The Effect of Non-Optimal pH on the Growth of Licmophora abbreviata

Yuchen Cheng, Celia Dossot, Vlada Mazur, Hadi Minai

Abstract

This paper aims to investigate the statistical relationships between varying pH levels and the growth rates of diatoms by using a hemocytometer to count cells, applying various graphing methods to observe growth rates and analyzing the significance of our results via a one-way ANOVA. It demonstrates that there is no difference in *Licmophora abbreviata* growth at pH 7.0, 8.0 and 9.0. This is a relevant discovery with respect to the ongoing acidification of oceans that is ultimately affecting the populations of diatoms and their primary consumers: the salmon near the west coast of British Columbia.

Introduction

Licmophora abbreviata (L. abbreviata) is a marine diatom that is about 70 μm in length and is yellow-brown in color (Guiry, 2015). It comes in multiple shapes, including triangular, circular and rectangular. The optimal pH for its growth is 7.8-8.5 (Ohgai, Tsukahara, Matsui, & Nakajima, 2008). Our strain was isolated from the Pacific Ocean near Vancouver, British Columbia. Studies indicate that coastal diatoms are more tolerant to changes in pH than oceanic species (Taraldsvik & Sverre, 2010). Researchers claim that despite all other nutrients still being supplied, a carbon limitation may be the reason photosynthesis rates become slower and growth eventually stops. But, this presumed carbon limitation may in fact be a pH effect (Taraldsvik & Sverre, 2010). Due to its increased resilience, we are interested in how well this diatom would grow in conditions outside of its optimal pH range.

This diatom forms an important part of the base of aquatic food webs in marine habitats. We predicted that growth would be slower in pH conditions outside the optimal range. *L.*

abbreviata have varying tolerance levels for different environmental variables, e.g., pH, temperature and light intensity. Given that our particular strain was isolated from Vancouver, it was expected to display insensitiveness to short-term pH variation (Scholz & Phycol, 2014).

Three pH conditions (7.0, 8.0, 9.0) were chosen to determine how well the diatom would grow outside of its optimal range and to better understand the adaptability of this species to changes in pH. The null hypothesis was that there is no difference in *L. abbreviata* growth rates at pH values 7.0, 8.0 and 9.0. The second condition, pH 8.0, falls in the optimal range and is expected to serve as a control in the study. The alternate hypothesis, on the other hand, predicts that there is a decrease in *L. abbreviata* growth rates at pH values 7.0, 8.0 and 9.0 as pH becomes more acidic, i.e., going from pH 9.0 to 7.0.

The recent changes in the pH and composition of the ocean can largely be attributed to the burning of fossil fuels throughout the last few decades which have resulted in a global rise in atmospheric CO_2 levels. This rise in CO_2 has made its way to the ocean, where the CO_2 can react with seawater to form carbonic acid and, in turn, lead to ocean acidification. The marine microorganism, *L. abbreviata*, is an important member of the food chain pyramid. It is responsible for nearly half of the primary production in the oceans (Yool & Toby, 2003). Primary production in the oceans is one of the main carbon sinks that helps lower the CO_2 concentration in the atmosphere (Thompson, 2012). Due to this, it is important to determine how well this organism will be able to adapt to its changing environment.

Following decreases in *L. abbreviata* populations, the bio life feeding off them are also likely to experience reduced growth. *L. abbreviata* is a phytoplankton; a crucial food source for juvenile salmon (Schmidt, 2013). As such, reduction in this organism's population could have negative implications for many species, particularly the salmon. A decline in *L. abbreviata*

would mean a decrease in food sources for the young fish. This paper aims to analyze if changing pH influences *L. abbreviata* growth and ultimately the salmon's population growth.

Methods

The *L. abbreviata* were grown in glass culture tubes using standard diatom media. Their growth rate was determined by calculating cell concentration per mL. This was obtained using a hemocytometer to count evenly suspended cells three times a week for two weeks.

