Ciliates along oxyclines of permanently stratified marine water columns

V.P. Edgcomb* and Maria Pachiadaki

1Department of Geology and Geophysics, Woods Hole Oceanographic Institution
Woods Hole, MA, 02543 USA

*Correspondence should be sent to VE at vedgcomb@whoi.edu

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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
to have evolved independently in many unrelated ciliate groups, including the karyorelictids, prostomatids, haptorids, trichostomatids, entodiniomorphids, suctorids, scuticociliatids, heterotrichids, odontostomatids, oligotrichids, and hypotrichids, some of which may be facultative anaerobes (Fenchel and Finlay 1995; Corliss 1979). Ciliates are one of the most conspicuous and best-studied taxa in many anaerobic communities (Fenchel and Finlay 1995). Aerobes and anaerobes are found within Ciliophora, and within anaerobes, energy metabolisms that include glycolysis and mixed acid fermentation have been described (Fenchel and Finlay 1995). Taxa found in anaerobic habitats all have mitochondria or mitochondria-like organelles called hydrogenosomes, and pyruvate oxidation through H₂-excretion appears central to their anaerobic lifestyle (Fenchel and Finlay 1991). Anderson et al. (2013) used RNA-SIP to demonstrate that prostomatid ciliates were among the active grazers of important chemolithoautotrophic epsilonproteobacteria found along pelagic oxyclines in the Baltic Sea. Protist grazing was found to balance cell production of this group of bacteria, indicating the importance of protist (including ciliate) grazing in regulating abundances of key redoxcline species, and in turn, influencing biogeochemical cycling.

Hypoxic (<20 μM O₂) and anoxic zones can appear in coastal regions and continental seas as a result of ecosystem responses to nutrient loading and/or coastal upwelling zones. Coastal eutrophication leads to decreases in dissolved oxygen as death of planktonic algae introduces increased organic material to fuel microbial respiration in underlying waters (Diaz and Rosenberg 2008). Such expanding oxygen depleted zones have serious implications for marine food webs, and one of the best ways to understand their impacts is to study permanently anoxic ‘endmember’ habitats. Here, we define ‘oxycline’ as the region of a stratified water column where oxygen approaches undetectable levels down to where sulfide starts to appear. We focus this paper on studies that report on ciliate communities along the oxycline and in anoxic waters of several contrasting endmember sites that vary in depth and salinity. Most of these studies are based solely on molecular data presenting small subunit ribosomal RNA gene (SSU rDNA) diversity detected in environmental samples. Due to high and highly variable copy numbers of this gene within ciliate taxa (Gong et al. 2013) we interpret relative abundance of different ciliate taxa with caution. While additional stable anoxic marine water column habitats exist, the ones discussed here represent the best studied for protist diversity. The sizes of these water masses vary, as does their degree of influence from riverine inputs, trophic responses to differential prey, temperature, and rates of primary production in their overlying waters. These differences are likely to select for unique communities in the oxyclines and anoxic waters of each site.

The Cariaco Basin, north of Venezuela, is the world’s largest truly marine anoxic system, which has remained anoxic for millions of years (Robertson and Burke 1989), although it probably experience periods of oxidation (Lin et al. 2008; Peterson et al. 2000) (Figure 1). The Black Sea is the largest brackish anoxic basin. A 20- to 40-m-thick suboxic transitional zone, characterized by low oxygen (<5 μM) and undetectable sulfide, persists throughout the basin between the surface oxic layer and the sulfidic anoxic deep water (≥100 m) (Jørgensen et al., 1991). On the other hand, Framvaren Fjord and Mariager Fjord in Northern Europe are coastal brackish features with stable oxyclines within the zone of significant light penetration (~10-20 m water depth), making them
interesting comparisons to aforementioned systems. Deep Hypersaline Anoxic Basins (DHABs) are located in the Eastern Mediterranean Sea, and most described DHABs were formed several thousand years ago through the dissolution of buried Messinian evaporitic deposits followed by brine accumulation in seafloor depressions (Cita 2006 and references therein). Their steep (typically narrow) and stable oxyclines (and haloclines) exist at more than 3000m below sea level (Figure 2).

