**Micro 493 Paper- Final Draft**

**The unique association between *Mesodinium rubrum* and its cryptophyte prey**

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**Introduction**

The common coastal ciliate, *Mesodinium* *rubrum* (= *Myrionecta rubra*) (Lohmann, 1908; Jankowski, 1976)*,* is among the marine microzooplankton that have been observed to harbor the plastids of their prey, allowing these predators to function as mixotrophs, capable of utilizing both phagotrophic and photosynthetic mechanisms to acquire carbon (Crawford, 1989). Though mixotrophic microzooplankton are now understood to be important primary producers in coastal systems (Stoecker et al., 1989), little is known about the specifics of these predator-prey relationships. The precise nature of the association between *M. rubrum* and its cryptophyte prey, in particular, has been highly debated, resulting in a number of recent studies investigating this mixotrophic predator-prey pair in both laboratory experiments, as well as in the field.

The discovery of the mixotrophic ability of *M. rubrum* was first facilitated by its capacity to form red-water blooms (Ryther, 1967; Lindholm, 1985). This obvious visual evidence of pigmentation prompted investigations into the photosynthetic capability of the ciliate, as well as its potential role as a primary producer. It was then determined that the bloom-forming *M. rubrum* has one of the highest primary production rates observed among marine phytoplankton, with a rate of over 2000 mg C m-3 hr-1 measured for a bloom off of the coast of Peru (Smith and Barber, 1979; Taylor, 1982). Based on the presence of phycoerythrin pigment, as well as morphological observations, the exogenous chloroplasts inside the ciliate were found to have originated from members of the cryptomonad group of algae, a group also known as being a common source of prey for a number of heterotrophic and mixotrophic protists (Barber et al., 1969; Taylor and Blackbourne, 1971). Additionally, it was through these early observations that the potential for a strong *M. rubrum*-cryptophyte association was first hinted at.

Though *M. rubrum’s* ability to photosynthesize has been hypothesized since the initial discovery of the red water blooms, its trophic categorization as a functional phytoplankton is more recent (Crawford, 1989). This is in part due to the differences among those studying *M. rubrum* as part of a red-water bloom vs. non-red-water *M. rubrum*. Since the evidence of photosynthetic ability in the ciliate was far less obvious to researchers investigating coastal plankton assemblages in non-red water, *M. rubrum* was often classified as being solely a microzooplankton, and excluded from phytoplankton counts in the field (Crawford, 1989). But by the 1980’s, the number of studies on *M. rubrum* acknowledging the organism’s role as both a primary producer and a microzooplankton grazer was steadily increasing, in part due to the recommendations made by Sieburth et al. (1978) (Crawford, 1989). Now that it has been determined that *M. rubrum*, even when non-bloom-forming, is an obligate mixotroph (Smith and Hansen, 2007) and a primary producer, the discussions surrounding this ciliate in the last several years have shifted towards understanding the specific mechanisms behind how it achieves this photosynthetic ability. This paper will review some of these recent studies focusing on the *M. rubrum*-cryptophyte relationship, placing them in the context of two model estuarine systems, the Chesapeake Bay and the Columbia River estuary.

**The nature of the *M. rubrum*/cryptophyte relationship**

The specifics of *M. rubrum’s* ability to photosynthesize, and whether it is conferred from an algal symbiont, stolen chloroplasts, or another mechanism (Figure 1), remain highly controversial. Though pigmented plastids and nuclei of cryptophyte origin were identified within *M. rubrum* cells early on (Barber et al., 1969; Taylor and Blackbourne, 1971), it was not until a cultured isolate of *M. rubrum* became available that more directed laboratory studies on the ciliate-cryptophyte relationship were able to be done (Gustafson et al., 2000). In culture experiments where *M. rubrum* was fed with the cryptophyte, *Teleaulax acuta*, it was shown that the ciliate required this cryptophyte prey to sustain its growth rates and that the number of cryptophyte nuclei within the ciliate increased when fed (Gustafson et al., 2000). These results were unexpected, given that the previously observed lack of a feeding vacuole and a greatly reduced cytostome (cell “mouth” that facilitates phagocytosis) suggested that *M. rubrum* may be incapable of ingesting prey (Lindholm et al., 1988; Taylor et al., 1971). But the results of these culturing experiments led to the hypothesis that *M. rubrum* “steals” the organelles of the ingested cryptophytes to become photosynthetic, a previously discovered process known as “kleptoplasty” (Figure 1) (Gustafson et al., 2000).

