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3 **Ciliates along oxyclines of permanently stratified marine water columns**

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Abstract

Studies of microbial communities in areas of the world where permanent marine water column oxyclines exist suggest they are 'hotspots' of microbial activity, and that these water features and the anoxic waters below them are inhabited by diverse protist taxa, including ciliates. These communities have minimal taxonomic overlap with those in overlying oxic water columns. Some ciliate taxa have been detected in multiple locations where these stable water column oxyclines exist, however, differences in such factors as hydrochemistry in the habitats that have been studied suggest local selection for distinct communities. We compare published data on ciliate communities from studies of deep marine water column oxyclines in Caricao Basin, Venezuela, and the Black Sea, with data from coastal, shallower oxycline waters in Framvaren and Mariager fjords, and from several deep-sea hypersaline anoxic basins (DHABs) in the Eastern Mediterranean Sea. Putative symbioses between Bacteria, Archaea, and ciliates observed along these oxyclines suggests a strategy of cooperative metabolism for survival that includes chemosynthetic autotrophy and exchanges of metabolic intermediates or end products between hosts and their prokaryotic partners.

Introduction

Around 1.8 billion years ago when deep ocean water masses were still mostly anaerobic (Schopf and Klein, 1992), eukaryotic life evolved on Earth, and over the last century anoxic marine habitats have provided fertile hunting grounds for novel protist taxa whose genetic signatures and cellular architecture have helped us to understand the evolution of single-celled eukaryotes. More recently, at least in part due to the global expansion of marine hypoxic and anoxic zones (Diaz and Rosenburg 2008), the microbiology of oxygen-depleted marine habitats has come under increased scrutiny from the perspective of needing to understand the likely impacts of increased oxygen depletion on marine food webs. Microbial eukaryotes are now recognized as pivotal members of aquatic microbial communities in numerical models of carbon cycling and in paradigms of surface and deep-ocean microbial ecology (Aristegui et al. 2009). They impact carbon and other nutrient cycles directly and indirectly, through grazing on prokaryotic prey and consequent regeneration of nutrients, and modification or re-mineralization of organic matter (particulate and dissolved) (Sherr and Sherr 2002; Taylor et al. 1986). In addition, they are known to affect the population dynamics, activity and physiological state of their prey (Lin et al. 2007). The main sources of mortality for marine microbes are phagotrophic protists and viruses (Aristegui et al. 2009; Suttle 2005) and the primary bacterial grazing is by flagellated protists and ciliates (Sherr and Sherr 2002; Frias-Lopez et al. 2009). The widespread application of culture-independent molecular approaches, primarily based on analysis of ribosomal RNA gene sequences amplified from environmental samples, and more recently advanced by introduction of Next Generation Sequencing methods, has revolutionized our understanding of the structure and complexity of marine microbial communities, including environments such as anoxic and deep-sea habitats. Genetic diversity detected within known protist taxa and also representing new taxa, is much greater than previously suspected using culture-based approaches, which are highly selective and appear currently capable of detecting only a fraction of taxa in environmental samples. Our understanding of eukaryotic microbial diversity along marine water column oxyclines, or transition zones between oxic seawater and anoxic/sulfidic waters, and within anoxic waters, however, lags far behind our knowledge of photic zone communities. These redox zones are found worldwide, and are now known to be hotspots of microbial activity. The steep physicochemical gradients typical of these redox zones make possible a wide range of microbial physiologies. The prokaryotic communities behind the intensive biogeochemical cycling that takes place in these habitats provide a type of microbial 'smorgasbord' for phagotrophic protists. Only recently have the activities and impacts of protist grazing been measured along such marine oxyclines (Anderson et al. 2012 Baltic Sea, Detmer et al. 1993 Baltic Sea, Lin et al. 2007 Cariaco Basin).
Ciliates are present in almost every habitat on Earth, and are commonly found in oxygen depleted and anoxic marine habitats (Lynn, 2008). They are distinguished by their dimorphic nuclei (large macronucleus accompanied by a small micronucleus), and conspicuous cilia that are present in at least some stage(s) of their life cycle. Ciliates are members of the protist superphylum Alveolata. Alveolates are among the most abundant and diverse groups of protists in marine environments (e.g., Lopez-Garcia et al. 2001; Moon-van der Staay et al. 2001; Edgcomb et al. 2011), and an anaerobic lifestyle appears