Cariaco Basin

The Cariaco Basin is a representative ‘endmember’ habitat for oxygen depleted marine water columns. A relatively stable oxycline exists there between approximately 250 and 350m water depth, and waters are anoxic and sulfidic down to the bottom of the basin at approximately 900-1200m depth. Early studies of Cariaco waters by Tuttle and Jannasch (1973, 1979) revealed active chemotrophic bacteria in and below the oxycline that can utilize reduced sulfur compounds for energy under both oxic and anoxic conditions. More recent studies have shown that chemolithoautotrophic activity in the redoxcline at times can match or even exceed rates of primary productivity in the surface water and support an active microbial food web at depth (Taylor et al. 2001). The first study of protist diversity in the Cariaco Basin revealed novel protist lineages in the anoxic portion of the water column, including signatures of what appeared to be a novel ciliate class (Stoeck et al. 2003) identified as ‘CAR_H’. Edgcomb et al. (2011) expanded on this previous work by sampling the Basin extensively at three stations, in two contrasting seasons, and at four depths including the oxycline and deep, sulfidic (30 µM sulfide) waters at 900m depth (Edgcomb et al. 2011; Orsi et al 2011).

The oxycline typically corresponds to a particle density maximum, and peaks in prokaryote and protist (including ciliates) cell numbers (Edgcomb et al. 2011; Lin et al. 2008). Phagotrophic protists, including ciliates, are able to chemically sense prey and will aggregate in water features with higher prey concentrations (Fenchel, 1987; Sherr and Sherr, 1994). Clone library and GS FLX 454 sequence data on small subunit ribosomal RNA (SSU rRNA) gene signatures recovered from these two habitats revealed a picture of diverse protist communities that were dominated by Alveolata (36-43% of eukaryotic signatures, predominantly the ciliate subphylum Intramacronucleata, and four dinoflagellate orders, Gymnodiniales, Prorocentrales, Syndiniales, and Gonyaulacales) and Rhizaria). Canonical Correspondence analysis showed that the eastern and western sub-basins of the Cariaco contain unique protistan communities, which is driven in part by differences in riverine inputs and primary production in the two parts of the Basin. Additionally, communities were unique in different seasons (Orsi et al. 2011). Ninety percent of detected protistan operational taxonomic units (OTUs) at 97% sequence similarity were unique between the oxic overlying water column samples and anoxic waters below (Orsi et al. 2011).

Approximately 20% of the 18S rRNA clone library (16,000 clones) data and ~28% of GS FLX 454 data captured signatures of Ciliophora (Edgcomb et al. 2011). Taxa (orders and top BLAST hit to genus) detected in the oxycline and anoxic water samples are presented in Table 1. Assignment of these genetic signatures (given the ~100-200 bp 454 pyrotags) to genera should be interpreted cautiously. The ciliate taxa detected within the oxycline of Cariaco included *Metopus* (Armophorida), *Frontonia*
(Peniculida), *Euplotes* (Euplotida), *Oxytricha* (Sporadotrichida), *Strombidium* (Oligotrichida), *Cariacothrix* (Cariacotrichida), and unclassified taxa affiliated with Colpodida and Scuticociliatia. Relatives of *Metopus*, *Cariacothrix*, and *Strombidium* were also observed in the underlying anoxic waters of Cariaco, as well as relatives of *Cyclidium* (Pleuronematida), *Epaxxella* (Odontostomida), *Prorodon* (Prorodontida), and unaffiliated members of Karyorelictida, Colpodida, and Scuticociliatia. This shift in ciliate taxa between the oxycline and anoxic/sulfidic waters is consistent with that observed along Baltic Sea redoxclines, although taxonomic composition of ciliate communities in the Baltic samples was different from Cariaco (Anderson et al. 2012).