Preparation of the 3 pH conditions

We received a prepared *L. abbreviata* culture flask that we then separated into 3 glass culture tubes containing 25 mL for each of our pH conditions. Next, we centrifuged the 3 tubes for a total of 5 minutes. The media was pipetted off from each of the 3 tubes, making sure not to disturb the pellet of diatom cells at the bottom. 1 mL of the varying pH media was added to each tube (pH 7.0, 8.0 and 9.0). The tubes were vortexed to ensure that the cells would be evenly suspended.

Diluting to proper cell concentration

From each tube, we pipetted 100 µL out and into separate Eppendorf counting tubes to determine cell concentration. Then, from each Eppendorf tube, we pipetted 20 µL out and onto a hemocytometer with a coverslip. We then counted the number of green squares on the hemocytometer (size 0.25 mm x 0.25 mm) that it took to obtain 100 cells (details on counting rules in next section). This step was repeated 3 times per Eppendorf tube. We then calculated the dilution factor needed to have 30 mL total for each of our conditions (so that we have 10 mL per sample) with a cell concentration of $3x10^4$ cells/mL. The appropriate volume was added to each of the 3 tubes.

Next, we filled each glass growth tube with 10 mL of the pH-adjusted cultures (3 samples for each of the 3 conditions), and the growth tubes were incubated at 20°C.

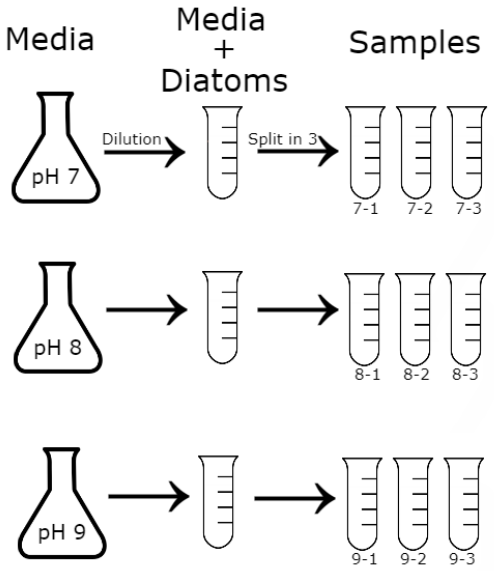

Figure 1. Showing the preparation of the 9 growth tube samples.

Counting of cells to determine cell concentration

Cell counts were taken every Mon/Wed/Fri (Oct 27th, Oct 30th, Nov 1, Nov 3, Nov 6th, Nov 8th and Nov 10th) for 2 weeks. On each counting day, 9 Eppendorf tubes were prepared by adding IKI fixative to the samples in a 1:10 ratio. Accordingly, we added 10 µL of fixative into each of the 9 Eppendorf tubes along with 100 µL from each of the 9 samples (which were vortexed prior to pipetting to ensure even cell distribution). Before pipetting from the growth tube samples, the opening of the tube was flamed to avoid contamination. In addition, a different pipette tip was used for each sample for the same reason. Each Eppendorf tube had a total of 110 µL of fixed cells.

Before counting, we pipetted the fixed samples in the Eppendorf tubes up and down a few times with a pipette to resuspend the cells homogeneously. We then took 20 µL out and deposited it against the edge of a coverslip placed on the hemocytometer so that the solution

would spread evenly over the grid. This was repeated 3 times per sample to ensure accurate determination of cell concentration.

On the hemocytometer, 100 *L. abbreviata* cells were counted and the number of green squares needed to reach that value were recorded. If the number of cells in a green square did not perfectly end at 100, the exact number of cells was recorded. To maintain consistency in our counting method, cells covering the left and/or bottom edges of a green square were not counted - while cells covering the right and/or top edges were.

