How does pH impact the growth of *Chlamydomonas reinhardtii*?

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Abstract

Ocean acidification impacts various marine species, including salmon. In order to understand the ecological effects of ocean and river acidification on salmon, it is essential to understand its effects on *Chlamydomonas reinhardtii*, as it is a primary producer in the salmon food chain. This study examined the impact of pH variation on the growth of *C. reinhardtii*. The experiment was conducted over a period of 11 days. On four set days, 10 μL of a fixative, iodine potassium iodide (IKI), was added to 100 μL samples containing *C. reinhardtii* from an acidic, basic, and control treatment. The number of cells per mL were counted on the last day using a hemocytometer and a compound microscope. The results show that the *C. reinhardtii* population increased from 2.0×10^5 cells/mL to 4.18×10^5 cells/mL in the basic medium of pH 8 and decreased to 0.46×10^5 cells/mL in the acidic medium of pH 5. Statistical analysis using a two-way analysis of variance (ANOVA) showed that the organism's growth between treatments was significant and that there is an interaction between the pH levels and time at $p < .05$, while the difference in growth over time was not significant at $p < .05$.

Introduction

Over the past century, oceans and rivers have absorbed high levels of carbon dioxide that have been released into the atmosphere through many means, such as the burning of fossil fuels (Calderia, 2003). The absorption of high levels of carbon dioxide results in a reduction of pH, thereby acidifying our oceans (Calderia, 2003). This impacts many marine species, as it changes their ecosystem and hinders their ability to interact with their environment (Ou et al., 2015). A reduction in pH can disable many marine species from being able to respond to olfactory cues from predators (Ou et al., 2015).

This study focuses on the impacts of river acidification on *Chlamydomonas reinhardtii* (*C. reinhardtii*), and explored the consequences this may have on salmon, a keystone species.

Salmon play an essential role in supporting food webs (Helfield, 2006) and are a valuable resource to British Columbia, which is why it is vital to investigate the effects of ocean and river acidification on the primary producer of their food chain, *C. reinhardtii.*

C. reinhardtii are microscopic, unicellular green algae with two flagella that can live in various environments, but are typically found in freshwater (Lustigman et al., 1994). *C. reinhardtii* have been used as a model organism in microbiology to study gene expression, protein synthesis, cell-cell recognition and even the cell cycle (Mamedov et al., 2011). These organisms are also capable of performing a buffering mechanism where they absorb hydrogen ions or release specific amino acids in order to resist pH changes (Brock, 1973). This is because a variation in pH can impact the metabolic processes of *C. reinhardtii* (Lustigman et al., 1994). The optimal pH for the growth of this primary producer is stated to be between 5.5 and 8.5 (Messerli et al., 2005).

This study will explore how pH impacts the growth of *C. reinhardtii.* We examined this by introducing an acidic and basic treatment to *C. reinhardtii* and comparing their growth to a control treatment. We predict that *C. reinhardtii* will show greater growth in the basic treatment (pH 8) than in the acidic treatment (pH 5). This prediction is supported by Messerli et al. (2005), who examined optimal growth of *C. reinhardtii* in a pH range of 5.5-8.5, with pH 8 falling within this range. This investigation is important because the abundance of *C. reinhardtii* impacts the abundance of salmon present in our oceans and consequently many other organisms in the food chain.

Our hypotheses with respect to our two-way ANOVA test are as the following:

H_{a1}: The mean number of *C. reinhardtii* counted in at least one pH medium is different

H_{01}: The means of the number of *C. reinhardtii* counted in each pH medium are equal

H_{a2}: The mean number of *C. reinhardtii* counted on at least one set day is different

H_{02}: The means of the number of *C. reinhardtii* counted on each set day are equal

H_{a3}: There is interaction between pH condition and time

H_{03}: There is no interaction between pH condition and time

Methods

Preparation

We began with a 90 mL flask of cultured *C. reinhardtii.* In order to determine whether our culture was too concentrated or too diluted, we counted the initial amount of *C. reinhardtii* present in the flask. This was done by pipetting 100 µL of the sample into an Eppendorf tube and then adding 10 µL of IKI fixative to kill the cells. From this mixture, 10 µL was pipetted onto a hemocytometer and then viewed under a compound microscope to count the initial number of *C. reinhardtii* in this volume. The initial number of cells required was 2.0×10^5 cells/ mL, but our sample was too concentrated, therefore we performed a dilution process after centrifuging.

