

The effect of pH on the growth of *Licmophora abbreviata*

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Abstract

Licmophora abbreviata is a species of diatom that is crucial in the salmon food chain as primary producers, and in its contribution to atmospheric oxygen. It is sensitive to a wide range of abiotic factors; pH was tested in this study. Our experiment was conducted over a two-week period, starting by diluting a stock solution of *L. abbreviata* to a concentration of 1.0×10^4 cells/mL in 3 treatments of different pH's: 7, 8 and 9, with three replicates of each. Every second day, a sample from each culture was fixed and counted to determine its concentration. Counts were done for 6 days over an 11-day period. Using daily observed counts, growth rates were calculated and a one-way ANOVA test was performed, yielding a p-value = 0.875, which found that the mean growth rates between treatments were not significantly different. The results may have been influenced by the changing pH of the media, resulting from diatoms' metabolic activities in vitro, as well as the relatively short time period over which the study was done. In conclusion, there was no association found between pH and the average growth rate of *Licmophora abbreviata*.

Introduction

As a keystone species, salmon are of extreme importance in British Columbia. Not only do they provide the rich nutrients that approximately 137 species depend on, they have also been a centerpiece to indigenous cultures (Rahr, 2016). Salmon distribution varies widely and is dependent on many different organisms, such as *Licmophora abbreviata*, a species of diatoms.

Diatoms play a substantial role in the salmon's ecosystem. They are at the bottom of the food chain and are often grazed on by other invertebrates, which eventually become salmon's food source. Indeed, diatoms' presence (or absence) can significantly affect salmon's growth, reproduction and morbidity. Research has shown that salmon nurseries, during growing seasons, are dominated by healthy diatom populations (Gregory-Eaves & Keatley, 2010). However, an excess of diatom biomass has been shown to be detrimental to salmon juveniles; a study found that the dead fish presented with hyperplasia, or thickening of the gill tissue, possibly caused by diatoms (O'Connor, 2002). Therefore, salmon population are very sensitive to diatoms

we have decided to study the effects of pH on the growth rate of *Licmophora abbreviata*. This particular species of diatoms is known to thrive at a range of pH 7.8 - 8.5 (Ohgai et al., 1984). It will be interesting and important to see how the changes in pH affect the diatoms, and since the pH of the ocean is slowly decreasing with global warming, this might be an indicator of what is to be expected for salmon populations.

We will be constructing growth curves based on concentrations of *Licmophora abbreviata* in media of three different pH's: 7, 8 and 9.

Our hypotheses are:

H₀: There is no association between pH and the average growth rate of *Licmophora abbreviata*.

H_A: There is an association between pH and the average growth rate of *Licmophora abbreviata*.

Based on our literature research, we predict that the highest average growth rate of *Licmophora abbreviata* will occur in the media of pH 8 (Ohgai et al., 1984). Seawater near the surface also has an average pH of 8.1 (Britton et al., 2016), supporting our prediction that our organism is best adapted to this environment.

Methods

We were provided with a stock solution of *Licmophora abbreviata*. To determine the concentration of our stock diatom solutions, we centrifuged 25mL of cell samples at different pH's. Then, 2mL of pH-specific media was added to each cell palate, and cells at each pH were counted to calculate their concentrations, as illustrated in **Figure 2**. We counted the cells using a hemocytometer, then using specific-pH media, diluted the cells to identical concentrations of

10,000 cells/mL, with a total starting volume of 10mL. We made four replicates of each treatment, however, we only used 3 for counting - the last replicates were stored as reserves.

