The effect of mineral oil on the CO_2 concentration of *Chlamydomonas reinhardtii*'s environment over time

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ABSTRACT

Oil is a common water pollutant that is released into the environment through spills which can harm marine ecosystems. As oil spreads out over the water surface, the diffusion of CO_2—which is required by *Chlamydomonas reinhardtii* for photosynthesis—becomes inhibited. This experiment investigated the effect of mineral oil on the CO_2 concentration of *C. reinhardtii*'s environment. We performed titrations on samples of *C. reinhardtii* that we treated with mineral oil to find the concentration of CO_2. The control group had the greatest change in CO_2 concentration over time, while the full oil treatment had the lowest. The mean CO_2 concentrations were 77.83 ppm for the control group, 53.67 ppm for the partial treatment, 49.08 ppm the half treatment, and 44.42 ppm for the full treatment. A two-way ANOVA revealed our results to be statistically significant for both time ($p=2.9 \times 10^{-4}$), treatment ($p=6.0 \times 10^{-5}$) and the interaction between the two (2.2×10^{-16}); thus, we all three null hypotheses. It is possible that the metabolic rates of *C. reinhardtii* were reduced due to the toxicity of aromatic hydrocarbons found in oil, although there was some growth over the 7-day period of the experiment.

INTRODUCTION

Oil remains a heavy pollutant that can drastically affect the marine ecosystem (Holcomb, 1969). Some ways oil can enter the marine ecosystem is through leaks and tanker accidents (Holcomb, 1969). The dispersion of oil in water will quickly spread as a thin layer on top of the surface (Holcomb, 1969). This oil film can negatively affect the survival of marine algae, such as *Chlamydomonas reinhardtii*.

Mineral oil has a negative impact on the molecular diffusion of carbon dioxide in oil. With high viscosity and highly refined hydrocarbons, the molecular bonds are stronger, which inhibits the diffusion of CO_2 molecules through the oil (Bakyani et al., 2016). Through this experiment, we wanted to see if *C. reinhardtii's* survival would be affected due to the oil layer on the surface preventing CO_2 from escaping the water and into the atmosphere.

Algae and plants, such as *C. reinhardtii*, support the majority of life on earth through oxygenic photosynthesis (Dent, Han & Niyogi, 2001). These marine algae are primary producers that can produce their own food and are a great source of nutrients to marine organisms in the higher trophic levels (Gende et al., 2002). Young salmon are dependent on the consumption of algae, such as *C. reinhardtii* as a food source (Orlov, Gerasimov & Lapshin, 2006). Salmon species act as an active link between ecosystems by providing marine-derived carbon and nutrients back into the rivers and land via fish carcass decomposition (Holmlund & Hammer, 1999). With the decrease in the abundance of *C. reinhardtii* due to oil pollution, we may see a decline in the salmon population and productivity in the surrounding ecosystem.

In our study, we exposed *C. reinhardtii* to three different concentrations of mineral oil and determined the CO_2 levels in each treatment. We have three proposed null hypotheses: that there is no difference in CO_2 concentrations in the presence of mineral oil, that there is no difference in CO_2 concentrations over time and that there is no interaction between the two factors (oil amount and time). The three alternative hypotheses state: that there is a difference in CO_2 concentrations in the presence of mineral oil, that there is a difference in CO_2 concentrations over time and there is an interaction between the two factor variables. We predict that with the increasing amount of oil there will be higher concentrations of CO_2 present in *C. reinhardtii's* environment. The layer of oil may prevent the diffusion of CO_2 to and from the water which leads to higher levels of CO_2 concentrations.