Treatment Set-up

To centrifuge, we split the 90mL of our culture into three centrifuge tubes. Each centrifuge tube contained 30 mL of the cultured *C. reinhardtii*. These tubes were centrifuged for ten minutes. Next, we removed the medium from each centrifuge tube until just the pellet remained.

After performing a dilution calculation, we pipetted a set volume of *C. reinhardtii* into 9 different test tubes to ensure the initial concentration in each test tube was $2.0x10^5$ cells/ mL. The

9 test tubes were further divided into different treatments; three samples for the acidic condition (pH 5), three samples for the control (pH 6.8), and three samples for the basic condition (pH 8). Next the medium for each treatment was pipetted into its respective test tube. Each test tube held a total of 10 mL of the *C. reinhardtii* and medium mixture. We examined the growth of *C. reinhardtii* in each treatment over a period of 11 days. Throughout these 11 days, the test tubes were incubated at 20°C and received 8 hours of daylight each day.

Adding Fixative

On set days within the 11 day period, 100 µL was pipetted out of all 9 test tubes and added into separate Eppendorf tubes. Ten µL of the IKI fixative was pipetted into each Eppendorf tube. Therefore, each treatment had a total of 3 Eppendorf tube samples after the addition of the fixative. These Eppendorf tubes were refrigerated until day 11, when all the samples were counted.

Data Collection: Counting Cells

To count the cells in the Eppendorf tubes, we pipetted 10 μL of the mixture onto a hemocytometer and counted cells using a compound microscope at a 400X magnification. When counting cells on the hemocytometer, we recorded how many 1x1 mm boxes of the hemocytometer it took to count 100 cells. Three counts were made for each Eppendorf tube to ensure accuracy. The average of the three counts is what was recorded as the final count of that Eppendorf tube sample. The average of the three Eppendorf tube samples from each treatment was recorded as the ultimate count for its respective treatment for that day. The cell counts of each sample from the different treatments were recorded and statistically analyzed. Since this study involved testing between two independent variables, time and pH levels, statistical analysis was performed using a two-way ANOVA. The test was done using 'data analysis' in excel.