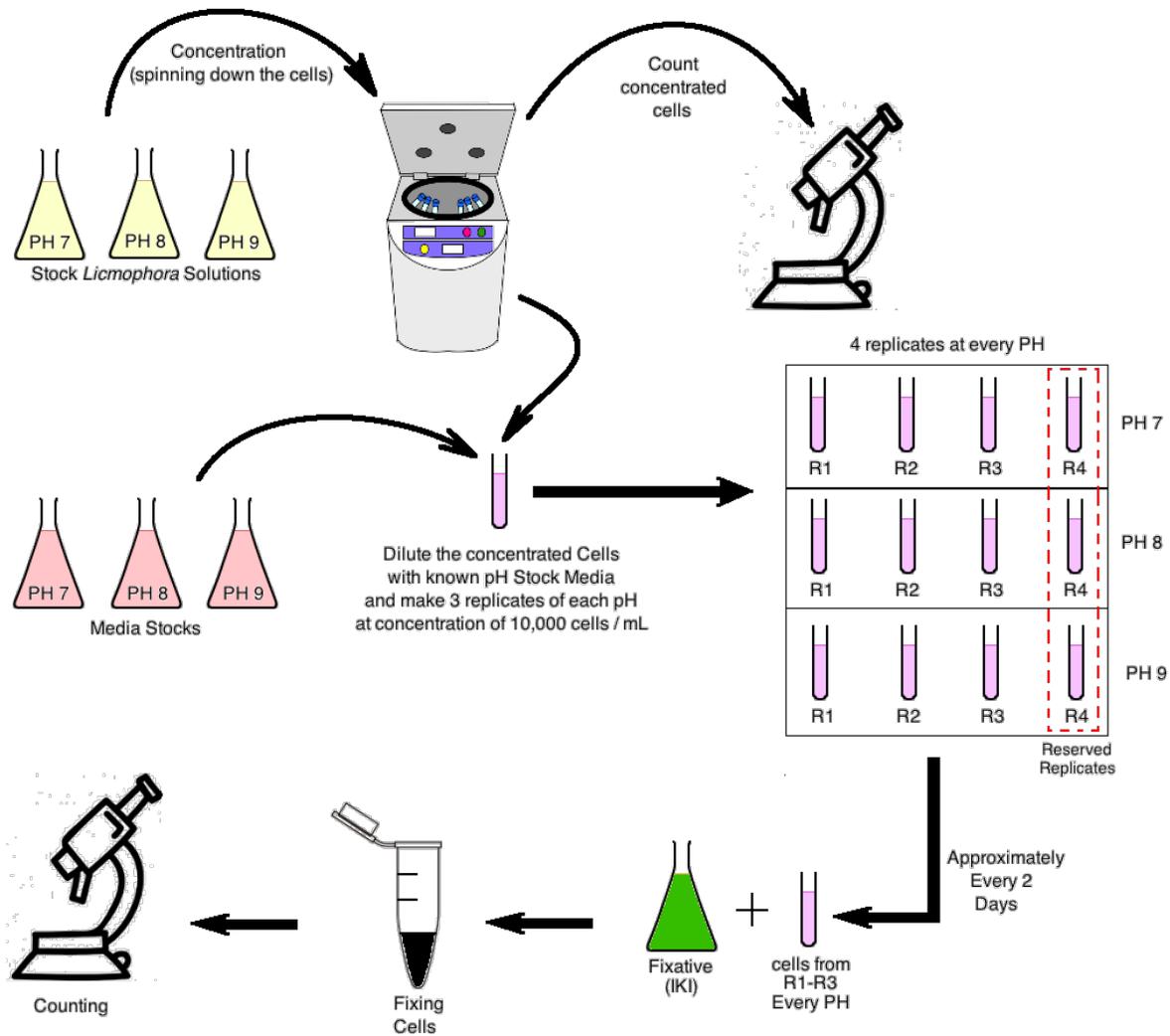


Figure 2. Flowchart of the steps taken, from the initial stock solutions to counting of cells for concentration and growth rate analysis.

We collected data over a period of 2 weeks, every Monday, Wednesday and Friday. Each time we collected data, we vortexed the replicates and sterilized test tube tips over a flame, prior to cell extraction. We then took 100 μ L of the samples from replicates R1-R3 at each pH and added it to sterilized Eppendorf tubes, fixed each sample with 10 μ L Lugol's Iodine solution,

resuspended the Eppendorf solution and then counted the cells by placing a 20 μ L sample of the solution on a sterilized hemocytometer. We counted the diatoms under a microscope, using 4mm x 4mm hemocytometer grid lines, resulting in a magnification of 3.125×10^2 . **Figure 3** shows our experimental setup.



Figure 3. From left to right: pipette tips, micropipettes, Eppendorf tubes containing fixed cell solutions, test tube replicates and vortexer.

We counted the cells starting in the upper left corner and worked across to the right, then repeated for each row on the grid; all cells within the 4mm x 4mm grid were counted and the total count was used for calculating concentrations. We measured the pH of our replicates three times during the entirety of our experiment: once on day 0 of the experiment, once halfway through the experiment, and a final time on the last day of counting the cells. In between counts we stored all cell replicates in an incubator at constant temperature (20°C), CO₂ and O₂ partial pressures, atmospheric pressure, light, and quantity of media available per diatom.

After our data was collected, we analyzed it using a one-way ANOVA test to determine if the mean rates were significantly different from each other.

