

Growth and domoic acid production of *Pseudo-nitzschia* on multiple scales

Kelsey Gaessner^{1,2}, Robin Kodner^{1,3}

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¹Friday Harbor Laboratories, University of Washington, Friday Harbor, WA 98250

²Department of Biology, University of Washington, Seattle, WA 98195

³School of Oceanography, University of Washington, Seattle, WA 98195

Contact Information

Kelsey Gaessner

University of Washington

Department of Biology

24 Kincaid Hall

Box 351800

Seattle, WA 98195-1800

kgaessne@u.washington.edu

Abstract

The toxin-producing diatom *Pseudo-nitzschia* has been monitored in a bloom for the last three years (2009-2011) in East Sound, WA. To expand the understanding of the dynamics of toxin production, especially in response to climate change and ocean acidification, two additional studies were conducted, including an ocean acidification mesocosm, and a laboratory growth experiment using low pH and low Silica conditions. Results from the mesocosm experiment were inconclusive. The *Pseudo-nitzschia multiseries* cultures grown in the laboratory were grown under low pH and low silica conditions. The pH was controlled by adding CO₂ saturated water to the cultures, instead of HCl, which has been previously done by other studies. Growth of *P. multiseries* was unaffected by low pH, and was negatively affected by silica limitation.

Keywords: *Pseudo-nitzschia*, East Sound, Ocean acidification, mesocosm, Silica, pH

Introduction

Global climate change and ocean acidification are two of the most important issues faced by the world today, and are consequences of anthropogenic activities. Of these issues, one of the most pressing is the rapid increase of CO₂ released into the atmosphere. Atmospheric CO₂ levels have risen almost 100 ppm over the past 250 years from 280 ppm to nearly 384 ppm in 2007 (Solomon et al. 2007), at a rate at least an order of magnitude faster than has been observed for that last million years (Doney and Schimel 2007). Anthropogenic CO₂ released into the atmosphere is absorbed by the ocean, changing the ocean's chemistry and causing a decline in pH. Ocean acidification has already caused a reduction in the world oceans' pH by an average of 0.1 units (Feely et al. 2008). This change in pH can have varying effects on marine organisms, both positive and negative. Ocean acidification is predicted to have large scale effects on the

carbon cycle, as it will alter the CO₂ speciation and alkalinity in seawater (Balch 2008). This in turn will have effect on organisms that utilize CO₂ for photosynthesis and bicarbonate for building calcium carbonate shells and skeletons.

Phytoplankton play a very important role in the carbon cycle, predominantly in primary production utilizing CO₂ in seawater and then carbon sequestration through the biological pump that can take that carbon and remove it from the cycle over geologic time scales (Berner 1991). In addition to their role in carbon biogeochemistry, phytoplankton primary production in marine environments is the foundation of all food webs, so it is important to know how the process of ocean acidification will affect these organisms. While each group, or even species of phytoplankton can have a different response to lower pH and higher pCO₂ levels in the ocean, it is important to understand how shifts in phytoplankton communities will affect ecosystems and food webs, and the net carbon cycle in general.

One observed change in phytoplankton communities that appears to already be occurring is an increase in harmful algal bloom (Halegraeff 1993, Anderson et al. 2002). Much of the documented drivers of this change are eutrophication and increased temperatures of the waters, though little is known specifically how changes in pCO₂ and pH will affect harmful algal blooms. Changes in harmful algal blooms may cause unexpected changes in planktonic and benthic communities, as well as the health of many larger marine organisms.

To investigate how phytoplankton, specifically harmful algal bloom species respond to higher pCO₂ levels, and a subsequent lower pH, we have explored phytoplankton dynamics on three scales: a field study in East Sound, Orcas Island, a

mesocosm study, and a laboratory culturing experiment. These studies focused primarily on the diatom genus *Pseudo-nitzschia* because it includes several harmful algal bloom species, and there has been a previously recorded toxic *Pseudo-nitzschia* bloom in East Sound during the spring of 2009 and 2010.

Pseudo-nitzschia is a group of pennate, chain-forming diatoms. At least nine species of *Pseudo-nitzschia* produce the neurotoxin domoic acid (Bates 2008). Bioaccumulation of domoic acid in animals can lead to amnesic shellfish poisoning, and is responsible for massive fish kills many sea lion and marine mammal deaths each year (Goldstein 2008).

A toxic *Pseudo-nitzschia* bloom in the East Sound, Washington was monitored throughout the spring of 2009 and 2010, and is considered predictable. East Sound is a fjord in Orcas Island with a sill at the mouth that restricts the water flow in and out. This allows sediments to settle to the bottom and be preserved. The preservation of sediment might allow *Pseudo-nitzschia* resting cells to persist in East Sound and bloom when environmental conditions become favorable.

For the second portion of this study, a mesocosm experiment was set up at the Friday Harbor Labs, Washington to investigate the effects of high pCO₂ on phytoplankton communities. Water saturated with CO₂ gas was added to 2000L bags hanging off the docks to simulate predicted future pCO₂ levels. The purpose of this study was to see not only how water chemistry changed through time in an enclosed environment, but to also see how an increase in pCO₂ affected the bacterial and phytoplankton communities.

The third portion of this study is a laboratory culturing experiment that aims to combine the East Sound field sampling with the mesocosm experiment. A harmful algal bloom species, *Pseudo-nitzschia multiseries* was selected as the study organism because of its dominant presence in previous East Sound blooms. Because *P. multiseries* produces domoic acid, and yearly toxic events have been recorded, it is important to learn more about the environmental factors that influence toxin production, especially as ocean acidification becomes a pressing issue.

