**Paper Draft 2**

**Working Title:** Dynamics of cryptophyte populations in the Columbia River Estuary

**Abstract**

In order to better understand the dynamics of the combination cryptophyte and *M. major* red water bloom occurring yearly in the Columbia River Estuary, we have used continuous flow cytometry to monitor the abundances and division rates of free-living cryptophyte algae during the September-October 2013 bloom. Continuous cryptophyte division rates were determined from flow cytometry data using a size structured division rate model (Sosik et al., 2003). These division rates were then compared to the daily abundances of the cryptophyte's predator, *M. major*, and showed no discernible relationship, thus pointing to the potential importance of the non-free-living prey cryptophytes in driving the dynamics of the *M. major* bloom.

**Introduction**

Every year, in the late summer-early fall, massive non-toxic red water blooms form in the Columbia River Estuary. These blooms consist of both cryptophyte algae and the mixotrophic ciliate microzooplankton, *Mesodinium major,* previously referred to as *Mesodinium rubrum* (*= Myrionecta rubra*)(Lohmann, 1908; Jankowski, 1976). This ciliate has been observed to harbor the plastids of their cryptophyte algae prey, *Teleaulax amphioexa*, allowing these predators to function as mixotrophs, capable of utilizing both phagotrophic and photosynthetic mechanisms to acquire carbon (Crawford, 1989; Herfort et al., 2011b).

The Columbia River Estuary (CRE) 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, along with coinciding neap tides, has been shown to correlate with the initiation of the yearly *Mesodinium sp.* bloom in the estuary (Herfort et al., 2011a).

Past research on the CRE has focused largely on understanding the dynamics of the *M. major*, and it has been hypothesized that the cryptophytes may be important in controlling the bloom, as they are both a source of prey and they confer photosynthetic ability to the ciliate. Weekly counts of *M. major* and cryptophyte cells during a red tide bloom in 2011 showed that the abundance of small (<5 um) “Teleaulax-like” cryptophytes declined just prior to an increase in *M. major* abundance, suggesting active predation by *M. major* or another zooplankton (Peterson et al., 2012). Though free-living *T. amphioexa* are overall low in number during the red tide blooms (Herfort et al., 2011b), the presence and growth dynamics of these cryptophytes have the potential to be key factors in the population growth of the ciliate, *M. major*.

To the best of our knowledge, this study is the first to monitor the dynamics of both the cryptophyte and ciliate populations in the Columbia River Estuary. More specifically, this study will ask these two interconnected questions: 1.) What are the effects of environmental conditions, such as nutrient availability, temperature, salinity, and light, on the growth of the cryptophyte populations? and 2.) How does the growth of the cryptophyte prey affect the dynamics of *M. major*?

To answer these questions, we have monitored the abundances and size distribution of a cryptophyte population continuously 5 days a week for 4 weeks in September-October 2013 during a red tide bloom in the Columbia River Estuary. We estimated daily-averaged division rates of the combined populations of cryptophytes based on the size distribution using a size structured division rate model (Sosik et al., 2003). This model has thus far, only been tested for Synechococcus and Prochlorococcus (Hunter-Cevera et al., 2014; Ribalet et al., in press?). We validated the use of this model for cryptophytes by comparing model estimates of division rate to estimates determined using cell-cycle analysis in a laboratory time course experiment. To monitor the bloom dynamics of the ciliate, daily M. major abundances were determined using FlowCAM. Abundances of *T. amphioexa* were estimated once a week using qPCR to determine their percent contribution to the total cryptophyte population.

**Methods**

**Study Area**

The Columbia River Estuary, located between Washington and Oregon (**fig. 1**), is a turbid and often highly stratified system. It is characterized by its dynamic physical processes and strong influence from diurnal and semi-diurnal tides (Jay, 1984). The estuary also has a short residence time, with flushing in the range of 0.5-5 days (Neal, 1972).

**Sample Collection**

A SeaFlow continuous flow cytometer (Swalwell et al., 2011) was stationed at SATURN 03 on a dock outside of Astoria, OR (**fig. 1**) and set up to run continuous measurements of surface water for up to five days each week for four weeks in September-October 2013. Discrete flow cytometry samples (1 mL fixed with 25% Glutaraldehyde) were collected for cell sorting on the cryptophyte population and were stored at -20 and analyzed 20 months later using Influx cell sorter (BD). 45mL samples fixed with 25% glutaraldehyde were collected for *M. major* counts, stored at 4 °C and analyzed blah months later using FlowCAM. 30mL surface water samples for nutrient analysis were taken in duplicate. Blah mL of water was filtered once a week to determine cryptophyte community composition using qPCR. All discrete samples were collected around 'slack' tide.

