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Review Article

Living at the Limits: Evidence for Microbial Eukaryotes Thriving under Pressure in Deep Anoxic, Hypersaline Habitats

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The advent of molecular tools in microbial ecology paved the way to exploit the diversity of microbes in extreme environments. Here, we review these tools as applied in one of the most polyextreme habitats known on our planet, namely, deep hypersaline anoxic basins (DHABs), located at ca. 3000–3500 m depth in the Eastern Mediterranean Sea. Molecular gene signatures amplified from environmental DHAB samples identified a high degree of genetic novelty, as well as distinct communities in the DHABs. Canonical correspondence analyses provided strong evidence that salinity, ion composition, and anoxia were the strongest selection factors shaping protistan community structures, largely preventing cross-colonization among the individual basins. Thus, each investigated basin represents a unique habitat ("isolated islands of evolution"), making DHABs ideal model sites to test evolutionary hypotheses. Fluorescence *in situ* hybridization assays using specifically designed probes revealed that the obtained genetic signatures indeed originated from indigenous polyextremophiles. Electron microscopy imaging revealed unknown ciliates densely covered with prokaryote ectosymbionts, which may enable adaptations of eukaryotes to DHAB conditions. The research reviewed here significantly advanced our knowledge on polyextremophile eukaryotes, which are excellent models for a number of biological research areas, including ecology, diversity, biotechnology, evolutionary research, physiology, and astrobiology.

1. Introduction

Ocean bottom surveys in the early 1980s observed abyssal depressions at a depth of more than 3000 m in the Eastern Mediterranean Sea showing unusual reflection profiles and backscatter images [1, 2]. Subsequent hydrochemical analyses of the water trapped in these depressions identified the respective environments as deep hypersaline anoxic basins (DHABs) [1, 3, 4]. With the most recent discovery [5], there are eight known DHABs in the Eastern Mediterranean Sea, distributed in the Strabo Trench (Tyro), the Mediterranean Ridge (Bannock, Kryos, Medee, and Thetis), and the Medriff Corridor (L'Atalante, Discovery, and Urania) ([6], Figure 1). The formation of DHAB brines is reviewed in Cita [7]. They originated by submarine dissolution of Messinian evaporites (late Miocene, ca. 6 MYA) thought to originate primarily from the dissolution of evaporites ~2000–176,000 years ago. Due to the fact that different minerals deposit in different orders depending on evaporation conditions, evaporites may contain different concentrations of halite (NaCl-mineral), kieserite ($MgSO_4$-mineral), and other minerals [8, 9]. Accordingly, the contemporary brine lakes, which formed in the dissolution process, differ from each other in their hydrochemical compositions [10]. To mention a few examples, salinity, which is ca. 35 PSU in "normsaline" seawater, varies from 240 PSU (Urania, [11]) up to 500 PSU (Discovery, [7]). Magnesium varies between 53 mmol in the Tyro basin [5] and 4995 mmol in Discovery [12, 13]. Sulfate

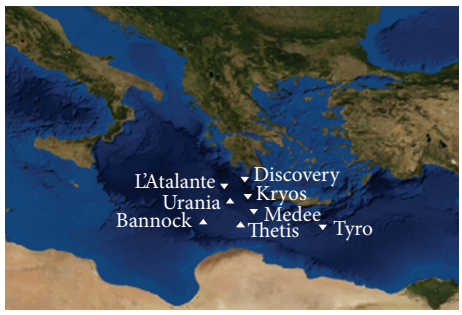

Figure 1: Location of the hitherto described deep-sea hypersaline anoxic basins (DHABs) in the Eastern Mediterranean Sea (modified map from http://visibleearth.nasa.gov). For details, see also [6].

varies between 53 mmol (Tyro, [5]) and 397 mmol (L'Atalante, [12]). Sodium has its minimum in Discovery (68 mmol, [12]) and its maximum in Tyro (5300 mmol, [5]). Concentrations of toxic hydrogen sulfide range between 0.7 mmol (Discovery, [12]) and up to 20 mmol (Urania, [14]).

