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Marine protist associations and environmental impacts across trophic levels in the twilight zone and below

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Abstract

Marine protists are integral to marine food webs and exhibit complex relationships with other microbial taxa. Phagotrophic protists contribute significantly to carbon turnover in the sunlit ocean and evidence suggests grazing in the dark ocean can be significant as well. New *in situ* sampling technologies hold great promise for more accurately accessing these impacts. The molecular signatures of parasitic protists comprise significant fractions of many high-throughput sequencing datasets, suggesting a major role in controlling populations of their host(s). The prokaryotic symbionts of free-living protists can be numerous, and, particularly in low-oxygen to anoxic marine habitats, their collective metabolisms may contribute significantly to biogeochemical cycling. This short review addresses principally planktonic communities in the meso- and bathypelagic dark ocean.

Introduction

Protists are unicellular eukaryotes that exhibit complex interactions with other protists, Metazoa, Bacteria, Archaea and viruses. This short review focuses on discussion of protist populations in the dark ocean, or that portion of the ocean below the photic zone. This realm contains a wide spectrum of habitats, including aquatic and benthic habitats, each of which can vary significantly in physico-chemical properties. For example, benthic habitats include organic-poor to organic-rich sediments that can range from oxic to anoxic, hardrock substrates, and hydrothermal vents and seeps. Pelagic habitats can also vary significantly in physico-chemical properties. Bottom bathymetry, rates of primary production in overlying waters, nutrient loading of overlying waters, currents, and temperature are all examples of factors that influence the chemistry of the water column. The pelagic water column is divided into several realms: the epipelagic or photic zone (0-200m depth), the mesopelagic (200-1000m), the bathypelagic (1000-4000m), the abyssopelagic (4000-6000m), and the hadal zone (below 6000m). Deep pelagic habitats commonly have near-saturated oxygen concentrations (~9 mg/L), but regions of the world oceans are increasingly common where partial to complete depletion of oxygen can be found at depth. Oxygen concentration is known to be a factor that shapes microbial community composition, and hence, trophic relationships. It therefore stands to reason that microbial interactions observed in widely varied pelagic and benthic marine habitats with variable concentrations and compositions of carbon sources and potential electron donors and acceptors, are likely to involve different players and different types of interactions.

Our understanding of marine protist diversity and ecology has expanded tremendously over recent decades in significant part due to information from high

throughput sequencing approaches based on bulk extracted DNA/RNA. Recent efforts on a large scale that have included data on marine eukaryotes include the circumglobal TaraOceans study of plankton in the photic zone [1] and Malaspina expedition that included analysis of protists in the bathypelagic [2]. Protists (or their molecular signatures) have been described from all marine habitats investigated, and are recognized as pivotal members of aquatic microbial communities in models of carbon cycling [3]. In near-surface, meso- and bathy-pelagic water columns and sediments, protists impact carbon and other nutrient cycles directly and indirectly, as primary producers, symbioses with other protists (including parasitic) and with prokaryotes, and through grazing on organic matter and prokaryotic prey, and consequent regeneration of nutrients (particulate and dissolved) (Figure 1). The main sources of mortality for marine microorganisms are phagotrophic protists and viruses [3,4]. Through grazing, protists actively contribute to the transfer of organic carbon from the dissolved fraction to higher trophic levels, in a heterotrophic food chain involving bacteria, small and large nanoflagellates (2-20 μ m), larger flagellates, and ciliates (e.g., [5,6]), that has been termed the “microbial loop” [7,8]. This review is restricted to perspectives on trophic impacts of marine protists in the less-studied but extensive habitats that exist in the mesopelagic (200-1000m), or twilight zone, and bathypelagic (1000-4000m), and on observed associations with other eukaryotic and prokaryotic microorganisms in those same environments. Considering that deep ocean waters represent the largest pool of dissolved organic carbon [9,10] and that respiration of this carbon in dark pelagic layers accounts for up to one third of oceanic biological CO₂ production [11,12] it is important to understand food web dynamics in the dark ocean.