Metopid ciliates are predators of bacteria that inhabit anoxic marine sediments, and members of this genus are known to have hydrogenosomes in close juxtaposition to endobiont methanogens. These endobionts are thought to play a role in conversion of hydrogenosomally produced hydrogen, carbon dioxide and acetate into methane and water (Fenchel and Finlay, 1991). Detection of these phagotrophic predators in the oxycline and anoxic waters of Cariaco suggests they are adapted to these low-oxygen/anoxic habitats. Species of *Frontonia* are commonly found in benthic and pelagic freshwater and marine habitats, and are voracious predators of bacteria, however they typically do not survive anoxia (see discussion in Yildiz and Senler 2013), explaining why they were not detected in the anoxic waters. The same pattern was observed for *Euplotes* and *Oxytricha*. Ciliates of the genus *Strombidium* are known dominant bacterivores along Baltic Sea redoxclines in suboxic zones, where their numbers reached up to 7 cells ml⁻¹ (Anderson et al. 2012). Members of *Cyclidium*, *Epaxxella*, scuticociliates, and karyorelictid ciliates are known to inhabit marine anoxic and sulfidic habitats (Dyer 1989; Lynn 2008). *Prorodon* are mostly described to tolerate hypoxia (facultative anaerobes) and not total anoxia (Fenchel and Finlay 1990), however it is possible that anoxic relatives inhabit the Cariaco.

Molecular data for ciliates based on SSUrDNA genes provide information on the content of ciliate communities, but another approach, such as, microscopy, is needed to determine relative abundance within an environmental sample. Scanning electron microscopy of anoxic water samples from Caricoa Basin indicated that ciliates were present at approximately 10⁴/L and that scuticociliates (belonging to the class Oligohymenophorea) and cells belonging to the recently described new ciliate class Cariacotrichhea (Orsi et al. 2012) were most abundant. Abundance of scuticociliate types is consistent with recovery of their SSU rRNA genes in surveys of the seasonally anoxic Saanich Inlet and the stratified Framvaren Fjord (see below and Orsi et al. 2012c). An interesting observation was that >90% of ciliates observed on filtered anoxic water samples from that study exhibited visible epibiotic microbes (Figure 3), whereas no such associations between ciliates and prokaryotes were observed on filters prepared from oxic water samples. The identity of these putative symbionts has not yet been determined, but given the prevalence of these associations among ciliates in the anoxic waters of Cariaco, this appears to play a role in the adaptation of these eukaryotes to their anoxic lifestyle in these waters.