Results*:*

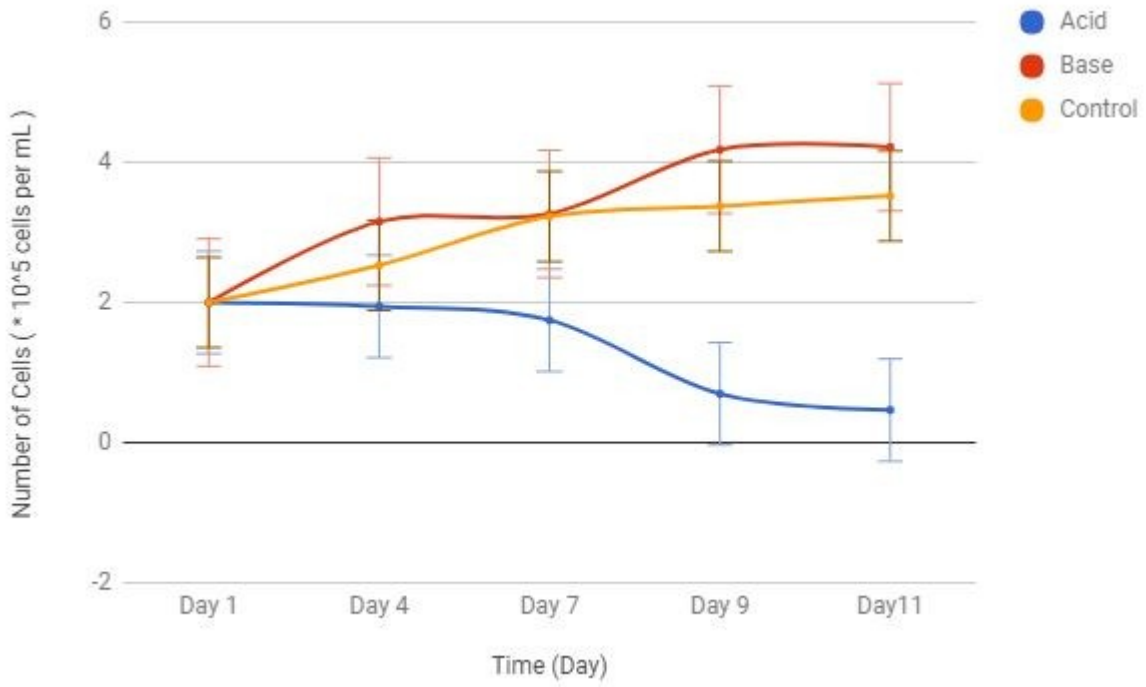

Figure 1. Average number of *C. reinhardtii* cells per mL. Each line represents the cell growth trend exhibited in the three different media, and each dot on the line indicates the number of the cells counted on each day. The bars represent the standard deviation (SD) for each treatment group. (Mean ± 0.73 SD for acid, ± 0.91 SD for base, and ± 0.64 SD for control).

Three different trends appear in Figure 1. The acidic medium of pH 5 shows an overall decrease in the number of cells. It starts decreasing gradually during day 1 - day 7, rapidly decreases during day 7 - day 9, and finally gradually decreases again during day 9 - day 11. This indicates zero growth during all intervals. On the other hand, the basic medium of pH 8, shows a rapid increase in reproduction during day 1 - day 4, a slight increase during day 4 - day 7, another rapid increase during the interval of day 7 - day 9, and no reproduction during the interval day 9 - day 11. This shows intervals of growth followed by intervals of no growth as time progressed. The control medium, at the optimal pH of 6.8, shows a constant increase in cell reproduction as time progressed.

The results from the two-way ANOVA test show that the *p*-value for H_{01} is 2.63×10^{-22} which indicate that the mean number of C. reinhardtii counted between treatments is significant at $p < .05$. However the *p*-value for H_{02} is 0.50, which is larger than .05, revealing the mean number of *C. reinhardtii* counted between days is not significant. Lastly, the *p*-value for H_{03} is 1.68×10^{-9}, which shows that the interaction between pH levels and time is significant at $p < .05$.

Discussion

We are able to reject our H_{01} and provide support for H_{a1} since the growth of *C. reinhardtii* was statistically significant between treatments, with a *p*-value of 2.63×10^{-22}, which is less than .05. This agrees with the findings of Messerli et al. (2005) and provides support for our prediction that *C. reinhardtii* would experience greater growth in a basic environment. However, we fail to reject our H_{02} as the *p*-value is 0.50, which is larger than .05. Therefore, we cannot conclude that there is a significant difference in growth over 11 days within the same treatment. Lastly, we

reject our H_{03} and provide support for H_{a3} as the *p*-value for interaction between pH and time is $1.68x10^{-9}$, which is less than .05.

As seen in Figure 1., the growth of *C. reinhardtii* drops below $2.0x10^{5}$ cells/mL in the acidic treatment of pH 5, indicating there is negative growth in *C. reinhardtii.* This implies cells of *C. reinhardtii* have disappeared. However, there is a plausible explanation for finding cell counts below the initial cell counts. One limitation to our study was evaporation. On day 4, a green ring was observed at the top of all test tubes containing *C. reinhardtii.* The green ring may have been cells of *C. reinhardtii* which adhered to the sides of the container when the medium evaporated, leaving the cells on the side of the container un-suspended in the medium, and discounted from our cell counts. Since all pH treatments contained the same volume, we expect that they experienced the same amount of evaporation, and therefore similar numbers of discounted cells. As a result, the growth ratios between treatments should still be accurate, but the amount of growth per treatment throughout the 11 days, as seen in Figure.1, is lower than the true values. This error may be the reason why we were unable to reject the H_{02}.

The basic pH of 8 shows intervals of growth, followed by intervals without growth. This trend may be correlating to a lag time in cell reproduction. In order to reproduce, *C. reinhardtii* needs to reach a desirable size before asexual or sexual reproduction (Mackean, 2004). As a result, while the new cells grow, they are temporarily unable to reproduce and thus growth does not increase. This explains the stabilized population of *C. reinhardtii* where growth is not observed in between growth intervals in the basic pH of 8.