The high densities of the brines (up to 1.33 kg L^{-1}, [5]) prevent mixing of the brine bodies with the overlying normsaline deep-sea water. Thus, the brines of all lakes, which are separated from the normsaline deep-sea water by a sharp 1–3 m interface (halocline), are anoxic. All these factors (elevated hydrostatic pressure, salt concentrations to saturation, extremely low water activity, chaotropicity, anoxia, and euxinia) make the DHABs some of the harshest and hostile environments on our planet. For a long time, such habitats were considered biochemical dead ends and devoid of life, especially when high concentrations of $MgCl_2$ were present [12]. However, this general notion changed with the appearance of the first study reporting bacterial and archaeal genes of the small ribosomal unit (16S rDNA) from brines of some DHABs [12, 15]. Further studies revealed that these genetic signatures were not only remnants of ancient preserved DNA that accumulated in the high-salt medium, but that they originated from highly active prokaryote communities thriving in the DHABs [16]. Fueled by the idea that such active deep-sea communities are able to initiate secondary food webs [17], providing the basis for phagotrophic unicellular eukaryotes (protists), a number of studies in recent years set out to study the diversity and ecology of protists in the DHABs. These studies addressed the following questions. Do DHABs in the Eastern Mediterranean Sea support eukaryote life? If so, how diverse is this yet hidden life? Do genetic signatures in the DHABs truly come from active polyextremophiles? Does the distinctive hydrochemistry of the individual basins select for unique protistan communities? Could the DHAB protistan communities play an important role in global biogeochemical cycles running in ocean's interior?

2. Genetic Eukaryote Signatures from DHABs

Molecular diversity studies of individual DHABs in the Eastern Mediterranean Sea targeted the basins L'Atalante [18], Bannock and Discovery [19], and Thetis [20]. These surveys compared genetic signatures obtained from brine and from the brine/seawater interface, with the exception of Discovery, for which only brine/seawater interface samples were analyzed. All studies employed the extraction of nucleic acids from filtered brine and interface samples, followed by PCR amplification of eukaryotic small subunit ribosomal RNA genes (18S rDNA), a genetic marker routinely and successfully applied in protistan diversity surveys [21, 22]. PCR products were then cloned, Sanger-sequenced, and phylogenetically analyzed.

The samples analyzed in these studies could be successfully amplified, producing high-quality 18S rDNA signatures. The results of the individual studies were congruent in the observation that phylotype richness (calculated with parametric stochastic abundance models and nonparametric coverage based estimators) as well as community memberships and community structures (calculated with Jaccard indices) differed notably between brine samples and interface samples. Protistan community richness, membership, and structure in a reference sample from normsaline deep-sea water overlying the brine basins differed markedly in both brine communities and interface communities [19]. The latter is not unexpected, because hypersaline environments require specific adaptations to cope with the physiological stress imposed by the low water availability and the high osmotic pressure [23]. Different communities in both habitats, which are only 2-3 m separated from each other, point to an additional salt barrier within the ion gradient. It is well known that freshwater-marine transitions are not very frequent [24, 25]; however, thus far only one study speculated about a second physiological barrier for protists at ca. 15% salt [26]. This study finds support in experimental data reported by Park and Simpson [27]. The authors cultured eight individual stramenopiles flagellates from marine and moderately hypersaline environments. Most of the flagellates grew readily to a salinity of up to 15%. Above this salinity, no growth was recorded, indicating a salt barrier at ca. 15% salinity. But in addition to salt, other hydrochemical variables with steep concentration gradients between the interface and brine may contribute to environmental selection (e.g., oxygen, sulfide, and methane concentrations).