**Black Sea**

The Black Sea is the world’s largest anoxic brackish water body. A strong density
stratification between lower salinity surface waters and higher salinity deeper waters results in a steep gradient of oxygen depletion below the halocline. Water below depths of about 100 m is anoxic and enriched with hydrogen sulfide (e.g. Jørgensen et al., 1991; Murray and Yakushev, 2006). A broad (20-30m) transitional zone is located between the oxic and anoxic/sulfidic water layers. In this stable redox gradient both oxygen and hydrogen sulfide are close to the detection limit (Jørgensen et al., 1991). The Black Sea has long attracted the interest of microbial ecologists (Sorokin, 1972) but the majority of studies have focused on prokaryotic communities and their function. The first microscopical – protist surveys of the suboxic and anoxic water layers of the Black Sea revealed a well-adapted community of flagellates and ciliates in the Black Sea redoxcline (e.g Zubkov et al., 1992). One group of ciliates consisting mainly of *Pleuronema marinus*, *Askenansia* sp. and species of the families Tracheliidae, Holophryidae and Amphileptidae inhabited the above the anoxic/upper boundary of H₂S, while ciliates within the order Scuticociliatida – many of which bore ectobionts – dominated the upper sulfidic zone. The first molecular profile of protist diversity across the water column including the redoxcline in the Black Sea used denaturing gradient gel electrophoresis (DGGE) of amplified eukaryotic SSU rRNA (Coolen and Shtereva, 2009), and in contrast to microscopical studies presented a picture of poor protist richness. A more recent study (Wylezich and Jürgens, 2011) resolved this discrepancy and revealed, using the same methodological approach, a complex community structure of metabolically active protists with distinct shifts in composition along the redox gradient. The majority of DGGE bands occurred for the first time around the chemocline and are believed to likely represent organisms that can survive and actively grow under anoxic, sulfide-influenced conditions. SSU rRNA libraries were constructed for two of the water features, the suboxic layer (130m) above the upper H₂S boundary, and the anoxic sulfidic layer, where the peak in dark CO₂ fixations was observed (155m). The ciliate OTUs detected were 20 in total (eight unique to the suboxic library and eight to the sulfidic library, and four were present in both). Most of the ciliate sequences were found to be closely related to known cultured representatives such as *Cryptocaryon*, *Prorodon*, *Euplotes*, *Pleuronema*, *Strombidium*, *Pseudocohnilembus*, *Mesodinium* and *Myrionecta*, or to environmental clones from other hypoxic marine systems (Gotland and Cariaco Basins, DHABs and Framvaren Fjord). However, some of the Black Sea clones were only distantly related (< 95% sequence similarity) to all known sequences from GenBank, and thus may represent new species or genera not detected by previous sequencing approaches. The sulfidic library produced the highest number of new sequence types, confirming the previously reported importance of this habitat for hitherto unknown microbial biodiversity (Stoeck et al., 2006). Scuticociliate sequences were common in clone libraries prepared using waters from both depths, but with a particular dominance in the sulfidic sample. Prostomatids were detected exclusively in the suboxic library, and plagiopylids exclusively in the sulfidic library. The OTU affiliated to *Pleuronema* was particularly dominant in the suboxic zone (70% of all clones), but was also present in the sulfidic zone. Another *Pleuronema*-like sequence was exclusively found within the suboxic clone library, while OTUs related to the oligohymenophorean genus *Cyclidium* and OTUs tightly clustered within the plagiopylids related to the genus *Epalxella*, were only detected in the sulfidic sample. The occurrence of plagiopylids and some of the scuticociliates (*Cyclidium*-related taxa) exclusively in sulfidic waters is in accordance with previous investigations.
for such habitats (e.g. Stoeck and Epstein, 2003; Behnke et al., 2006; Zuendorf et al., 2006; Stock et al., 2009). Most of the plagiopylid-like sequences were affiliated to the hydrogenosome-bearing genera *Trimyema* and *Epalxella*, described from submarine hydrothermal vents and a meromictic alpine lake (Baumgartner et al., 2002; Stoeck et al., 2007a). The *Cyclidium*-related phylotypes clustered together with the anaerobic *Cyclidium porcatum* (Clarke et al., 1993; Guggiari and Peck, 2008) and with other environmental sequences originating from anoxic habitats (e.g. Framvaren Fjord, Cariaco Basin). Species of this genus are known for having high intraspecific genetic divergence (Fenchel and Finlay, 2006), and have often been detected in anoxic habitats using morphological identification methods (Fenchel et al., 1990; Clarke et al., 1993; Guhl et al., 1996) but also using molecular approaches; for example, in the sulfidic zone of the Framvaren Fjord and the Gotland Deep redoxclines (Behnke et al., 2006; Stock et al., 2009). Finally, one OTU related to the parasitic ciliate *Cryptocaryon irritans* was detected. Although this parasite is able to survive in a free-living stage for a short while, it seems to be only sporadically active in redoxclines and was previously detected in libraries from Gotland Deep (Stock et al., 2009), Mariager Fjord (Zuendorf et al., 2006) and Bannock interface (Edgcomb et al. 2009).

**Mariager and Framvaren Fjords**

Mariager and Framvaren Fjords are the most studied, permanently-stratified fjords in terms of eukaryotic diversity. Both have shallow oxycline layers at 10-15m within the photic zone. The first study of protist vertical composition in the Mariager Fjord, located in the northern Denmark, was in 1990; microscopical observations revealed stratification of the ciliate assemblages (Fenchel et al., 1990). *Cyclidium citrilus* and *Pleuronema* sp. were found to dominate the oxycline; other common species were *Prorodon* sp., *Euplotes* sp., *Uronychia transfuga* and *Peritromus* sp. In the deeper, anoxic water layers, *Plagiophora frontata* and *Caenomorpha* sp. formed the highest population densities; *Metopus controtus*, *Saprodyrium halophile*, *Lacrymaria* sp and a *Cyclidium*-like morphotype were detected. A later study (Zuendorf et al, 2006) of a single water layer below the oxycline (18m) using a DNA-based approach detected the molecular signatures of almost all the above taxa, as well as riboclones from the anaerobe trichostomatid ciliates *Lechriopyla* and the oligotrich ciliate *Srombidium purpureum*.