METHODS

To set up our experiment, we started with a stock solution of *C. reinhardtii* that we needed to dilute down to $1.5x10^5$ cells/mL. Mindy Chow grew the stock solution in *C. reinhardtii* media which had an initial pH of 6.5 and incubated the cells at 25.7^0C. First, we had to count the number of cells in our stock solution using a haemocytometer and a compound microscope. In order to count the cells easily, we fixed a small sample of the *C. reinhardtii* with IKI. Once we knew the concentration of our stock solution ($3.66x10^5$ cells/mL), we could dilute it to $1.5x10^5$ cells/mL. To dilute the stock solution, we used the $C_1V_1 = C_2V_2$ equation and found that we needed 410 mL of the stock solution and 550 mL of the *C. reinhardtii* media. After diluting the stock solution, we counted the cells, once more, so that we had an accurate initial cell count. The final dilution had $3.1x10^4$ cells/mL, less than we wanted but since we were not looking for a growth curve, it was not detrimental to the experiment. Any time we transferred cells between flasks, we followed sterile technique to ensure that the only organism in our flasks was *C. reinhardtii*.

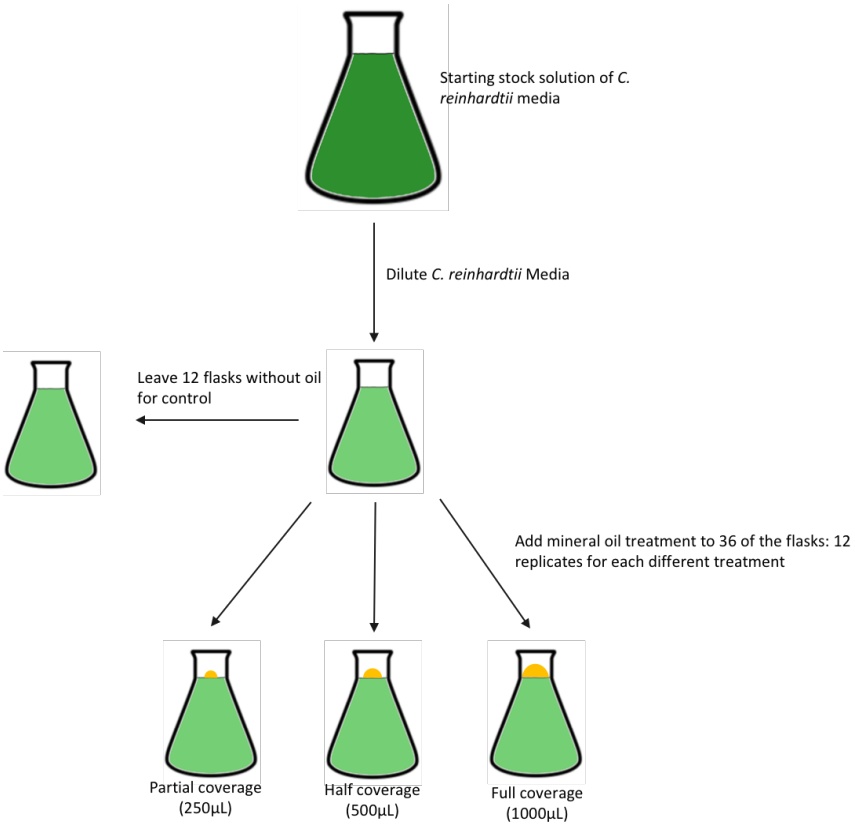

Figure 1. A flow chart of the dilution of *C. reinhardtii* solution and set up of treatments. The final dilution had 3.1x104 cells/mL

Next, we had to set up our flasks with our oil treatments. We added 20mL of the dilute *C. reinhardtii* solution to 48 flasks: we had 12 flasks for each treatment but only three replicates per treatment per day. After each treatment, we had to discard the sample due to the titration. We also used different flasks for each day's samples because we did not want to disturb the oil layer multiple times over the course of the experiment to retrieve sample. Thus, we distributed sample over a larger number of flasks so that each day had its own flask and corresponding sample. After we filled the flasks with the solution, we used a random number generator to label which flasks would be given which treatment and sampled on which day. We set up 3 different

treatments of oil coverage and a control by micropipetting oil onto the *C. reinhardtii* solution: a control without oil, partial (250µL oil), half (500µL oil), and full (1000µL oil).