Taxonomically, the phylotypes retrieved from the brines and interfaces of the basins under study were affiliated with most major lineages in the eukaryotic tree of life (Figure 2). Alveolates, particularly ciliates and dinoflagellates, are dominant components of the protistan communities in all brine samples. Fungi are also major components of all brine communities, with the exception of L'Atalante, where choanoflagellates were the most diverse eukaryotic taxonomic group [18]. In Thetis brine, stramenopiles accounted for 20% of all detected phylotypes [20], and this group was largely missing in L'Atalante and Bannock brines [18, 19]. Many stramenopile sequences are highly divergent to previously described and deposited sequences, pointing to a high degree of as-yet-undetected diversity in DHABs, some of which appears to be at a high taxonomic level (possibly class- or order-level). Other, less abundant taxonomic groups that were detected with the primer sets used in the individual studies included chlorophytes, jakobids, cryptophytes, haptophytes, radiolaria, and euglenozoans. Even though present in

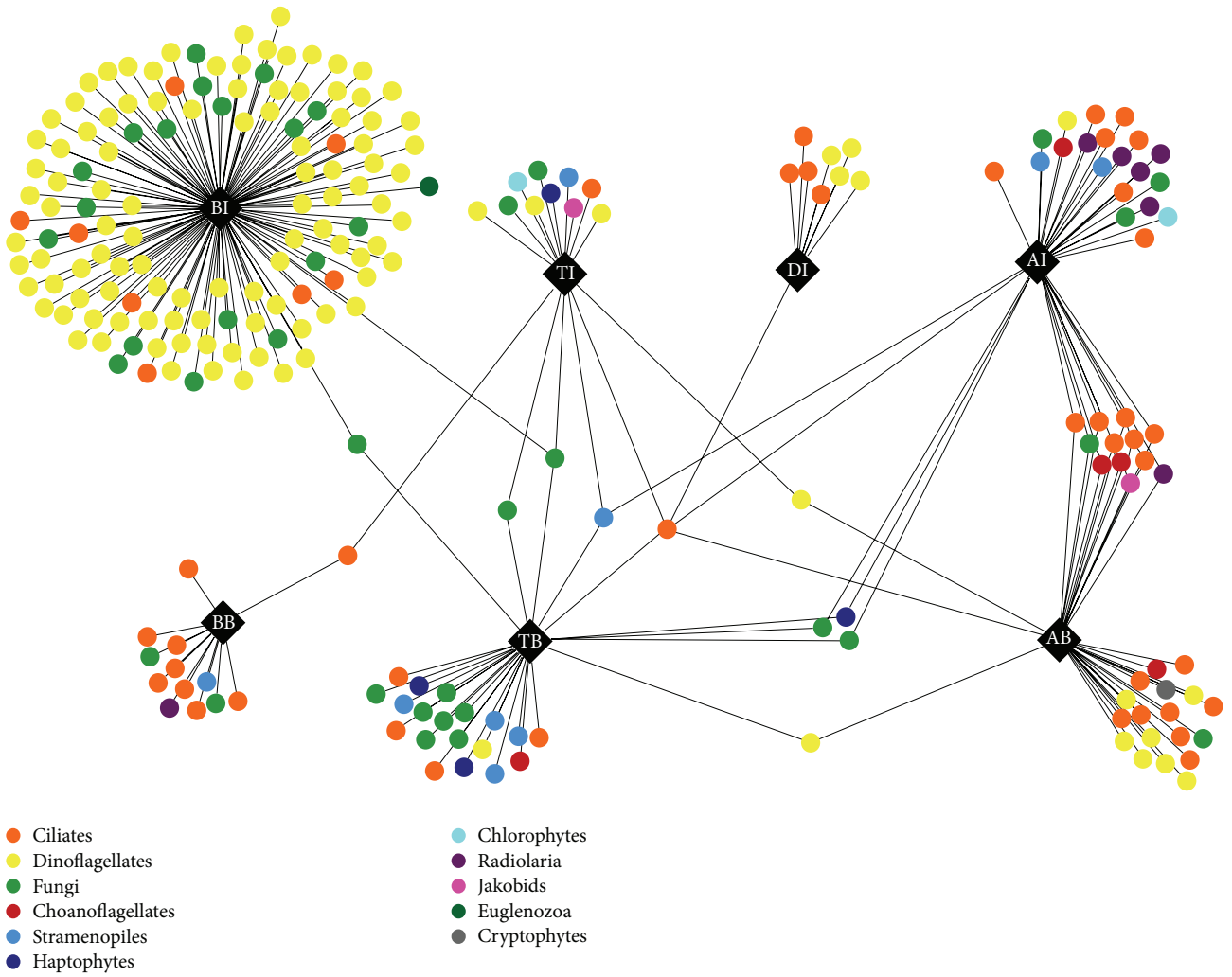

Figure 2: Partitioning of protistan phylotypes (called at 98% sequence similarity) between the compared 18S rDNA clone libraries from the interfaces (I) and brines (B) of the L'Atalante (A) [18], Bannock (B) [19], Discovery (D) [19], and Thetis (T) [20] DHABs. Corresponding sequences were retrieved from GenBank's nucleotide database based on the accession numbers given in the aforementioned publications. Sequences were then clustered in QIIME [95] at 98% sequence similarity before conducting a network analysis in QIIME. For visualization of the network we used Cytoscape [96]. Taxonomic assignment of sequences was conducted with JAguc [97].

clone libraries with only few representatives, these examples show the broad extent of taxonomic eukaryotic diversity in the extreme habitat under study. At first sight, the observation of chlorophytes may be surprising considering the general notion that chlorophytes are photoautotrophs. However, even among chlorophytes mixotrophic life style is common [28].

Fungi, ciliates, dinoflagellates, and stramenopiles are all taxon groups known from studies of high-salt environments, such as solar salterns, to contain members that have successfully adapted to high-salt conditions (e.g., [29–38]). Since fungi are mostly saprobic organisms, they benefit from the accumulation of organic material from the upper water column in the high-density brine. The brine/seawater interface with its abundant prokaryotes could represent a decked out table for phagotrophic protists like some ciliates, dinoflagellates, and stramenopiles.