89   to have evolved independently in many unrelated ciliate groups, including the
90   karyorelictids, prostomatids, haptorids, trichostomatids, entodiniomorphids, suctorids,
91   scuticocilliatids, heterotrichids, odontostomatids, oligotrichids, and hypotrichids, some of
92   which may be facultative anaerobes (Fenchel and Finlay 1995; Corliss 1979). Ciliates are
93   one of the most conspicuous and best-studied taxa in many anaerobic communities
94   (Fenchel and Finlay 1995). Aerobes and anaerobes are found within Ciliophora, and
95   within anaerobes, energy metabolisms that include glycolysis and mixed acid
96   fermentation have been described (Fenchel and Finlay 1995). Taxa found in anaerobic
97   habitats all have mitochondria or mitochondria-like organelles called hydrogenosomes,
98   and pyruvate oxidation through H_2-excretion appears central to their anaerobic lifestyle
99   (Fenchel and Finlay 1991). Anderson et al. (2013) used RNA-SIP to demonstrate that
100 prostomatid ciliates were among the active grazers of important chemolithoautotrophic
101 epsilonproteobacteria found along pelagic oxyclines in the Baltic Sea. Protist grazing was
102 found to balance cell production of this group of bacteria, indicating the importance of
103 protist (including ciliate) grazing in regulating abundances of key redoxcline species, and
104 in turn, influencing biogeochemical cycling.
105
106   Hypoxic ($< 20\mu M$ O_2) and anoxic zones can appear in coastal regions and
107 continental seas as a result of ecosystem responses to nutrient loading and/or coastal
108 upwelling zones. Coastal eutrophication leads to decreases in dissolved oxygen as death
109 of planktonic algae introduces increased organic material to fuel microbial respiration in
110 underlying waters (Diaz and Rosenberg 2008). Such expanding oxygen depleted zones
111 have serious implications for marine food webs, and one of the best ways to understand
112 their impacts is to study permanently anoxic 'endmember' habitats. Here, we define
113 'oxycline' as the region of a stratified water column where oxygen approaches
114 undetectable levels down to where sulfide starts to appear. We focus this paper on studies
115 that report on ciliate communities along the oxycline and in anoxic waters of several
116 contrasting endmember sites that vary in depth and salinity. Most of these studies are
117 based solely on molecular data presenting small subunit ribosomal RNA gene (SSU
118 rDNA) diversity detected in environmental samples. Due to high and highly variable
119 copy numbers of this gene within ciliate taxa (Gong et al. 2013) we interpret relative
120 abundance of different ciliate taxa with caution. While additional stable anoxic marine
121 water column habitats exist, the ones discussed here represent the best studied for protist
122 diversity. The sizes of these water masses vary, as does their degree of influence from
123 riverine inputs, trophic responses to differential prey, temperature, and rates of primary
124 production in their overlying waters. These differences are likely to select for unique
125 communities in the oxyclines and anoxic waters of each site.
126   The Cariaco Basin, north of Venezuela, is the world's largest truly marine anoxic
127 system, which has remained anoxic for millions of years (Robertson and Burke 1989),
128 although it probably experience periods of oxidation (Lin et al. 2008; Peterson et al.
129 2000) (Figure 1). The Black Sea is the largest brackish anoxic basin. A 20- to 40-m-thick
130 suboxic transitional zone, characterized by low oxygen (<5 μM) and undetectable sulfide,
131 persists throughout the basin between the surface oxic layer and the sulfidic anoxic deep
132 water (≥ 100 m) (Jørgensen et al., 1991). On the other hand, Framvaren Fjord and
133 Mariager Fjord in Northern Europe are coastal brackish features with stable oxyclines
134 within the zone of significant light penetration (~10-20 m water depth), making them

135   interesting comparisons to aforementioned systems. Deep Hypersaline Anoxic Basins
136   (DHABs) are located in the Eastern Mediterranean Sea, and most described DHABs were
137   formed several thousand years ago through the dissolution of buried Messinian evaporitic
138   deposits followed by brine accumulation in seafloor depressions (Cita 2006 and
139   references therein). Their steep (typically narrow) and stable oxyclines (and haloclines)
140   exist at more than 3000m below sea level (Figure 2).
141
142   **Cariaco Basin**
143
144   The Cariaco Basin is a representative 'endmember' habitat for oxygen depleted marine
145   water columns. A relatively stable oxycline exists there between approximately 250 and
146   350m water depth, and waters are anoxic and sulfidic down to the bottom of the basin at
147   approximately 900-1200m depth. Early studies of Cariaco waters by Tuttle and Jannasch
148   (1973, 1979) revealed active chemoautotrophic bacteria in and below the oxycline that
149   can utilize reduced sulfur compounds for energy under both oxic and anoxic conditions.
150   More recent studies have shown that chemolithoautotrophic activity in the redoxcline at
151   times can match or even exceed rates of primary productivity in the surface water and
152   support an active microbial food web at depth (Taylor et al. 2001). The first study of
153   protist diversity in the Cariaco Basin revealed novel protist lineages in the anoxic portion
154   of the water column, including signatures of what appeared to be a novel ciliate class
155   (Stoeck et al. 2003) identified as 'CAR_H'. Edgcomb et al. (2011) expanded on this
156   previous work by sampling the Basin extensively at three stations, in two contrasting
157   seasons, and at four depths including the oxycline and deep, sulfidic (30 µM sulfide)
158   waters at 900m depth (Edgcomb et al. 2011; Orsi et al 2011).
159   The oxycline typically corresponds to a particle density maximum, and peaks in
160   prokaryote and protist (including ciliates) cell numbers (Edgcomb et al. 2011; Lin et al.
161   2008). Phagotrophic protists, including ciliates, are able to chemically sense prey and will
162   aggregrate in water features with higher prey concentrations (Fenchel, 1987; Sherr and
163   Sherr, 1994). Clone library and GS FLX 454 sequence data on small subunit ribosomal
164   RNA (SSU rRNA) gene signatures recovered from these two habitats revealed a picture
165   of diverse protist communities that were dominated by Alveolata (36-43% of eukaryotic
166   signatures, predominantly the ciliate subphylum Intramacronucleata, and four
167   dinoflagellate orders, Gymnodiniales, Prorocentrales, Syndiniales, and Gonyaulacales)
168   and Rhizaria). Cannonical Correspondence analysis showed that the eastern and western
169   sub-basins of the Cariaco contain unique protistan communities, which is driven in part
170   by differences in riverine inputs and primary production in the two parts of the Basin.
171   Additionally, communities were unique in different seasons (Orsi et al. 2011). Ninety
172   percent of detected protistan operational taxonomic units (OTUs) at 97% sequence
173   similarity were unique between the oxic overylying water column samples and anoxic
174   waters below (Orsi et al. 2011).
175   Approximately 20% of the 18S rRNA clone library (16,000 clones) data and
176   ~28% of GS FLX 454 data captured signatures of Ciliophora (Edgcomb et al. 2011).
177   Taxa (orders and top BLAST hit to genus) detected in the oxycline and anoxic water
178   samples are presented in Table 1. Assignment of these genetic signatures (given the
179   ~100-200 bp 454 pyrotags) to genera should be interpreted cautiously. The ciliate taxa
180   detected within the oxycline of Cariaco included *Metopus* (Armophorida), *Frontonia*