Next, we took the pH of 3 replicates of each treatment group by wetting pH strips with 50µL of solution. Once we took the pH measurement, we used the remaining sample from the pH tests to run a titration to find the amount of CO_2 in ppm. We used titration kits that used phenolphthalein as the indicator and chemical Reagent B as the titrant. As we ran the titration, the phenolphthalein should create a pink colour when the reaction reached the endpoint, but in our sample, it looked brown due to the green colour of the sample. With the remaining samples, we incubated them at 25.7^{0}C over a week and collected subsequent pH and CO_2 data on day 3, day 6, and day 7. Finally, we also counted the cells on day 7 to take into account any difference in CO_2 due to the amount of *C. reinhardtii*.

All data values were tabulated into laboratory notebooks and then later input into a Microsoft Excel spreadsheet. We conducted all data analyses through the Rstudio statistical program (Rstudio Team, 2016). Since both oil amount (treatment) & time (day) influenced CO_2 concentration (the response variable), we planned to use a two-way ANOVA to determine if the means of the groups differ. If the data proved to be statistically significant (i.e. $p<0.05$), then a Tukey Kramer test can determine which groups, specifically, differed.

RESULTS

Initial cell counts for all treatments are roughly the same since the starting solution was diluted as mentioned in the methods above. Final cell counts on day 7 showed that the control group had the most number of cells, as shown in Figure 2 below. The half and partial treatments were not too far behind in cell count; the half treatment had the second most cells followed by

the partial treatment. The full oil treatment proved to have a remarkably lower number of cells. Furthermore, we observed that the control solution was the darkest green in colour. The green color was lighter for the partial treatment and the half oil treatment was lighter than the partial. The full treatment had the faintest color.

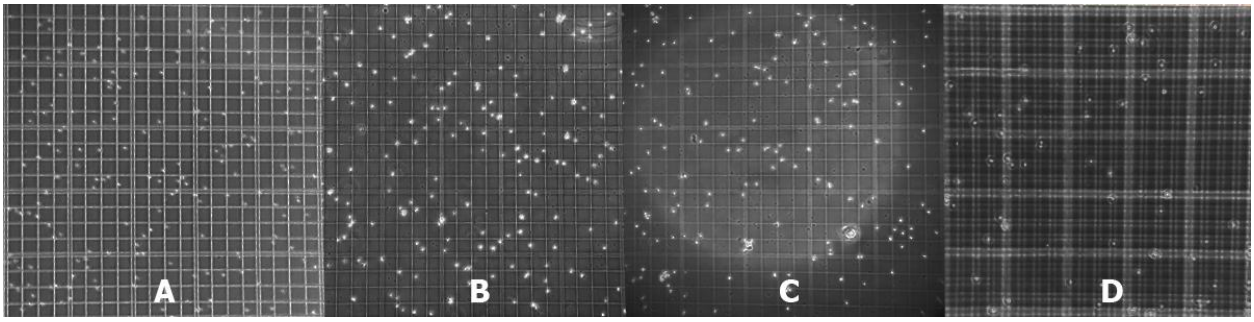

Figure 2. Pictures taken with an iPhone SE through the lens of a compound light microscope. The image labelled A is the control group, B the partial treatment, C the half treatment, and D the full treatment. The final cell counts were 4.2×10^7 cells in control, 3.7×10^7 cells in partial, 4.1×10^7 cells in half, and 1.1×10^7 cells in full.

In regards to CO_2, the data collected met the assumptions of normality, large sample size (n=48), independence, and random sampling so a two-way ANOVA was used for analysis. Figure 3 shows the averaged results of data from all days. On average, the control group shows to have the greatest variance in average CO_2 concentration. There was less variance in the other three treatments (partial, half, and full).