Protist Predatory interactions

Phagotrophic protists can significantly impact the structure of prey populations, as well as the activity and physiological state of their prey [13,14], sometimes by exhibiting prey preferences (e.g., [5,14]). Although protist communities in the dark ocean can be diverse (e.g., [15,16]), abundances are typically observed to decrease with depth, with the exception of hotspots of abundance that can exist along oxyclines and redoxclines [17-19] (Figure 2). An overview of protist abundance in the dark ocean is provided by the recent Malaspina circumnavigation that sampled heterotrophic protists in the mesopelagic and bathypelagic ocean down to 4000m. That study reported that protist numbers decreased from an average of ~72 cells/ml in mesopelagic waters to ~11 cells/ml in bathypelagic waters, with a concomitant decrease in total biomass [20]. It should be noted, however, that this study and almost all studies published to date that provide protist cell counts, base count data on samples collected with Niskin bottles, and commonly incorporate a prefilter. Prefilters are likely to remove an unknown fraction of larger protists as well as protists associated with particulate matter, and depending on volumes of water filtered, prefilters may also start to trap smaller protists as well as prokaryotes. Collection of water samples from the deep ocean using Niskin bottles may also cause sensitive protists to lyse during transit to the ocean surface [21]. As a result, published studies of protists in the mesopelagic and below may underestimate protist abundance and diversity to an unknown degree. Similarly, studies of the activities of phagotrophic protists have been conducted mainly within the photic zone (e.g., [22-27]) and primarily using water samples collected with Niskin bottles. Little is known about the

93 grazing impact of protists below the upper mesopelagic zone.

94 A few studies have been conducted using waters collected from deep marine
95 redoxclines. Protist grazing was found to control the growth and potentially, the
96 distribution of the major epsilonproteobacterial chemoautotrophic denitrifier along the
97 Black Sea oxic/anoxic interface, *Sulfurimonas* (subgroup GD17) [28]. Cho et al [29]
98 examined grazing rates of heterotrophic nanoflagellates on bacteria in the epipelagic and
99 mesopelagic zones of the East China Sea in Korea using fluorescently-labeled prey.
100 Despite lower bacterial abundance and production in the mesopelagic relative to the
101 epipelagic, clearance rates were equivalent and sometimes higher in the mesopelagic
102 samples. This observation highlights the potentially important role of protists in carbon
103 turnover in the dark ocean.

104 Recently, two studies of protist grazing were conducted that included samples
105 from the oxic bathypelagic, also utilizing fluorescently-labeled bacterial prey. Using
106 shipboard experiments, as is typical for grazing studies, Rocke et al. [30] found that
107 protist grazing removed 3.8 to 31% of the standing stock of planktonic (free-living)
108 prokaryotes from Antarctic Intermediate Water (750-875m depth) and North Atlantic
109 Deep Water (2500m depth). Pachiadaki et al [31] was the first to employ new technology
110 that allowed grazing studies to be conducted *in situ* instead of returning samples to the
111 ocean surface prior to initiating experiments. The use of new *in situ* incubation
112 technologies minimizes the potential biases introduced to such studies because the protist
113 grazers are not subjected to (often significant) pressure changes and other physico-
114 chemical parameters, such as, redox, temperature, and ambient light during transport of
115 seawater to the surface and initiation of shipboard incubation experiments. Such

alterations have the potential to either kill some fraction of the protist grazer community, or alter their behavior. The use of these new incubation technologies is therefore expected to provide a more accurate assessment of *in situ* activities of microbiota. As expected, a decline in abundances of prokaryotes and protists in the Eastern Mediterranean Sea was observed with depth from the euphotic zone (40m) to the mesopelagic (200, 500, and 950m depth) realm. At 3000m prokaryotes averaged $\sim 9 \times 10^4$ per mL and protists (predominantly small flagellates) ~ 223 per mL, and the percentage of prokaryotes removed daily by grazing was estimated at $\sim 9\%$. These protist counts are within the same order of magnitude as reported in other bathypelagic samples (~ 14 per mL) in the global ocean (discussed in Pernice et al. [2]). In such relatively dilute environments, protist grazing may be focused primarily on particles that offer hotspots of prey abundance, however this remains to be confirmed. Illustrating the hotspot nature of deep oxyclines, at the oxycline/halocline of the deep hypersaline anoxic basin Urania (3450m depth at the same location in the E. Mediterranean), prokaryotic counts increased to $\sim 2 \times 10^6$ cells mL⁻¹ and protists (primarily small flagellates and some ciliates) to ~ 1071 cells mL⁻¹. In comparison to daily prokaryotic removal due to grazing in the oxic photic zone at 40 m depth ($\sim 31\%$), $\sim 20\%$ of the *in situ* abundance was removed within the oxycline at 3450m depth. These two studies suggest that in spite of generally lower abundances, protist grazing may still contribute significantly to carbon turnover in the dark ocean.