Several studies have attempted to understand the triggers for domoic acid production, including silicate, nitrate, and phosphate limitation, as well as the effects of a higher pH in the water (Lundholm 2004). One study (Bates et al. 1991) found that silicate limitation induced toxin production by *P. multiseries*. However, these studies have failed to address the potential effects of ocean acidification on toxin production.

To investigate these effects, a *P. multiseries* isolate, ES9, from the East Sound 2010 bloom was cultured under low silica, low pH, and a combination of both treatments. A low silica treatment was used to not only confirm Bates' findings, but to also provide a comparable value of domoic acid for the low pH treatment. The dual treatment was tested to see if there was an additive effect of low silica and low pH to toxin production. Data from the 2010 East Sound *Pseudo-nitzschia* (Kodner 2010, unpublished) was also used to show environmentally relevant levels of domoic acid production by the same strain of *P. multiseries*.

Methods

East Sound Field Sampling

Plankton tows were taken off a small boat at several locations in East Sound. A transect was taken with locations just outside, at the mouth, Rosario Point near the center, and in the upper region of East Sound. Tows were taken for 5 minutes with a 15 μM mesh net. A flow meter was attached to the mouth of the net to measure the amount of water filtered. Samples were preserved with lugols and counted using a Palmer counting chamber.

Cell counts were performed at 20x, and all diatoms were identified to genus. The volume in one field of view was calculated to be 0.343 μL , and the cell concentration in the field was calculated using cell counts and flow meter values.



Mesocosm Experiment

A dock was built and attached to the existing docks at the Friday Harbor Labs, Washington. Six-2000L bags were made of cereal-grade plastic, and supported with metal rings at the surface. The bags were hung off the dock and covered with plastic domes to keep out environmental pollutants. The bags were filled with ambient seawater. Brine was added to the bags to increase the salinity by approximately 1 psu. This allowed the bags to have a higher osmotic pressure than the surrounding water, allowing them to stay full.

CO₂ saturated filtered seawater was added to three of six bags as a treatment. The pCO₂ of the ambient water was approximately 600 ppm, and the treatment was approximately 1200 ppm. Samples were taken daily using a nicking bottle to measure the DIC, DOC, pCO₂, alkalinity, and nutrients. Samples were also taken using an integrated water sampler to measure changes in bacterial and phytoplankton communities. Chlorophyll extractions were taken to measure total changes in phytoplankton biomass.

Culturing Experiment

To test the response of *P. multiseriis* and domoic acid production, four batch culture treatments were tested in triplicate. The four cultures included a control, low pH, silicate limitation, and low pH with silicate limitation.

The cultures were grown in 500 mL polycarbonate bottles, filled with 400 mL of artificial seawater with F/4 nutrients (Guillard 1975). The low silica treatment was grown with F/20 silicate, at a concentration 5.3 uM. The controls were grown with 53 uM silicate.

P. multiseriis was isolated (ES9) from the 2010 East Sound bloom, and maintained in F/2 medium at 13°C under a 16h light, 8h dark cycle. For the experiment, the culture was transferred to artificial seawater (Berges et al. 2001), and maintained at 15°C under a 12h light, 12h dark cycle for two weeks. The parent culture was in stationary growth phase prior to inoculating to the experimental treatments.

Growth Measurements

Daily growth measurements were taken 2 hours into the light cycle. Two mL of culture was removed from the bottle, and allowed to sit for at least 15 minutes in a plastic cuvette prior to reading. The cuvette was shaken prior to readings to suspend any cells that may have settled out of the water. Raw Fluorescence measurements were taken using Turner Designs Trilogy Laboratory Fluorometer. The fluorometer was turned on an hour prior to taking measurements to allow it to warm up and for the readings to stabilize. Three measurements were taken for each sample and averaged.

pH

The pH of the cultures was initially lowered to 8.07 for control pH conditions, and to 7.60 for low pH conditions prior to inoculation. The pH was lowered to 8.07 because it reflects current environmental averages (Royal Society, 2005). The pH was lowered using artificial seawater that was bubbled with CO₂ to lower its pH. The pH was measured using a Thermo Scientific Orion pH probe. The pH was measured daily, and adjusted to 7.6 for the treatments if the pH had increased. The pH was not adjusted after the initial value for the controls.

Domoic Acid

To test for the initial domoic acid content of the cells, 8.5 mL of the parent culture was filtered at the beginning of the experiment. Samples were also filtered (25 mL) from each culture at day 8, when the cultures were just beginning to reach exponential phase, day 11, when the low silica cultures were just reaching stationary phase, and when the high silica treatments were in mid exponential phase, and days 16 and 18 for the low silica treatment and high silica treatments, respectively. These final days were chosen for domoic acid analysis because they both coincide with the fifth day of stationary phase. An ELISA antibody kit was used to measure the amount of domoic acid produced in each of the cultures, and samples were run in triplicate on the plate.

Results

East Sound

There was a large amount of variability in the total concentration of phytoplankton throughout the sampling period of the East Sound bloom. The variability is apparent through time at each station in the transect, as well as between each station on any given day (Fig. 1). Days with no cell counts indicate no data.