Results

From the observed cell counts, the cell concentrations for each treatment were calculated and presented in **Figure 4**. For the first 7 days, we have an increase in concentration in all samples. This is followed by a significant decrease in growth of the sample at pH 7 and 9, while cells in pH 8 media continue to grow relatively undisturbed. On day 11, cells in pH 9 show signs of recovery and increasing numbers, while cells in pH 7 continue to decline at a very shallow slope.

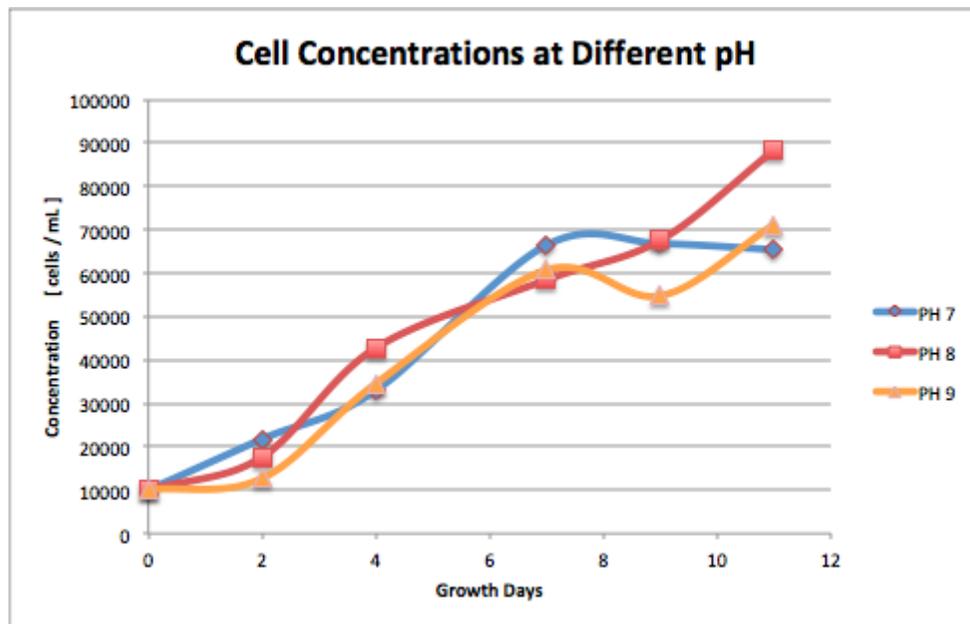


Figure 4. Growth curves for *Licmophora abbreviata* in media of pHs 7, 8 and 9.

Given that all three treatments showed a relatively linear growth curve within the first 7 days of incubation, rates of for each replicate were calculated over this period.

Based on the linear approximation rates calculated from each replicate, we performed an ANOVA test to conclude if there was any significant difference between the growth rates of the three pH treatment groups. The p-value of the ANOVA test was 0.875. Our $F_{\text{calculated}}$ was equal to 0.101 and the F_{critical} was 5.143. This was calculated using the following formulas:

$$F_{\text{calculated}} = \frac{n \left(\frac{\sigma_x^2}{n} + \text{variance} [\mu_i] \right)}{\sigma_x^2} \quad \text{and} \quad F_{\text{calculated}} = \frac{MS_{\text{group}}}{MS_{\text{error}}} =$$

$$\frac{323937.6078}{3198486.098} = 0.10127.$$

Qualitatively, we observed that the diatoms were aggregated at the bottom of each test tube after retrieving them from the incubator. The pHs of solutions were observed to change during the experimental period. On the first day (day 0), the media pH's were measured to be 7, 8 and 9 on a pH paper. Halfway through the experiment the media pHs were read as 8, 9 and 9 respectively and on the last day of counting, all media pHs were read as 9, 9, 9.

Using the replicate rates, average growth rates were calculated for each starting pH treatment, as illustrated in **Figure 5**. Cells cultured in a starting media of pH 7 showed the greatest variance within their replicates, and have the largest standard error of the mean (error bar).