Pathogens

Parasitic protist taxa are taxonomically diverse and likely play a major ecological role in both coastal and open ocean habitats. The Alveolata comprise one of the largest

139 eukaryotic lineages, which includes four protist classes, the Ciliophora, Dinoflagellata,
140 Protalveolata, and Apicomplexa, the last two of which are comprised primarily of
141 parasites. Parasitic protists are a major source of mortality for other microbial eukaryotes
142 as well as Metazoa, and hence, are important components of marine food webs [32].
143 Parasitic protists are parasites of mollusks as well as other protistan taxa including
144 dinoflagellates, and taxa such as the novel marine alveolate group (MALV) Group II,
145 may play a major role in controlling ‘red tide’ blooms [32]. Apicomplexa are comprised
146 solely of obligate parasites that include the causative agents of malaria, *Plasmodium*, and
147 of toxoplasmosis, *Toxoplasma*. Discussion here will focus on a group of exclusively
148 marine parasitoid protists found within the Protalveolata, which figures prominently in
149 high throughput molecular studies of marine protist diversity in all marine environments
150 investigated to date, from ocean surface waters to the benthos. Their ribosomal DNA
151 gene (rDNA) sequences are retrieved from the picoplankton (< 3 micron) fraction from a
152 wide range of habitats, although some clades appear to be restricted to specific habitats,
153 including coastal waters, deep-sea hydrothermal vents, and anoxic habitats [33]. The
154 majority, or at least a highly significant fraction of rDNA clone library sequences are
155 assigned to novel marine alveolate (MALV) Groups I and II that belong to the
156 Syndiniales [17,34-38]. MALV Group II was one of four taxonomic groups that
157 dominated protist pyrotag signatures from oxic bathypelagic water samples in the
158 Malaspina global survey [2]. In the TaraOceans oxic photic zone samples, 36% of OTUs
159 were from parasites, almost half of which were MALV Group I [1]. Syndiniales are
160 generally known to obligately kill their hosts. Known hosts include other Syndiniales,
161 photosynthetic dinoflagellates, ciliates, rhizarians and metazoans [33, 39, 40]. Lower

clonal abundances in RNA-based libraries, however, suggest these groups may play a more minor role in parasitism than what is implied by DNA-based diversity surveys [41].

The seasonally stratified water column of Sannich Inlet, Vancouver B.C. will be used here to illustrate the potential environmental importance of the Syndiniales. Saanich Inlet is a coastal habitat for which a picture of protist diversity over a complete 12-month cycle is available, and where protist populations occupy different niches along the redoxcline at different times during the year [42]. Marine oxygen minimum zone formation can have a strong influence on protistan communities, with different populations being selected for as a result of changes in dissolved oxygen. In Saanich Inlet during November, deep waters are less stratified due to earlier seasonal inputs of upwelled water from coastal sources. During this time an increase in Dinoflagellates (80% of which affiliated to Syndiniales) was observed at 200m depth relative to abundances in July and April, when waters are maximally stratified [42]. The upwelling water that replaces deeper waters at 200m originates from coastal sources. Syndiniales have been detected previously in coastal waters (e.g., [43]). In oxic waters at 10m depth, sequences of Syndiniales represented ~90% of all Dinophyceae-affiliated sequences in samples from all seasons. Different members of this group likely increase in abundance contemporaneously over the seasons with abundances of potential hosts, e.g., during particular bloom events. Abundances at depth may reflect release of parasites from infected hosts sinking from surface layers. It is unknown the extent to which Syndiniales may participate in different deep host-specific parasite relationships or may live a different free-living life-style at depth. Specific interactions between many Syndiniales and their hosts are known to exist. Interesting correlations between Syndiniales and

known bloom taxa in Saanich Inlet and elsewhere are already emerging from network analyses (Edgcomb, unpublished data).

Associations with Bacteria and Archaea.