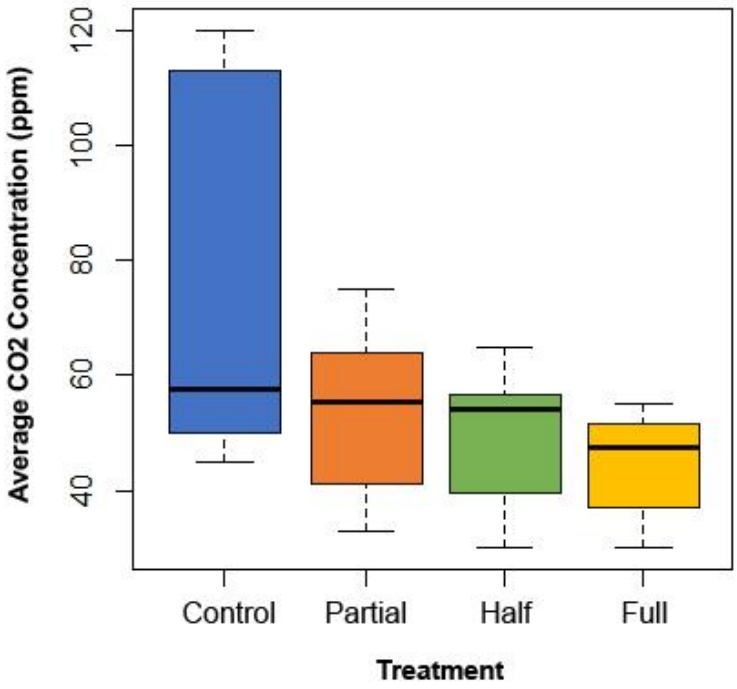

Figure 3. Box plot showing the average CO_2 concentration (ppm) present in each treatment (control, partial, half, and full). We averaged all of the values of CO_2 concentration collected over seven days into their perspective treatment groups. Figure does not account for change in CO_2 over time. The means for the four treatment groups above were 77.83 ppm (control), 53.67 ppm (partial), 49.08 ppm (half), and 44.42 ppm (full). The 95% confidence intervals were 18.57, 7.66, 6.64, and 5.18, respectively. Compared to the other treatments, we saw a wider range of CO_2 concentrations in the control treatment. The centre division of the boxplot represents the median outcome and error bars represent standard error. No outliers are visible.

Figure 4 below shows the change in CO_2 concentration measured over time within the control and 3 treatments. All treatments showed a slight decrease in CO_2 concentration from day 1 to day 3. But overall, results indicated an increase in CO_2 concentration among treatments. The control group presented the greatest increase in CO_2 concentration followed by partial oil, then half oil, and full oil being the lowest CO_2 concentration. In general, a greater amount of oil resulted in a smaller increase in CO_2 concentration.