**SeaFlow Data Analysis**

All SeaFlow data was analyzed using the R package, popcycle (<https://github.com/uwescience/popcycle>). The sds file from SeaFlow was converted to an sfl format in order to be compatible with the popcycle software. Background “noise” (detritus and other non-cell particles) in the evt files were filtered out using a notch of 0.8 and a width of 0.5 for the Columbia River Estuary field data. The same parameters were used for all evt files for the field data. Manual clustering of beads and “cryptophyte” population was applied to a single time point file and then used for all files. Clustered particle counts were then compiled into a stats table located within the popcycle database. The same method was used for the laboratory experiment SeaFlow data, with a notch of 1 and a width of 0.5 applied to all evt files.

**Flow Cytometry Sorting – Light microscopy**

A 1mL surface water sample was run on an Influx flow cytometer, and 100 cells from the gated population of supposed “cryptophytes” were sorted onto a glass slide. The cells were then examined under a Nikon Eclipse 80i epifluorescent microscope at 20x magnification and photographed using a Qimaging MicroPublisher 3.3 RTV camera. The gated population was confirmed as consisting of cryptophytes after observations of the sorted cells revealed the orange autofluorescence, characteristic of the cryptophyte group, when viewed under epifluorescent microscopy (image as supplemental data). The size of the cells viewed (<5 μm) agreed with past observations of cryptophytes in the Columbia River Estuary (Peterson et al., 2012) and were teardrop-shaped.

**Size-structured Division Rate Model**

FR writes this

-based off of model from Sosik et al., 2003

-”matrix population model” based on light and cell size

-assumptions of the model

**Division Rate Lab Verification**

For verification of the division rates found using the size-structured growth model, non-axenic 150 mL cultures of the cryptophyte, *Rhodomonas sp*. (CCMP 755), were grown in flasks at 13 °C in f/2 seawater media under a (16:8 hr) light-dark cycle. The abundances of daily 1mL samples of Rhodomonas sp. were determined via cell counts using a Sedgewick-Rafter slide. Growth rates were calculated using the equation below:

Where t₁ is the time of the start of the growth cycle and t₂ is the time at the end. P₁ and P₂ are the concentrations of Rhodomonas (in cells mL⁻¹) at t₁ and t₂, respectively.

A single replicate of the *Rhodomonas sp.* cultures was brought to a volume of 20L in a Nalgene carboy with a concentration of 50 cells mL⁻¹ and mixed using a magnetic carboy stirbar. SeaFlow was run on the culture under a (16:8) light-dark cycle for 4 days. During the first day running SeaFlow, 1mL triplicate samples of the *Rhodomonas sp.* culture were taken every 2 hours for 28 hours, preserved with 20μL of 50% gluteraldehyde, flash frozen, and stored at -80 °C for cell cycle analysis.

The 1mL samples of *Rhodomonas sp.* were later thawed over ice, and run on an Influx flow cytometer...

-I might need some help with describing the cell cycle analysis (mostly the processing of the flow cytometery data)

***M. major* Enumeration**

TP writes this

**qPCR for *Teleaulax* abundance**

KM writes this

**Nutrient Analysis**

JN writes this

**Results**

**Lab Verification**

The hourly division rates estimated using both cell cycle analysis and the model ranged from 0.012 to 0.042 divisions per hour. The estimates for each method appear to follow the same general trend and are closely coupled throughout the time course, with the exception of the first few hours of the experiment (supplemental fig). The average daily division rates estimated using the two methods were blah for the cell cycle analysis and blah for the model.

**Environmental Data**

Throughout the survey, the average surface water temperature and salinity were 17.45 °C and 11.90 psu, respectively. In the first and third weeks, the salinity is higher and the temperature lower than the rest of the time course (**fig. 2, a**). Week 4 had both the lowest surface water salinity (mean=5.965 psu) and lowest temperature (mean=15.659 °C), compared to the other weeks. Surface water temperature and salinity appear to be anti-correlated and oscillate with the tidal cycle. High tide is characterized by an influx of colder, more saline water, and low tide by an increase in warmer freshwater.

The daily maximum PAR fell between 116.6-802.9 µE m-2 s -1 (**fig. 2, b**). A 5 fold change of daily PAR was observed during the survey. Weeks 2 and 3 exhibited the greatest amount of variation in PAR, with days within each week where PAR did not exceed 200 µE m-2 s -1, and other days where it reached up to ~800 µE m-2 s -1.

