Final Report for OLI Project: Delaware Bay as model system for tracking the source, transport and activity of marine subsurface fungi

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Background

Marine sediments cover more than two thirds of the Earth's surface and have been estimated to contain as much as one-third of Earth's prokaryotic biomass (Whitman et al., 1998), yet relatively little is known about this habitat, in particular of the microbial eukaryotes. In terrestrial environments fungi encompass a significant portion of total microbial diversity and biomass and include key biological components in ecologically important symbioses, chemical cycles and food webs (Bass et al. 2007), yet very little is known about fungi in marine sediments. Microbial eukaryotes, including fungi are presumed to be important in the cycling of organic matter in all phases (particulate and dissolved) but their specific roles in these processes are not well constrained, and more knowledge is required to inform accurate models of marine sedimentary carbon and nitrogen cycling. The biogeochemical activities, composition, and temporal and spatial dynamics of marine subsurface communities are an emerging central topic in marine sciences and biogeochemistry.

An earlier analysis of eukaryotic small subunit ribosomal RNA gene (18S rRNA) sequences obtained from deep (up to 35mbsf) marine subsurface sediments showed that fungi within the Basidiomycota were the dominant sequence types in all cDNA-based 18S rRNA clone libraries (Edgcomb et al. 2010). Together with a metagenome and a cultivation study (Biddle et al. 2005b, Biddle et al. 2008), these results suggest that fungi dominate eukaryotic life in the buried marine subsurface. The recovery of ribosomal RNA (and not just DNA) of fungi at these depths suggest that these sequences come from living cells, however many of the sequences detected to date from surface sediments (Bass et al. 2007) and deeper samples (Edgcomb et al. 2010) are close relatives of terrestrial fungi. The boundaries between terrestrial and marine fungi are currently not clear. There is some evidence that terrestrial/surface-dwelling fungi may be capable of colonizing deep-sea habitats due to their ability to alter their membrane composition to accommodate high hydrostatic pressure (Simonato et al. 2006).

Original Research Questions

The aim of this study was to use the Delaware Bay system to track and enumerate fungi from a terrestrial environment out to a more pure marine system using molecular methods to confirm whether previously recovered fungal sequences come from living cells and to determine whether the marine sedimentary fungal biosphere is seeded by adaptable, opportunistic, terrestrial organisms or fungi that are truly marine in origin. If these sequences are coming from intact fungal cells that are abundant in marine sediments then this has global implications for ocean carbon and nitrogen cycling, given the extent of the marine sedimentary biosphere.

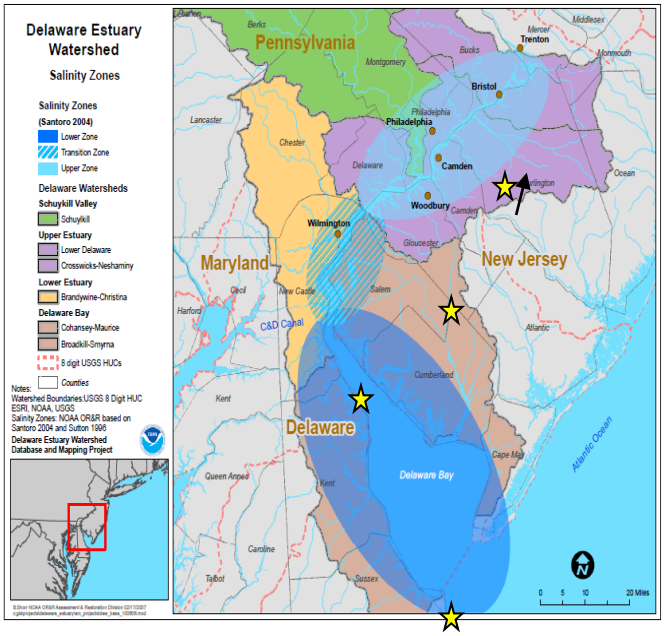

Fig 1: Sampling sites in different Delaware Bay salinity zones, map courtesy of NOAA

Sampling, Location, and Nature of Experimental Work

The Delaware Bay was used as a model estuarine system in which to study the microbial changes that occur along a transect from a terrestrially influenced section of water and sediment to a higher salinity, truly marine system. The Delaware Bay is well

studied and has three major salinity zones (Fig. 1), representing an ideal transect for studying terrestrial input into the marine system. Hydrographically, it is one of the simplest estuaries to study with one major source, the Delaware River, and a single bay in which the river input mixes with saline waters.