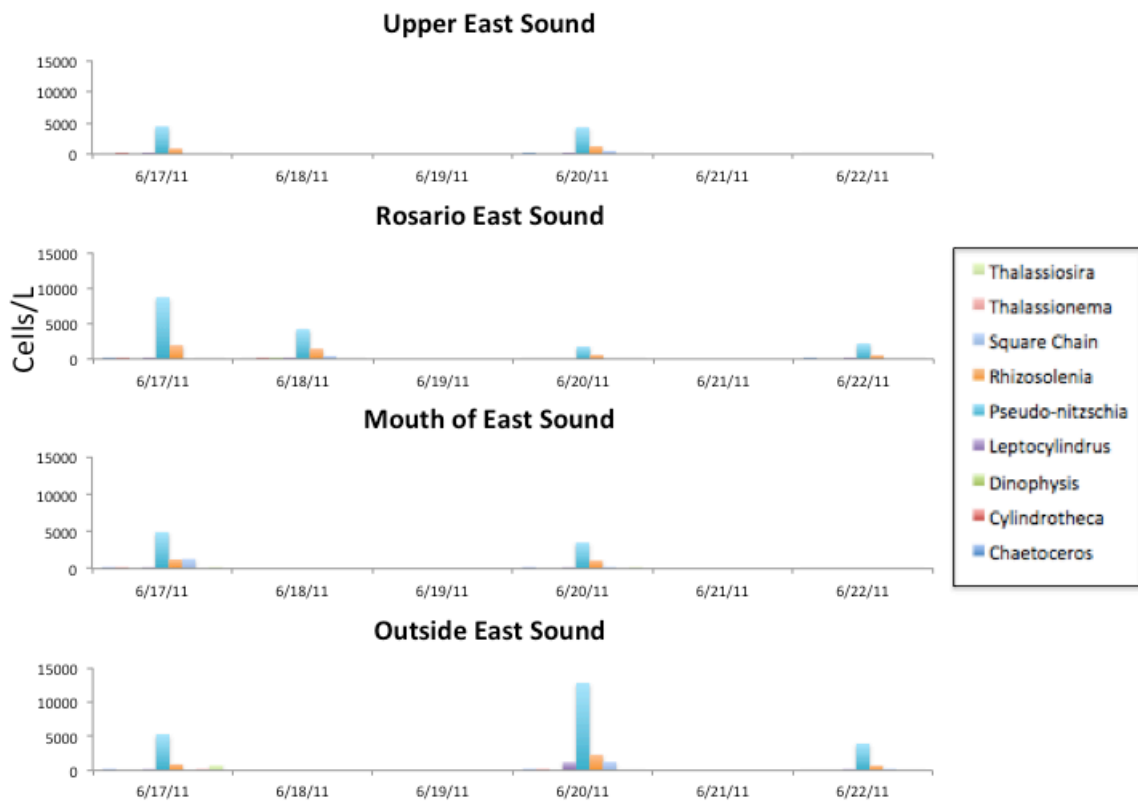


Figure 1. Present genera in East Sound throughout the bloom, in Cells/L. The graphs are shown in the order of the vertical transect, with Upper East Sound being the northern most sample station, and Outside East Sound being the southern most sample station.

The percentage of *Pseudo-nitzschia* was relatively constant throughout the bloom sampling period (Fig. 2), even though the total biomass was variable. The percentage of *Rhizosolenia* was slightly different between sights on 6/17/11 and 6/20/11, when a complete transect was taken.

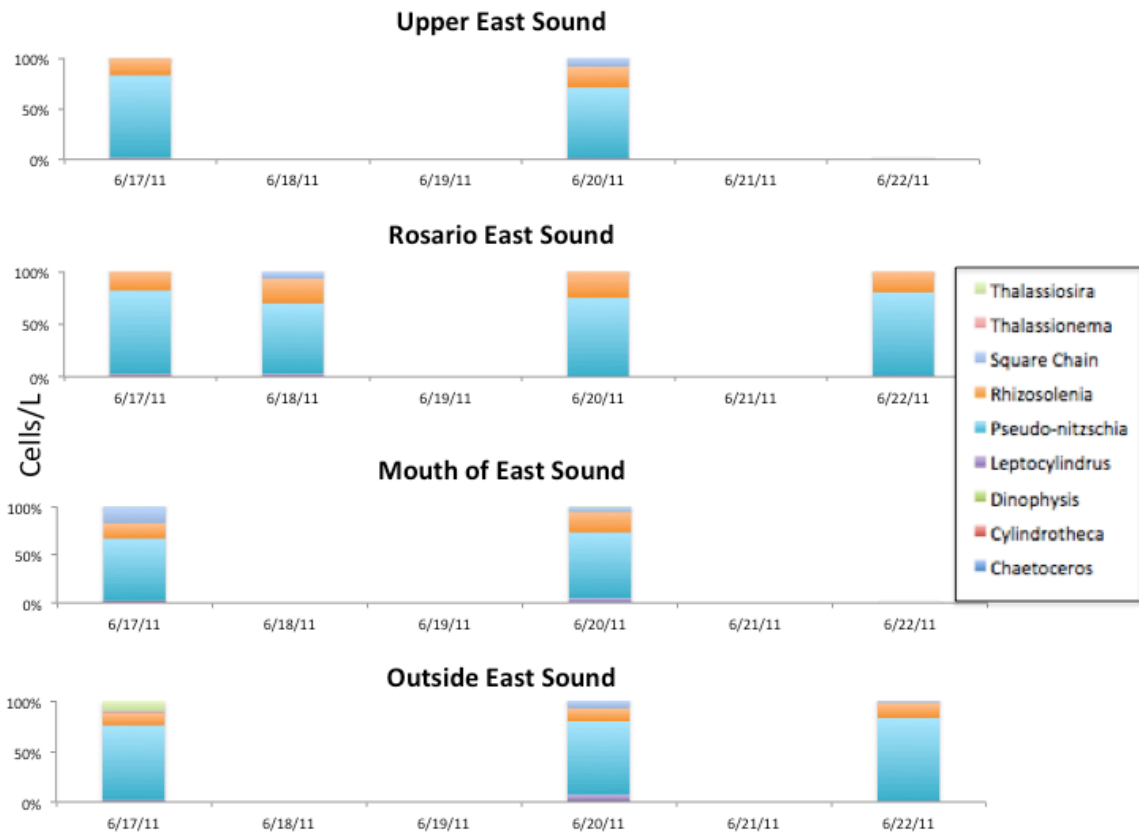


Figure 2. Percent of present genera across location and time. The graphs are shown in the order of the vertical transect, with Upper East Sound being the northern most sample station, and Outside East Sound being the southern most sample station.

