MEDEA-II CARD FISH cell counts

CARD FISH Eukaryote, Fungi, Labyrinthomycete, Kinetoplastid counts Medea 2

Excerpt from Bochdansky, A. B., M. A. Clouse, G. J. Herndl (2016). Eukaryotic microbes, principally fungi and labyrinthulomycetes, dominate biomass on bathypelagic marine snow. ISME J (in press)

MATERIAL AND METHODS

Sample collection. Seawater was collected during the MEDEA-2 research expedition to the North Atlantic and Arctic Basin between June 22 and July 22, 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. The cartridge was loaded with a 25 mm diameter, 30 μm pore-size polycarbonate filter membrane (type PCT30025100, Sterilitech Corp.). 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 μm or 0.8 μm filters is limited to much smaller volumes (~100 ml and ~1l on 25 mm diam. 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 fashion (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 conc.) formaldehyde solution in 0.2 μm filtered deep-sea water. The filter was gently rinsed with sterile 1x phosphate buffered saline (PBS) and ultrapure water by attaching another set of syringes to the cartridge and by gently moving the water in the 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 GTTP) using a standard filtration manifold. Filters were also rinsed with 1x PBS

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). In order to minimize losses from the filter surface, we covered the filter with agarose according to the CARD-FISH procedure (Teira *et al*., 2004; Morgan-Smith *et al*., 2013) prior to 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 catalyzed reporter deposition fluorescence in situ hybridization (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 (e.g., 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). Signals were only considered positive (regarded as a 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 μm 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, U-LH100HG APO mercury burner, 100 x UPlanSApo objective lens, and 20 x 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), an index insensitive to zero counts, and 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 x 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 ($\pi/6$ x width^2 x length) for eukaryotic microbes (Pernice *et al*., 2015), and on the equation ($\pi/4$) x width^2 x ((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.

Data Processing:
Raw lengths were converted into micrometers using a calibration curve with relative object distance. The calibration equation is =uncorrected length/(1+0.00893*(object distance/1000)).

References:

Alldredge AL, Silver MW. (1988). Characteristics, dynamics and significance of marine snow. *Prog Oceanog* **20**: 41-82.

Bar-Zeev E, Passow U, Castrillon SR, Elimelech M. (2015). Transparent exopolymer particles: From aquatic environments and engineered systems to membrane biofouling. *Environ Sci Technol* **49**: 691-707.

Bochdansky AB, Bollens SM. (2004). Relevant scales in zooplankton ecology: Distribution, feeding, and reproduction of the copepod *Acartia hudsonica* in response to thin layers of the diatom *Skeletonema costatum*. *Limnol Oceanogr* **49(3)**: 625-636.

Bochdansky AB, Huang L. (2010). Re-evaluation of the EUK516 probe for the domain Eukarya results in a suitable probe for the detection of kinetoplastids, an important group of parasitic and free-living flagellates. *J Eukaryot Microbiol* **57(3)**: 229-235.

Bochdansky, A.B., M. A. Clouse, G. J. Herndl (2016). Dragon kings of the deep sea: major deviation of particles from the common number - size spectrum. Scientific Reports 6, doi 10.1038/srep22633

Bratbak M. (1985) Bacterial biovolume and biomass estimations. *Appl Environ Microbiol* **49(6)**: 1488-1493.

Cavaliere R, Ball JL, Turnbull L, Whitchurch CB. (2014). The biofilm matrix destabilizers, EDTA and DNasel, enhance the susceptibility of nontypeable *Hemophilus influenza* biofilms to treatment with ampicillin and ciprofloxacin. *MicrobiologyOpen* **3**: 557-567.

Hugenholtz P, Tyson GW, Blackall L. (2001). Design and evaluation of 16S rRNA-targeted oligonucleotide probes for fluorescence *in situ* hybridization. In: Lieberman, BA (ed). Methods in Molecular Biology, vol. 176: Steroid Receptor Methods: Protocols and Assays. Humana Press Inc: Totowa, NJ, pp 29-41.

Jackson, G. A. *et al.* Particle size spectra between 1 µm and 1 cm at Monterey Bay determined using multiple instruments. *Deep-Sea Res. I* **44,** 1739-1767 (1997).

Jobard M, Rasconi S, Solinhac L, Cauchie H-M, Sime-Ngando T. (2012). Molecular and morphological diversity of fungi and the associated functions in three European nearby lakes. *Environ Microbiol* **14(9)**: 2480-2494.

Lloyd M. (1967). Mean crowding. *J Anim Ecol* **36**: 1-30.

Morgan-Smith D, Clouse MA, Herndl GJ, Bochdansky AB. (2013). Diversity and distribution of microbial eukaryotes in the deep tropical and subtropical North Atlantic Ocean. *Deep-Sea Res I* **78**: 58−69.

Mukherjee I, Hodoki Y, Nakano S. (2015). Kinetoplastid flagellates overlooked by universal primers dominate in the oxygenated hypolimnion of Lake Biwa, Japan. *FEMS Microbiol Ecol* **91(8)**: 1-11.

Pernice MC, Giner CR, Logares R, Perera-Bel J, Acinas SG, Duarte CM *et al*. (2016). Large variability of bathypelagic microbial eukaryotic communities across the world's oceans. *ISME J* **10(4)**: 945-958.

Stemmann, L. *et al.* Volume distribution for particles between 3.5 to 2000 µm in the upper 200 m region of the South Pacific Gyre. *Biogeosciences* **5,** 299-310 (2008).

Teira E, Reinthaler T, Pernthaler A, Pernthaler J, Herndl GJ. (2004). Combining catalyzed reporter deposition-fluorescence in situ hybridization and microautoradiography to detect substrate utilization by bacteria and archaea in the deep ocean. *Appl Environ Microbiol* **70**: 4411-4414.

van Aken, H. M. The hydrography of the mid-latitude northeast Atlantic Ocean I: The deep-water masses. *Deep-Sea Res. I* **47,** 757–788 (2000).