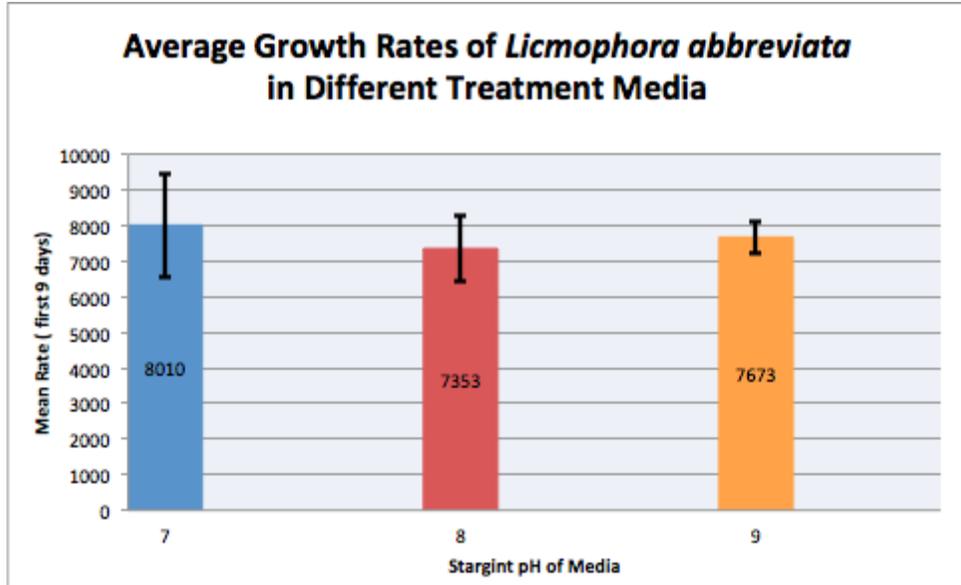


Figure 5. Average growth rates for each pH treatment, calculated based on the mean of three replicates for each pH.

Discussion

Based on our results, we fail to reject the null hypothesis since our p-value is above 0.05 and, therefore, fail to provide support for our alternative hypothesis. While conducted in a controlled environment, some aspects of the experiment may have served as confounding variables. For instance, our small number of data points could have affected the statistical analysis and significance of our experiment and tampered with its validity.

While we did our best to reduce any potential sources of error, different human errors could have also affected our results. Despite using three replicates for each treatment and having consistent group members performing the same tasks, some mistakes may still have arisen, such as counting errors using the hemocytometer.

Another potential source of human error might come from the lack of vortexing and homogeneity in our test tubes when pipetting our samples. Diatoms often aggregated at the

bottom of each test tube and required a lot of mixing before they would detach and resuspend in the media (**Figure 6**). To minimize counting errors, we worked with the same equipment during every day of measurements: the same microscope, hemocytometer, fixative solution, and micropipettes.



Figure 6. A culture tube with an aggregate of diatoms suspended in solution.

The greatest source of error in our experiment may have been the changes of pH in our treatment media. As mentioned previously, while the starting media pH's were different (7, 8, 9), by the end of the experiment, those replicates labelled as pH 7 and 8, had gradually increased to pH 9, while test tubes labelled as pH 9 remained the same, as seen in **Figure 7**. This change reduces the significance of our results since our goal was to see how *L. abbreviata* grew under different pH conditions.

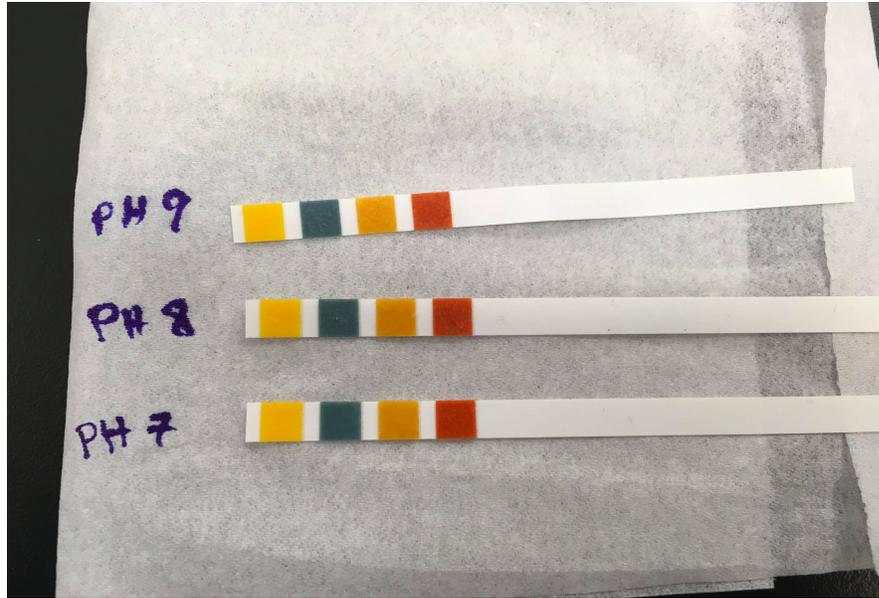


Figure 7. The pH of all three treatment groups on the last day of measurements.