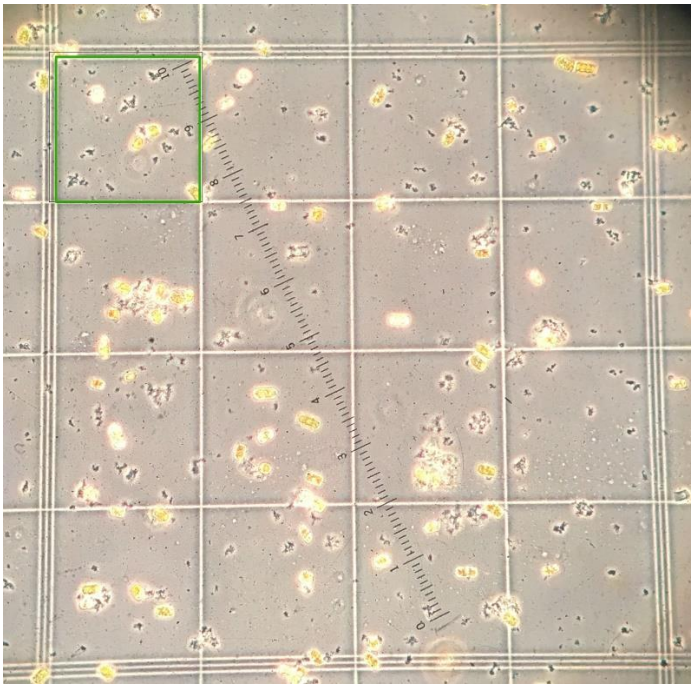

Figure 2. Direct view of a hemocytometer through a compound microscope lens (10x) showing the "green square" used for counting. *L. abbreviata* is shown under pH 7 conditions.

Data analysis methods

The total cell concentration was averaged over 3 samples, and we used these results to plot cell concentration (cells/mL) over time (days). After all the data had been collected, an average trend line was used to determine slope, which is the growth rate of the cells under each pH condition. A one-way ANOVA was run to determine if any of the growth rates were

statistically different from one another.

Results

According to Figure 3, the concentration of *L. abbreviata* cells at the end of the experiment, under both pH 8.0 and pH 9.0 conditions, were relatively similar. These concentrations were both higher than the concentration of *L. abbreviata* cells measured at pH 7.0. The final concentration of cells, at pH 7.0, was 8.73 x 10^4 cells/mL.

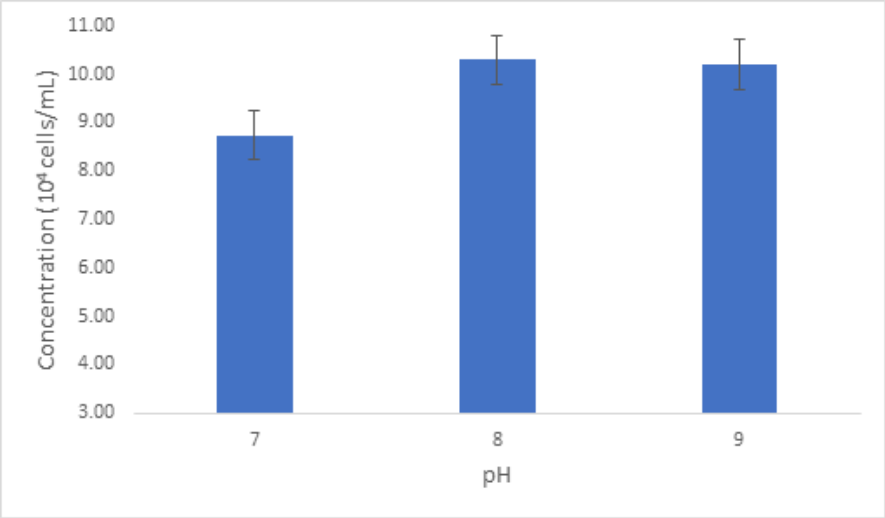

Figure 3. Initial & Final cell concentrations (cells/mL x 10^4) on day 0 and 14 for *L. abbreviata* at different pH values (7.0, 8.0 and 9.0). All initial cell concentrations were 3 x 10^4 cells/mL.

