**Paper Draft 1**

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

**Abstract** will happen eventually...

**Introduction**

Every year, in the late summer or 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, *Mesodinium major,* previously referred to as *Mesodinium rubrum* (*= Myrionecta rubra*)(Lohmann, 1908; Jankowski, 1976). *M. major* and *M. rubrum* are among the marine microzooplankton that have been observed to harbor the plastids of their cryptophyte algae prey, allowing these predators to function as mixotrophs, capable of utilizing both phagotrophic and photosynthetic mechanisms to acquire carbon (Crawford, 1989). The specifics of the *M. major*-cryptophyte relationship though, have not yet been determined with certainty. It is unclear as to whether the cryptophytes inside *M. major* remain endosymbionts (Hansen and Fenchel, 2006; Hansen et al., 2012), or if the organelles are being “stolen” from the cryptophytes, as in the processes of kleptoplasty and karyoklepty (Gustafson et al., 2000; Johnson et al., 2007). But without an *M. major* isolate from the Columbia River Estuary to use in controlled laboratory experiments, investigations into the details of this specific predator-prey relationship remain limited to field studies on the bloom.

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 *Mesodinium sp.* bloom in the estuary (Herfort et al., 2011a). But reasons for the timing, patchiness, and persistence of the bloom are still not yet fully understood.

Past research on the estuary has focused largely on understanding the dynamics of the *M. major*, but it has been hypothesized that the cryptophytes may be important in controlling the bloom, as they are both a source of prey and photosynthetic ability to the ciliate. Chloroplasts originating from the cryptophyte, *Teleaulax amphioexa,* are found inside the *M. major* in the Columbia River Estuary, conferring photosynthetic capabilities (Herfort et al., 2011b). 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 proliferation of their ciliate predator.

This intent of this study is to monitor the dynamics of the cryptophyte populations in the Columbia River Estuary in an attempt to better understand their role the bloom. The specific questions that this study will address include:

-What are the effects of environmental conditions, such as nutrient availability, temperature, salinity, and light, on the physiology of the cryptophyte populations, and how does their physiology change over both tidal cycles, and the course of the bloom?

-Is there a relationship between cryptophyte physiology and the dynamics of *M. major*?

To answer these questions, we have monitored the abundances of a cryptophyte population continuously during a red tide bloom in the Columbia River Estuary from September-October 2013, and have estimated division rates of the combined populations of cryptophytes using a size structured division rate model from Sosik et al. (2003). In addition, *M. major* abundances were determined using FlowCAM, with daily counts of the ciliate. The abundances of *T. amphioexa* were estimated weekly, using qPCR.

**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 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. 1mL samples of whole water for (how do I specify “regular” flow cytometery from SeaFlow?) flow cytometry, 45mL samples for *M. major* counts via FlowCam, and 30mL surface water samples for nutrient analysis were taken in duplicate. The samples for flow cytometery and *M. major* counts were taken at three depths, and preserved with 20μL and 1mL of 25% gluteraldehyde, respectively. The samples for flow cytometry and nutrient analysis were then stored at -20°C, the samples for *M. major* counts refrigerated at 4°C.

**Division Rate Lab Verification**

For verification of the division rates found using the size-structured growth model, 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 blah equation (insert equation here).

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 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

**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.

**Nutrient Analysis**

JN writes this

**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.

**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

**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**

Surface water temperature and salinity (**fig. 2, a**) 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. In the first and third weeks of the time course, the average surface water salinity is higher and the temperature lower than the rest of the time course. Week four had both the lowest surface water salinity and lowest temperature, compared to the other weeks. (Compare these values for temperature and salinity to data from past red tides?)

During the time course, the daily maximum PAR fell between 116.6-802.9 (units) (**fig. 2, b**). The average daily PAR was between 28.9-177.

Surface water concentrations of ammonium ranged from 2.3 μM-74.4 μM, concentrations of nitrate from 3.5 μM-16.1 μM, and of phosphate from 0.4μM-1.5 μM (fig. 2, c). Changes in phosphate and nitrate appeared to be coupled throughout the first three weeks of the time course. A peak in the surface water concentrations of all nutrients occurred on 9/16.

**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 some of the highest concentrations occurring within the first week (**fig. 3, a**). 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 (units?) (**fig. 4**), with weeks three (**fig. 4, c**) and four (**fig. 4, b**) exhibiting dramatically increased rates as compared to the first two weeks.

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. Additionally, there was no relationship between cryptophyte abundance and division rate, pointing to the importance of loss processes (both physical and biological).

***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 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 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 time series, 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.

**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.

The cryptophyte abundances additionally change over the course of the month. The weekly average of cryptophyte abundance is highest during the first week, decreases in the middle of the month, and then increases again during the final week of the experiment (**table 1**).

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

Though the cryptophyte division rates that we have estimated from the model (0.023 to 9.78) appear to conflict with our laboratory estimates for the cryptophyte, *Rhodomonas sp.* (average daily?), this does not necessarily suggest that our model estimates are incorrect. Growth rates for *M. major* found in the Columbia River Estuary 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.

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 the high growth rates observed. 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). 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.

**Environmental Influences on Cryptophyte Division Rate 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, as well as surface water nutrients, to our cryptophyte division rate estimates did not reveal any limiting factors to the growth of the cryptophyte bloom (**fig 5**). 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 limited by overabundance of 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 division rate estimates did not show any correlation with PAR, negative or otherwise. Comparisons of environmental data with daily average cryptophyte production also did not result in any significant correlations (supplemental fig), though it should be noted that the regression lines were skewed by the presence of a singular outlying point that represented the last day in the time course.

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, we did not find a correlation (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. A major difference in the overall pattern of division rate over the course of the experiment is unlikely though, as the environmental factors influencing the change in division rate and physiology of the total population of cryptophytes will likely have the same effect on *T. amphioexa*.

Though our data set contains only four time points, comparisons 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. 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, but without having measured grazing rates in the field, we cannot determine this.

A number of other potential explanations as to the question of *M. major* bloom proliferation, despite low prey abundance, remain. A previous study on the 2011 bloom in the Columbia River Estuary documented *M. major*'s ability to retain cryptophytes attached to the ciliate's cirri, finding up to ~50 cryptophytes captured by these external structures (Peterson et al., 2012). Our measurements of *T. amphioexa* abundance are limited to those that are free-living, and it is likely 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 division rates of the cryptophyte population.

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