This change in acidity can be explained by the fact that diatoms' cell wall take up H^+ from the environment, making the solution more basic (Milligan, 2002), explaining why the ocean's pH is locally elevated during intense primary production periods - i.e. a diatom bloom (Trimborn et al., 2007). In nature, however, oceanic pH is buffered due to high concentrations of bicarbonate and also due to the symbiotic activities of other marine organism which contribute to environmental pH. In vitro, there was not buffering mechanism to counter the H^+ uptake by diatoms. This change in pH could have altered the growth of the *L. abbreviata* and thus affected the growth rates to what we observed. We were unable to continuously titrate the media to achieve a steady pH due to time and resource limitations. Furthermore, we used pH paper to measure the acidity of our samples which relies on visual judgement. Our limitation to judge pH of media based on very slight colour changes of pH paper, also meant that detection of minute pH changes was not practical. That is why our team tested the pH only 3 times during the experiment, knowing that any daily pH changes of media would be too small to be detected by pH paper.

As seen in the results, some of the counts decreased after day 7 (pH 7 and pH 9); this could be due to some of the human errors mentioned above, or due to the fact that we noticed some of the diatoms lysing. Indeed, during the last 2 days of counting (days 9 and 11), when counting the cells on the hemocytometer, many cells were observed to have undergone lysis and were therefore not counted. As illustrated in **Figure 8**, when diatoms were observed to still maintain their cell wall, they were counted as part of the population, regardless of their shapes. Indeed, diatoms have a well-known “pizza shape” morphology, we can see from **Figure 8** that some were also observed as round or wedge-shaped cells.