Marine protists commonly exhibit associations with bacteria and archaeal whereby symbionts can be associated with their protist host as ectobionts (on the outside of their host) or as endobionts (inside the host cell membrane). Symbioses between protists and bacterial and archaeal partners are common in oxic as well as oxygen-depleted marine habitats [44]. These partnerships appear to be based on complementation of aspect(s) of host metabolism by the prokaryotic associate(s), thus potentially expanding the niche of the host. Such associations have been integral to the evolution of eukaryotes (e.g., the primary symbioses that lead to the acquisition of plastids and mitochondria in eukaryotes). There are many modern day examples of acquisition of photosynthetic partners by protists, such as amoebae, and ciliates in the oxic photic zone, suggesting their continued importance for the ecology and evolution of protists [44]. Discussion here is focused on the dark ocean, and in particular, on associations between protists and prokaryotes in oxygen-depleted and anoxic habitats.

Despite their discovery over three decades ago, we still know little about the mechanics and dynamics of metabolic interactions between protist hosts and their prokaryotic partners in the deep ocean, and in anoxic marine benthic and oxycline and anoxic water column habitats. Protist symbioses with methanogens as well as sulfate-reducing and sulfur- or sulfide-oxidizing bacteria are commonly observed in these habitats. Anaerobic ciliates affiliated with the genera *Metopus*, *Trimyema*, and *Cyclidium*

are examples of free-living taxa known to host methanogens [44]. In symbioses involving intracellular methanogens and/or sulfate-reducing and sulfur-oxidizing bacteria in low-oxygen to anoxic habitats it is thought that the symbionts facilitate anaerobic host metabolism of microbial prey by removing fermentative end products, thus enabling metabolism of microbial prey by the host without the use of oxygen [45-48] however, this has not been directly demonstrated. Protists in these habitats employ anaerobic metabolisms involving modified mitochondria or mitochondrion-derived organelles called hydrogenosomes that allow generation of ATP via fermentation of the glycolysis product pyruvate, producing molecular hydrogen and other fermentation end-products (e.g., [49-51]). The physical association of cytoplasmic endosymbionts – sometimes up to 10,000 in a single protist – with putative hydrogenosomes (e.g., [45, 47]) (Figure 3) may permit consumption of the end products of host fermentation (e.g., the hydrogen), thereby increasing the thermodynamic favorability of host metabolism [45, 48]. Such partnerships would permit host niche expansion into anoxic waters/sediments. In deep anoxic marine sedimentary environments not only protist-prokaryote but metazoan-prokaryote associations are commonly observed, and it should be noted that flagellate taxa are among protist morphotypes observed to host putative symbionts [52]. Aside from a few exceptions, little is known about the identity of the prokaryotic partners of protists in these deep habitats. Some flagellates, such members of the euglenozoan subclade Symbiontida, inhabiting both shallow and deep anoxic marine sediments, host sulfur- or sulfide-oxidizing epsilonproteobacterial epibionts [53]. It was hypothesized that these epibionts may not only enhance host fermentation of prey by consuming hydrogen

produced by host hydrogenosomes, but they may also serve a detoxification role for the host by taking up hydrogen sulfide which is toxic to most eukaryotes.

While there are abundant examples of protist-prokaryote and metazoan-prokaryote symbioses, it should be noted that protists may also occur as endosymbionts of other protists and of metazoans. The most common type of protist-protist symbiosis involves short- or long-term acquisition of photosynthetic algal symbionts by various protist taxa living in the photic zone (reviewed in Nowack and Melkonian [44]). Some foraminiferal protists in these deep sedimentary habitats are also known to sequester plastids, the function of which is largely unknown [54]. Little is known about protist-protist symbioses in the dark ocean. Protist-metazoan symbioses are known to occur in association with hydrothermal vent bivalves, some portion of which may be parasitic (discussed in Saudavet et al. [55]). These protist symbionts include various ciliate taxa and putative parasitic kinetoplastids, including members of the Syndiniales Group II that are also known to infect other protists. It is quite likely that future investigations of deep-sea communities will reveal many other examples of associations of protists with other protist and metazoan taxa.