Considering the high degree of protistan diversity in DHABs and the high degree of diversity of detected sequences in these habitats, halotolerance, and perhaps even halophily, may be far more widespread among protistan lineages than assumed previously [39]. Also successful adaptation to anoxia and euxinia is common throughout major eukaryotic evolutionary lineages [40]. These initial studies in DHAB brines indicated that the limits of eukaryotic life are far from being established.

3. DHABs as Models for Environmental Selection and Species Sorting in Polyextremophile Protistan Communities

Despite a limited depth of sequencing, Sanger-sequenced clone libraries in protistan diversity research have invaluably advanced our knowledge in this field [41–54]. One main constraint of the Sanger 18S rDNA approach is the relatively low number of clones that can be analyzed with a reasonable

financial effort. Considering the high complexity of protistan communities revealed by next-generation sequencing [55–59] it is now known that Sanger-sequenced 18S rDNA clone libraries only record the most abundant gene templates in a sample, and rare taxa typically escape detection due to undersampling. Therefore, comparative analyses of clone-library datasets are significantly biased towards dominant templates, even with the use of statistical tools developed to account for this shortcoming ([60], but see [61] for performance of these strategies). Furthermore, direct comparisons of the individual studies are biased by different molecules considered (e.g., environmental RNA for L'Atalante and Thetis and DNA for Bannock and Discovery, [18–20]). Previous studies have demonstrated that DNA- and RNA-based environmental diversity surveys uncover different subsets of the protistan communities under study [62, 63]. Also different PCR primers were employed for gene amplification in these studies, which hinders direct comparisons of results [58, 64–66]. Therefore, for a solid comparison of the protistan communities, a consistent approach is warranted.

While Filker and colleagues [67] used a molecular fingerprinting technique (terminal restriction fragment length polymorphism—TRFLP) to analyze biogeographic patterns and environmental selection of DHAB protists, Stock et al. [68] applied a next-generation sequencing strategy (pyrosequencing of the hypervariable V4 region of the 18S rDNA) to address the same question for a specific subset of the DHAB communities, namely, ciliates. Within each of these studies, the same lab protocols were used to minimize biases. Filker et al. [67] included the basins Medee, Tyro, Urania, Thetis, and Discovery and Stock et al. [68] targeted Medee, Tyro, Urania, and Thetis.