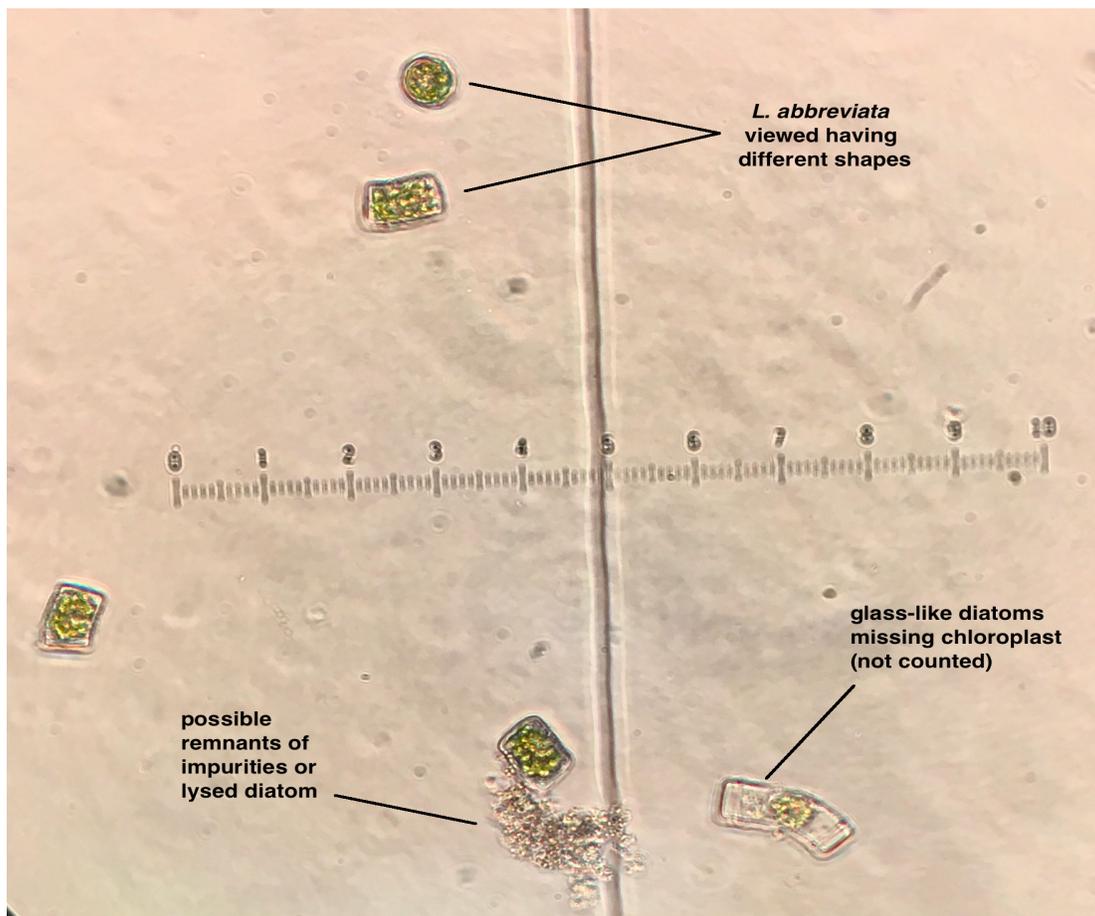


Figure 8. Morphological differences encountered when counting cells

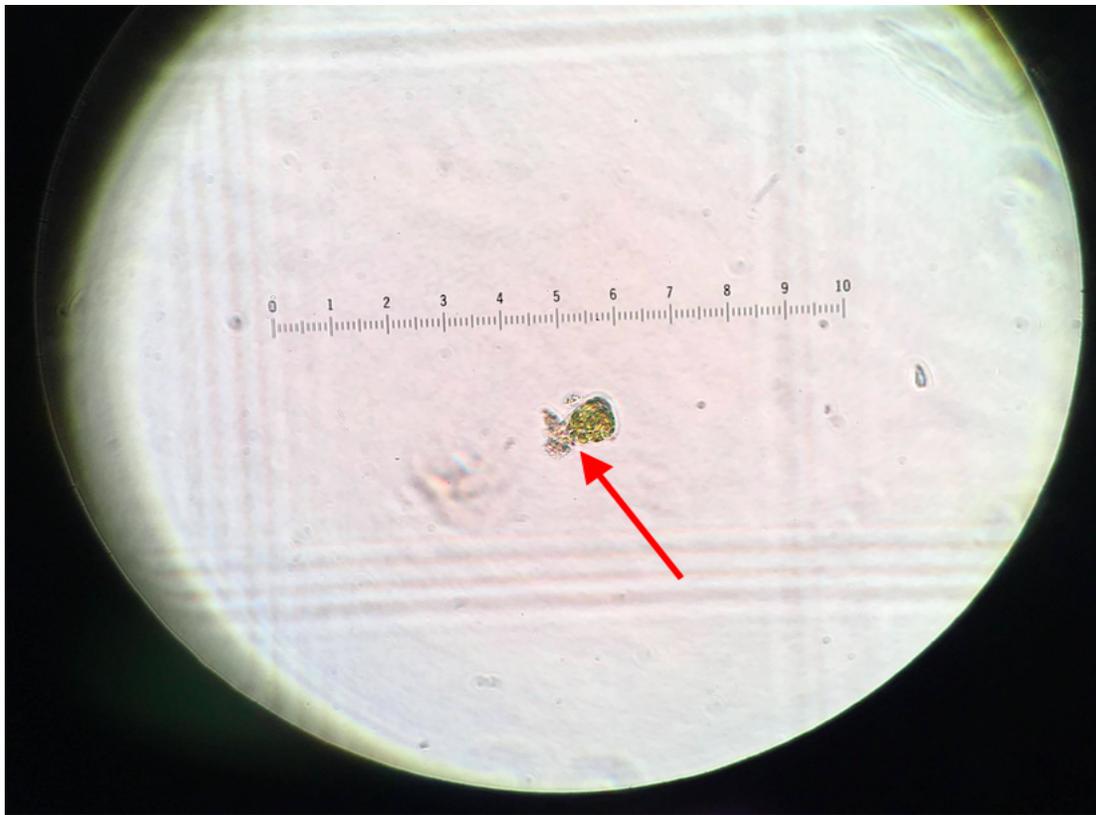


Figure 9. Microscope view of lysing diatom.

We did not count lysed parts of diatoms, such as clusters of internal granules with no defining cell wall, as seen in **Figure 8** and **Figure 9**. Cell death in laboratory cultures has been shown to be triggered by the release of chemical compounds such as polyunsaturated aldehydes and oxygenated fatty acids. Programmed cell death in diatoms can be found in the environment as well, where sudden decline in diatoms happen and leads to the export of carbon and silica to the bottom of the ocean (Gallo et al., 2017). This unpredictable lysing can also be considered as a source of declined number of cells towards the final counting stages of this study.

Following this experiment, and observing the changing pH's of media, future studies can significantly benefit from a study design where not only cells can be monitored for a longer growth period, but also media pH can be monitored and maintained on a continuous basis throughout the study.