The average concentration for a cell was calculated using the following method.

Green square dilution factor: 8 x 10^4

For pH 7 cell concentration:

Average green squares count/ 100 cells = (152 + 126 +225)/3 = 167.7

100 cells/167.7 green squares = 0.5963 cells/green square

0.5963 cells/green square x (8 x 10^4) = 4.77 x 10^4 cells/mL

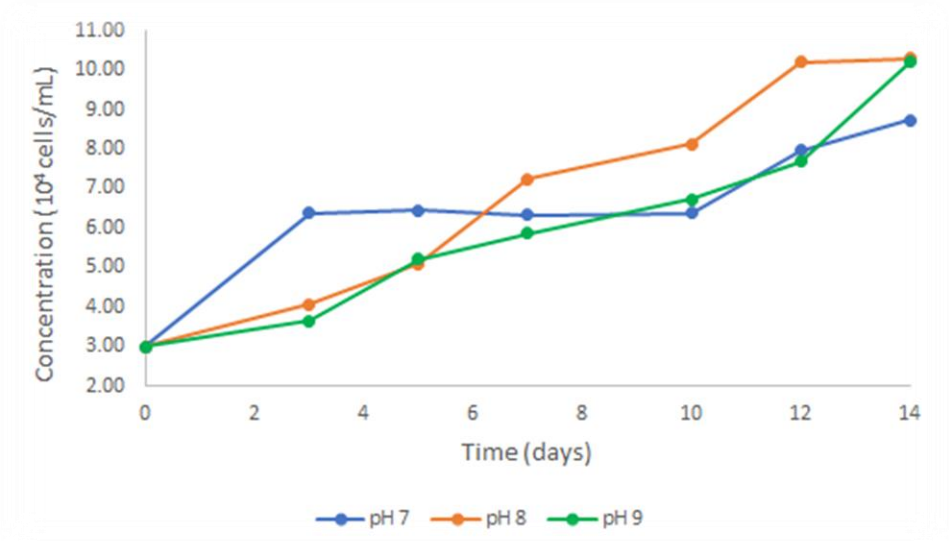

Figure 4. Cell concentrations (cells/mL x 10^4) of *L. abbreviata* cells, under different pH values, from days 0-14.

The concentrations of *L. abbreviata* cells, under all pH conditions, increased as time progressed. The initial growth of cells was highest for pH 7.0 (6.37 x 10^4 cells/mL). The growth rates at the end for pH 8.0 and pH 9.0, respectively, were larger than the growth rate for cells grown in pH 7.0 media (Fig. 3). Cells grown under pH 8.0 condition had a higher slope for their growth curve. The growth curves for cells grown in pH 7.0 and pH 9.0 conditions were lower in comparison. To determine if these differences were statistically significant, a one-way ANOVA was performed (Table 1).

Table 1: One-way ANOVA to test if at least one of the means is different.

SUMMARY				
Groups	*Count*	*Sum*	*Average*	*Variance*
pH 7.0	7	452300	64614.29	3.24 x 10^8
pH 8.0	7	480400	68628.57	8.41 x 10^8
pH 9.0	7	423300	60471.43	6.03 x 10^8

ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	2.33 x 10^8	2	1.16 x 10^8	0.198	0.822	3.55
Within Groups	1.06 x 10^{10}	18	5.89 x 10^8			
Total	1.08 x 10^{10}	20				

Discussion

Our findings indicate that the diatom, *L. abbreviata*, grew under all 3 varying pH conditions throughout the course of the experiment. As expected, the cells grown in pH 8.0 had the highest cell concentration (Fig. 3).