181 (Peniculida), *Euplotes* (Euplotida), *Oxytricha* (Sporadotrichida), *Strombidium*
182 (Oligotrichida), *Cariacothrix* (Cariacotrichida), and unclassified taxa affiliated with
183 Colpodida and Scuticociliatia. Relatives of *Metopus, Cariacothrix,* and *Strombidium*
184 were also observed in the underlying anoxic waters of Cariaco, as well as relatives of
185 *Cyclidium* (Pleuronematida), *Epalxella* (Odontostomida), *Prorodon* (Prorodontida), and
186 unaffiliated members of Karyorelictida, Colpodida, and Scuticociliatia. This shift in
187 ciliate taxa between the oxycline and anoxic/sulfidic waters is consistent with that
188 observed along Baltic Sea redoxclines, although taxonomic composition of ciliate
189 communities in the Baltic samples was different from Cariaco (Anderson et al. 2012).
190 Metopid ciliates are predators of bacteria that inhabit anoxic marine sediments,
191 and members of this genus are known to have hydrogenosomes in close juxtaposition to
192 endobiont methanogens. These endobionts are thought to play a role in conversion of
193 hydrogenosomally produced hydrogen, carbon dioxide and acetate into methane and
194 water (Fenchel and Finlay, 1991). Detection of these phagotrophic predators in the
195 oxycline and anoxic waters of Cariaco suggests they are adapted to these low-
196 oxygen/anoxic habitats. Species of *Frontonia* are commonly found in benthic and pelagic
197 freshwater and marine habitats, and are voracious predotors of bacteria, however they
198 typically do not survive anoxia (see discussion in Yildiz and Senler 2013), explaining
199 why they were not detected in the anoxic waters. The same pattern was observed for
200 *Euplotes* and *Oxytricha*. Ciliates of the genus *Strombidium* are known dominant
201 bacterivores along Baltic Sea redoxclines in suboxic zones, where their numbers reached
202 up to 7 cells ml^{-1} (Anderson et al. 2012). Members of *Cyclidium*, *Epalxella,*
203 scuticociliates, and karyorelictid ciliates are known to inhabit marine anoxic and sulfidic
204 habitats (Dyer 1989; Lynn 2008). *Prorodon* are mostly described to tolerate hypoxia
205 (facultative anaerobes) and not total anoxia (Fenchel and Finlay 1990), however it is
206 possible that anoxic relatives inhabit the Cariaco.
207 Molecular data for ciliates based on SSUrDNA genes provide information on the
208 content of ciliate communities, but another approach, such as, microscopy, is needed to
209 determine relative abundance within an environmental sample. Scanning electron
210 microscopy of anoxic water samples from Caricao Basin indicated that ciliates were
211 present at approximately 10^4/L and that scuticociliates (belonging to the class
212 Oligohymenophorea) and cells belonging to the recently described new ciliate class
213 Cariacotrichea (Orsi et al. 2012) were most abundant. Abundance of scuticociliate types
214 is consistent with recovery of their SSU rRNA genes in surveys of the seasonally anoxic
215 Saanich Inlet and the stratified Framvaren Fjord (see below and Orsi et al. 2012c). An
216 interesting observation was that >90% of ciliates observed on filtered anoxic water
217 samples from that study exhibited visible epibiotic microbes (Figure 3), whereas no such
218 associations between ciliates and prokaryotes were observed on filters prepared from oxic
219 water samples. The identity of these putative symbionts has not yet been determined, but
220 given the prevalence of these associations among ciliates in the anoxic waters of Cariaco,
221 this appears to play a role in the adaptation of these eukaryotes to their anoxic lifestyle in
222 these waters.
223
224 **Black Sea**
225
226 The Black Sea is the world's largest anoxic brackish water body. A strong density