In contrast, other feeding experiments using a Danish isolate of *M. rubrum* showed that there was only a minor decrease in the number of chloroplasts in the ciliate after multiple cell divisions in the absence of free-living cryptophyte prey in the cultures, which supported the idea of a permanent, self-replicating endosymbiont (Hansen and Fenchel, 2006). In addition, morphological observations suggested that the exogenous organelles inside *M. rubrum* were not derived from the same cryptophtyes that the ciliate was fed (Hansen and Fenchel, 2006). But in the Antarctic strain of *M. rubrum*, the organelles were found to originate from the same cryptophyte prey species added to cultures in experiments using a fluorescence *in situ* hybridization (FISH) probe (Johnson et al., 2007). In addition, the cryptophyte nuclei remained transcriptionally active in the Antarctic strain, despite the dissolution of the cryptophyte membrane and other structures after being ingested (Johnson et al., 2007). The sequestered chloroplasts inside *M. rubrum* were able to divide, with the cryptophyte nuclei aiding in their maintenance- without these nuclei, the chloroplasts could not replicate and decreased in number. From this data, it was hypothesized that *M. rubrum* needed to repeatedly ingest cryptophyte prey primarily in order to obtain new prey nuclei that maintain the chloroplasts and sustain its high growth rates, coining the term “karyoklepty” (Figure 1) (Johnson et al., 2007).

This idea of sequestration of both nuclei and chloroplasts, as opposed to the preservation of a whole endosymbiont, was not only a major development in understanding the *M. rubrum*-cryptophyte relationship, but it also represented a novel mechanism that has not yet been observed among any other organisms. Karyoklepty in *M. rubrum* may additionally be of interest to those studying the evolutionary history of eukaryotic algae. Controversy regarding the origin of the cryptophyte organelles remains. Initially, it was believed that some of the contrasting results found among the different culture studies (Hansen and Fenchel 2006; Gustafson et al., 2000; Johnson and Stoecker, 2005; Johnson et al., 2007) were due to the use of differing *M. rubrum* strains (Hansen and Fenchel, 2006; Park et al., 2007). However, molecular analyses indicated that the chloroplasts inside the Danish strain of *M.rubrum* were, in fact, originally from the prey species of cryptophyte, just like the Antarctic strain (Hansen et al., 2012). Differences among strains likely still play a role in the ciliate-cryptophyte relationship, especially in regards to the continually-debated question of whether the prey organelles are being sequestered karyokleptically or remaining as part of a temporary endosymbiont.

The specific species of cryptophyte used for feeding *M. rubrum* in culture has also been established to influence the growth rates of the ciliate. Prey from the Teleaulax/Plagioselmis/Geminigera (TPG) clade confers the highest growth rates in its predator (Park et al., 2007; Hansen et al., 2012) and all laboratory strains of *M. rubrum* are now fed from this group. It has been thought that much of the difficulty involved in attempts to culture *M. rubrum* have been due to the use of prey that was not the isolate’s preferred cryptophyte prey type (Park et al., 2007). It is also possible that the specific prey species could be one of the determining factors for other aspects of this unique predator-prey relationship. For example, the retention time of the sequestered cryptophyte nuclei/full endosymbiont could potentially vary by cryptophyte species (Park et al., 2007; Myung et al., 2013). Because of this possibility for a strong prey influence, it is becoming evident that the species of cryptophytes found among local phytoplankton assemblages are an important consideration when investigating how *M. rubrum* functions in the environment.

**Case study- Chesapeake Bay**

The Chesapeake Bay is the largest estuary in the United States (Hack, 1957), featuring a number of tributaries, and the circulation of the bay is strongly influenced by river flow (Pritchard, 1952). A number of local initiatives and monitoring programs have maintained yearly and seasonal counts, as well as preserved samples of *M. rubrum* and other plankton in the main body of the bay and some of the major tributaries from as early as 1992 (Johnson et al., 2013). Though *M. rubrum* rarely forms blooms within this estuary, it is a common member of the plankton communities in the Chesapeake Bay, making this estuary a model system for investigating predator-prey dynamics *in situ*. But until recently, most of the published literature on the ciliates of this region have focused on the purely heterotrophic species (Dolan and Coats, 1990).