Using UPGMA clustering of beta-diversity indices, both studies confirmed the previous assumptions that all interface communities are indeed more similar to each other than to any of the brine communities (Figure 3). Within the brine communities, protists inhabiting Medee and Discovery are most distinct from those in all other DHABs. These results imply that the geochemical gradients within the DHAB interfaces act as dispersal barriers that prevent not only ciliates, but also many other protists from vertical migrations (with the exception of few widely adapted taxa such as the heterotrophic flagellate *Bodo saltans* [11]). Previous molecular diversity surveys identified redox gradients in aquatic habitats as strong biogeographic barriers to protistan dispersal [49, 53, 54, 69–71]. In addition, Elloumi et al. [26] suggested that, in a gradient of elevated salt concentrations, a salinity of around 15% is a barrier that is difficult to cross for protists, selecting "moderate halophiles" from "extreme halophiles." However, this suggestion remains to be confirmed. Support for this idea would fuel new interesting research subjects, namely, uncovering the physiological processes that result in halotolerance and halophily.

A significant effect of spatial distance between DHABs on the speciation of ciliates was not observed. Rather, their unique hydrochemistries were identified to be significant in shaping the unique communities. As expected, the best models of canonical correspondence analyses (CCA) identified oxygen, sulfate, and salinity as the strongest factors

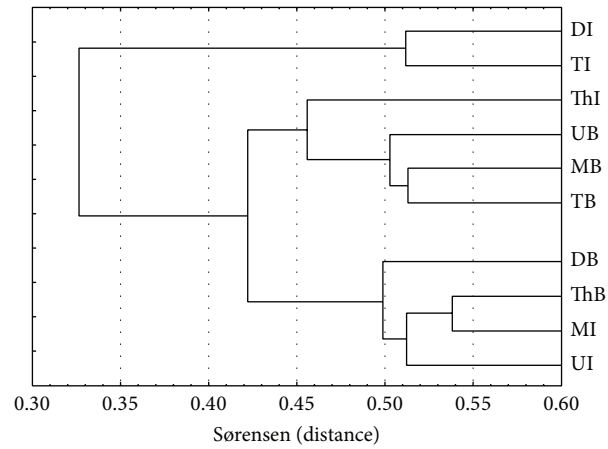

Figure 3: Beta-diversity hierarchical clustering (Sørensen distance) of the brines and interfaces from the DHABs Discovery (D), Tyro (T), Thetis (Th), Urania (U), and Medee (M) based on terminal restriction fragment patterns of eukaryote 18S rDNA fragments. Data from Filker et al. [67].

separating interface from brine communities [67, 68]. For marine-freshwater transitions it is well known that gradients in ion concentrations and osmotic pressure are strong environmental barriers that are difficult to cross for higher organisms [72] as well as for some protists [24], including ciliates [25]. Likewise, morphological studies (e.g., [36, 38]) as well as molecular diversity surveys (e.g., [73]) indicate notable changes in community structures within salt gradients. The energetic costs of osmoregulation and the evolution of adaptations to high salt concentrations are possibly among the most important factors determining the distribution of organisms along salt gradients [74, 75].

Oxygen gradients are also a dispersal barrier for protistan plankton [18–20, 49, 57, 70, 71, 76, 77], including ciliates [25, 40]. While for many protists oxygen is a prerequisite for survival, numerous other protists have different adaptations to an anaerobic lifestyle, which are reviewed in Müller et al. [78]. Facultative anaerobes have the possibility to cross the oxygen barrier and thrive in habitats with and without oxygen [40].