Changes in phosphate and nitrate appeared to be coupled throughout the first three weeks of the time course (**fig. 2, c**). In the first week, all surface water nutrient concentrations remained relatively stable. A peak in all nutrients occurred on 9/16, the beginning of week 2. After this peak, phosphate and nitrate began to decrease, then slowly increased again starting at the beginning of week 3. Nitrate continued to increase during week 4.

**Abundances**

Data from SeaFlow showed the abundances of the surface water cryptophytes to be between <0.01x10⁶ - 19.2x10⁶ cells L⁻¹ throughout the time course, with an average of 0.26x10⁶ cells L⁻¹ (**fig. 3, a**). A dramatic peak in abundance occurred during the first day (9/11), reaching up to 19.2x10⁶ cells L⁻¹. Week 1 also had the highest average cryptophyte abundance at 0.52x10⁶ cells L⁻¹. Weeks 2 and 3 had the lowest average abundances at 0.08x10⁶ cells L⁻¹ and 0.09x10⁶ cells L⁻¹, respectively, and did not exhibit any of the strong peaks in abundance that characterized week 1. Week 4 had an average abundance of 0.24x10⁶ cells L⁻¹ and did have at least one peak on 10/1, reaching up to 2.85x10⁶ cells L⁻¹. Cryptophyte abundance did not appear to be correlated with tidal cycle, though oscillations in abundance did occur within individual days.

**Division Rate**

Model estimates of the mean daily division rate of the surface water cryptophytes ranged from 0.023 to 9.78 (**fig. 4**), with weeks 3 (**fig. 4, c**) and 4 (**fig. 4, b**) exhibiting dramatically increased rates as compared to the first two weeks. Week 4 had the highest weekly average mean daily division rate at 4.83, and week 2 the lowest at 0.47.

The model estimates of the mean daily division rates did not have a significant correlation with any of the surface water nutrient concentrations or mean PAR (**fig. 5**), though it is important to note that nutrient concentrations were determined from single time point samples, whereas division rate was continuous.

***M. major* Counts**

Abundances of *M. major* ranged from between 21-323 cells mL⁻¹, with the some of the highest values occurring during weeks 2 and 3 (table 1). The highest weekly average abundance occurred during week 2 at 142.75 cells mL⁻¹, and coincided with decreasing nutrient concentrations (fig. 2, c). The lowest average was during week 1 with 101 cells mL⁻¹, and coincided with the highest measurements of cryptophyte abundance (fig. 3, a). The most dramatic change in abundance occurred between 9/12 and 9/13, with a decrease from 175 to 27 cells mL⁻¹, within 24 hours. The M. major abundances showed no relationship with cryptophyte division rate, nor any significant correlation with surface water nutrient concentrations or mean PAR (supplemental fig).

**qPCR for *Teleaulax* abundance**

The percent of the total cryptophytes that were *T. amphioexa*, as estimated from qPCR data, was very low across the entire survey, ranging from 0.0615% - 0.397% (**table 1**), and agreed with observations made in previous years (Herfort et al., 2011b). When applied to abundances measured via SeaFlow, this percent translated to between 5.03 and 93.18 *T. amphioexa* cells mL⁻¹ in the surface waters. The smallest percent of the cryptophyte population consisting of *T. amphioexa,* and the lowest abundance of *T. amphioexa* occurred during the second week, which was also the week when *M.* *major* was at it's highest averarage abundance. The largest percent of the cryptophyte population consisting of *T. amphioexa* occurred in the day just prior to the start of the survey (9/10).

**Discussion**

**Cryptophyte Abundances**

Though past data has suggested that the cryptophytes in the Columbia River Estuary are of freshwater origin (Peterson, unpublished data?) and thus increase in number with the outgoing tide, our data do not support this finding. While a distinct oscillation in cryptophyte abundance occurs within each day of the bloom, upon closer look this does not appear to be correlated with the tidal cycle. Though no discernible pattern emerges in our data set, this does not eliminate the possibility of a relationship between tidal cycle and cryptophyte abundance. It may be that co-occurring biological processes, such as growth and grazing, are obscuring this relationship. Other non-tidal physical processes may also have an influence on cryptophyte abundance.