The Framvaren Fjord, located in southwest Norway, contains the highest concentration of H$_2$S ever reported for an open anoxic basin reaching up to 6mM in the deepest anoxic layers (Millero, 1991). Using SSU rDNA clone libraries Behnke and colleagues (2006) were the first to provide molecular evidence of protist stratification along this O$_2$/H$_2$S gradient. Three water features were investigated: the photic microoxic interface (18 m), the lower redox transition zone/upper H$_2$S boundary (23 m), and a highly sulfidic layer with low microbial abundance (36 m). The highest protist OTU richness was noted in the upper H$_2$S boundary was in accordance with observations obtained by light and fluorescence microscopy that revealed remarkably diverse morphologies. It was hypothesized that chemoautotrophy, the dominant microbial process in such habitats, supports a secondary microbial food web that stimulates the growth of bacterivorous protists. As expected, the OTU richness in the high sulfide, anoxic layer was the lowest. The most abundant protist group was the Alveolata and
within that, the ciliates. Sequences of known groups of anaerobic and micro-oxic ciliates
such as the families Plagiopylidae, Strombiidae, Nyctotheridae, Cyclidiidae, and
Prorodontidae, were retrieved from anoxic Framvaren waters. In a later study, the same
group (Behnke et al., 2010) studied the spatio-temporal variation of protist communities
in the aforementioned water features of the Framvaren Fjord. In all nine clone libraries (3
habitats in 3 seasons), ciliates and stramenopiles accounted for the largest proportion of
the total eukaryotic clones. Yet, as expected, at the OTU level, the protistan communities
from distinct habitats differed significantly, with the number of shared OTUs between
any two habitats being as low as 18%. This confirmed previous notions that
environmental factors along the stratification gradient shape biodiversity patterns.
Surprisingly, the intra-habitat community composition and structure varied at a
comparable order of magnitude over time, with only 18–28% phylotypes shared within
the same habitat. Regarding ciliates, 63% of phylotypes were present in only one of the
libraries. According to the authors the observations provided support for the seed bank
hypothesis (Pedros-Alio, 2006; Pedros-Alio, 2007), which states that taxa within the ‘rare
biosphere’ provides the seed for shifts in community composition in response to changes
in physicochemical conditions.

Deep Hypersaline Anoxic Basins in the Eastern Mediterranean Sea

Deep hypersaline anoxic basins (DHABs) found in the Eastern Mediterranean Sea
are another example of stable marine oxycline habitats, however they are even more
challenging environments for ciliates (and other eukaryotes) due to their hypersalinity
and extreme depths. All of the basins that have been studied have unique
hydrochemistries that result from the dissolution of different evaporitic strata laid down
during the Messinian salinity crisis (examples shown in Table 2). For example, Mg\(^{2+}\)
concentrations in Discovery Basin can reach up to 5000mM compared with 300-650 mM
in other basins, sodium concentrations can range from 70 mM (Discovery Basin) to 4700
mM, methane concentrations are variable, and sulfide can be as high as 16 mM (Urania
Basin) (van der Wielen et al. 2005). The oxyclines (and haloclines) of these basins are
typically found more than 3000m below sea level (Table 2), and the extremely high
densities of these basins (typically ranging from 1.13 to 1.35 x 10\(^3\) kg m\(^{-3}\)) relative to
Mediterranean seawater (1.03 x 10\(^3\) kg m\(^{-3}\)) lead to a stable and steep halocline that
minimizes mixing with overlying normal salinity seawater (van der Wielen et al. 2005).
Hypersaline environments are characterized by a low water content or water activity
because of the high-salt concentrations, presenting challenges for organisms living in
these habitats. Microbiota typically cannot tolerate water activity at or below 0.72, where
there is not enough free water available for general metabolic processes, and for
hydrating proteins and nucleic acids (Brock, 1994). Some DHABs have brines that
originate from seawater (thalassohaline) and are dominated by sodium chloride, while
others (e.g., Discovery Basin) have brines that originate from other ions (athalassohaline)
(Litchfield 1998).