Samples were collected in September 2010 and January 2011 using the R/V *Hugh Sharp* by collaborator Dr. Jennifer Biddle (U. DE) (Table 1). Water samples were collected using a submersible pump 1 m below the surface at 3 different stations representing the 3 major salinity zones of the Delaware Bay (Table 1). Dr. Gaetan Burgaud, an expert on culturing marine fungi came to WHOI to assist with culture-based investigations. Water samples were collected and used by Dr. Burgaud in January 2011 for cultivation-based approaches and *in situ* hybridization. Regarding cultivation, water samples were stored at 4°C for a few hours before filtration and plating. For *in situ* hybridization, 200ml of water samples were directly fixed with paraformaldehyde (2% final concentration) for 2 hours at 4°C in the dark before filtration on polycarbonate filters, with a pre-filtration through 200 micron mesh (Pernthaler et al. 2001).

Bottom sediment samples were obtained on the same cruises using a KC Denmark HAPS corer, which recovers undisturbed samples of the sediment-water interface. The cores were extruded vertically aboard the ship and sectioned in 2-cm intervals. The sediment was placed in Whirl-pak sterile bags and frozen at -80 degrees C. Samples were processed from the 2-4 centimeters below seafloor (cmbsf) depth horizon in order to avoid seawater contamination.

Sediment samples were used for cultivation-based approaches, *in situ* hybridization and Calcofluor staining, and analyses of small subunit ribosomal RNA (SSU rRNA) and internal transcribed spacer region 1 (ITS1) sequences. For *in situ* hybridization, multiple aliquots of 100 mg sediment samples were fixed in 2% paraformaldehyde (final concentration) for at least 2 hours at 4°C in the dark. After fixation, sediment samples were washed 2 times with sterile 1X PBS and stored at -20°C in 50:50 1X PBS/96% ethanol (Pernthaler et al. 2001). Multiple 2g aliquots were directly frozen at -80°C for extraction of nucleic acids. A summary of samples collected and methods applied to each sample can be found in Table 1.

After plating samples, all subsequent culturing activities, molecular investigations (sequencing of ribosomal RNA and Internal Transcribed Spacer region), and microscopy (fluorescent *in situ* hybridization and Calcofluor staining of fungal cells) work was conducted at WHOI, primarily by Burgaud and Edgcomb between January 2011 and summer 2011. Equipment involved included Edgcomb's Zeiss Axioscope fluorescence microscope, laminar flow hoods for culturing activities, autoclave, centrifuges, PCR machines, and incubators. Results subsequently analyzed and were submitted for publication in January 2012.

Station	Sample Type	Latitude (N)	Longitude (W)	Salinity (PSU	Date Sampled	Study performed
Station 23	water	40° 08.96	74° 43.31	0,1	3/11/2010	FISH
Station 11	water	39° 26.916	75° 33.125	6,9	3/11/2010	FISH
Station 1	water	38° 46.94	74° 55.76	31,5	3/10/2010	FISH
2H	sediment	39° 48.714	75° 24.167	0,19	3/12/2010	FISH, ITS DNA, ITS RNA
9H	sediment	39° 40.442	75° 31.774	1,5	3/12/2010	FISH, ITS DNA, ITS RNA
20H	sediment	39° 21.143	75° 27.175	9,2	3/11/2010	FISH, ITS DNA, ITS RNA
Station 23	water	40° 08.96	74° 43.31	0,1	12/14/2010	culture
Station 11	water	39° 26.916	75° 33.125	2,3	12/13/2010	culture
Station 1	water	38° 46.94	74° 55.76	29,7	12/13/2010	culture
Station 23	water	40° 08.96	74° 43.31	0,08	6/4/2011	culture
Station 11	water	39° 27.04	75° 33.125	2,58	6/3/2011	culture
Station 1	water	38° 46.83	74° 54.22	29,23	6/3/2011	culture

Table 1. Delaware estuary sample collection metadata.

Major Findings

Success in fungal isolation was dependent on the salinity of the different sites since a decreasing number of fungal strains, both yeasts and filamentous fungi, were harvested as we transitioned from freshwater to marine sites. Another correlation was observed when comparing isolation sources and physiology, showing that yeasts from marine sites exhibited growth consistent with a strong preference for higher salinity, while isolates from brackish sites were haloterant, and isolates from freshwaters were clearly non halophiles. Indeed, based on culturing results, each site/salinity condition harbored a mostly distinct fungal community. Interpretation appeared more complex with filamentous fungi and showed that those organisms are more able to adapt to saline conditions.

Few fungi were retrieved in culture at more than one site, in spite of using the same enrichment media. While it is possible that expanded culturing efforts using additional filtered water samples as inoculum would have produced a greater number of overlapping cultures between sites, we think the observed pattern of culture recovery suggests very different active fungal populations in waters of significantly different salinity. This is particularly striking when one compares the successful isolates from station 23 (0.008-0.01% salinity) to those from station 1 (2.92-3.15%). If our cultures were being seeded primarily by inactive spores, it is likely that we would observe a greater overlap in our culturing results for the different stations. Molecular results from SSU rRNA and ITS1 region analyses support the idea of a transition in fungal community structure along the salinity gradient since a much narrower spectrum of taxonomic signatures was recovered from the fully marine sediments.