227 stratification between lower salinity surface waters and higher salinity deeper waters
228 results in a steep gradient of oxygen depletion below the halocline. Water below depths
229 of about 100 m is anoxic and enriched with hydrogen sulfide (e.g. Jørgensen et al., 1991;
230 Murray and Yakushev, 2006). A broad (20-30m) transitional zone is located between the
231 oxic and anoxic/ sulfidic water layers. In this stable redox gradient both oxygen and
232 hydrogen sulfide are close to the detection limit (Jørgensen et al., 1991). The Black Sea
233 has long attracted the interest of microbial ecologists (Sorokin, 1972) but the majority of
234 studies have focused on prokaryotic communities and their function. The first –
235 microscopical – protist surveys of the suboxic and anoxic water layers of the Black Sea
236 revealed a well-adapted community of flagellates and ciliates in the Black Sea redoxcline
237 (e.g Zubkov et al., 1992). One group of ciliates consisting mainly of *Pleuronema*
238 *marimus, Askenansia* sp. and species of the families Tracheliidae, Holophryidae and
239 Amphileptidae inhabited the above the anoxic/upper boundary of H_2S, while ciliates
240 within the order Scuticociliatida – many of which bore ectobionts – dominated the upper
241 sulfidic zone. The first molecular profile of protist diversity across the water column
242 including the redoxcline in the Black Sea used denaturing gradient gel electrophoresis
243 (DGGE) of amplified eukaryotic SSU rRNA (Coolen and Shtereva, 2009), and in contrast
244 to microscopical studies presented a picture of poor protist richness. A more recent study
245 (Wylezich and Jürgens, 2011) resolved this discrepancy and revealed, using the same
246 methodological approach, a complex community structure of metabolically active protists
247 with distinct shifts in composition along the redox gradient. The majority of DGGE bands
248 occurred for the first time around the chemocline and are believed to likely represent
249 organisms that can survive and actively grow under anoxic, sulfide-influenced conditions.
250   SSU rRNA libraries were constructed for two of the water features, the suboxic
251 layer (130m) above the upper H_2S boundary, and the anoxic sulfidic layer, where the
252 peak in dark CO_2 fixations was observed (155m). The ciliate OTUs detected were 20 in
253 total (eight unique to the suboxic library and eight to the sulfidic library, and four were
254 present in both). Most of the ciliate sequences were found to be closely related to known
255 cultured representatives such as *Cryptocaryon, Prorodon, Euplotes, Pleuronema,*
256 *Strombidium, Pseudocohnilembus, Mesodinium* and *Myrionecta*, or to environmental
257 clones from other hypoxic marine systems (Gotland and Cariaco Basins, DHABs and
258 Framvaren Fjord). However, some of the Black Sea clones were only distantly related (<
259 95% sequence similarity) to all known sequences from GenBank, and thus may represent
260 new species or genera not detected by previous sequencing approaches. The sulfidic
261 library produced the highest number of new sequence types, confirming the previously
262 reported importance of this habitat for hitherto unknown microbial biodiversity (Stoeck et
263 al., 2006). Scuticociliate sequences were common in clone libraries prepared using waters
264 from both depths, but with a particular dominance in the sulfidic sample. Prostomatids
265 were detected exclusively in the suboxic library, and plagiopylids exclusively in the
266 sulfidic library. The OTU affiliated to *Pleuronema* was particularly dominant in the
267 suboxic zone (70% of all clones), but was also present in the sulfidic zone. Another
268 *Pleuronema*-like sequence was exclusively found within the suboxic clone library, while
269 OTUs related to the oligohymenophorean genus *Cyclidium* and OTUs tightly clustered
270 within the plagiopylids related to the genus *Epalxella,* were only detected in the sulfidic
271 sample. The occurrence of plagiopylids and some of the scuticociliates (*Cyclidium*-
272 related taxa) exclusively in sulfidic waters is in accordance with previous investigations