**Cryptophyte Division Rate Estimates and Evaluating the Model**

Though, to the best of our knowledge, no growth rates for the cryptophytes in the CRE have been previously determined, laboratory estimates of the growth rates of various strains of cryptomonads have found rates up to an average of 0.85 d⁻¹ (Park et al., 2007), which agrees with our overall average rate of 1.00. Our highest estimates of mean daily division rate, as found in the final week of the survey, reach up to nine times greater than the average. This is unsurprising though, as growth rates for *M. major* found in the CRE in both 2011 and 2012 greatly exceeded the growth rates determined for laboratory cultures, at times by up to a seven-fold difference (Yih et al., 2004; Herfort et al., 2011; Peterson et al., 2012). It is possible that the environmental conditions and specific biological community found in the Columbia River Estuary allow for the growth of both ciliates and cryptophytes at a rate not yet replicated in culture.

Measuring growth in the field is difficult, but this new approach, utilizing a model, eliminates many of the known problems associated with traditional methods that rely on bottle incubations (Landry and Hassett, 1982; Landry et al., 1995) and is less labor-intensive. The model does have some limitations though, as it relies on a number of important assumptions. One of these assumptions that is likely to have been violated, is that the cryptophytes population consists of a single taxonomic group. It is possible that multiple taxonomic groups of cryptophytes with different physiologies are represented within the population of cryptophytes that we observe with SeaFlow. But we do not actually have any evidence that connects this possible model assumption violation to errors our division rate estimates. Our laboratory verification of the model worked (better way of saying this?) and the only indication of any problems associated with the model in our field study occurred in the last two weeks, with parameter optimization reaching extremes (supplemental figure).

**Environmental Influences on Cryptophyte Production and *M. major* Abundances**

In the turbid waters of the Columbia River Estuary, light is generally considered to be the factor most limiting to phytoplankton growth (Herfort et al., 2012). Comparing measurments of PAR to our cryptophyte production estimates did not result in any statistically significant relationship (**fig 5, d**). But because the photosynthetic machinery of cryptophyte cells are well adapted to conditions of low light (Bergman et al., 2004), it makes sense that they are not limited by PAR during the time of our field study. It would actually be more likely that the cryptophytes would be photoinhibited by light, as they are not among the phytoplankton that produce light-protective compounds that shield cells from the damaging effects of radiation (Vernet et al., 1994; Herfort et al., 2012). But again, our production estimates did not show any correlation with PAR, negative or otherwise.

Comparisons of nitrate concentration with daily average cryptophyte production revealed a potentially weak positive correlation (R²=0.231, p-value=0.048) between the two (**fig. 5, a**). This possible relationship could be interpreted in a multiple ways. It may be that nitrate is the limiting factor for cryptophyte production. But, *M. major* also requires a nitrogen source and appears to have a weak anti-correlation with cryptophyte abundance (potentially due to grazing). An additional possibility is that the positive relationship between cryptophyte production and nitrate concentration is actually dependent onthe abundance of the ciliate.

Past studies of the blooms occurring from 2007-2010 showed a negative correlation between ammonium, *M. major'*s preferred nitrogen source (Crawford et al., 2007), and the abundance of the ciliate in the estuary's main channel (Herfort et al., 2012). In our comparison of *M. major* abundance and surface water ammonium, no significant correlation was found (supplemental fig). There was also no relationship between abundance and nitrate, phosphate, or PAR, suggesting that *M. major* may potentially be limited by non-environmental factors.

***M. major* Abundances in Relation to Cryptophyte Data**

Because division rate can be considered somewhat of a proxy for the “health” of a cell, it would seem likely that a rapidly dividing prey population would correspond to an increase in the number of its predator- especially when the interactions are as specific as with *M. major* and *T. amphioexa.* But with our data set, we were unable to find a distinct relationship between the abundances of *M. major* and the division rates of the cryptophytes. Again, these division rate estimates are determined for the population of cryptophytes, as a whole. *M. major's* preferred prey, *T. amphioexa*, makes up <1% of the total cryptophyte population, and *T. amphioexa* may exhibit division rates that are very different from the rest of the population. But, it is possible that there would not any major difference in the overall pattern of division rate over the course of the experiment, as the environmental factors influencing the change in division rate and physiology of the total population of cryptophytes could potentially have the same effect on *T. amphioexa*.