Conclusion

In conclusion, we fail to reject the null hypothesis and we cannot provide support for our alternative hypothesis. Our prediction, in which the highest average growth rate of *Licmophora abbreviata* would be at a pH of 8, was incorrect (as illustrated in **Figure 5**). The treatments starting at pH 7 had the highest average growth rate, while also having the greatest standard error of their mean. Based on the calculated p-value and the overlapping standard error bars, we did not find any statistical difference between the growth rates of *Licmophora abbreviata*, which were cultured in different pH media (pH 7, 8 and 9).

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Literature Cited

Britton, D., Cornwall, C. E., Revill, A. T., Hurd, C. L., & Johnson, C. R. (2016). Ocean acidification reverses the positive effects of seawater pH fluctuations on growth and

photosynthesis of the habitat-forming kelp, *Ecklonia radiata*. *Scientific Reports*, 6, 26036. doi: 10.1038/srep26036

Gallo, C., d'Ippolito, G., Nuzzo, G., and Sardo, A. (2017). Autoinhibitory sterol sulfates mediate programmed cell death in a bloom-forming marine diatom. *Nature Communications*, 8(1), 1-11. Doi: 10.1038/s41467-017-01300-1

Gregory-Eaves, Irene & E. Keatley, Bronwyn. (2010). Tracking fish, seabirds, and wildlife population dynamics with diatoms and other limnological indicators. *The Diatoms: Applications for the Environmental and Earth Sciences*, Second Edition. 497-513. doi:10.1017/CBO9780511763175.027

Milligan, A. J. (2002). A Proton Buffering Role for Silica in Diatoms. *Science*, 297(5588), 1848-1850. doi:10.1126/science.1074958

Munday, P., McCormick, M., Meekan, M., et al. (2012). Selective mortality associated with variation in CO₂ tolerance in a marine fish. *Ocean Acidification*, 1, pp. 1-5. Retrieved 14 Nov. 2017, from doi:10.2478/oac-2012-0001

O'Connor, W. (2002). An Assessment of Diatom Biomass on Atlantic Salmon (*Salmo salar* L.) Spawning Habitat in the River Bush, County Antrim. *Biology and Environment: Proceedings of the Royal Irish Academy*, 102B(2), 105-111. Retrieved from <http://www.jstor.org/stable/20500153>

Ohgai, M., Tsukahara, H., Matsui, T., & Nakajima, K. (1984). The Effect of the Environmental Factors on the Growth of two Epiphytic Diatoms *Licmophora abbreviata* AGARDH and *L. paradoxa* (LYNGBYE) AGARDH in vitro. *Bulletin of the Japanese Society of Scientific Fisheries*, 50(7), 1157-1163. doi:10.2331/suisan.50.1157

Rahr, G. (2016, January 13). Why Protect Salmon. Retrieved from <https://www.wildsalmoncenter.org/work/why-protect-salmon/>

Saade, A. and Bowler, C. (2009). Molecular Tools for Discovering the Secrets of Diatoms. *BioScience*, 59(9), 757-765. doi:10.1525/bio.2009.59.9.7

Theriot, E., Herbarium, D., Round, F. E., Crawford, R. M., & Mann, D. G. (1992). The Diatoms. Biology and Morphology of the Genera. *Systematic Biology*, 41(1), 97-98. doi:10.2307/2992511.

Trimborn, S., Lundholm, N., Thoms, S., Richter, K.-U., Krock, B., Hansen, P. J. and Rost, B. (2008). Inorganic carbon acquisition in potentially toxic and non-toxic diatoms: the effect of pH-induced changes in seawater carbonate chemistry. *Physiologia Plantarum*, 133, 92–105. doi: 10.1111/j.1399-3054.2007.01038.x

Appendix**Raw Data Table 1.** Table of raw hemocytometer counts, recorded based on readings from the hemocytometer

pH		Cells counted per 4mm / 4mm Hemocytometer Grid					
		Oct. 30th, 2017	Nov. 1st, 2017	Nov. 3rd, 2017	Nov. 6th, 2017	Nov. 8th, 2017	Nov. 10th, 2017
7	Replicate 1	29	55	87	144	226	200
	Replicate 2	29	63	116	245	197	157
	Replicate 3	29	72	86	191	160	214
8	Replicate 1	29	63	139	209	207	261
	Replicate 2	29	46	128	155	220	256
	Replicate 3	29	45	107	146	163	254
9	Replicate 1	29	40	106	162	186	179
	Replicate 2	29	25	81	194	164	221
	Replicate 3	29	46	116	174	128	221
days		0	2	4	7	9	11

Table 2. Includes calculated data concentrations (extrapolated from the hemocytometer counts) which were used to construct graphs in Figure 1 of Appendix, also Mean Slopes of Replicates (Mean Growth Rate of Replicate) pertaining to Figure 5.