A principal components analysis including relevant environmental variables for 7 of the sample days was analyzed. These factors include time, temperature, pH, salinity, dissolved oxygen, and chlorophyll. The biological factors included in the analysis are *Pseudo-nitzschia* and total cells. Samples without a complete environmental data set were not included in the analysis. The number following the sample location indicates the day

the sample was taken. Chlorophyll was the driving variable for the differences between sites (Fig. 3). Differences in the physical environment of each sample location were not significant enough to explain the variation between the sites. There is a grouping of OES1, MES1, UES1, and RES2, indicating that these samples are similar. RES4 and OES3 are outliers, indicating that they are different than the other samples.

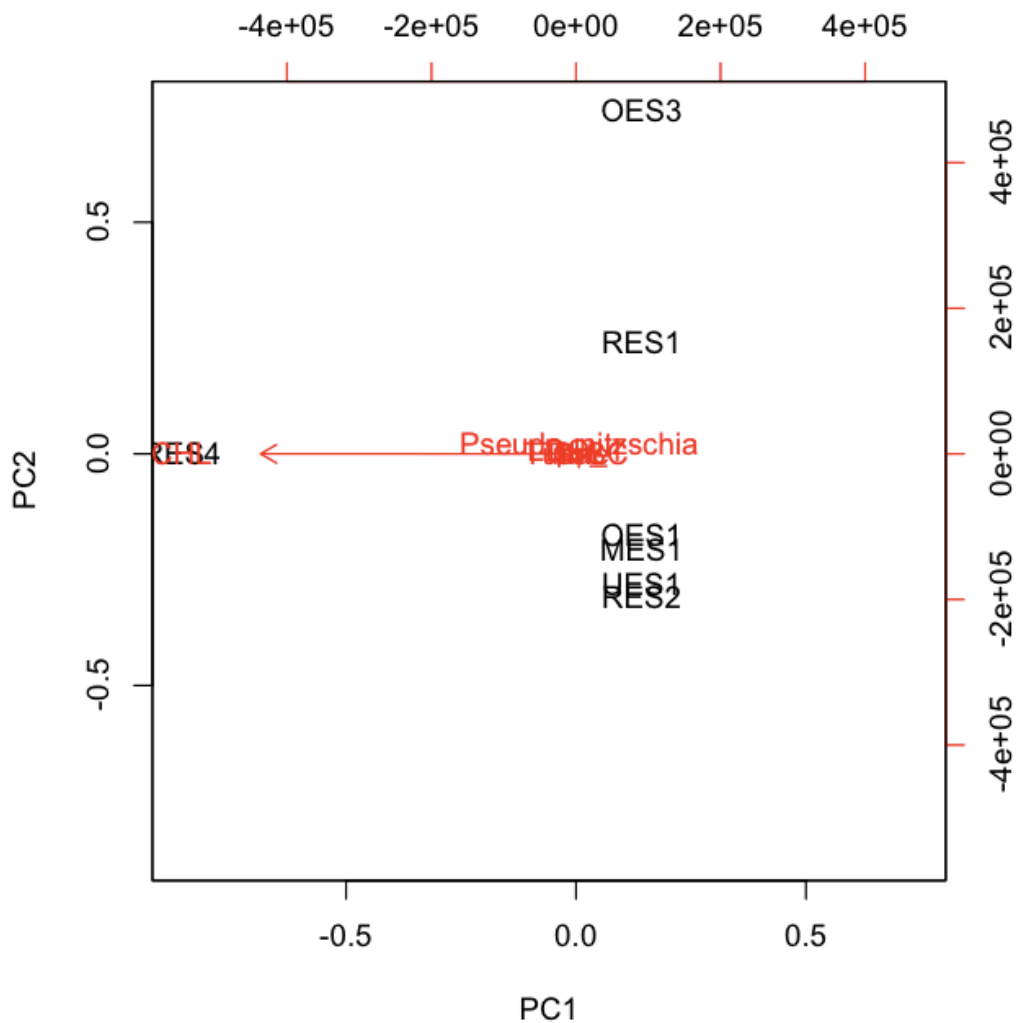


Figure 3. Principal components analysis including most of the samples taken, and relevant environmental variables. Chlorophyll is the driving variable of the differences found in the samples, as indicated by the red arrow.

There was a decline in the Shannon-Weiner Diversity Index for RES and OES throughout the bloom. There was an increase in diversity at UES. The diversity was constant at MES throughout the bloom (Fig. 4). Diversity is calculated using the relative abundance and evenness of present species in a sample. The same pattern was found during the 2010 *Pseudo-nitzschia* bloom, which was sampled only at RES. There was a similar pattern between years, with a decline in diversity as the bloom progressed (Fig. 5). The red box on the graph indicates the period of the *Pseudo-nitzschia* bloom. While the sampling resolution is much lower for the 2011 data, the initial and final values seen in the 2011 data are comparable to the values found in 2010.