The recent synthesis of the historical data available on *M. rubrum* provides a relatively comprehensive look at how the ciliate functions in the Chesapeake Bay under a variety of seasonal and environmental conditions (Johnson et al., 2013). It was found that, overall, the abundance of *M. rubrum* was positively correlated with temperature and cryptophyte abundance, and negatively correlated with salinity, but these associations do not necessarily hold true on a seasonal basis. In autumn, *M. rubrum* abundance was negatively correlated with temperature. Blooms of the ciliate were found to primarily occur in the sub-estuaries of the bay’s tributaries, for reasons not yet entirely understood- though it is suggested that the lower levels of light penetration in the Chesapeake Bay may be driving the photosynthetic ciliates to the upper surface layer. Though not necessarily a yearly occurrence, these blooms appeared in spring or fall under various hydrodynamic conditions, which suggests that there may be different variants of *M. rubrum* with distinct seasonal niches (Johnson et al., 2013).

This study also included an investigation into the cryptophytes in the Chesapeake Bay, finding that an overall high abundance was maintained throughout the year, in agreement with the previous studies in this region (Marshall et al., 1996; Adolf et al., 2006; Johnson et al., 2013). The abundances of the cryptophytes are positively correlated with abundances of *M. rubrum*, and in one year, peaks in *M. rubrum* abundance were followed by a sharp decline in cryptophytes, which could suggest grazing by the ciliate on the cryptophytes (Johnson et al., 2013). It remains unclear why, despite the high abundance of cryptophytes, *M. rubrum* rarely forms blooms in the Chesapeake Bay.

Dilution experiments in one of the sub-estuaries of the bay’s tributaries have shown that microzooplankton grazing likely constrains the net growth of the ciliate (Dolan et al., 2000), but a number of other factors, including the specific species of cryptophytes, competition for cryptophtye prey among small grazers and mixotrophs, and even the strain and genetic variants of *M. rubrum* could be also contributing to the lack of red tide blooms in the Chesapeake Bay (Johnson et al., 2013). The ability to maintain its presence (even in low numbers) in the bay throughout the year may be facilitated by the capacity to retain cryptophyte plastids for up to 80 days when starved of prey, as shown in a recent laboratory study on *M. rubrum* (Myung et al., 2013). But additional research, including molecular investigations into the local diversity of *M. rubrum*, is needed to better understand which environmental and biological factors prevent the ciliate from forming blooms in the Chesapeake Bay.

**Case study- Columbia River Estuary**

In contrast to the Chesapeake Bay, the *M. rubrum* found in the Columbia River Estuary forms impressive red-tide blooms each year in the late summer or early fall. Although this estuarine system does not have as much historical observational data as does the Chesapeake Bay, a number of studies have been recently published on *M. rubrum* in the Columbia River Estuary. These blooms are not only visually significant, but they also play an important role in shaping the biogeochemistry of the region by shifting the estuary from net heterotrophy to net autotrophy during the fall (Herfort et al., 2012). Despite *M. rubrum’s* ubiquitous late summer/early fall presence in the Columbia River Estuary, little is known about their bloom dynamics and much of the current research on the ciliate in this system has focused on understanding what causes the yearly bloom’s initiation and its end.

Similar to both the Southampton Estuary (Crawford et al., 1997) and the Chesapeake Bay (Johnston et al., 2013), the physical properties of an estuarine environment are likely to play an important part in structuring the dynamics of a bloom in the Columbia River Estuary. The Columbia River Estuary is highly influenced by both tidal forcing and the seasonality of freshwater discharge, which results in an extended summer saltwater intrusion (Chawla et al., 2008). This saltwater intrusion and subsequent temporary decrease in turbulence has recently been shown, along with coinciding neap tides, to correlate with the initiation of the yearly *M. rubrum* bloom in the estuary (Herfort et al., 2011a). In the Columbia River Estuary, the bloom development is composed of two distinct phases (Herfort et al., 2011a). The initiation phase of the bloom appears to occur in Baker Bay, one of the lateral bays in the estuary, with high abundances and fast growth rates of *M. rubrum* first emerging here. The retentive nature of Baker Bay, conferred in part by its shallow depth, likely plays an important role in the initiation of the bloom. After a period of delay, the bloom then becomes established within the main estuary (Herfort et al., 2011a).