Explanations for the highly divergent brine communities are more obscure. The main structuring factor in some cases seems to be related to magnesium [67]. Magnesium (present as $MgCl_2$) is a divalent cation, which has different physiological effects than sodium, which is a monovalent cation [79]. Magnesium ions strongly affect the cellular machinery through destabilization of proteins by increasing their solubility [80]. Therefore, it is reasonable to assume that microbes require special adaptation strategies to withstand elevated concentrations of magnesium ions. However, the Discovery basin is the only DHAB discussed here in detail that is characterized by high loads of $MgCl_2$, and therefore this factor explains only a small proportion of the total variation among the protistan brine communities [67, 68]. Further efforts are necessary to unlock additional environmental factors (in addition to salinity, sulfide, and

oxygen, which are all known to structure the communities), which exert selective pressure on DHAB communities. While dispersal among interface communities is at least to some extent possible, dispersal barriers are stronger for the brine communities. As a consequence, the DHAB brines could be habitats with an "island character," each with an independent evolutionary history of its inhabitants, at least partly subjected to environmental selection through specific hydrochemistries. Genetic exchange among the individual brines seems very limited.

4. Are There Truly Active Extremophiles behind Environmental Sequences Obtained from DHAB Samples?

Reports in the literature show evidence that nucleases are inhibited in high-salt media under laboratory conditions, resulting in enhanced accumulation of extracellular DNA [81]. Therefore, it was unclear whether the sequences published in molecular DHAB diversity surveys [18, 19] were indeed (at least mostly) from active cells rather than ancient preserved extracellular nucleic acids [79, 82].

Strong evidence for the integrity of indigenous microorganisms behind obtained environmental gene fragments comes from (a) fluorescence *in situ* hybridization (FISH) assays and (b) an *in situ* sampler designed for deep-sea studies. FISH is a technique that takes advantage of unique genetic signatures of specific (microbial) target organisms, which are hybridized with a complementary fluorochrome-labeled oligonucleotide probe [83]. The so-called rRNA-approach takes advantage of unique gene regions of the SSU rRNA gene, the design of specific probes hybridizing to this region [84], a hybridization assay as described, for example, in [85], and epifluorescence microscopy. Applying this strategy, Edgcomb et al. [11] performed a phylogenetic analysis of kinetoplastid (heterotrophic euglenozoan flagellates) sequences, obtained from different DHAB samples. The authors identified a novel environmental sequence clade within this taxon group, whose closest relative with a described 18S rDNA sequence belonged to *Neobodo saliens*, with a sequence similarity of only 87% to this environmental sequence clade. After probe-design targeting this unknown taxon group, FISH not only visualized a novel morphotype, but also revealed that kinetoplastids may account for up to 10% of the total protistan cell abundance in the Eastern Mediterranean DHABs. This raised the question why this taxon group was hardly represented in previous molecular diversity surveys. The answer to this question lies in the unusual ribosomal RNA gene primary structure of kinetoplastids, which deviates from most other eukaryotes [86], and is largely incompatible with universal eukaryote PCR primers. Specific PCR primers designed for kinetoplastids are therefore required [87]. Assuming that other taxa may escape the detection of universal eukaryote-specific primers, the study of Edgcomb et al. [11] concluded that the taxonomic diversity in the investigated DHABs is even larger than revealed through the conducted diversity surveys. We note that the detection of active kinetoplastids with specifically designed oligonucleotide probes does not necessarily allow for the general conclusion that also all other sequences detected in the DHAB protistan diversity surveys [18–20, 67, 68] originate from indigenous polyextremophiles. However, the detection of ciliates in the DHABs with phylum-specific probes [20] suggests that at least some of the non-kinetoplast sequences are of autochthonous nature.

Figure 4: Microbial sampler-submersible incubation device (MS-SID), designed and manufactured by C. Taylor and V. Edgcomb, Woods Hole Oceanographic Institution, Woods Hole, MA, together with McLane Research Laboratories, Falmouth, MA, USA, in action during cruise to the Eastern Mediterranean DHABs with RV *Atlantis* (WHOI). MS-SID allows both fixation of samples at depth and incubation experiments at depth and has started to provide insights into the life thriving in DHABs.

