Mineral oil may impede maximum growth rates and exacerbate carbon dioxide build-up in *Chlamydomonas reinhardtii*

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Abstract

Chlamydomonas reinhardtii is a photosynthetic unicellular green algae. Due to its relatively simple genome, *C. reinhardtii* is a model organism for observing various biological processes. This study focused on how mineral oil impacted its growth rate, since mineral oil prevents gas exchange from occurring on water surfaces. We expected both change in CO_2 concentration (Δ[CO2]) of the media and maximum growth rate to decrease throughout the duration of the experiment. We had four different experimental groups containing equal amounts of *C. reinhardtii* in each with different volumes of mineral oil added: 0 μL (control group), 250 μL, 500 μL, and 1000 μL. A one-way ANOVA (d.f. = 3, F = 3.32) on maximum growth rates yielded $p = 0.115$ ($\alpha = 0.05$), so we failed to observe any significant difference in the maximum growth rates of *C. reinhardtii*. Additionally, a one-way ANOVA (d.f. = 3, F = 2.13) yielded $p = 0.175$ for Δ[CO2] , so we also failed to see any significant differences in the Δ[CO2]. Despite the results, the maximum growth rate may have shown a decline as more oil was added, but Δ[CO2] may have shown an increasing trend with increasing amounts of mineral oil. We believe multiple sources of errors may have played a role in the results that we obtained as they did not match with our prediction.

Introduction

The unicellular *Chlamydomonas reinhardtii* lives in a widespread distribution worldwide, in both soil and water. As a primary producer, the C. *reinhardtii* forms the base of the food chain, making it essential for other marine species such as salmon. Salmon serve as a source of carbon and nutrients and form a link between ecosystems through decomposition of fish carcasses (Holmlund & Hammer, 1999). A decrease in *C. reinhardtii* populations is known to decrease overall ecosystem productivity as this decreases food bioavailability and oxygen production, leading to decreases in the salmon population. (Carter et al., 2005). Carter et al. (2005) also concluded that embryonic and larval stage salmon have an increased sensitivity to dissolved oxygen levels as compared to mature salmon. Additionally, juvenile salmon are also dependent on the consumption of algae as a food source (Orlov, Gerasimov & Lapshin, 2006).

The amount of algae a fish consumed had a positive effect on growth and the intake of other feed (Norambuena et al., 2015). Embryonic and larval salmon do not have the ability to move to more favourable conditions with higher C. *reinhardtii* and oxygen levels (Carter et al., 2005). We may expect to see a decline in overall ecosystem productivity and salmon populations as *C. reinhardtii* populations are negatively impacted by the effect of oil pollution.

Oil is a common water pollutant that may originate from spills during transportation, drilling, or fuel usage (Brussaard et al., 2016). Large oil spills have toxicological effects on the marine ecosystem it affects (Gros et al., 2014), as well as affect environmental processes important for marine algae such as *C. reinhardtii.* In a study of the effect of oil-films on the gas exchange in sea water, Anikiev et al. (1988) concluded that oil spills prevent the exchange of gases in its area, trapping O_2 under the oil and inhibiting the diffusion of CO_2 into the ocean water.

Mineral oil is a liquid by-product of refining crude oil to form gasoline and petroleum (Marinescu et al., 2004). Its composition consists of highly refined hydrocarbons, alkanes and cycloalkanes (Rocchini, 1952). These components give mineral oil high viscosity and a low density; inhibiting the diffusion of CO_2 molecules through the oil (Bakyani et al., 2016). This inhibition of gas exchange is expected to cause a build up of O_2 as all the present CO_2 will be used for photosynthesis by *C. reinhardtii*.

Through this experiment, we wanted to investigate if *C. reinhardtii's* maximum growth rate would be impacted due to the mineral oil layer on the surface. To further solidify our findings, we also examined the effect of mineral oil on concentration of CO_2 in the growth media of *C. reinhardtii*. In this study, we exposed cultured *C. reinhardtii* to four different levels of