		Concentration (cells / mL)						Linear Approx . of Slope	Mean Slopes of Three Replicate s	Stand. Err. of Mean (+/-)
		Oct. 30th, 2017	Nov. 1st, 2017	Nov. 3rd, 2017	Nov. 6th, 2017	Nov. 8th, 2017	Nov. 10th, 2017			
7	Replicate 1	10000	18906	29906	49500	77688	68750	5679	8010	1458
	Replicate 2	10000	21656	39703	84219	67719	53969	10693		
	Replicate 3	10000	24750	29391	65656	55000	73563	7657		
8	Replicate 1	10000	21656	47781	71844	71156	89719	9184	7353	933
	Replicate 2	10000	15813	43828	53281	75625	88000	6744		
	Replicate 3	10000	15469	36781	50188	56031	87313	6129		
9	Replicate 1	10000	13750	36266	55688	63938	61531	6966	7673	450
	Replicate 2	10000	8594	27672	66688	56375	75969	8508		
	Replicate 3	10000	15813	39703	59813	44000	75969	7544		
days		0	2	4	7	9	11			

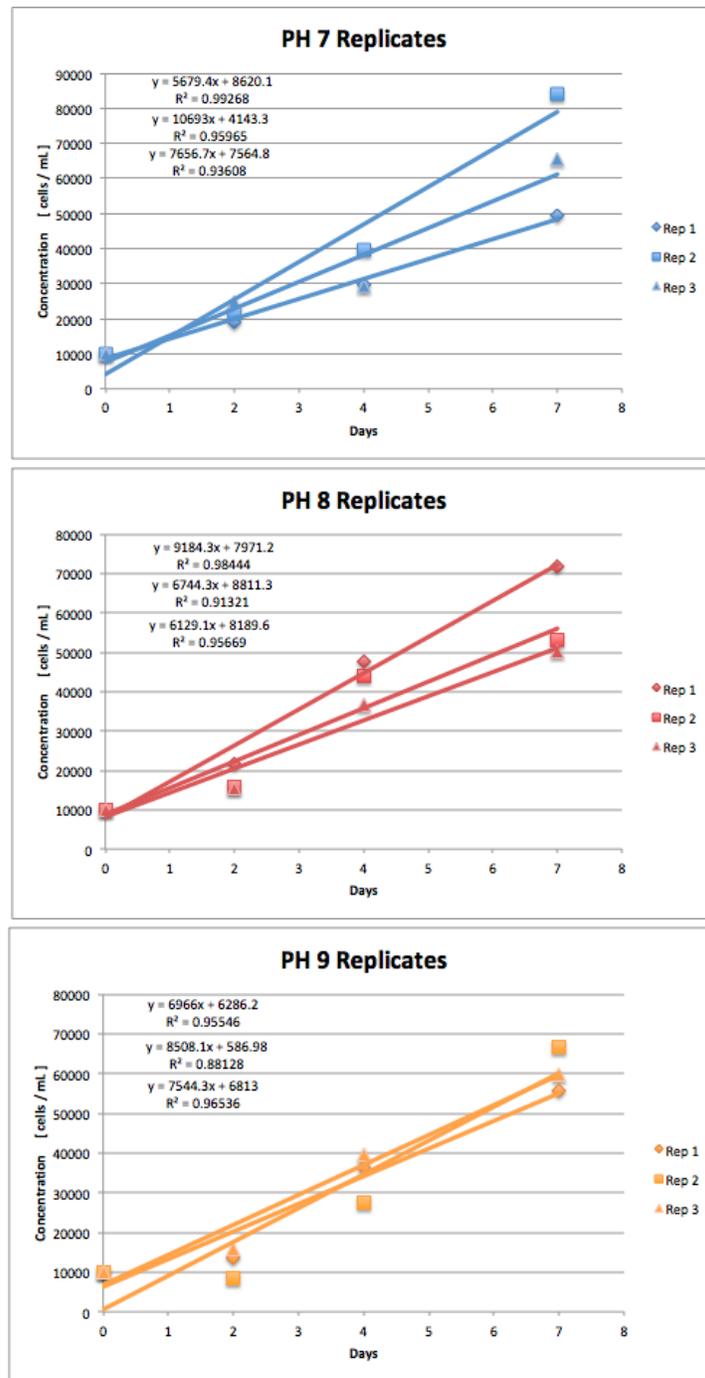


Figure 2. Growth rates (linear approximation slopes) of three replicates for each pH media
 Data points for these graphs can be found in Table 3 of Appendix.