Comparisons of our estimates of *T. amphioexa* abundance and percent composition to *M. major* counts point to the potential importance of the prey community composition in *M. major* bloom development, despite only having four time points. Of these four coinciding time points, the highest *M. major* count (179) occurs when the percent of the total cryptophytes that are *T. amphioexa* is the lowest (0.0615%), and vice versa (**table 1**). This could potentially be considered evidence of selective grazing on *T. amphioexa*, though again, additional time points and multiple replicates of *M. major* counts would be needed to be able to draw any concrete conclusions. The estimated abundances of *T. amphioexa* are generally low throughout the bloom. The very first time point, taken during week one of the study at the beginning of the bloom, is the only instance in which the abundance of *T. amphioexa* exceeds that of the ciliate. This finding is curious, as grazing experiments using laboratory cultures of *M. rubrum* have estimated ingestion rates between ~3.5 and 8.9 cryptophytes ciliate⁻¹ day⁻¹ (Yih et al., 2004; Hansen and Fenchel, 2006). Additionally, using a FISH probe for *T. amphioexa*, we have been able to observe up to >20 prey within a single *M. major* cell (pic as supplemental fig). It is possible that the low abundances of the prey cryptophyte that we observe in the Columbia River Estuary are the result of the result of grazing by *M. major* and other microzooplankton. Dilution experiments to determine grazing rates of M. major on cryptophytes in the field should be considered to test this hypothesis.

An alternative explanation could be related to *M. major*'s ability to retain cryptophytes attached to the ciliate's cirri, as it this has been observed in the CRE during the 2011 red water events (Peterson et al., 2012). Our measurements of *T. amphioexa* abundance are limited to those that are free-living, and it is possible that a number of prey are living attached to the outside of *M. major* cells. These captured prey could be what sustains M. major throughout the bloom, despite low numbers of the free-living *T. amphioexa*. Another possibility could be that the ingested *T. amphioexa* remain a full or partial endosymbiont and are able to divide inside the ciliate, allowing *M. major* to essentially “farm” the cryptophytes as a source of chloroplasts. But these explanations remain pure speculation and require more in depth molecular investigations into the predator-prey relationship between *M. major* and *T. amphioexa* in the Columbia River Estuary.

**Conclusion**

Past studies on the *M. major* bloom in the Columbia River Estuary have hypothesized that the dynamics of the ciliate's cryptophyte prey may play a role in the proliferation of the bloom, but until now, the cryptophyte population within the bloom had not yet been investigated. This study is the first to show continuous abundances and division rates of the cryptophyte population in the estuary, as well as near-daily *M. major* counts over the course of the bloom. From our data, no clear relationship appears between the dynamics and physiology of the free-living cryptophytes and *M. major*, pointing to the importance of the non free-living cryptophyte prey, either attached to or within the ciliate. Future studies should focus on using molecular approaches to better understand the specific interactions between *M. major* and *T. amphioexa*, in combination with *in situ* measurements of grazing rates. Additional investigations into the cause of the exceptionally high growth rates of phytoplankton in the Columbia River Estuary, as found in this and other studies (Herfort et al., 2011; Peterson et al., 2012), may also help to reveal any unique properties of this system- some of which could be contributing to the dynamics of this bloom.

**Figure Captions**

**Fig. 1** Map of the Columbia River estuary with the sampling site location marked.

**Fig. 2** Time series of environmental data from the sampling site including (a) temperature and salinity in surface waters, overlain with a low-pass filter represented by the grey and black lines, (b) post-low-pass filtered PAR (?), and (c) measurements of ammonia, nitrate, and phosphate concentrations in surface waters. Temperature, salinity (a), and PAR measurements (b) were determined via moored sensors (?). Nutrient measurements (c) were taken from discrete water samples.

**Fig. 3** Time series of SeaFlow measurements of cryptophyte cell abundance, with each plot showing data collected for a specific week. Grey points represent individual time points and the black line overlain represents the data after a low-pass filter was applied. Shading within the plot shows the tidal cycles, with the beginning of each grey region corresponding to low tide and the beginning of each white region corresponding to high tide.

**Fig. 4** Time series of the model estimates of mean daily division rate for the cryptophyte population separated by week. Vertical bars represent the standard error of the average of 24 model estimates. Shading within the plot shows the tidal cycles, with the beginning of each grey region corresponding to low tide and the beginning of each white region corresponding to high tide.

**Fig. 5** Plots of nitrate (a), phosphate (b), ammonia (c), and PAR (d) vs. the mean daily production rates of the cryptophyte population, determined using mean daily abundance and average mean daily division rate data from SeaFlow. P-values were found to be 0.048 (a), 0.1416 (b), 0.4221 (c), and 0.4221 (d).

**Table 1.** *M. major*, total cryptophyte weekly average, and *T. amphioexa* abundances (cells mL⁻¹ ), the estimated percent of *T. amphioexa* as determined via qPCR, and daily mean cryptophyte division rate. *T. amphioexa* abundances calculated using the estimated percent.