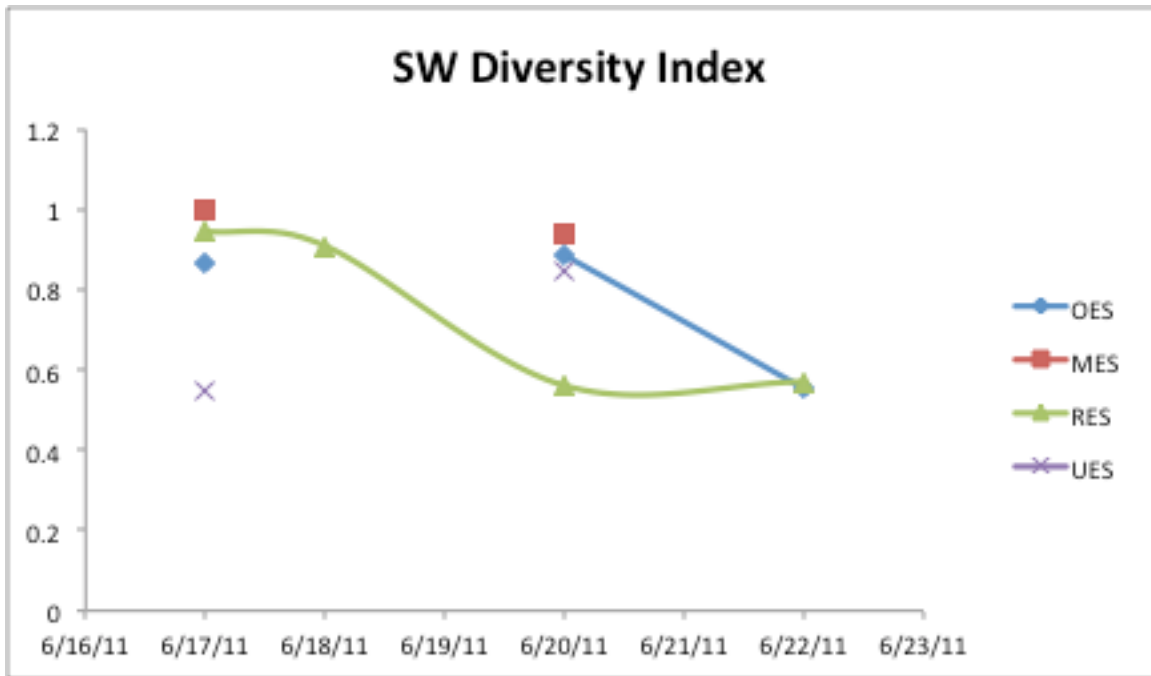


Figure 4. Shannon-Weiner diversity index through time for samples taken at each sample site.

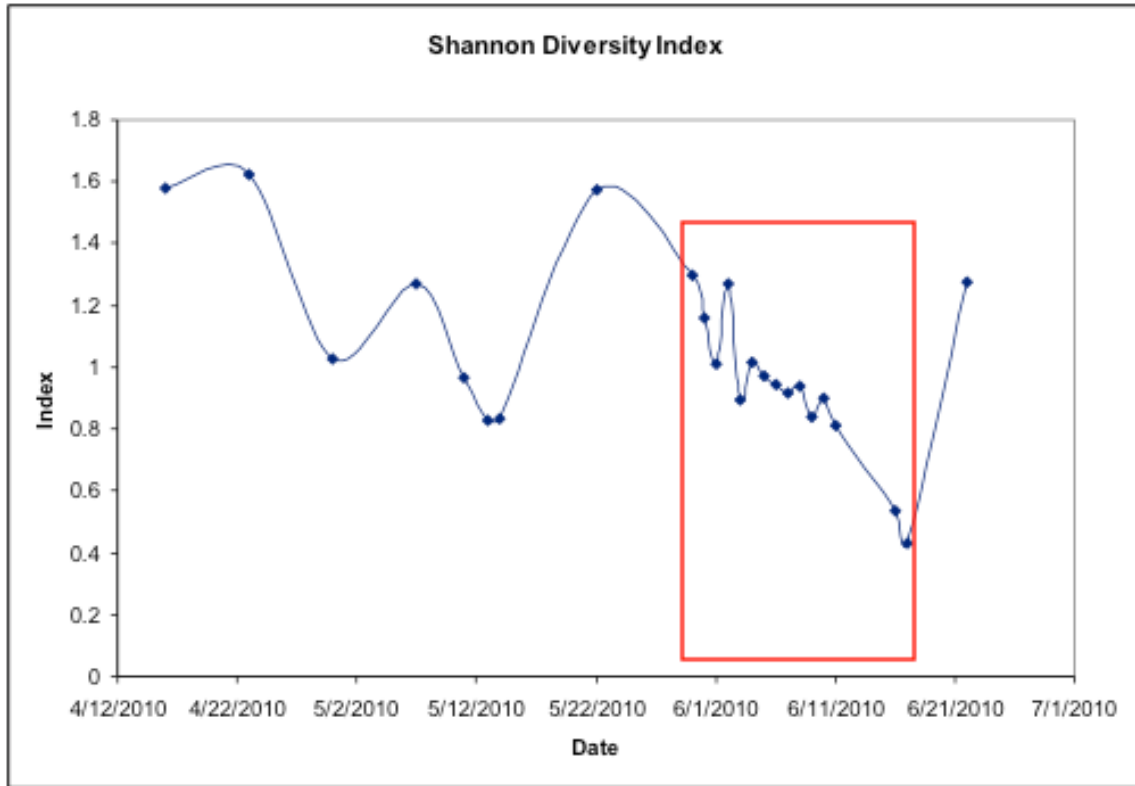


Figure 5. Shannon-Weiner Diversity index for samples taken during the East Sound 2011 *Pseudo-nitzschia* bloom (modified from Gaessner 2011, presentation).

Mesocosm

Daily chlorophyll measurements were averaged for the triplicate treatments, and graphed together for each day (Fig. 6). There was no statistical difference between the total chlorophyll of the control and the treatment samples ($p > 0.05$ for all days after the start of the experiment).