Ciliates are one group of protists that have long been known to be able to adapt to
life in hypersaline environments (e.g., Post 1983). The first studies of protist diversity in
several Eastern Mediterranean DHABs using DNA-based (Edgcomb et al. 2009) and
RNA-based (Alexander et al. 2009) molecular approaches suggested that these habitats
and different basins harbored diverse and distinct protistan communities that included
ciliates. While ciliates were relatively rare in the overlying normal seawater above
Bannock Basin, the oxycline/halocline and brine water samples of Discovery and
Bannock produced 75% SSU rDNA signatures (at 98% sequence similarity) affiliating
with Alveolata, 12% of which represented ciliates, and 62% dinoflagellates (Edgcomb et
al. 2009). Signatures of the strictly anaerobic Armophorea were unique to the chaotrophic
Discovery sample, while heterotrich ciliate signatures occurred exclusively in the
Bannock brine. While many ciliate signatures were detected that had no close sequenced
affiliations in public databases, datasets from Bannock and Discovery oxycline/halocline
and brine included known taxa such as, Trimyema, Strombidium, Metopus, and
Peritrichus. One clade of signatures was recovered from the thalassohaline Bannock
interface that was highly divergent to Cryptocaryon irritans, an enigmatic parasite
loosely affiliated with the class Prostomatea (Wright and Colorni 2002). When
community membership was compared for Bannock and Discovery, Jaccard indices
suggested that the communities were unique from one another and shared little (0.8-
2.8%) in species composition with overlying waters with typical marine salinity and
oxygen (Edgcomb et al. 2009).

It was presumed that the ciliates, which are successful phagotrophs, were likely
feeding on the abundant bacteria present, particularly along the halocline, however being
a DNA-based study it was difficult to infer activity from these signatures. The RNA-
based study by Alexander et al. (2009) provided another line of evidence for active
ciliates in the oxycline/halocline of a different basin, L’Atalante. This study compared
18S rRNA gene signatures recovered from the upper (3499 m) and lower (3501 m)
halocline of L’Atalante basin, where it was found that ciliates represented the largest
proportion (18 in upper halocline and 21 in lower) of phylotypes (43 and 42, respectively,
sharing 99% sequence similarity) in both libraries. Furthermore, only 12 phylotypes
(including 7 ciliate) were shared between the two halocline samples collected only ~1.5
m apart. The different community compositions are likely driven by some combination of
the steep gradient in electron donors and acceptors, salinity, and ammonia concentrations
(5.5 µM in the upper halocline to 3000 µM in the lower). In addition to ciliate signatures
that could not be assigned to any described riboclasses, representatives of
Oligohymenophorea, Spirotrichea and Prostomatea were found in both upper and lower
halocline libraries from L’Atalante, Plagiopylea were found only in the lower,
hypersaline library, and Phyllopharyngea occurred only in the upper halocline (Alexander
et al. 2009).

Thetis basin has one of the highest salt concentrations reported for DHABs
(348‰), its brine is 80% halite and 12% bischofite (La Cono et al. 2011), yet it supports
protist counts of ca. 0.6 x 10^4 per liter of anoxic brine (Stock et al. 2012). This RNA-
based study revealed that ciliates accounted for 20% of phylotypes, many of which were
closely related to sequences detected in surveys of other DHABs, suggesting specific
adaptations to these deep, hypersaline habitats. Ciliate signatures in the
oxycline/halocline were dominated by those affiliating with the scuticociliate
Pleuronema coronatum. This facultative anaerobe (Fenchel and Bernard 1996) taxon was
also detected in the brine, although at a lower abundance, suggesting this is a halotolerant
(not halophilic) taxon (Stock et al. 2012). Similar sequences were also found in the
interfaces of bischofite (MgCl_2) dominated Discovery Basin and thalassohaline
L’Atalante (Alexander et al. 2009; Edgcomb et al. 2009). In the brine of Thetis, signatures of the strict anaerobe *Trimyema compressum* were found, although signatures of this presumably halophilic taxon (also found in Bannock and L’Atalante basins) were phylogenetically distinct from other known marine forms, suggesting the potential for allopatric speciation in these relatively isolated brine habitats (Stock et al. 2012). Also detected in the brine and/or oxycline/halocline of Thetis were relatives of *Strombidium* and *Cyclidium* (previously described from hypersaline habitats) and *Pseudotontonia* (not previously described from hypersaline habitats).