The decline in fungal diversity from more freshwater to fully marine samples is also reflected by the results of the culture-based studies. Isolations from the freshwater station 23 produced 14 different isolates, the brackish waters at station 11 produced 12, and the fully marine station 1 produced only 5. Very little overlap in taxonomic composition was observed between isolates from different sites. Together with molecular results, this suggests that the active fungal community transitions in composition along this salinity gradient, and that many fungi found in fresh waters are not adapted for survival in the fully marine environment. This is consistent with previous findings of a relatively simple fungal community composition in marine systems (based on fungal-specific clone library analyses) with relatively few phylotypes in total relative to terrestrial systems (Bass et al. 2007, Cathrine & Raghukumar 2009, Le Calvez et al. 2009, Edgcomb et al. 2010).

The finding of halotolerant fungi of likely terrestrial origin is consistent with other studies of deep-sea and subsurface marine sediments where many signatures affiliated with clades of known terrestrial fungi (Edgcomb et al. 2010, Takishita et al. 2006). Focusing on the degree to which cultured fungi appeared in the ITS1 and SSU rRNA clone libraries indicates a poor overlap between the fungi revealed using molecular vs. culture-based methods, since only *Phoma* and *Trichoderma* were detected using both methods. Rarefaction curves (Fig. 4B) indicated that the sequencing effort was clearly insufficient to cover *in situ* diversity, and hence, only a fraction of the molecular diversity was revealed. Moreover, some technical biases are also inherent in cultured based-studies, *i.e.* unculturable fungi, endophytes, cryptic species, dormant spore germination, fast/slow growing strains, cryoconservation of sediment samples etc. that explain this lack of overlap. Only 2 culture media were used in this study, and fungi occurring in macro-aggregates may have difficulties to grow in Petri dishes or marine broth (Damare & Raghukumar, 2007). As a first investigation into the transitions within occurring and active fungal communities along a salinity gradient, this study does indicate that fungal communities are not the same along this gradient, and it does provide evidence of active, truly marine or halotolerant fungi. Coupling a deeper sequencing effort and a more complex culture-based experimental plan in the future will certainly increase the overlap in fungal types detected using culture-based and molecular methods.

On the basis of our culture studies, microscopy using FISH and Calcofluor staining, and molecular work, we conclude that living and active fungal communities do exist in the water column and sediments of Delaware Bay along a salinity gradient from freshwater to fully marine environments, that communities in

different salinity regimes are different, and that there appear to be a majority of metabolically active marine fungi of terrestrial origin. Indeed, there appear to be quite a few fungi that may originate from terrestrial sources and get dispersed to fully marine water columns and sediments, and are capable of surviving there. These include *Penicillium, Thysanophora*, and *Aspergillus* confirming previous studies (Raghukumar et al. 2004, Damare et al. 2006, Pindi 2012). In buried marine sediments their osmotrophic lifestyle is advantageous, as attachment to larger physical substrates, such as buried organic material, and osmotrophy via secreted enzymes is much easier there than in the water column where needed nutrients and secreted enzymes can be lost more easily by diffusion.

The results of this study are presented in a manuscript published in *Aquatic Microbial Ecology* (Burgaud et al. 2013).

Significance

The mounting evidence for successful filamentous fungi and yeast forms in marine environments suggests that fungi may play a much more significant role in carbon cycling in the buried marine subsurface than previously thought. This has potentially major implications for our understanding of marine carbon cycles. Currently, the activities of fungi are not factored into equations and models that project the rates of recycling of buried organic carbon and the impacts on the marine carbon pump. Most buried carbon is considered to be highly refractory, but it may turn out that a much larger fraction of buried carbon is remineralized by fungi than previously expected.

Our findings from this OLI-funded study fueled increased interest in this field. The preliminary findings from this study made it possible for me to garner postdoctoral fellowship funding from NSF (CDEBI program) for William Orsi in 2011 to investigate fungal populations and activities in different IODP deep subsurface sediment cores. Assisted by some nominal DOEI funds for research monies, we completed that project which yielded two publications (Orsi *et al*. 2012 *PLoS One*; Orsi *et al.* 2013 *Nature*). As a result, there is now great interest in the community regarding the potential role/impacts of subsurface fungi in carbon and other nutrient cycling, and I plan to leverage these results to garner further funding for subsurface investigations from NSF.

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