273 for such habitats (e.g. Stoeck and Epstein, 2003; Behnke et al., 2006; Zuendorf et al.,
274 2006; Stock et al., 2009). Most of the plagiopylid-like sequences were affiliated to the
275 hydrogenosome-bearing genera *Trimyema* and *Epalxella*, described from submarine
276 hydrothermal vents and a meromictic alpine lake (Baumgartner et al., 2002; Stoeck et al.,
277 2007a). The *Cyclidium*-related phylotypes clustered together with the anaerobic
278 *Cyclidium porcatum* (Clarke et al., 1993; Guggiari and Peck, 2008) and with other
279 environmental sequences originating from anoxic habitats (e.g. Framvaren Fjord, Cariaco
280 Basin). Species of this genus are known for having high intraspecific genetic divergence
281 (Fenchel and Finlay, 2006), and have often been detected in anoxic habitats using
282 morphological identification methods (Fenchel et al., 1990; Clarke et al., 1993; Guhl et
283 al., 1996) but also using molecular approaches; for example, in the sulfidic zone of the
284 Framvaren Fjord and the Gotland Deep redoxclines (Behnke et al., 2006; Stock et al.,
285 2009). Finally, one OTU related to the parasitic ciliate *Cryptocaryon irritans* was
286 detected. Although this parasite is able to survive in a free-living stage for a short while,
287 it seems to be only sporadically active in redoxclines and was previously detected in
288 libraries from Gotland Deep (Stock et al., 2009), Mariager Fjord (Zuendorf et al., 2006)
289 and Bannock interface (Edgcomb et al. 2009).
290
291 **Mariager and Framvaren Fjords**
292
293   Mariager and Framvaren Fjords are the most studied, permanently-stratified fjords
294 in terms of eukaryotic diversity. Both have shallow oxycline layers at 10-15m within the
295 photic zone. The first study of protist vertical composition in the Mariager Fjord, located
296 in the northern Denmark, was in 1990; microscopical observations revealed stratification
297 of the ciliate assemblages (Fenchel et al., 1990). *Cyclidium citrilus* and *Pleuronema* sp.
298 were found to dominate the oxycline; other common species were *Prorodon* sp., *Euplotes*
299 sp., *Uronychia transfuga* and *Peritromus* sp. In the deeper, anoxic water layers,
300 *Plagiophora frontata* and *Caenomorpha* sp. formed the highest population densities;
301 *Metopus controtus*, *Saprodynium halophile*, *Lacrymaria* sp and a *Cyclidium*-like
302 morphotype were detected. A later study (Zuendorf et al, 2006) of a single water layer
303 below the oxycline (18m) using a DNA-based approach detected the molecular signatures
304 of almost all the above taxa, as well as riboclones from the anaerobe trichostomatid
305 ciliates *Lechriopyl*a and the oligotrich ciliate *Strombidium purpureum*.
306   The Framvaren Fjord, located in southwest Norway, contains the highest
307 concentration of H_2S ever reported for an open anoxic basin reaching up to 6mM in the
308 deepest anoxic layers (Millero, 1991). Using SSU rDNA clone libraries Behnke and
309 colleagues (2006) were the first to provide molecular evidence of protist stratification
310 along this O_2/H_2S gradient. Three water features were investigated: the photic microoxic
311 interface (18 m), the lower redox transition zone/upper H_2S boundary (23 m), and a
312 highly sulfidic layer with low microbial abundance (36 m). The highest protist OTU
313 richness was noted in the upper H_2S boundary was in accordance with observations
314 obtained by light and fluorescence microscopy that revealed remarkably diverse
315 morphologies. It was hypothesized that chemoautotrophy, the dominant microbial
316 process in such habitats, supports a secondary microbial food web that stimulates the
317 growth of bacterivorous protists. As expected, the OTU richness in the high sulfide,
318 anoxic layer was the lowest. The most abundant protist group was the Alveolata and

319 within that, the ciliates. Sequences of known groups of anaerobic and micro-oxic ciliates
320 such as the families Plagiopylidae, Strombiidae, Nyctotheridae,Cycliidae, and
321 Prorodontidae, were retrieved from anoxic Framvaren waters. In a later study, the same
322 group (Behnke et al., 2010) studied the spatio-temporal variation of protist communities
323 in the aforementioned water features of the Framvaren Fjord. In all nine clone libraries (3
324 habitats in 3 seasons), ciliates and stramenopiles accounted for the largest proportion of
325 the total eukaryotic clones. Yet, as expected, at the OTU level, the protistan communities
326 from distinct habitats differed significantly, with the number of shared OTUs between
327 any two habitats being as low as 18%. This confirmed previous notions that
328 environmental factors along the stratification gradient shape biodiversity patterns.
329 Surprisingly, the intra-habitat community composition and structure varied at a
330 comparable order of magnitude over time, with only 18–28% phylotypes shared within
331 the same habitat. Regarding ciliates, 63% of phylotypes were present in only one of the
332 libraries. According to the authors the observations provided support for the seed bank
333 hypothesis (Pedros-Alio, 2006; Pedros-Alio, 2007), which states that taxa within the 'rare
334 biosphere' provides the seed for shifts in community composition in response to changes
335 in physicochemical conditions.
336
337 **Deep Hypersaline Anoxic Basins in the Eastern Mediterranean Sea**
338
339   Deep hypersaline anoxic basins (DHABs) found in the Eastern Mediterranean Sea
340 are another example of stable marine oxycline habitats, however they are even more
341 challenging environments for ciliates (and other eukaryotes) due to their hypersalinity
342 and extreme depths. All of the basins that have been studied have unique
343 hydrochemistries that result from the dissolution of different evaporitic strata laid down
344 during the Messinian salinity crisis (examples shown in Table 2). For example, Mg^{2+}
345 concentrations in Discovery Basin can reach up to 5000mM compared with 300-650 mM
346 in other basins, sodium concentrations can range from 70 mM (Discovery Basin) to 4700
347 mM, methane concentrations are variable, and sulfide can be as high as 16 mM (Urania
348 Basin) (van der Wielen et al. 2005). The oxyclines (and haloclines) of these basins are
349 typically found more than 3000m below sea level (Table 2), and the extremely high
350 densities of these basins (typically ranging from 1.13 to 1.35 x 10^3 kg m^{-3}) relative to
351 Mediterranean seawater (1.03 x 10^3 kg m^{-3}) lead to a stable and steep halocline that
352 minimizes mixing with overlying normal salinity seawater (van der Wielen et al. 2005).
353 Hypersaline environments are characterized by a low water content or water activity
354 because of the high-salt concentrations, presenting challenges for organisms living in
355 these habitats. Microbiota typically cannot tolerate water activity at or below 0.72, where
356 there is not enough free water available for general metabolic processes, and for
357 hydrating proteins and nucleic acids (Brock, 1994). Some DHABs have brines that
358 originate from seawater (thalassohaline) and are dominated by sodium chloride, while
359 others (e.g., Discovery Basin) have brines that originate from other ions (athalassohaline)
360 (Litchfield 1998).
361   Ciliates are one group of protists that have long been known to be able to adapt to
362 life in hypersaline environments (e.g., Post 1983). The first studies of protist diversity in
363 several Eastern Mediterranean DHABs using DNA-based (Edgcomb et al. 2009) and
364 RNA-based (Alexander et al. 2009) molecular approaches suggested that these habitats

