Title:

Dynamics of *Teleaulax*-like cryptophytes during red water blooms in the Columbia River Estuary.

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**ABSTRACT (one page)**

The annual red water bloom that occur in the late summer/early fall in the Columbia River Estuary (Oregon, USA) consists of the mixotrophic ciliate *Mesodinium major* and its cryptophyte algae prey *Teleaulax amphioexa*. 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. Daily cryptophyte division rates were determined from flow cytometry data using a previously developed size structured division rate model.

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We have found that there is a significant positive correlation between the abundances of the cryptophyte and *M. major* populations during the bloom, which 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.

MORE TO COME.

Key words: cryptophytes; *Teleaulax; Mesodinium;* growth 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 *Mesodinium* populationsare now understood to be important primary producers in coastal and estuarine systems (Stoecker et al., 1989, Herfort et al., 2012), little is known about the ecology and physiology of the *Teleaulax* cryptophyte prey and how it impacts the dynamics of *Mesodinium*.

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), free-living “*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 TLC is an important factor for the initiation of the blooms. In an Antarctic saline lake, abundance of *M. rubrum* was enhanced following increases of cryptophyte populations (van den Hoff et al., 2015). The underlying hypothesis of the present study is that the availability of free-living 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. The estimation of cryptophyte division rates is a critical component of our overall understanding of the interactions between *M. major* and its prey. 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 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 for 1 month, 4 days a week, 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?). All discrete samples were collected during the turn of the high tide.

**Hydrological conditions**

Water temperature, salinity, pH were measured continuously using XXX. Photosynthetic Active Radiations were obtained from a mooring located X miles away from SATURN03.

**Nutrient concentrations**

30 mL surface water samples for nutrient analysis were taken in duplicate.

JN writes this

***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 ºC. 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. A day 3, 1mL sample 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.

*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.

**Crytophyte community composition**

KM and PZ write this

***Mesodinium rubrum* cell abundance**

45 mL samples fixed with XX% glutaraldehyde were collected for *M. major* counts, stored at 4 °C and analyzed XX months later using FlowCAM.

TP 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 = 0.60 (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. High tide was characterized by an influx of colder seawater, and low tide by an increase of warmer freshwater. In week 1 and 3, the salinity was 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. The pH ranged from 7.8 to 8.4, with the lowest values observed on day 3, and increased progressively during the survey (**Fig. 2B**). The variations of pH were correlated with tidal cycles, with values increasing during flood tides. A negative correlation was observed between pH and DIN and phosphorus concentrations (R2 = 0.38 and 0.33, p< 0.05, p< XX respectively). 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 as the sum of nitrate, nitrite and ammonium) appeared to be coupled throughout the survey (**Fig. 2C**). During the first week, nutrient concentrations remained relatively stable, increased in week 2 and decreased in week 3 and 4.

**Cell abundances**

Hourly-averaged cell abundances of TLC ranged from 0.02 x 106 to 1.8 x 106 cells L-1 throughout the survey, with an average of 0.29 x 106 cells L-1 (**Fig. 3**). Variations in cell abundance did not appear to be correlated with tidal cycle, though oscillations in abundance did occur within individual days. No significant correlation was observed between TLC abundances and salinity or temperature (data not shown). The highest abundances of TLC were observed during the first two days of the survey, with daily-averaged abundance of 0.52 x 106 and 0.XX x 106 cells L-1, respectively. 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. TLC abundances during the survey were not significantly correlated with environmental conditions such as nutrient concentrations (data not shown).

The abundances of M. major during the survey ranged from 0.021 x 106 to 0.32 x 106 cells L-¹. The highest abundances occurred during week 2, with a weekly average of 0.14 x 106 cells L-¹, and coincided with high DIN concentrations (**Fig. 2C**), however no significant correlation was observed between abundances of *M. major* and DIN concentrations (data not shown). The lowest weekly averages occurred in weeks 1 and 4 with 0.10 x 106 and 0.11 x 106 cells L-1, respectively (Fig. 3B and C). Despite the apparent dissimilarity between the weekly averages of *M. major* and TLC, of which the greatest weekly average occurred in week 1, a positive correlation between abundances of TLC and *M. major* was observed during the survey (R2 = 0.51, p < 0.001) (**Fig. 5**). This is due to the use of corresponding hourly estimates of TLC abundance, as opposed to daily or weekly averages, in comparisons with M. major abundance. This difference in results highlights the importance of high time resolution sampling.

**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, with the highest division rates observed at day 3 of the survey, which coincided with the lowest values of pH. While no significant correlation was observed between division rates and temperature, salinity, PAR and inorganic nutrient concentrations (**Fig. 6A-C, E-F**), division rates were negatively correlated with pH during the survey (R2 = 0.41, p < 0.05) (**Fig. 6D**), with decreasing division rates observed with daily-averaged pH > 8.0. Cell production was correlated negatively with pH (R2 = 0.53, p < 0.05) and positively with concentration of dissolved inorganic nitrogen (R2 = 0.58, p < 0.01) (**Fig. 6G, H**).

**Crytophyte community composition**

The percent of *Teleaulax amphioexa* to the total cryptophytes ranged from 0.06% - 0.40% (**Table 1**), with the highest and lowest percent of *T. amphioexa* occurring during the first and second week of the survey, respectively, similar to the abundances of TLC and in direct contrast to the weekly averages of *M. major* abundance (**Fig. 3**).