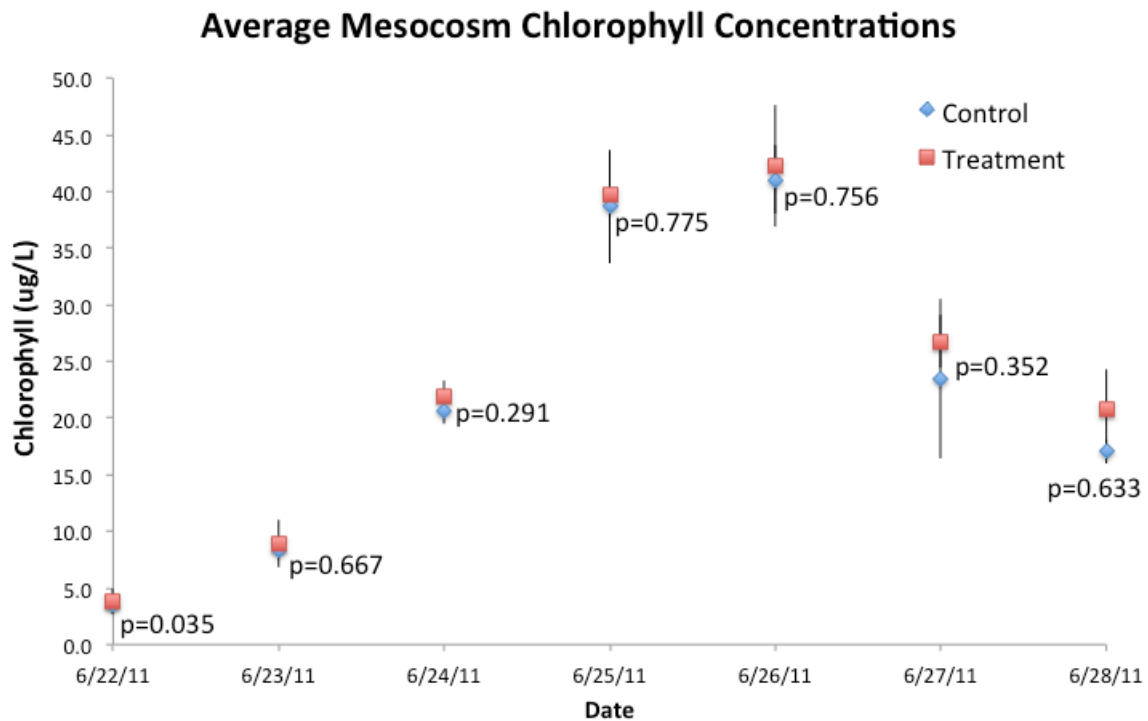


Figure 6. Chlorophyll data from the mesocosms, measured in $\mu\text{g/L}$. A t-test was used to obtain a p-value. Values of $p < 0.05$ show significant variation between samples.

Growth Experiment

There was a distinct acclimation period from days 1-8, a distinct exponential period for the low silica treatments for days 9-11, and days 9-13 for the high silica treatments, and a stationary phase for all of the cultures following the exponential period (Fig. 7). There was a decline in the raw fluorescence for the control and low silica treatments at the end of the experiment, for days 16-18. There was a distinct separation between the low and high silica cultures ($p \ll 0.001$) in terms of the raw fluorescence. There was no statistical difference between the low pH and control treatments on any of the days of the experiment ($p > 0.05$), or between the low silica and low silica + low pH treatments ($p > 0.05$).

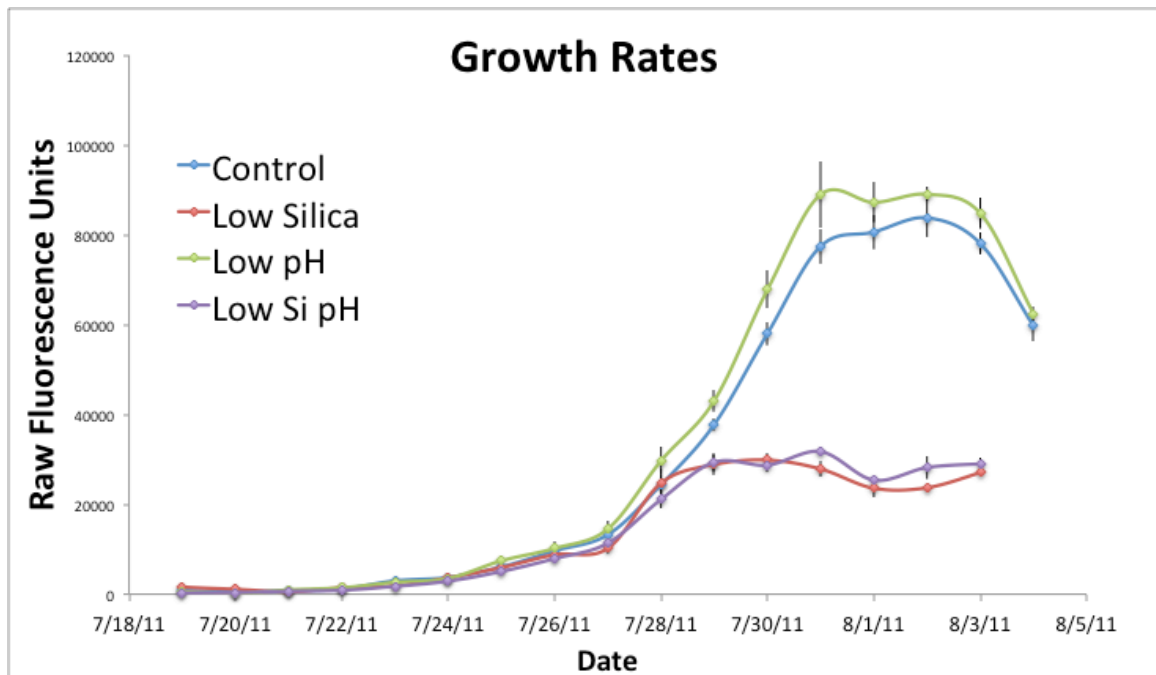


Figure 7. Growth rates of the average Raw Fluorescence Units for each treatment. Treatments were run in triplicate. Fluorescence is measured in Relative Fluorescent Measurements.