Figure 3 shows that there is less overall growth in *L. abbreviata* cells grown in pH 7.0 and 9.0 as opposed to pH 8.0. A decrease in growth rate at pH > 8.0 can be attributed to a decrease in the rate of certain biochemical reactions within the cell, along with changes in cell membrane proteins (Taraldsvik & Sverre, 2010). As pH rises, toxicity increases. This happens because more ammonium ($NH_4^{+)}$ is being converted into ammonia (NH_3). Ammonia is toxic to fish because it causes neurons to be depolarized, resulting in an influx of Ca^{2+}. This eventually leads to cell death in the central nervous system (Randall, 2002).

Diatoms play a vital role in oceans by reducing total ammonia production through the prevention of toxic algal growth; they are responsible for increasing oxygen in the water (Towers, 2013). A decrease in diatom population would result in higher pH conditions, as more NH_3 would be formed. It would also negatively impact fish populations. Salmon, as mentioned earlier, rely on *L. abbreviata* as a source of nutrients.

When analyzing the concentration of cells at different time intervals, the initial growth seen in *L. abbreviata* cells grown in pH 7.0 was higher than the growth seen in pH 8.0 and 9.0 respectively (Fig. 4). This suggested that *L. abbreviata* cells initially bloomed faster in pH 7.0. The cells had a shorter exponential phase and longer stationary phase with regards to cells grown in pH 8.0 and pH 9.0. As stated earlier, diatoms undergo different changes in short-term versus long-term exposures to pH variation. Our experiment was conducted over 14 days and may explain why significant differences in growth rates were not seen between the varying pH

conditions. During long-term exposure (30 days or more), changes in the composition of amino acids, and a decrease in chlorophyll a concentrations may be seen within the cells (Scholz, B., 2014). This could have given different results.

Alternatively, a possible explanation for the stationary phase in the pH 7.0 samples could be due to media changes. At the beginning of the experiment, the pH media were verified using pH paper and were found to be within the given pH range. However, at the end of the experiment, the pH media had increased to pH 8.5. Research has shown that media made following classical protocols (containing bicarbonate as part of the buffer) can increase pH to 8.5 within a matter of hours (Lelong & Rebel, 1998). Since our cells were grown in standard diatom media, this could explain why the media increased to pH 8.5 by the end of the experiment. We did not measure pH every counting day because we assumed it to be controlled. We are also unsure how the changes in pH were reflected in the growth rates. Our prediction is that as the pH increased to 8.5 in the pH 7.0 samples, the growth rate experienced a new peak at the end of the counting period (Fig. 4).

A one-way ANOVA allowed us to determine if at least one of the three mean growths were different than the others. If $F > F_{crit}$, we can reject the null hypothesis. As shown in Table 1, $0.198 < 3.55$. Therefore, we failed to reject the null hypothesis and cannot accept the alternative hypothesis (Ha). A t-test on each pair of means would have been conducted if our F value was larger than F_{crit}. This means that there was no statistical difference in the growth rates of *L. abbreviata* at pH 7.0, 8.0 and 9.0. This result can be reasonably attributed to the media pH changing to the same value of 8.5 - which would result in no difference in growth rates among the samples.

During the experiment, we observed many different diatom shapes (Figure 5). These included triangular, rectangular and circular shapes, each of which is circled for clarity in the figure. Since these shapes were spotted under all pH conditions, it is hard to tell what caused the differences in shape.

