Title:

Dynamics of cryptophyte populations during red water blooms in the Columbia River Estuary.

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**ABSTRACT**

The annual red water bloom that occurs in the late summer/early fall in the Columbia River Estuary consists of both the mixotrophic *M. major*, as well as its cryptophyte algae prey. In order to better understand the dynamics of this bloom, we have used continuous *in situ* flow cytometry to monitor the abundances and division rates of free-living cryptophyte algae for 1 month during the September-October 2013 bloom. Continuous cryptophyte division rates were determined from flow cytometry data using a previously developed size structured division rate model. We have found that there is a significant positive correlation between the abundances of the cryptophyte and *M. major* populations during the bloom, which may potentially suggest a strong coupling between prey availability and *M. major* proliferation. Additionally, cryptophyte cell production was determined to have a significant positive correlation with dissolved inorganic nitrogen (DIN), revealing its role as a possible driver of cryptophyte growth. Overall, these results suggest that the dynamics of the combination cryptophyte-*M. major* bloom are likely influenced by the bottom-up processes (i.e. DIN availability) affecting the cryptophyte prey, whose abundance then impacts its predator, *M. major*.

Key words: cryptophytes; *Teleaulax; Mesodinoium;* division rates; SeaFlow

**INTRODUCTION**

The common coastal ciliate, *Mesodinium major*, previously referred to as *Mesodinium rubrum* (=*Myrionecta rubra*) (Lohmann, 1908; Jankowski, 1976), is among the marine microzooplankton that have been observed to harbor the plastids of their cryptophyte algae prey, *Teleaulax amphioexa* (Herfort et al., 2011b). This unique association allows the ciliate to function as a mixotroph, 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.

In the Columbia River Estuary (CRE), massive *M. major* blooms occur annually and last for several months during late summer through early fall (Herfort et al., 2011a). These blooms play an important role in shaping the biogeochemistry of the region by shifting the estuary from net heterotrophy to net autotrophy (Herfort et al., 2012). The 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, along with coinciding neap tides, have recently been shown to correlate with the initiation of the yearly *M. major* bloom in the CRE (Herfort et al., 2011a). The initiation phase of the bloom occurs in Baker Bay, where its shallow depth and retention nature favor high abundances (>100 cells L-1) and fast growth rates (1.2-3.1 d-1) of *M. major;* which then spread a few weeks later throughout the main estuary (Herfort et al., 2011a). The decline in abundance of small (<5 um) “*Teleaulax*-like” cryptophyte (TLC) cells prior to the increase in *M. major* abundance observed in 2011 (Peterson et al., 2012) suggest that the abundance of *T. amphioexa* is an important factor for the initiation of the blooms. A cyclic relationship between the *M. rubrum* and cryptophyte populations found in Ace Lake, with *M. rubrum* responding positively to increases in cryptophyte abundance, has also been determined for this Antarctic saline lake (van den Hoff et al., 2015). The underlying hypothesis of the present study is that the availability and type of cryptophyte prey is an important driver for the development of *M. major* blooms in the CRE.

Interpretations of the abundance patterns of cryptophytes are complicated due to the influence of cell division, cell mortality and strong physical transport in the CRE that can add or remove cells. Thus, the estimation of cryptophyte division rates is a critical component to our overall understanding of the interactions between *M. major* and its prey in the field. Division rates are commonly derived from dilution experiments (Landry and Hassett, 1982) or measures of cell cycle progression of the diel cycle (Carpenter and Chang, 1988). Both approaches are complicated, labor intensive and thus limited in their broad-sale applicability (Laws, 2013). Our understanding of the interactions between *M. major* and the cryptophyte prey is therefore extrapolated from relatively few measurements. In an important innovation, Sosik et al. (2003) adapted a matrix population model to estimate division rates (Caswell, 1989) based on the change of size distribution over the course of a day. Studies with both laboratory cultures and natural populations of cyanobacteria indicate that the model accurately estimates cell division rates (Sosik et al., 2003; Hunter-Cevera et al., 2014; Ribalet et al., 2015), and its effectiveness with cryptophytes is tested in the present study.