Conclusions

Grazing by phagotrophic protists is emerging as a potentially significant source of deep-ocean carbon turnover, especially where redoxclines exist. Until recently, biological oceanographers have relied on hydrocasting for collecting water samples for grazing studies as well as studies of biomolecules of interest, typically using Niskin-bottles. Once on the ship's deck, these waters are typically transferred into incubation bottles. While this

process is less likely to introduce artifacts associated with sample collection for near-surface water samples, similar handling of deeper samples exposes microorganisms to significant pressure changes and other potential alterations (e.g., temperature, redox) that may either destroy cells in those samples or influence their physiological poise. Such alterations can introduce artifacts into grazing studies and analyses of labile molecules such as mRNA [56, 57]. Regardless of the degree to which water samples are affected by traditional Niskin bottle retrieval, it makes sense that the best strategy going forward for evaluating marine microbial trophic interactions is to minimize those sources of artifact by conducting process rate measurements *in situ*. Further advances in technologies for conducting such studies is needed, and possibly creation of regional instrument pools for interested researchers. Additional studies of protist phagotrophy in meso- and bathypelagic waters globally using *in situ* approaches will provide a more complete understanding of ocean carbon cycling and of how grazing impacts abundances of key prokaryotic populations involved major elemental cycling and greenhouse gas production.

Viral lysis, protist phagotrophy, and parasitism are the major causes of mortality of eukaryotic and prokaryotic microorganisms in the marine environment. Parasitic protist groups such as the Syndiniales are emerging as a significant component of communities in global molecular surveys. Given the potentially significant numbers of parasitic protist taxa it will be intriguing to investigate the degree to which certain groups regulate the intensity and duration of plankton (including toxic) blooms in specific areas. Their contributions to dissolved and particulate organic carbon pools may be significant.

The ubiquity of protist-prokaryote associations throughout the marine realm, particularly in low-oxygen habitats [53] suggests we may be overlooking a significant fraction of biogeochemical cycling by symbiotic prokaryotes, at least in some habitats, by measuring key processes using methods that exclude most protists (e.g., prefilters). Nano-secondary ion mass spectrometry (Nano-SIMS) or new Raman atomic force microscopy (Raman-AFM) approaches hold great promise for increasing our understanding of the underpinnings of symbioses. For example, a halogenated in situ hybridization nanometer-scale secondary ion mass spectrometry (HISH-SIMS) was recently used to demonstrate a mutualistic partnership between a nitrogen-fixing cyanobacterium and algal protist (a prymnesiophyte), whereby the protist receives fixed nitrogen in exchange for fixed carbon from the host [58]. Direct evidence for the metabolic exchange between host and symbionts using methods that can track exchange of metabolites at the subcellular level will increase our understanding of symbioses involving protists, including in the ocean's twilight zone and below..

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Figure legend

Figure 1. Roles of protists in the oxic marine water columns and underlying sediments in both particle-associated and free-living fractions. Increasing and decreasing arrows indicate general, non-quantitative trends in relative abundance of each activity/role based on published studies to date. Width of arrows corresponds to known or hypothesized relative importance. Question marks indicate realms where only hypotheses can be made because little or no published data exist.

Figure 2. General trends in relative abundance and grazing activity/impact of protists in a stratified watercolumn. There is a general decrease with ocean depth in protist abundance and grazing activity, however peaks are observed again at depth along oxyclines (typical of oxygen minimum zones and seasonally- or permanently-stratified water columns, concomitant with peaks in oxycline prokaryotic populations. Increasing and decreasing arrows indicate general, non-quantitative trends in relative abundance or grazing activity/impact based on published studies to date. Width of arrows corresponds to hypothesized relative importance. Question marks indicate realms where only hypotheses can be made because little or no published data exist.

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525 Figure 3. Light micrograph (A) and Transmission electron micrograph (B) of a
526 karyorelictid ciliate from Santa Barbara Basin sediments. Red oval indicates area of
527 ciliate body where membrane-bound vesicles contain structured arrangements of
528 endobiont morphotypes are located. Black arrows indicate putative hydrogenosomes.
529 TEM image taken by J. Bernhard, and light micrograph by V. Edgcomb, images adapted
530 from Edgcomb et al. 2011c. Scale bar in A=10 μ M, B=50 μ M.

Figure 1

Roles of protists in the oxic marine water column and underlying sediments in both free-living and particle-associated fractions

Figure2

Protist relative abundance and grazing activities along stratified marine water columns

Figure3