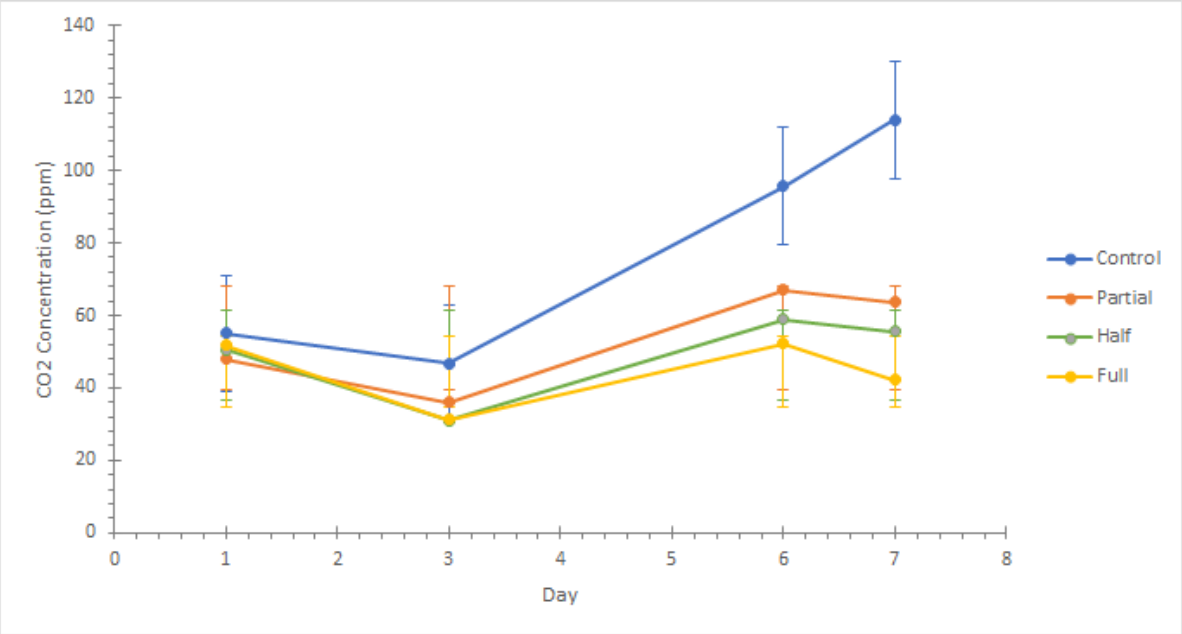

Figure 4. Line graph showing the change in CO_2 concentration (ppm) over the course of 7 days. We collected values on days 1, 3, 6, and 7. The blue line represents the control, orange represents partial oil, green represents half oil, and yellow represents full oil. The means for the four treatment groups above were 77.83 ppm (control), 53.67 ppm (partial), 49.08 ppm (half), and 44.42 ppm (full). The 95% confidence intervals were 18.57, 7.66, 6.64, and 5.18, respectively. All treatment groups started at roughly the same CO_2 concentration on day 1. The control group showed to have the greatest increase in CO_2 concentration; increasing from an average CO_2 concentration of 55 ppm on day 1 to 114 ppm on day 7.

The first null hypothesis states that there is no statistical difference in CO_2 concentrations in the presence of mineral oil. The two-way ANOVA determined a p-value of 6×10^{-5} for the difference in CO2 concentration between treatments (df=3). We reject the null hypothesis. The second null hypothesis states that there is no statistical difference in CO_2 concentrations over time. The two-way ANOVA determined a p-value of 2.9×10^{-4} for a difference in CO2 concentration over time (df=1). We also reject this null hypothesis. The third null hypothesis

states that there is no statistical interaction between the two factors (oil amount and time). The way-way ANOVA determined a p-value of 2.2×10^{-16} for the third null hypothesis, which was also rejected.

All data analyses had an α-value of 0.05 and since $p<0.05$, we reject the null hypothesis. Both time (day) and oil amount (treatment) are statistically significant, with amount of oil added being the most significant factor variable. A Tukey Kramer test was then conducted to determine which groups differed. The results indicated the full treatment ($p = 2.8 \times 10^{-6}$), the half treatment ($p = 4.1 \times 10^{-5}0$), and the partial treatment ($p = 5.3 \times 10^{-4}$) differed significantly from the control group. The difference between other groups were not statistically significant according to the Tukey Kramer test.

DISCUSSION

The two-way ANOVA analysis resulted in a p-values less than 0.05, meaning the results were significant. Therefore, we reject all three null hypotheses as there was a difference in the CO_2 concentration of *C. reinhardtii*'s environment due to the presence of mineral oil. There was a decreasing trend in the average CO_2 concentration for each treatment as seen in Figure 3. Also, Figure 4 shows that the CO_2 concentration increased over time for each treatment. These results do not coincide with our original prediction that the amount of CO_2 would increase with the addition of more oil. The flasks were sealed during the course of the experiment and some CO_2 may have escaped when they were opened in order to take measurements, but we cannot account for this amount as it is probably too small. It is likely that the metabolic activities of *C. reinhardtii* played a more important role than the diffusion of CO_2 out of the flasks. For reference, reported oxygen uptake for *C. reinhardtii* is 0.53 mmol O_2 per gram per hour

(Kliphuis et al., 2012). Based on the molar ratio of the respiration equation, $C_6H_{12}O_6 + 6O_2 \rightarrow 6CO_2 + 6H_2O + ATP$ (Johnson & Alric, 2013), the rate of CO_2 produced during respiration may be similar, but we can only predict how the metabolic rates would be affected by oil.