The aim of this study is to assess how environmental conditions affect the division rates of the cryptophyte populations and how the fitness of the cryptophyte prey affect the dynamics of *M. major.*

To do so, a 4 week-survey was conducted in 2013 during which nutrients, salinity, temperature, light irradiance, and abundances of TLC and *M. major* were also measured during red water blooms in the CRE. Daily division rates of TLC were estimated using the size-structured division rate model (Sosik et al., 2003).

**METHODS**

**Study Area**

The Columbia River Estuary 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). Samples were collected at 3 m depth from September 11th to October 2nd 2013 using a continuous seawater flow-through system of SATURN03, a scientific station located on a dock near Astoria, OR (**fig. 1**) (REF?).

**Sample Collection**

A continuous flow cytometer, SeaFlow, (Swalwell et al., 2011) sampled 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 °C 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 XX 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.

***Teleaulax*-like cryptophyte abundance**

Continuous measurements of cryptophyte abundances and cell size were made using SeaFlow, (Swalwell et al. 2011). The instrument was equipped with a 457-nm 300-mW laser (Melles Griot). Forward light scatter (a proxy for cell size), red and orange fluorescence were collected using a 457–50 bandpass filter, 572–27 bandpass filter and 692–40 band-pass filter, respectively. Seawater was prefiltered through a 100-μm stainless steel mesh (to eliminate large particles) prior to analysis. The flow rate of the water stream was set at 15 mL min−1 through a 200-µm nozzle for both cruises and for the laboratory experiments; this corresponded to an analysis rate of 15 μL min−1 by the instrument. A programmable syringe pump (Cavro XP3000, Hamilton Company) continuously injected fluorescent microspheres (1 μm, Polysciences) into the water stream as an internal standard. Data files were created every three minutes. Data were analyzed using the R package Popcycle version 0.2, which uses a SQLite relational database management system to retrieve flow cytometry data (<https://github.com/uwescience/popcycle>). A sequential bivariate manual gating scheme was used to cluster cryptophyte population based on orange fluorescence and forward light scatter measurements.

For the identification of *Teleaulax*-like cryptophytes (TLC) cells, discrete flow cytometry samples were collected once a day during slack tide, fixed with 0.025% glutaraldehyde and stored at -80 °C. Six months after sample collection, fixed samples were analyzed with a BD Influx cell sorter. 100 cells from the gated population of high-orange particles (assumed to represent TLC) 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 small size (<5 µm in length) and teardrop shape of the cells (Fig. S1) agreed with past observations of TLC (Peterson et al., 2012).

**Estimates of division rates**

We used a size-structured matrix population model developed by Sosik et al. (2013) to estimate population division rates of TLC. We implemented Sosik’s original Matlab model in an R package ssPopModel version 0.1.1, available on Github (https://github.com/armbrustlab/ssPopModel). The model is based on the assumptions that 1) cell growth is determined by light exposure, with other abiotic factors such as nutrient availability and temperature operating at longer time scales, 2) the probability of a cell dividing depends on size, 3) all cells within a discrete size class have the same probability to change to another size class, and 4) a cell divides into two daughter cells, each half the size of the mother cell. The model predicts the cell size distribution over the course of the day using the cell size/cell division relationships and the light-dependence of cell division. Note that the model does not take into account intrinsic cell death. Intrinsic cell death will only affect the estimate of division rate if the probability of cell death varies among the different TLC size classes. This has not yet been observed in the field or in cultures. In our study, cell death of *Rhodomonas* grown in cultures was low (< less than 1%) during the experiment (see below). For these reasons, we did not implement intrinsic death in the size-based division rate model.

To establish the accuracy of size distribution-based division rate estimates using SeaFlow measurements of forward-angle light scattering converted to cell volume using an empirical relationship (Ribalet et al. 2015), we compared size-based estimates of cryptophyte division rates (h-1) with cell-cycle based estimates of division rates.