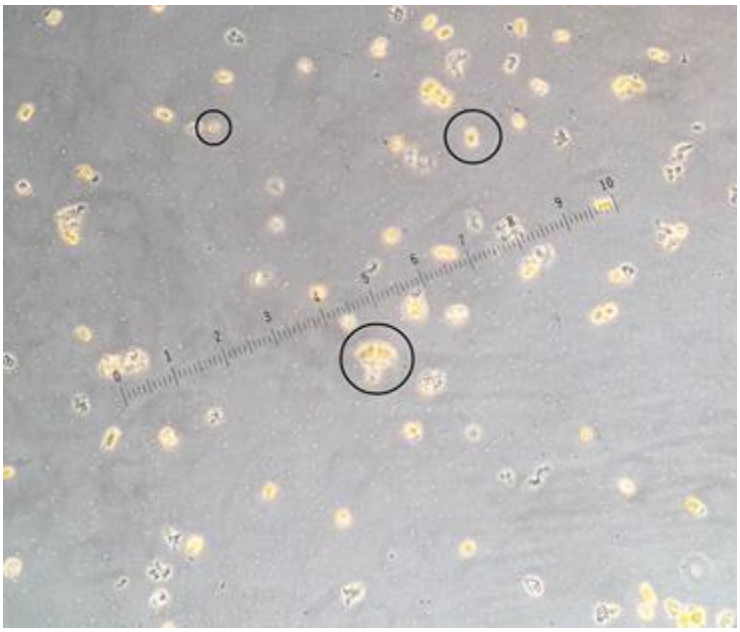

Figure 5. Showing the diversity of *L. abbreviata* cells at pH 8.0 in sample 2.

Further studies focusing specifically on shape occurrence at different pH values may provide clarity as to whether pH is one of the factors affecting the shapes of diatoms. Additionally, future studies may also consider studying long-term exposures across a wider range of pH values, for instance, pH 6.0 – 10.0. Lastly, growing diatoms in media with low bicarbonate content, such as Hank's formula-derived media, will allow pH to be more controlled, since a less important rise in pH has been observed in such media (Lelong I, et al., 1998).

Sources of error

As stated earlier, research on *L. abbreviata* is very novel. We are limited in the amount of information made available to us by other literature. Thus, there were several potential sources of error in our experiment. Firstly, while preparing the slides, some cells would cluster together,

making counting difficult. Second, errors in pipetting would have occurred due to uneven cell distribution. In addition, we had to remove the samples from the incubator before every count. This could have exposed the cells to varying light intensities and temperatures. Lastly, as discussed earlier, the initial pH values taken at the beginning of the experiment were lower than the pH values recorded after the experiment was finished (due to the instability of the media).

Conclusion

Our findings indicate that *L. abbreviata*, at pH 8.0, had the highest concentration. After conducting a one-way ANOVA, we obtained an F value smaller than the F_{crit} value. Consequently, we failed to reject the null hypothesis. Therefore, there is no difference in *L. abbreviata* growth rates at pH values 7.0, 8.0 and 9.0.

Acknowledgments

A big thank you to Mindy Chow for conceptual and material assistance. Thank you to our lovely professor, Celeste Leander for all her help and for answering any questions we had along the way. Thank you to both the TAs (Megan and Jordan) and finally, a big thank you to the University of British Columbia (UBC) for giving us the opportunity to take this course.

References

Gregory-Eaves, I., Smol, J. P., Douglas, M. S. V., & Finney, B. P. (2003). Diatoms and sockeye salmon (Oncorhynchus nerka) population dynamics: Reconstructions of salmon-derived nutrients over the past 2,200 years in two lakes from Kodiak Island, Alaska. *Journal of Paleolimnology, 30*(1), 35-53. http://doi.org/10.1023/A:1024792831385.

Guiry, M. D. (2107). World Register of Marine Species. *Licmophora abbreviata*. [Data file]. Retrieved from http://www.marinespecies.org/aphia.php?p=taxdetails&id=157062

Lelong, I. H., & Rebel, G. (1998). pH drift of "physiological buffers" and culture media used for cell incubation during vitro studies. *Journal of Pharmacological and Toxicological Methods, 39*(4), 203-210. https://doi.org/10.1016/S1056-8719(98)00019-7

Ohgai, M., & Tsukahara, H., & Matsui, T., & Nakajima, K. (2008). The Effect of the Environmental Factors on the Growth of two Epiphytic Diatoms Licmophora abbreviata

AGARDH and L. paradoxa (LYNGBYE) AGARDH in vitro. *J-Stage*, *50*(7), 1157-1163. https://doi.org/10.2331/suisan.50.1157

Randall, D. J. (2002). Ammonia toxicity in fish. *Marine Pollution Bulletin*, *45*(1-12), 17-23. Retrieved from https://www.sciencedirect.com/science/article/pii/S0025326X02002278

Schmidt, L. J. (2013). Bloom or Bust: The Bond Between Fish and Phytoplankton. *NASA: Earth Observing System Data and Information System (EOSDIS)*. Retrieved from http://cdn.earthdata.nasa.gov/conduit/upload/1255/NASA_SOP_2005_Bloom-or-Bust-The-Bond-Between-Fish-and-Phytoplankton.pdf.