Further evidence for the uniqueness of protist communities in different DHABs came from a comparison of ciliate communities in the brines and haloclines of four different DHABs based on SSU rDNA pyrotag analysis (Stock et al. 2013). The interface communities from Urania, Medee, Thetis, and Tyro basins were relatively similar to each other, however there were significant differences in the brine ciliate communities from each site. This suggests that there is some connectivity between the halocline communities (via mixing with overlying seawater) but little between brine communities, creating an ‘island character’ of those habitats and allowing for evolution of unique assemblages. All four of these basins have thalassohaline brines, however important distinctions in ionic compositions include that Medee, Tyro, Thetis, and Urania brines have 792, 71, 604, and 315 mM Mg^{2+}, respectively, and Urania has 15 mM sulfide as opposed to 2.1-2.9 mM in the other basins (Stock et al. 2013). The brine of Medee was dominated almost entirely (~89%) by relatives of the genus *Anoplophyra* (Astomatida), whereas the brines of Tyro and Thetis were similar, but dominated (45% and 65%) by relatives of *Strombidium* and *Novistrombidium* (30% in Tyro and 9% in Thetis brine). A few taxa were found only in one of the two basins, such as *Laboea* (in Thetis brine only), and a tintinnid ciliate taxon *Salpingella* (in Tyro brine only). In Urania, *Pseudotontonia*-related amplicons dominated (40%). A metadata-analysis found salt and oxygen to be the largest contributing environmental factors driving differentiation of ciliate communities (Forster et al. 2012), so it is not surprising that different salt ion concentrations in different basins would impose different physiological challenges that would select for unique communities. As Stock et al. (2013) discuss, the degree to which differences in ciliate communities are shaped by top-down or bottom-up factors, or by differences in initial ‘seed’ communities, remains to be determined.

It is difficult based on DNA- or RNA-based markers to prove that signatures represent active/living cells. Scanning electron microscopy was therefore instrumental in demonstrating the presence of intact and presumably living ciliates in several of these DHAB brine and halocline habitats (Orsi et al. 2012b; Stock et al. 2013). The dominant ciliate morphotype present on filters prepared from Discovery Basin halocline samples (>50% of total protists observed) was a narrowly fusiform ciliate present at a concentration of ~3.7 x 10^5 cells L^{-1} and >80% of these cells had 10-20 µm-long, slightly curved bacterial cells attached to their cortex (Orsi et al. 2012b) (Figure 4a). The observation of these attached, organized arrangements of epibiotic prokaryotes supported the notion that these ciliates were living. Fluorescence in situ hybridization (FISH) confirmed that these epibionts were deltaproteobacteria (Orsi et al. 2012b). The reduction of the oral cavity of this ciliate morphotype suggests that this taxon may rely less on heterotrophic grazing, and more on their putative symbionts for nutrition (Orsi et al. 2012b). Such nutritional symbioses have been observed in other ciliates, such as the...
karyorelictid ciliate *Kentrophoros fistulosus*, which is dependent on its sulfate-reducing bacteria (Gast et al. 2009). In contrast to the fusiform ciliates observed in Discovery basin halocline samples, 95% of all ciliate morphotypes observed in Urania basin oxycline/halocline samples (and >50% of total eukaryotic cells) were similar to the scuticociliate morphotype observed in Cariaco anoxic waters, were covered with similar epibionts, and were present at a concentration of $\sim 9.7 \times 10^4$ L$^{-1}$ (Orsi et al. 2012b) (Figure 4b). The identity of these epibionts is still unknown beyond their hybridization to general bacterial FISH probes.