*Estimated division rates in cultures.*

A non-axenic culture of the cryptophyte *Rhodomonas sp*. (CCMP 755) was grown in in the laboratory at 13 °C with a 16:8 light-dark cycle under 100 µE m-2 s-1 provided by white fluorescent tubes. Natural seawater amended with f/2 nutrients was used as a medium. The culture was grown for 4 days in a 20-L batch culture and mixed with a magnetic carboy stirbar and analyzed with SeaFlow. At the start of day 3, 1mL samples of the culture was collected in triplicate every 2 hours for 28 hours, fixed with 0.01% glutaraldehyde and stored in liquid nitrogen. 1 month after sample collection, fixed samples were stained with 0.01% green-fluorescing DNA stain SYBR Green I (diluted with dimethylsulfoxide) for 15 minutes at room temperature in the dark. Following the addition of fluorescent microspheres (1 μm, Polysciences) used as internal standard, stained samples were analyzed with a BD Influx flow cytometer. Data were obtained using the Spigot Operating Software version 5.0 (BD Biosciences) and analyzed using FlowJo version 9.7.2 (Tree Star). A minimum of 10,000 cells wascollected per sample. DNA frequency distributions were analyzed using FlowJo cell cycle platform to obtain cell fractions in G1, S, and G2+M phases. Division rates based on DNA distributions were computed as described previously (Carpenter and Chang, 1988). Division rates based on measures of DNA content were compared to division rates based on the size-structured matrix population model.

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*Estimated division rates in the field.*

The method used to estimatehourly division rates of *Rhodomonas sp.* in culture was applied to TLC populations in the field. Parameters fell into the following ranges: xx < *dmax* < xx; xx < *gmax* < xx; xx < *b* < xx; xx < *E\** < xx µE m-2 s-1. Daily-averaged division rates were calculated as the sum of hourly division rates over a 24-h period.

**Fluorescent In Situ Hybridization of *Teleaulax amphioexa***

KM and PZ write this

**Mesodinium cell abundance**

TP 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 h-1. The estimates for each method appear to follow the same general trend and are closely coupled throughout the survey. The coefficients of determination R2 = 0.597 (p < 0.001) indicate that the division rate estimates from the model agree with those from DNA distributions (Fig. S2).

**Environmental Data**

Throughout the survey, the average surface water temperature and salinity were 17.5 °C and 12 psu, respectively. In week 1 and 3, the salinity is higher and the temperature lower while week 4 had both the lowest surface water salinity (mean=6 psu) and lowest temperature (mean=16 °C), compared to the other weeks. Surface water temperature and salinity appear to be anti-correlated and oscillate with the tidal cycle. High tide was characterized by an influx of colder seawater, and low tide by an increase of warmer freshwater.

A 6 fold change of daily PAR was observed during the survey, with daily maximum values ranging from 100-600 µE m-2 s -1 (**Fig. 2B**). Weeks 2 and 3 exhibited the greatest amount of daily PAR.

Changes in phosphate and dissolved inorganic nitrogen (DIN) appeared to be coupled throughout the survey (**Fig. 2C**). In the first week, nutrient concentrations remained relatively stable. Higher nutrient concentrations were observed at the beginning of week 2.

**Cell abundances**

Hourly-averaged cell abundances of TLC ranged from 0.02 to 1.8 x 106 cells L-1 throughout the survey, with an average of 0.29 x 106 cells L-1 (**Fig. 3A**). Cell abundance did not appear to be correlated with tidal cycle, though oscillations in abundance did occur within individual days.

The highest abundances were observed during the first two days of the survey, with daily-averaged abundance of 0.52 x 106 cells L-1, while week 2 and 3 exhibited the lowest abundances, with an average of 0.08 x 106 cells L-1 and 0.09 x 106 cells L-1 respectively.