**DISCUSSION**

**Ecophysiology of the *Teleaulax*-like cryptophyte during the survey**

The cryptophyte *Teleaulax amphioexa* is a marine species distributed in coastal habitats worldwide. During our survey, no consistent increase in TLC abundance was observed with seawater intrusion, and variations in TLC abundances were not shown to be related to tidal cycle. These results suggest that TLC distribution is very patchy in the CRE, which could be caused by strong physical processes in the estuary, obscuring the effect of tidal cycle on TLC abundances. The absence of a correlation between TLC division rate and abundance (data not shown) similarly suggest that these physical processes play a key role in determining the distribution of TLC abundances.

To the best of our knowledge, this study is the first to attempt to estimate division rates of *Teleaulax* species in the field. Laboratory estimates of *Teleaulax amphioexa* division rates under nutrient replete conditions range from 0.7 to up to 1.57 d-1 (Nishitani et al. 2008; Berge et al. 2010; Rial et al. 2012). This is in good agreement with our highest estimate of 1.67 d-1 observed on day 3 of the survey, suggesting that cells at that time were growing near optimal growth conditions.

In the turbid waters of the Columbia River Estuary, light is generally considered to be an important factor limiting phytoplankton growth (Herfort et al., 2012). However, we did not observe a significant correlation between light intensity and division rates for *Teleaulax*-like cryptophytes estimated at 3 m depth during the survey, which support previous studies that showed that the photosynthetic machinery of cryptophytes is well adapted to low light conditions (Bergman et al., 2004). Instead, we observed a negative correlation between division rates of TLC and pH during the survey. The negative effect of pH values above 8 on the division rates of TLC was unexpected, considering that a previous study has shown that the growth of *Teleaulax amphioexa* was only affected by low pH values (pH < 6.1) and did not observed any negative effects on their growth at pH > 8 (Berge et al. 2010). One explanation for this apparent discrepancy could be that nutrient availability may have still been in excess during their experiment despite the reduced solubility of nutrients at higher pH. During our survey, pH was negatively correlated with nutrient concentrations and cell production was positively correlated with DIN concentrations. These results suggest that elevated pH reduced the availability of DIN, whose concentrations became the dominant factor limiting the growth of *Teleaulax amphioexa* during the survey.

**Impact of *Teleaulax*-like cryptophyte on *M. major* abundances during the survey**

The abundances of *Teleaulax*-like cryptophytewere positively correlated with the abundances of *M. major* and both were of similar order of magnitude. Assuming an ingestion rates between ~3.5 and 8.9 cryptophytes ciliate-¹ day-¹ by *M. rubrum* (Yih et al., 2004; Hansen and Fenchel, 2006), our result suggest that abundances of free-living *T. amphioexa* were limiting the abundance of *M. major*. Using a FISH probe for the *T. amphioexa* 28S DNA, up to >20 *T. amphioexa* cells were observed either within or attached to a single *M. major* cell (Peter Zuber, personal communication). This finding potentially supports previous observations of *M. major* during red water blooms in the CRE having the ability to retain cryptophytes attached to the ciliate's cirri (Peterson et al., 2012). Alternatively, it may be possible that the ingested *T. amphioexa* cells are able to divide inside the ciliate. While the division of whole endosymbiont cells has not yet been demonstrated in culture for *M. rubrum*, it has been observed in other single-celled endosymbiont-baring organisms (Kodama and Fujishima, 2009; Johnson, 2011). But without a cultured representative of *M. major*, explanations as to the specific fate of *T. amphioexa*, either as a whole endosymbiont or as sequestered organelles, inside the ciliate remain speculative and are constrained by the limitations of field investigations.

The approach used in this study circumvents many of the limitations associated with traditional methods of monitoring plankton populations in the field. Continuous flow cytometry allows for high-resolution sampling of TLC abundance. In a dynamic system, such as the CRE, the abundance of a population is highly influenced by physical process and can shift dramatically over the course of just a few hours. This is exemplified in our data set, where a statistically significant positive correlation between TLC and *M. major* abundance only becomes apparent when hourly estimates of TLC abundance are used. This relationship is obscured with the use of daily or weekly estimates of abundance, yet previous studies on the CRE have drawn their conclusions based low time resolution sampling schemes. Despite the avoidance of many of the common pitfalls of *in situ* monitoring, our methods are not completely free of constraint. The model utilized to determine division rates relies on a number of important assumptions, including the assumption that the population of interest 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, despite the consistent morphology seen among the sorted population. But we do not actually have any evidence that connects this possible model assumption violation to any potential errors in our division rate estimates. Furthermore, our validation of the model on a laboratory culture of cryptophytes confirms the model’s ability to accurately estimate the division rates of these eukaryotic phytoplankton.

TO INCLUDE (?):

-lack of correlation between TLC division rates and *M. major* abundance (also in results?)

- potential explanations for the two points above

-limitations of the model

- discussion of cryptophyte community composition

- there is no *M. major* in culture

**Conclusions**

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 our understanding of the ecophysiology of *Teleaulax* remains limited. 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.

**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 Radiations (PAR, µE m2 s-1, black line) in surface waters and pH (grey line), 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 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), D) pH, and concentrations of E) dissolved inorganic nitrogen (DIN, µM) and F) phosphate (µM); relationship between cell production (106 cells L-1 d-1) and G) pH, H) dissolved inorganic nitrogen (DIN, µM) and I) phosphate concentrations (µM) during the survey. Dashed lines represent model II linear regression of plotted data and R2 represents the coefficient of determination.