Biological Mechanisms: ATP consumption

To understand why pH impacts the growth of *C. reinhardtii*, it is essential to understand the biological mechanisms of *C. reinhardtii* growth. Like most plants, *C. reinhardtii* generates its own food supply by the process known as photosynthesis (Messerli et al., 2005). For photosynthesis to work, dissolved carbon, salts, and light must be available for the chloroplast to create starch, a carbohydrate responsible for creating essential nutrients (Messerli et al., 2005). Importantly, *C. reinhardtii* require a cytosolic pH of 7-7.1 for their organelles and other internal processes to function (Messerli et al, 2005). To maintain this cytosolic pH, membrane proteins use ATP to prevent external environments from creating an H+ gradient and entering the cell (Messerli et al, 2005). In acidic external conditions extra ATP is consumed to maintain the cytosolic pH of 7, and thus *C. reinhardtii* is less energy efficient in acidic environments (Messerli et al., 2005). As extra ATP is used up, less energy can be used toward cell growth and reproduction, which could explain why growth was not observed in the pH of 5 treatment.

Ecological Applications

A growing concern in British Columbia is the decline in salmon, a keystone species. Although there are many factors that play a role in the decline of wild salmon populations, the relationship between *C. reinhardtii* and salmon in freshwater environments is crucial. The increasing amount of carbon dioxide released into the atmosphere increases the amount of carbon stored in the oceans (Calderia, 2003). Acidification of freshwater can be damaging to the ecosystem. Infact, according to our results *C. reinhardtii* can grow in a pH range of 6.8-8, but growth is inhibited at a pH of 5. If freshwater acidifies to a pH of 5, we expect *C. reinhardtii*

populations to decrease, as reproduction is required for a stable population. As a primary producer in freshwater ecosystems, this result could impact many other marine organisms.

Once salmon lay eggs, the young salmon develop in freshwater until they are large enough to hunt prey and travel to the ocean. While salmon are in this developing stage, they eat algae such as *C. reinhardtii* and plankton (Mackean, 2004). If freshwater bodies acidify into acidic ranges (pH 5 and below) that are not suited for *C. reinhardtii,* growth of *C. reinhardtii* could be inhibited, and thus salmon will have a limited food supply in developing stages. We expect the limitation of *C. reinhardtii* to decrease the number of salmon that survive and develop in freshwater, therefore decreasing the population of salmon who make it to the ocean. As a keystone species, a decrease in their abundance impacts many other species in the food chain.

Alternatively, too much algae can also be problematic for salmon and the surrounding aquatic ecosystem in freshwater. In many urbanized areas, nitrogen runoff into freshwater streams causes algae blooms (Plouviez, 2017). Since algae like *C. reinhardtii* metabolize nitrogen, they can grow in extremely large numbers in areas where they have access to a lot of nitrogen. Algae blooms deplete dissolved oxygen levels in the water and this has the potential to kill or cause sub lethal effects in the aquatic organisms which reside there (Plouviez, 2017). As a result, an excess of *C. reinhardtii* could also be problematic to salmon and the ecosystem. Our study may help provide a method to treat algal blooms of *C. reinhardtii* by altering external factors in the environment which impact *C. reinhardtii* growth, such as pH level.

Limitations

Several limitations and procedural errors may have led to misleading results. Firstly, asmentioned earlier, we could not prevent evaporation from occurring during the 11 day growth period. This may have led to an accumulation of dried *C. reinhardtii* on the sides of test tubes, discounting the number of suspended cells, and resulting in cell counts lower than initial cell counts.