Scholz, B., & Phycol, J. (2014). Purification and culture characteristics of 36 benthic marine diatoms isolated from the Solthörn tidal flat (Southern North Sea). *Journal of Phycology: An International Journal of Algal Research, 50*(4), 685-697. http://doi.org/10.1111/jpy.12193

Taraldsvik, M., & Sverre, M. (2000). The effect of pH on growth rate, biochemical composition and extracellular carbohydrate production of the marine diatom skeletonema costatum. *European Journal of Phycology*, *35*(2), 189-194. http://doi.org/10.1080/09670260010001735781.

Thompson, A. (2012, December 21). What is carbon sink? *Live Science*. www.livescience.com/32354-what-is-a-carbon-sink.html.

Towers, L. (2013, April 30). Diatoms Help Prevent Harmful Algal Blooms in Fish Ponds. *The Fish Site*. Retrieved from http://thefishsite.com/articles/diatoms-help-prevent-harmful-algal-blooms-in-fish-ponds.

Yool, A., & Toby, T. (2003). Role of diatoms in regulating the ocean's silicon cycle. *Global Biogeochemical Cycles*, *17*(4), 1103. http://doi.org/10.1029/2002GB002018.

Appendix

Appendix 1: Day 0, setting up, green squares count for 100 cells, from 20 uL:

Samples	Count 1	Count 2	Count 3	Average	Concentration (cells/ mL)
pH 7	35	40	25	33.3	$2.4x10^5$
pH 8	29	28	22	26.33	$3.04x10^5$
pH 9	25	27	28	26.66	$3.0x10^5$

<u>Sample Calculation for original cell concentration from average green square count:</u>
Green square dilution factor = $8x10^4$

For pH7 cell concentration:

Average green squares count for 100 cells = (35 + 40 + 25)/3 = 33.3

100 cells/ 33.3 green squares = 3.0 cells/ green square

3.0 cells/ green square x ($8x10^4$) = $3.04x10^5$ cells/mL

Appendix 2: Dilutions to make each pH condition have a cell concentration of $3x10^4$:

pH conditions	Amount of Diatom to add (mL)	pH Media to add (mL)
pH 7	1.250	8.750
pH 8	0.987	9.013
pH 9	1.000	9.000

Sample calculation for dilution recipe:

Wanted cell concentration (C2) = $3x10^4$ cells/mL

For amount of pH7 diatom solution to add to each tube:

$C1 \times V1 = C2 \times V2$

$(2.4x10^5)\ V1 = (3x10^4)\ (10)$

$V1 =$ 1.250 mL

For amount of pH7 media to add to each tube:

10 - 1.250 = 8.750 mL

Appendix 3: Day 1, green squares for 100 cells count (Oct 30):

Samples	Count 1	Count 2	Count 3	Average	Cell Concentration
7-1	*45 cells in 256	109	108	108.5	7.37×10^4
7-2	152	126	225	167.6	4.77×10^4
7-3	121	56	167	114.6	6.98×10^4
8-1	182	217	177	192	4.17×10^4
8-2	223	186	160	189.7	4.22×10^4
8-3	160	248	215	207.7	3.85×10^4
9-1	224	202	203	209.7	3.82×10^4
9-2	186	*94 cells in 256	256	221	3.62×10^4
9-3	202	256	*84 cells in 256	229	3.49×10^4