Abundances of *M. major* ranged from between 0.021-0.58 x 106 cells L-¹, with the highest values occurring the second day of the survey that coincided with the highest values of TLC abundances (**Fig. 3**). The highest weekly average abundance occurred during week 2 at 0.14 106 cells L-¹, and coincided with decreasing nutrient concentrations (**Fig. 2C**). The lowest average was during week 2 and 4 with 0.13 and 0.09 x 106 cells L-1 , respectively, which coincided with the lowest abundances of TLC (**Fig. 3A**). A positive correlation between abundances of TLC and *M. major* was observed during the survey (R2 = 0.63, p < 0.001) (**Fig. 4**)

**Division rates and cell production**

Estimates of the daily division rates of *Teleaulax*-like cryptophytes ranged from 0.30 ± 0.08 d-1 to 1.67 ± 0.13 d-1 (**Fig. 5**), which correspond to 0.43 and 2.4 division per day, respectively. The highest division rates were observed during the first two weeks, when concentrations of inorganic nutrients were the highest (Fig. 2C). While no significant correlation was observed between division rates and environmental conditions such as temperature, salinity, PAR and nutrient concentrations (Fig. 6A-E), there was a positive correlation between cell production (i.e., cell abundance x division rate) and concentration of dissolved inorganic nitrogen (R2 = 0.57, p < 0.01) (Fig. 6F).

**DISCUSSION**

**Dynamics of *Teleaulax*-like cryptophytes**

To the best of our knowledge, this study is the first to attempt to measure abundance of *Teleaulax*-like cryptophytes over tidal cycles. While important variations in cell abundances occurred within the tidal cycles, no general correlation between tidal cycle and abundance of Teleaulax-like cryptophytes patterns emerged from our survey. Although co-occurring biological and physical processes may obscure this relationship, our result indicate that *Teleaulax*-like cryptophytes was not associated with any particular water masses (i.e., marine or freshwater) suggesting that *Teleaulax*-like cryptophytes are estuarine… Need to rephrase that.

## THIS IS WHERE I STOPPED TODAY ###

No *in situ* division rates for the cryptophytes have been previously estimated in the CRE or elsewhere. Laboratory estimates of the division 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 by the black dot.

**Fig. 2** Hydrological conditions during the 4 week-survey in the CRE. A) Salinity (psu, black line) and temperature (ºC, grey line) at 3 m depth. B) Photosynthetic Active Radiation (PAR, µE m2 s-1) in surface waters, and C) concentrations of dissolved inorganic nitrogen (DIN, µM, open circle), and phosphate (µM, black circle) at 3 m depth.

**Fig. 3** Hourly-averaged cell abundances of *Teleaulax*-like cryptophytes (TLC, 106 cells L-1) and daily abundance of *Mesodinium major* (106 cells L-1) for each week of the survey in the CRE. Vertical bars represent the standard deviation of the hourly-mean cell abundance (n=20). Grey regions represent flood tide. The discontinuity of TLC data is the result of frequent clogging of the flow cytometer caused by suspended particle in the water.

**Fig. 4** Relationship between hourly-average cell abundances of *Teleaulax*-like cryptophytes (TLC, 106 cells L-1 d-1)and abundances of *Mesodinium major* (*M. major*, 106 cells L-1) during the survey in the CRE. A positive correlation between the cryptophyte and *Mesodinium* abundances was observed (R2 = 0.63, p < 0.01).

**Fig. 5** Daily division rates (d-1) of *Teleaul*ax-like cryptophytes during the survey in the CRE. Vertical bars represent the propagated standard error of the sum of hourly estimates during a 24 h-period.

**Fig. 6** Relationship between division rates (d-1) of *Teleaulax*-like cryptophytes with A) daily-averaged salinity (psu), B) daily-averaged temperature (ºC), C) daily-averaged Photosynthetic Active Radiation (µE m-2 s-1) and concentrations of D) dissolved inorganic nitrogen (DIN, µM) and E) phosphate (µM); relationship between cell production (106 cells L-1 d-1) and concentrations of F) dissolved inorganic nitrogen (DIN, µM) and G) phosphate (µM) and during the survey in the CRE. A positive correlation between cell production and DIN was observed (R2 = 0.57, p < 0.01).