There was a change in pH for all of the cultures every day (Fig. 8). There was an increase in pH for the majority of cultures each day. The greatest change in pH was found during the exponential phase for the all cultures.

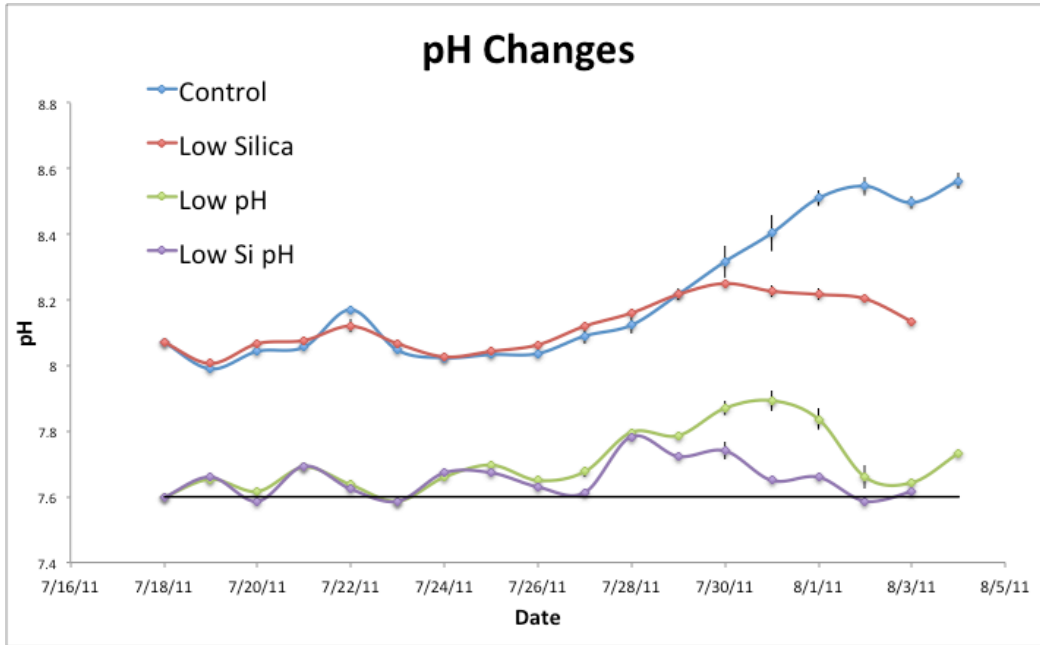


Figure 8. The pH of the samples measured from each bottle daily. Treatments were run in triplicate. The top two lines represent the pH of cultures without a low pH treatment. The bottom two lines represent the pH of cultures with a low pH, and were lowered to 7.60 daily, after measurements were taken, as indicated by the solid black line.

There is a correlation between the pH and growth, with a linear increasing trend (Fig. 9). The R^2 value for the control is 0.99017, indicating a strong relationship between the growth and pH. The relationship between growth and pH weakens for the treatments.

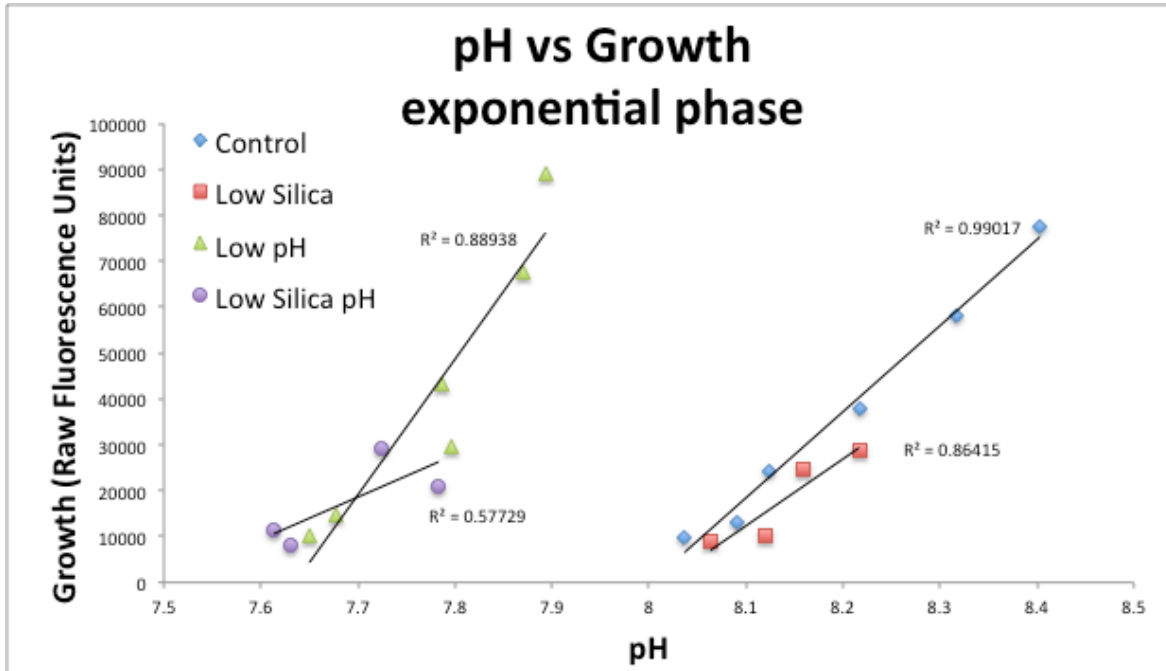


Figure 9. The pH vs the growth (Raw Fluorescence Units) during the exponential growth phase of the cultures.