**Looking Forward**

Steep chemoclines along the stable oxyclines discussed in this paper, with their gradients in available electron donors and acceptors, likely select for different ciliate communities and for types of symbioses among ciliates. Similarly, the community structures of the “deep” water anoxic bodies appear to be shaped by the yet not fully understood local physicochemical and biotic characteristics of each anoxic water entity, e.g. hydrogen sulfide is widely known to be toxic to eukaryotes, and hence is a strong selective force. On the other hand, common ciliate populations are observed in deep oxyclines and anoxic waters and shallow fjord oxyclines and anoxic waters, which are in relative close proximity to the photic zone. In spite of the difficulties in making direct comparisons between the protist communities in the different locations, given the limited number of samples in some of these studies and physicochemical differences between them, a common observation is that all these oxycline habitats are inhabited by ciliates, and that ciliate communities in most cases have distinct compositions of the dominant taxa. Molecular and/or microscopical approaches have detected ciliates related to *Cyclidium*, *Strobilium*, *Euplotes* and *Prorodon* in all of the above mentioned sites; for studies that include abundance data the first two appear to be among the most dominant taxa within ciliates. Taxa related to *Metopus*, *Mesodinium/Myrionecta*, *Cardiostomatella* and *Pleuronema* were also detected in most of the sites, and have also been found in a variety of anoxic environments usually in putative symbiotic association with prokaryotes. Others, such as *Cariacothrix*, were only detected in one study.

Syntrophy with bacteria and archaea appears to a dominant strategy among ciliates living along oxyclines and in anoxic water columns, who likely cooperate in catabolism of organic matter. This is consistent with what is known of prokaryotic syntrophies in water column and sedimentary anoxic habitats, including studies of protist-prokaryote interactions (see detailed discussions in Fenchel and Finlay 1995 and Hackstein 2011; 2010). Further exploration of the frequently observed putative symbioses between ciliates and prokaryotes along oxycline water samples and in anoxic waters will shed light on their role in marine biogeochemical cycling. While the symbionts of many free-living ciliates in anoxic marine habitats are known to be methanogens living in close association with host hydrogenosomes, and suggestive of a cooperative metabolism centered around hydrogen transfer (e.g. Fenchel and Finlay, 1991; van Hoek et al. 2000; Embley and Finlay, 1993; 1994), other types of associates and metabolic exchanges are also likely.

Seasonal variation of the ciliate communities in permanently anoxic marine water bodies was not expected but, interestingly, it appears to be significant (Behnke et al. 2010,
Edgcomb et al. (2011). This may result from seasonal changes in organic matter inputs to deeper, anoxic waters. Further studies of seasonal changes in the ciliate inhabitants of the permanent anoxic marine water bodies that show no or little variation in the physicochemical conditions, would give exciting insights in the drivers succession of abundant taxa. Seasonal studies of such communities together with their prokaryotic associations have not yet been performed, and these would further elucidate if biotic factors can shape anoxic ciliate assemblages, or perhaps, how such associations can shape the environment through their microbial transformations.

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References


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Table and Figure Legends

Table 1. Signatures of ciliate taxa recovered in studies of Cariaco Basin, Black Sea, DHABs, Framvaren Fjord, and Mariager Fjord oxycline and anoxic waters.

| Table 2. Physicochemical data for several Eastern Mediterranean Sea DHABs illustrating variations in hydrochemistry. Using the conventional sensor mounted on CTD rosette, the measurement of conductivity is not reliable in athalassohaline brines enriched by divalent cations. |

Figure 1. Map of Cariaco Basin, Venezuela. Stars indicate positions of sampling stations. Adapted from Edgcomb et al. 2011.

Figure 2. Image of the deep hypersaline anoxic basin Discovery. Top of oxycline/halocline in vicinity of light ‘beach,’ and dark brine to the right. Image taken with ROV Jason.

Figure 3. Scanning electron micrographs of scuticociliates with different epibiotic bacteria recovered from the Cariaco Basin (a-c) (Caribbean Sea) B: Bacteria, Scale bar in a applies to b: 9 µm, Images a-b are modified from Orsi et al., 2012b. Photographs by W. Orsi.

Figure 4. Scanning electron micrographs of ciliates with different epibiotic bacteria recovered from Urania and Discovery basins (Eastern Mediterranean Sea); a) ~9µm long scuticociliate morphotype from Urania Basin (adapted from Edgcomb and Orsi 2013); b) fusiform ciliate from Discovery Basin halocline (adapted from Orsi et al. 2012b) scale bar 5 µm. Photographs by W. Orsi.