365 and different basins harbored diverse and distinct protistan communities that included
366 ciliates. While ciliates were relatively rare in the overlying normal seawater above
367 Bannock Basin, the oxycline/halocline and brine water samples of Discovery and
368 Bannock produced 75% SSU rDNA signatures (at 98% sequence similarity) affiliating
369 with Alveolata, 12% of which represented ciliates, and 62% dinoflagellates (Edgcomb et
370 al. 2009). Signatures of the strictly anaerobic Armophorea were unique to the chaotrophic
371 Discovery sample, while heterotrich ciliate signatures occurred exclusively in the
372 Bannock brine. While many ciliate signatures were detected that had no close sequenced
373 affiliations in public databases, datasets from Bannock and Discovery oxycline/halocline
374 and brine included known taxa such as, *Trimyema, Strombidium, Metopus*, and
375 *Peritromus*. One clade of signatures was recovered from the thalassohaline Bannock
376 interface that was highly divergent to *Cryptocaryon irritans*, an enigmatic parasite
377 loosely affiliated with the class Prostomatea (Wright and Colorni 2002). When
378 community membership was compared for Bannock and Discovery, Jaccard indices
379 suggested that the communities were unique from one another and shared little (0.8-
380 2.8%) in species composition with overlying waters with typical marine salinity and
381 oxygen (Edgcomb et al. 2009).
382   It was presumed that the ciliates, which are successful phagotrophs, were likely
383 feeding on the abundant bacteria present, particularly along the halocline, however being
384 a DNA-based study it was difficult to infer activity from these signatures. The RNA-
385 based study by Alexander et al. (2009) provided another line of evidence for active
386 ciliates in the oxycline/halocline of a different basin, L'Atalante. This study compared
387 18S rRNA gene signatures recovered from the upper (3499 m) and lower (3501 m)
388 halocline of L'Atalante basin, where it was found that ciliates represented the largest
389 proportion (18 in upper halocline and 21 in lower) of phylotypes (43 and 42, respectively,
390 sharing 99% sequence similarity) in both libraries. Furthermore, only 12 phylotypes
391 (including 7 ciliate) were shared between the two halocline samples collected only ~1.5
392 m apart. The different community compositions are likely driven by some combination of
393 the steep gradient in electron donors and acceptors, salinity, and ammonia concentrations
394 (5.5 µM in the upper halocline to 3000 µM in the lower). In addition to ciliate signatures
395 that could not be assigned to any described riboclasses, representatives of
396 Oligohymenophorea, Spirotrichea and Prostomatea were found in both upper and lower
397 halocline libraries from L'Atalante, Plagiopylea were found only in the lower,
398 hypersaline library, and Phyllopharyngea occurred only in the upper halocline (Alexander
399 et al. 2009).
400   Thetis basin has one of the highest salt concentrations reported for DHABs
401 (348‰), its brine is 80% halite and 12% bischofite (La Cono et al. 2011), yet it supports
402 protist counts of ca. 0.6×10^4 per liter of anoxic brine (Stock et al. 2012). This RNA-
403 based study revealed that ciliates accounted for 20% of phylotypes, many of which were
404 closely related to sequences detected in surveys of other DHABs, suggesting specific
405 adaptations to these deep, hypersaline habitats. Ciliate signatures in the
406 oxycline/halocline were dominated by those affiliating with the scuticociliate
407 *Pleuronema coronatum*. This facultative anaerobe (Fenchel and Bernard 1996) taxon was
408 also detected in the brine, although at a lower abundance, suggesting this is a halotolerant
409 (not halophilic) taxon (Stock et al. 2012). Similar sequences were also found in the
410 interfaces of bischofite ($MgCl_2$) dominated Discovery Basin and thalassohaline