Discussion

East Sound

The cell count data from the East Sound field sampling shows that there is high variability between the sample locations each day samples were taken. There was also a decline in total cell concentration throughout the sampling duration. It is unclear where the sampling events fall into the progression of the bloom because the sampling period did not include the entire bloom. It seems as though the end of the bloom was sampled.

Based on observational data, the *Pseudo-nitzschia* bloom was followed by a *Heterosigma* bloom. Plankton tows taken during the *Heterosigma* bloom were not useable because the plankton net became clogged with what seemed like extracellular

polysaccharide substances, perhaps secreted by the *Heterosigma*. This prevented any further quantification of the *Pseudo-nitzschia* bloom.

The relative abundance of each phytoplankton species may be the most important thing to consider with this data. The variability of the total cell abundance between sites on any given day indicates that the bloom was patchy, and may not be an accurate representation of the bloom. One belief is that the community composition is more important than the total biomass in the water (*Per. Comm.*) for the gene expression, toxin production, and behavior of the phytoplankton.

Another belief is that the total biomass is the driving factor for community behavior because the phytoplankton have a greater affect on the environment, predominantly with nutrient and resource consumption. Changes in the physical environment may influence the toxin production and behavior more than the community composition for *Pseudo-nitzschia*.

To look closer into the community composition and behavior of *Pseudo-nitzschia*, a metatranscriptome analysis of all the RNA expressed during the field sampling of 2010 is being analyzed. Comparing gene expression of *Pseudo-nitzschia* between two different days of the bloom will begin to answer questions about it's behavior. Continued sampling of East Sound during the Spring is expected to continue studying the bloom dynamics and behavior of *Pseudo-nitzschia*.

Growth Experiment

Because the data from the mesocosm experiment is incomplete and inconclusive, the data will not be used for considering the effects of pH on phytoplankton. Instead, the

data from the pilot growth experiment will be used. The data from the growth experiment shows a linear relationship between the pH and growth. As expected, increases in growth rate lead to increases in pH. As CO₂ is removed from the water, the water chemistry changes, and the pH increases. The control showed a very strong correlation between growth rate and rate of increase in pH or CO₂ drawdown, while that relationship is not as strong in the treatment cultures. This is likely because other factors are probably influencing the growth rate

An expected, silica appears to be the limiting factor for phytoplankton growth. Silica limitation played a larger role in the final concentration of phytoplankton in the cultures. It is unclear if the limiting factor for the high silica treatment was also silica, but nutrient data analyzed in the future will indicate the final nutrient levels of the control and low pH cultures.

The pCO₂, and subsequently low pH didn't play a role in total phytoplankton growth ($p > 0.05$) throughout the duration of the experiment. This indicates that the cultures were never limited by carbon, and weren't given a competitive advantage with excess available CO₂. If larger conclusions are drawn from these data, it suggests that ocean acidification will not affect diatom growth rates negatively. In order to confidently make this statement, further experimentation on growth and physiology must be conducted in a number of settings, including mesocosm experiments mimicking environmental conditions.

Despite not being able to make overarching conclusions about phytoplankton communities and their response to climate change, this experiment was valuable. It

provides as pilot study for future research, and many things can be learned from it, especially from the mistakes and errors encountered in the experiment.

There were several human and experimental errors in this experiment. First, there is the issue of contamination. Sterile technique was not used throughout the experiment, and bacterial or fungal contamination occurred at the beginning of the experiment. Visible pieces of yellow organic material were in the bottles a few days into the experiment, and disappeared after a few days. This contamination could have had major effects on the growth and behavior of *P. multiseriis*, and could have affected the growth measurements if there was chlorophyll in the contaminant.

Another source of error could be from the pH meter that was used. Measuring pH with a probe is not the most accurate method. In addition, the pH meter was temperature-adjusted, but the thermometer was left at room temperature (~25°C) for the first week of sampling. This is likely responsible for some of the variation in pH data from day to day. While pH meters are not as accurate as spectrophotometer (cite), the scale of this experiment was too small to remove 50 mL of culture every day to measure and adjust the pH. Using a spectrophotometer also encounters accuracy issues when thick algal cultures are measured. However, filtering the phytoplankton out of a water sample changes the pH.

Another source of error for pH is the air-water interface. The pCO₂ of the water was higher than that of the air in the bottles, which allowed for gas exchange, and potentially a change in pH. Conducting an ocean acidification experiment is difficult, and must be carefully designed under specific chemical parameters. If this experiment were to be conducted again, it would be beneficial to improve culturing conditions to

maintain somewhat steady chemical conditions. In addition, this experiment didn't work with cultures that were acclimated to a low pH setting. Ideally, the cultures would be acclimated to the low pH and low silica media, having the same growth rate for three consecutive generations.

Conclusions

Despite all of the problems associated with this experiment, it is a useful pilot study showing a relationship between growth and pH. When domoic acid, lipid, and biogenic silica data become available, a more complete picture can be drawn. More questions will be raised about the relationship between CO₂ uptake in association with Silica uptake.

The fate of CO₂ in the world's oceans still remains at large, but future lipid data from this study will begin to draw a picture of the potential changes in nutritional content of phytoplankton. If the total lipid per cell increases with the low pH condition, it is possible this could happen in the environment, potentially changing food web dynamics.

This three-scale study only begins to look at the dynamics of harmful algae and phytoplankton communities. Continued field sampling of the recurring toxic bloom in East Sound, WA will allow more complicated questions about *Pseudo-nitzschia* to be asked in the future, especially about the gene expression of *Pseudo-nitzschia* during toxin production.

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