Secondly, the cell count technique was not ideal. Although we counted each sample three times to ensure accuracy, counting the entire hemocytometer grid on the microscope became problematic. Since some samples had such few cells, instead of using one 1 x 1 mm grid box, we had to use the entire hemocytometer grid to count enough cells (100 cells per sample) for accuracy. Additionally, our microscope required 400X magnification to see the cells, so we had to move the hemocytometer slide around to count the nine 1 x 1 mm grid boxes since only one 1 x 1 mm grid box was within microscopic view. Furthermore, specs of dust on the microscope light resembled cells at times, meaning errors in miscounting dust as cells was likely. Our technique to reduce this was moving the slide to see which remained stationary (the dust) and which moved with the slide (real cells). In future studies, an air blower should be used to eliminate dust particles, and a higher initial concentration of *C. reinhardtii* should be used to prevent counting entire hemocytometer. Finally, *C. reinhardtii* was vortexed and pipetted up and down to ensure mixing, difficulty was experienced in distinguishing individual cells in a congregate, causing the counting of cells in these congregates to be less precise. Additionally, counting large congregates of cells on areas where the distribution of cells were scarce may have skewed our count results in some cases. Lastly, we were unable to track each treatment's pH as growth occurred. According to a study

on buffering effects of *C. reinhardtii*, it was reported that "changes in pH of batch cultures are a common phenomenon" (Lustigman, 1995). As a result, the pH in our treatments may have changed from the original pH we introduced to *C. reinhardtii*.

Conclusion

The results indicate that *C. reinhardtii* growth is optimal at a pH of 8 and 6.8, while at a pH of 5, *C. reinhardtii* does not grow. We reject H_{01} and H_{03} since there is no difference in the mean number of *C. reinhardtii* in each pH medium and there is no interaction between pH treatment and time, while H_{02} is not rejected since there is no difference in the mean number of *C. reinhardtii* over time. This concludes that the effect of pH on *C. reinhardtii* growth is statistically significant, and there is a significant interaction between time and pH with respect to the growth of *C. reinhardtii*. The main implication of these results demonstrate how acidification of freshwater could decrease abundance of *C. reinhardtii* and consequently, decrease the abundance of salmon, a keystone species.

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Appendix:

Table 1. Raw data - number of cells counted per 1mm x 1mm box on hemocytometer

	Number of cells		
Date	Aci	Bas	Contr
Nov 3rd	18	23	22
	20	25	23
	16	21	21
	15	25	26
	18	29	24
	12	21	25
	43	42	24
	49	40	21
	48	37	25
Nov 6th	2	29	14
	2	26	20
	2	33	19
	19	42	42
	20	38	43
	18	40	42
	9	20	32
	9	20	30
	9	20	28
Nov. 8th	8	45	26
	7	47	28
	8	41	26
	3	44	29
	3	46	24

Appendix:	2	44	29
	5	33	32
	5	31	33
	6	32	32
Nov. 10th	7	38	34
	7	37	33
	7	38	34
	6	30	30
	6	30	30
	6	30	30
	3	51	32
	3	44	28
	3	46	34

Table 2. Average number of cells per 1mm x 1mm box on hemocytometer

Nov 3rd	Aci	Base	Control
Rachael	18	23	22
Gurkim	20	40	23
Sally	15	25	24
Count	194333.333	322666.666	253000
Nov 6th	Aci	Base	Control
Rachael	19	40	42
Gurkim	20	29	18
Sally	9	20	32
Count	174625	326333.333	337333.3333
Nov 8th	Aci	Base	Control
Rachael	3	38	29
Gurkim	8	44	27
Sally	5	32	32

Appendix: Count	69666.6666	418000	322666.6667
Nov	Aci	Base	Control
Rachael	28	47	32
Gurkim	56	38	34
Sally	31	47	30
Count	46444.4444	53777.7777	352000

Table 3. Number of cells ($x10^5$) / mL in each medium over time

	Acid	Bas	Control
Oct. 31st	2	2	2
Nov. 3rd	1.94333	3.226666667	2.5
Nov. 6th	1.74625	3.263333333	3.22666666
Nov. 8th	0.69666	4.1	3.3733333
Nov.	0.46444	1.3	3.5

Table 4. Statistical results from a two-way ANOVA test

ANOVA						
Source of Variation	SS	df	MS	F	P-	F*(F
Between Days	135.213	3	45.0709	0.7935 8	0.50039	2.699393
Betwee n	9899.68	2	4949.84	87.154 3	2.63E-2	3.091191
Interaction	3842.31	6	640.385	11.275 5	1.68E-0	2.194516
Within	5452.22	96	56.7939			
Tot	19329.4	107				