Appendix 4: Day 2, green squares for 100 cells count (Nov 1):

Samples	Count 1	Count 2	Count 3	Average	Cell Concentration
7-1	136	128	143	135.6	5.90×10^4
7-2	105	161	127	131	6.11×10^4
7-3	92	123	113	109.3	7.32×10^4
8-1	137	139	159	145	5.51×10^4
8-2	182	141	120	147.6	5.42×10^4
8-3	184	191	173	182.6	4.38×10^4
9-1	81	121	136	112.6	7.10×10^4
9-2	192	236	176	201.3	3.97×10^4
9-3	182	179	165	175.3	4.56×10^4

Appendix 5: Day 3, green squares for 100 cells count (Nov 3):

Samples	Count 1	Count 2	Count 3	Average	Cell Concentration
7-1	128	104	140	124	6.45×10^4
7-2	122	132	117	123.6	6.47×10^4
7-3	125	128	141	131.3	6.09×10^4
8-1	116	106	144	122	6.56×10^4
8-2	128	120	84	110.6	7.23×10^4
8-3	112	95	96	109.3	7.92×10^4
9-1	137	90	134	120.3	6.65×10^4
9-2	147	110	149	135.3	5.91×10^4
9-3	204	115	*37 cells in 256	159.5	5.02×10^4

Appendix 6: Day 4, green squares for 100 cells count (Nov 6):

Samples	Count 1	Count 2	Count 3	Cell Concentration
7-1	101 cells in 135	139	101 cells in 235	5.06×10^4
7-2	88	122	192	5.97×10^4

7-3	102 cells in 86	102 cells in 105	113	$8.11x10^4$
8-1	101 cells in 80	110	89	$8.79x10^4$
8-2	101 cells in 116	101 cells in 81	102 cells in 135	$7.66x10^4$
8-3	78	106	101 cells in 137	$7.90x10^4$
9-1	97	101 cells in 103	112	$7.77x10^4$
9-2	118	128	137	$6.27x10^4$
9-3	141	101 cells in 128	120	$6.22x10^4$

Appendix 7: Day 5, green squares for 100 cells count (Nov 8):

Samples	Count 1	Count 2	Count 3	Cell Concentration
7-1	101 cells in 70	105	92	$9.29x10^4$
7-2	132	109	103 cells in 104	$7.11x10^4$
7-3	94	98	128	$7.50x10^4$
8-1	65	68	75	$1.15x10^5$
8-2	66	77	86	$1.05x10^5$
8-3	101 cells in 95	93	101 in 97	$8.48x10^4$
9-1	96	101 in 94	82	$8.89x10^4$
9-2	101 cells in 118	101 cells in 118	115	$6.88x10^4$
9-3	87	115	128	$7.27x10^4$

Appendix 8: Day 6, green squares for 100 cells count (Nov 10):

Samples	Count 1	Count 2	Count 3	Cell Concentration
7-1	104 cells in 112	101 cells in 93	110	$7.80x10^4$
7-2	102 cells in 109	102 cells in 137	101	$7.12x10^4$
7-3	102 cells in 89	88	101 cells in 52	$1.13x10^5$
8-1	69	45 cells in 256	59	$1.26x10^5$
8-2	128	56	101 cells in 83	$1.01x10^5$

8-3	90	103 cells in 134	101 cells in 102	$8.24x10^4$
9-1	34	103 cells in 138	217	$1.11x10^5$
9-2	124	99	110	$7.21x10^4$
9-3	92	101 cells in 87	102 cells in 42	$1.25x10^5$

Appendix 9. Average concentrations of *L. abbreviata* cells (cells/mL x 10^4) under varying pH conditions.

Day	pH 7.0	pH 8.0	pH 9.0
0	3.00	3.00	3.00
3	6.37	4.08	3.64
5	6.44	5.10	5.21
7	6.34	7.24	5.86
10	6.38	8.12	6.74
12	7.97	10.2	7.68
14	8.73	10.3	10.2