In situ sample collection and preservation allow a closer examination of a deep-ocean community without many of the potential artefacts introduced during sample recovery and are one avenue by which we can start to address some of our outstanding questions about the activities of DHAB microbiota and processes. The recent design and application of a deep-sea sampler to DHAB studies that allows both fixation of samples at depth and incubation experiments have started to provide insights into these habitats. The microbial sampler-submersible incubation device (MS-SID) (Figure 4), designed and manufactured by C. Taylor and V. Edgcomb, Woods Hole Oceanographic Institution, Woods Hole, MA, together with McLane Research Laboratories, Falmouth, MA, USA, brought to light a number of previously unseen protists from DHABs. A previous model of a similar sampler helped to discover the novel ciliate class Cariacotrichea [88] and was used to report novel ciliates from Mediterranean DHABs that are enveloped with bacteria as ectosymbionts [89] (Figure 5). Putative symbioses between protists and prokaryotes are known to occur in anoxic marine habitats, and such partnerships are also observed in hypersaline habitats. For example, Filker et al. [90] and Foissner et al. [91] discovered and described a novel ciliate in a brine sample from solar salterns in Portugal, which was covered with prokaryotes. The latter were identified as a novel genus of Archaea belonging to the family Halobacteriaceae [90]. These discoveries give reason to assume that prokaryote-eukaryote partnerships in

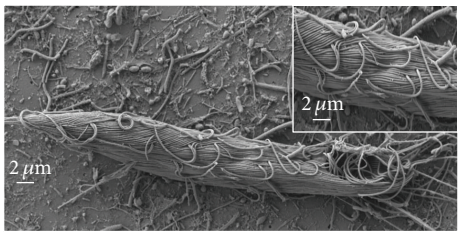

Figure 5: Ciliate from the interface of Discovery brine covered with bacterial or archaeal ectosymbionts. This partnership, also found in hypersaline ciliates from solar salterns [90], may be a survival strategy for the two partners to thrive under high-salt conditions. Modified reprint from [89].

hypersaline systems such as the DHABs may be a successful strategy for survival.

In conclusion, the research described above (integrity of extracellular DNA and DNA from cell debris, FISH assays, and the preservation of organisms at depth using state-of-the-art sampling devices) provides strong evidence not only for the existence of active polyextremophile eukaryotes in one of the most hostile (from human point of view) environments, but also for a flourishing microbial diversity.

5. Outlook

Eastern Mediterranean DHABs have now been identified as excellent sites for the discovery of novel eukaryotic and prokaryotic taxa, as model systems for astrobiology, and as habitats suitable for addressing outstanding fundamental questions in plankton ecology. Further research efforts could study biogeographic patterns, comparing DHAB communities in the Eastern Mediterranean to those in the Red Sea [92] and the Gulf of Mexico [93]. Resulting data would inform another fundamental question in ecology, namely, about the global dispersal capabilities of microbes [94]. A further interesting field of research includes the development of novel cultivation strategies for polyextremophile protists. Isolates would greatly assist in the formal description of new protist taxa, the analysis of their specific adaptation strategies (including the analysis of symbiotic partnerships between protists and prokaryotes, Figure 5), and exploitation of their genomic potential. Metatranscriptome analyses may provide insights into novel pathways that are required to function in these polyextreme habitats. Studies of the ecological role of protists in these habitats (e.g., do interfaces and brines represent hot spots of carbon turnover in a secondary microbial deep-sea food web? What are specific interactions in the complex microbial web? What is the influence of protists on dark-carbon fixation in the deep-sea?) can now be addressed through the application of specifically designed sampling devices such as MS-SID (Figure 4). DHABs represent an ideal habitat for addressing these important questions in aquatic microbial ecology, and future studies targeting DHABs will likely result in significant advances in polyextremophile research.

Disclosure

Thorsten Stoeck and Sabine Filker share first authorship, contributing equally.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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