplonemids for deep-sea environments (Lara *et al.*, 2009; Morgan-Smith *et al.*, 2013; Lukeš *et al.*, 2015; Pernice *et al.*, 2016). In contrast, group 2 alveolates that represent a large portion in deep-sea clone libraries (for example, Countway *et al.*, 2005; Pernice *et al.*, 2016) make a relatively smaller fraction in deep-sea samples when enumerated by CARD-FISH (Morgan-Smith *et al.*, 2013). Ciliates are dominant in clone libraries of the deep sea (for example, Countway *et al.*, 2005; Pernice *et al.*, 2016). Ciliate abundances are high in oxygen-depleted waters where they either form symbiotic relationships with prokaryotes (Edgcomb *et al.*, 2011b; Orsi *et al.*, 2013) or feed on them (Anderson *et al.*, 2012). However, ciliates are rare in the typical high-oxygen deep-sea samples and when counts are based on morphological criteria (Aristegui *et al.*, 2009). There are considerable biases that lead to overrepresentation of some groups in the analysis of 18S rRNA gene libraries discussed in detail in Koid *et al.* (2012), including multiple genome copies found especially in alveolates (for example, Dinophycea and Ciliophora). Unless the actual cell numbers can be independently verified by direct cell counts using suitable CARD-FISH probes, results of the relative dominance of some eukaryotic groups in deep-sea samples need to be treated with great caution.

Conclusions

There is growing evidence that neutrally buoyant or slowly-sinking particles augmented by an active community of chemosynthetic and carbon-fixing microorganisms need to be considered in budget calculations of the deep sea (Baltar *et al.*, 2009, 2010; Bochdansky *et al.*, 2010; Burd *et al.*, 2010; Herndl and Reinthaler, 2013). The high TEP content of deep-sea macroscopic particles, especially those in the

range of 400 μm to several mm in size, could contribute to that buoyancy (Atsetsu-Scott and Passow, 2004; Bochdansky *et al.*, 2016). Slowly-sinking and neutrally buoyant particles would allow sufficient time for complex eukaryotic microbial communities to form on the high organic nutrients available in particle matrix and pore water. The ratios between eukaryotes and prokaryotes in our 0.2 μm filter samples were low and similar to those typically found in the deep ocean (Aristegui *et al.*, 2009; Morgan-Smith *et al.*, 2013; Pernice *et al.*, 2015). In contrast, the bathypelagic marine snow samples abounded with eukaryotic microbes with eukaryote:prokaryote ratios much higher and closer to those found in surface waters. At these high biomass levels, eukaryotic microbes could significantly contribute to particle solubilization and remineralization, and to the control of prokaryote abundances through grazing, all of which strongly suggests that eukaryotic microbes can no longer be considered sideshows to ecosystem processes of the deep sea.

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgements

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