Field studies of the *M. rubrum* bloom in the Columbia River Estuary have also been able to highlight a number of unique characteristics of the ciliate, some of which may potentially be contributing to its successful bloom formation. The maximum growth rates of this species in the Columbia River Estuary are incredibly fast- 7-11 fold times faster than the highest growth rates previously found for laboratory cultures of the Korean isolate of *M. rubrum* (Herfort et al., 2011a; Peterson et al., 2012). Though this bloom develops rather rapidly, it is likely to be self-limiting, as the growth rates of *M. rubrum* are actually lower in red water patches compared to non-red water, suggesting that self-shading occurs at high densities (Herfort et al., 2011a).

Studies in the Columbia River Estuary have utilized molecular approaches to identify the species composition of the cryptophytes present during red water blooms. Analysis of the 16S RNA of the associated chloroplast revealed the ciliate’s prey to be the cryptophyte, *Teleaulax amphioxeia,* though there were very few 18S RNA sequences related to this cryptophyte, suggesting an absence of *Teleaulax amphioxeia* nuclei inside the predator(Herfort et al., 2011b). This potential lack of cryptophyte nuclei found within *M. rubrum* in the Columbia River Estuary conflicts with the observations of both the Antarctic and Danish strains adding to the controversy surrounding the specificity of predator-prey interactions (Johnson et al., 2007; Hansen et al., 2012). Additionally, 18S DNA sequence analyses of the bloom patches showed a virtual absence of free-living versions of the specific prey species of cryptophyte, another unique feature of this estuary that is not yet understood (Herfort et al., 2011b). Studies using advanced imaging technology have shown that large amounts of cryptophytes (up to ~50) could be residing on the cirri (bristle-like outer structures used for motility and prey capture) of the ciliate (Peterson et al., 2012). But it is unknown whether these attached cryptophytes are *Teleaulax amphioxeia*, the actual prey species. Additionally, the fate of the cryptophytes attached to the cirri of *M. rubrum*, whether or not attached prey is ingested, and the role that this potential prey capture mechanism might play in the overall *M.rubrum*-cryptophyte relationship remain largely unstudied.

Examinations of the ‘18S-28S’ rDNA region have determined that there are at least five genetic variants of *M. rubrum* in the estuary, though only one of them is actually associated with the formation of these blooms, variant B (Herfort et al., 2011b). Clearly, the bloom-forming variant possesses some sort of genetic advantage that allows it to bloom in the estuary. This is a critical finding in regards to the overall understanding of the *M. rubrum* species, potentially even suggesting that the question of whether the ciliate utilizes kleptoplasty, karyoklepty, or a temporary endosymbiont for photosynthesis could be answered by looking at genetic variability and identifying key genes involved in each mechanism.

**Future Directions**

The possibility that genetic differences among *M. rubrum* can contribute to the variety of life strategies, such as the ability to form blooms, seen among this species in the field is emerging as a potential explanation as to the controversy surrounding laboratory studies of the ciliate and its cryptophyte prey. Recent investigations comparing both morphology and genomic analyses of the Danish strain and the five Columbia River Estuary variants of *M. rubrum* support the idea of a species complex, even going as far as to propose the need for a new separate species to classify the un-culturable variants, now called *Mesodinium major* (Garcia-Cuetos et al., 2012). But further studies on the genetics of Antarctic, Korean, and Chesapeake Bay isolates of this ciliate are needed to understand the specifics of the relationship between the type of predator-prey interaction (e.g. karyoklepty, temporary endosymbiont) and the organism’s genetics. These investigations will undoubtedly be complicated by the potential for prey type and other factors to contribute to how the ciliate functions, and thus, supplemental field studies focusing on initial interactions between free-living cryptophytes and ciliates, including prey capture mechanisms, will also be necessary.

Ultimately, though the current understanding of *M. rubrum’s* relationship with its cryptophyte prey is still relatively limited, future studies on the unique characteristics of this organism pair have the potential to reveal much about predator-prey interactions, eukaryotic phytoplankton evolution, and mixotrophy in general. The need for further genomic investigations into *M. rubrum* is also a prime example of the push towards the use of molecular approaches in biological oceanography to find better and more specific answers to persistent questions in the field.

**Figure 1.** Schematic illustrating three potential variations of the *M. rubrum*-cryptophyte relationship.



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