411 L'Atalante (Alexander et al. 2009; Edgcomb et al. 2009). In the brine of Thetis,
412 signatures of the strict anaerobe *Trimyema compressum* were found, although signatures
413 of this presumably halophilic taxon (also found in Bannock and L'Atalante basins) were
414 phylogenetically distinct from other known marine forms, suggesting the potential for
415 allopatric speciation in these relatively isolated brine habitats (Stock et al. 2012). Also
416 detected in the brine and/or oxycline/halocline of Thetis were relatives of *Strombidium*
417 and *Cyclidium* (previously described from hypersaline habitats) and *Pseudotontonia* (not
418 previously described from hypersaline habitats).
419   Further evidence for the uniqueness of protist communities in different DHABs
420 came from a comparison of ciliate communities in the brines and haloclines of four
421 different DHABs based on SSU rDNA pyrotag analysis (Stock et al. 2013). The interface
422 communities from Urania, Medee, Thetis, and Tyro basins were relatively similar to each
423 other, however there were significant differences in the brine ciliate communities from
424 each site. This suggests that there is some connectivity between the halocline
425 communities (via mixing with overlying seawater) but little between brine communities,
426 creating an 'island character' of those habitats and allowing for evolution of unique
427 assemblages. All four of these basins have thalassohaline brines, however important
428 distinctions in ionic compositions include that Medee, Tyro, Thetis, and Urania brines
429 have 792, 71, 604, and 315 mM Mg^{2+}, respectively, and Urania has 15 mM sulfide as
430 opposed to 2.1-2.9mM in the other basins (Stock et al. 2013). The brine of Medee was
431 dominated almost entirely (~89%) by relatives of the genus *Anoplophyra* (Astomatida),
432 whereas the brines of Tyro and Thetis were similar, but dominated (45% and 65%) by
433 relatives of *Strombidium* and *Novistrombidium* (30% in Tyro and 9% in Thetis brine). A
434 few taxa were found only in one of the two basins, such as *Laboea* (in Thetis brine only),
435 and a tintinnid ciliate taxon *Salpingella* (in Tyro brine only). In Urania, *Pseudotontonia*-
436 related amplicons dominated (40%). A metadata-analysis found salt and oxygen to be the
437 largest contributing environmental factors driving differentiation of ciliate communities
438 (Forster et al. 2012), so it is not surprising that different salt ion concentrations in
439 different basins would impose different physiological challenges that would select for
440 unique communities. As Stock et al. (2013) discuss, the degree to which differences in
441 ciliate communities are shaped by top-down or bottom-up factors, or by differences in
442 initial 'seed' communities, remains to be determined.
443   It is difficult based on DNA- or RNA-based markers to prove that signatures
444 represent active/living cells. Scanning electron microscopy was therefore instrumental in
445 demonstrating the presence of intact and presumably living ciliates in several of these
446 DHAB brine and halocline habitats (Orsi et al. 2012b; Stock et al. 2013). The dominant
447 ciliate morphotype present on filters prepared from Discovery Basin halocline samples
448 (>50% of total protists observed) was a narrowly fusiform ciliate present at a
449 concentration of ~3.7 x 10^5 cells L^{-1} and >80% of these cells had 10-20 µm-long, slightly
450 curved bacterial cells attached to their cortex (Orsi et al. 2012b) (Figure 4a). The
451 observation of these attached, organized arrangements of epibiotic prokaryotes supported
452 the notion that these ciliates were living. Fluorescence *in situ* hybridization (FISH)
453 confirmed that these epibionts were deltaproteobacteria (Orsi et al. 2012b). The reduction
454 of the oral cavity of this ciliate morphotype suggests that this taxon may rely less on
455 heterotrophic grazing, and more on their putative symbionts for nutrition (Orsi et al.
456 2012b). Such nutritional symbioses have been observed in other ciliates, such as the