A similar study by Soto et al. (1975) investigated the effect of naphthalene and crude oil extracts on the growth rate of another *Chlamydomonas* species, *Chlamydomonas angulosa*. *C. reinhardtii* use CO_2 for photosynthesis and release it during respiration, so we are considering these two processes represent their metabolic activity. Soto et al. (1975) reported a significant decrease in growth over time with the addition of naphthalene, made from crude oil. Our results showed a trend of decreasing average CO_2 concentrations with increasing amounts of oil, which may show a reduction in metabolic activity. This may be due to the high aromatic content of the oil making it toxic to marine algae (O'Brien & Dixon, 1976). Aromatic hydrocarbons are more soluble and thus more bioavailable, increasing their toxicity (Swigert et. al., 2014). After a week, the control had the darkest green colour and there was a lighter green colour in the treatments with increasing amounts of oil. The CO_2 concentration for each treatment likely increased over time due to the growth of *C. reinhardtii* cells in the media. The cell counts taken on the final day of the experiment showed the highest cell count for the control and less for each of the treatments. It appears that some metabolic activity was maintained as cells grew over the course of the experiment but growth was overall inhibited in the flasks containing more oil. As *C. reinhardtii* populations diminish due to oil pollution, the salmon populations in the surrounding ecosystem will suffer from some loss of an essential food source (Orlov, Gerasimov & Lapshin, 2006).

The bicarbonate buffer equation shows that CO_2 reacts with water, (reversibly) forming carbonic acid. Carbonic acid may lose protons, resulting in the formation of bicarbonate and carbonate. With an increase in protons in the water, the pH can lower resulting in a more acidic environment (Greenwood et al., 1997). However, the fact that pH did not change throughout the experiment indicates that the dissolved CO_2 did not dissociate; thus, using the CO_2 titration kit was an accurate method of measuring CO_2 concentration. This is consistent with literature stating that most CO_2 does not dissociate into carbonic acid and thus does not the affect the pH due to the hydration constant (Rau & Caldeira, 1999; Garg & Maren, 1971). For reference, the pH was measured as 6 or 7 for our treatments, which is within the range of 5.5 to 8.5 for optimal growth for *C. reinhardtii* (Messerli et al., 2005).

A considerable amount of variation arises from our CO_2 measurements. Since our solutions were of a green color, it was challenging to determine the color of the titration endpoint as the solutions did not turn a light pink but rather a brownish-green; thus, we may have had a wide range of data. To improve this, it would have been efficient to centrifuge the solutions and perform titration measurements only on the supernatant. The overlap in our confidence intervals may arise from human errors regarding insufficient mixing of our cell culture when preparing the flask and inconsistent counting of cells.

CONCLUSION

We found the CO_2 concentration in the environment of *C. reinhardtii* to be affected by mineral oil. The results show a statistically significant decrease in CO_2 concentrations as oil amounts increased. The results also show an overall increase in CO_2 concentration over time. Although there was an overall growth of *C. reinhardtii*, the metabolic rates of these cells may

have been impaired due to being exposed to increasing amounts of aromatic hydrocarbons found in the mineral oil. The presence of mineral oil in aquatic environments may cause a decrease in *C. reinhardtii* populations, causing a subsequent decrease in salmon populations relying on this marine algae as a food source.

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APPENDIX

Day	Treatment	pH	CO_2 (ppm)
1	control	7	57
1	control	7	58
1	control	7	50
3	control	6	45
3	control	6	50
3	control	6	45
6	control	6	56
6	control	7	120
6	control	6	111
7	control	6	118
7	control	6	109
7	control	6	115
1	partial	7	44
1	partial	6	50
1	partial	6	50
3	partial	7	38

3	partial	7	37
3	partial	7	33
6	partial	6	75
6	partial	7	65
6	partial	7	61
7	partial	7	63
7	partial	6	62
7	partial	6	66
1	half	6	47
1	half	6	50
1	half	7	55
3	half	6	32
3	half	7	30
3	half	7	31
6	half	7	57
6	half	7	55
6	half	6	65
7	half	6	56

7	half	6	53
7	half	6	58
1	full	7	50
1	full	6	55
1	full	6	50
3	full	7	34
3	full	7	30
3	full	7	30
6	full	7	53
6	full	7	50
6	full	6	54
7	full	7	45
7	full	6	40
7	full	6	42