457 karyorelictid ciliate *Kentrophoros fistulosus*, which is dependent on its sulfate-reducing
458 bacteria (Gast et al. 2009). In contrast to the fusiform ciliates observed in Discovery basin
459 halocline samples, 95% of all ciliate morphotypes observed in Urania basin
460 oxycline/halocline samples (and >50% of total eukaryotic cells) were similar to the
461 scuticociliate morphotype observed in Cariaco anoxic waters, were covered with similar
462 epibionts, and were present at a concentration of ~9.7 x 10^4 L^{-1} (Orsi et al. 2012b) (Figure
463 4b). The identity of these epibionts is still unknown beyond their hybridization to general
464 bacterial FISH probes.
465
466 **Looking Forward**
467
468   Steep chemoclines along the stable oxyclines discussed in this paper, with their
469 gradients in available electron donors and acceptors, likely select for different ciliate
470 communities and for types of symbioses among ciliates. Similarly, the community
471 structures of the "deep" water anoxic bodies appear to be shaped by the yet not fully
472 understood local physicochemical and biotic characteristics of each anoxic water entity,
473 e.g hydrogen sulfide is widely known to be toxic to eukaryotes, and hence is a strong
474 selective force. On the other hand, common ciliate populations are observed in deep
475 oxyclines and anoxic waters and shallow fjord oxyclines and anoxic waters, which are in
476 relative close proximity to the photic zone. In spite of the difficulties in making direct
477 comparisons between the protist communities in the different locations, given the limited
478 number of samples in some of these studies and physicochemical differences between
479 them, a common observation is that all these oxycline habitats are inhabited by ciliates,
480 and that ciliate communities in most cases have distinct compositions of the dominant
481 taxa. Molecular and/or microscopical approaches have detected ciliates related to
482 *Cyclidium Strobidium*, *Euplotes* and *Prorodon* in all of the above mentioned sites; for
483 studies that include abundance data the first two appear to be among the most dominant
484 taxa within ciliates. Taxa related to *Metopus*, *Mesodinium/Myrionecta*, *Cardiostomatella*
485 and *Pleuronema* were also detected in most of the sites, and have also been found in a
486 variety of anoxic environments usually in putative symbiotic association with prokaryotes.
487 Others, such as *Cariacothrix,* were only detected in one study.
488   Syntrophy with bacteria and archaea appears to a dominant strategy among
489 ciliates living along oxyclines and in anoxic water columns, who likely cooperate in
490 catabolism of organic matter. This is consistent with what is known of prokaryotic
491 syntrophies in water column and sedimentary anoxic habitats, including studies of protist-
492 prokaryote interactions (see detailed discussions in Fenchel and Finlay 1995 and
493 Hackstein 2011; 2010). Further exploration of the frequently observed putative
494 symbioses between ciliates and prokaryotes along oxycline water samples and in anoxic
495 waters will shed light on their role in marine biogeochemical cycling. While the
496 symbionts of many free-living ciliates in anoxic marine habitats are known to be
497 methanogens living in close association with host hydrogenosomes, and suggestive of a
498 cooperative metabolism centered around hydrogen transfer (e.g. Fenchel and Finlay,
499 1991; van Hoek et al. 2000; Embley and Finlay, 1993; 1994), other types of associates
500 and metabolic exchanges are also likely.
501   Seasonal variation of the ciliate communities in permanently anoxic marine water
502 bodies was not expected but, interestingly, it appears to be significant (Behnke et al. 2010,

503 Edgcomb et al 2011). This may result from seasonal changes in organic matter inputs to
504 deeper, anoxic waters. Further studies of seasonal changes in the ciliate inhabitants of the
505 permanent anoxic marine water bodies that show no or little variation in the
506 physicochemical conditions, would give exciting insights in the drivers succession of
507 abundant taxa. Seasonal studies of such communities together with their prokaryotic
508 associations have not yet been performed, and these would further elucidate if biotic
509 factors can shape anoxic ciliate assemblages, or perhaps, how such associations can shape
510 the environment through their microbial transformations.
511
512

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728   **Table and Figure Legends**
729
730   **Table 1.** Signatures of ciliate taxa recovered in studies of Cariaco Basin, Black Sea,
731   DHABs, Framvaren Fjord, and Mariager Fjord oxycline and anoxic waters.
732   n.d.=not detectable, n.r.=not reported
733    $^{\$}$ 6 montly samplings, oxycline ranged from ~11-23 m in April to ~13-17m in October
734   and anoxic layer from ~23-25 to ~17-25m
735    $^{\#}$ cannnot be converted in μM due to the lack of temperature data
736   Type of data M=microscopy counts, D=SSU rDNA, R=SSU rRNA; *Abundance (of
737   signatures or cells) data provided in source study.
738   +=present, ++=present and noted in study as relatively abundant.
739
740   **Table 2**. Physicochemical data for several Eastern Mediterranean Sea DHABs illustrating
741   variations in hydrochemistry. [1]Using the conventional sensor mounted on CTD rosette,
742   the measurement of conductivity is not reliable in athalassohaline brines enriched by
743   divalent cations.
744
745   **Figure 1.** Map of Cariaco Basin, Venezuela. Stars indicate positions of sampling stations.
746   Adapted from Edgcomb et al. 2011.
747
748   **Figure 2.** Image of the deep hypersaline anoxic basin Discovery. Top of
749   oxycline/halocline in vicinity of light 'beach,' and dark brine to the right. Image taken
750   with ROV *Jason*.
751
752   **Figure 3**. Scanning electron micrographs of scuticociliates with different epibiotic
753   bacteria recovered from the Cariaco Basin (a-c) (Caribbean Sea) B: Bacteria, Scale bar in
754   a applies to b: 9 µm, Images a-b are modified from Orsi et al., 2012b. Photographs by W.
755   Orsi.
756
757   **Figure 4.** Scanning electron micrographs of ciliates with different epibiotic bacteria
758   recovered from Urania and Discovery basins (Eastern Mediterranean Sea); a) ~9µm long
759   scuticociliate morphotype from Urania Basin (adapted from Edgcomb and Orsi 2013); b)
760   fusiform ciliate from Discovery Basin halocline (adapted from Orsi et al. 2012b) scale
761   bar 5 µm. Photographs by W. Orsi.