mineral oil, in order to cover the culture to different extents. During the study, both the growth rate of *C. reinhardtii* and the CO_2 concentration are measured. Our proposed null hypotheses (H_0) are: there will be no difference in the means of *C. reinhardtii* maximum growth rates due to the effect of mineral oil ($H_{0,1}$) and there will be no significant difference in the means of change of CO_2 concentrations ($\Delta[CO_2]$) in all groups due to the effect of mineral oil ($H_{0,2}$). Our proposed alternative hypotheses are that: there will be a difference in the means of *C. reinhardtii* maximum growth rates due to the effect of mineral oil ($H_{1,1}$) and there will be a significant difference in the means of $\Delta[CO_2]$ in all groups due to the effect of mineral oil ($H_{1,2}$). From previous literature, we predict that the maximum growth rate of *C. reinhardtii* will decrease with increasing amounts of mineral oil added as the supply of CO_2 needed for metabolism will be limited by mineral oil (Bakyani et al., 2016); we also predict there will be a lower $\Delta[CO_2]$ with increasing amounts of mineral oil since *C. reinhardtii* will grow less and subsequently produce less CO_2 from cellular respiration (Brar et al., 2019).

Methods

To set up the experiment, Mindy Chow provided us with *C. reinhardtii* stock solution which she grew at 25.7°C at a pH of 6.5. We aimed to have a diluted solution of 2.0×10^5 cells/mL, but did not have enough stock solution so we diluted the *C. reinhardtii* stock solution to 9.9×10^4 cells/mL. Then we transferred 30 mL of diluted *C. reinhardtii* to each 50 mL Erlenmeyer flask. Throughout the experiment, we used sterile technique any time we transferred cells between flasks. For our treatment groups, we applied 250 µL, 500 µL, and 1000 µL of mineral to the surface of the diluted solutions in the flasks to form a uniform oil layer with three replicates per treatment group (Figure 1). These amounts of mineral oil we chose were based on

a previous study that investigated the effect of mineral oil on CO_2 concentrations of *C. reinhardtii*'s environment (Brar et al., 2019). We kept three flasks with 0 µL of mineral oil applied as our control group. In total, we had twelve Erlenmeyer flasks with *C. reinhardtii* cells.

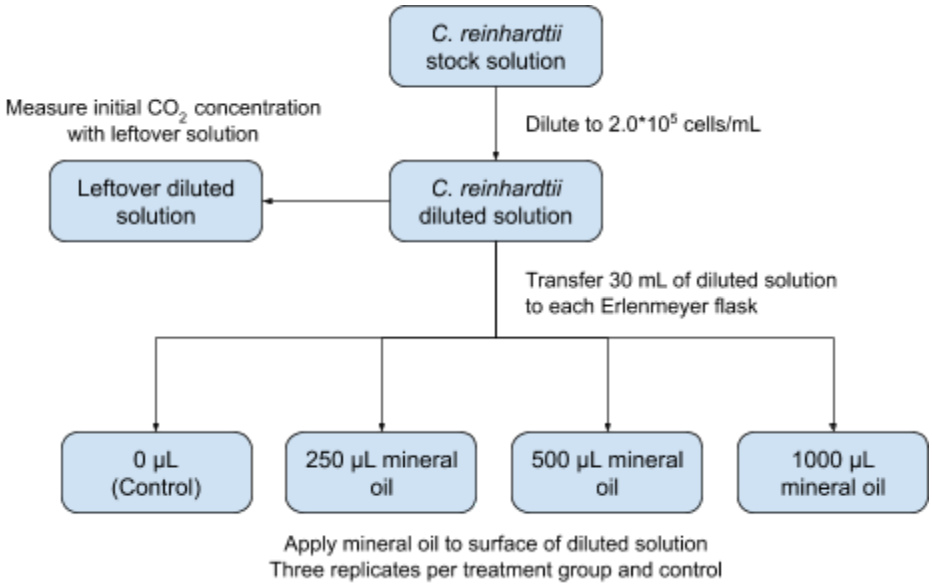

Figure 1. Flow chart of the planned experiment set-up. Actual diluted solution concentration was 9.9×10^4 cells/mL.

We incubated the *C. reinhardtii* cultures at 25°C for 10 days with a 12-hour light cycle. Every 48 hours with the exception of a weekend, we mixed and sampled each flask carefully to break up cell clumps while minimizing disturbance to the oil layers. After mixing, we sampled 100 µL from each replicate, which we fixed with 10 µL of IKI. Then we pipetted 10 µL of our fixed samples onto a hemocytometer and determined the number of cells in a 1×1 mm quadrant with a compound microscope. We counted to a minimum of 100 cells within the hemocytometer space, which had nine 1×1 mm quadrants to account for cell count variability. Each sample was counted three times to account for sampling variability.

We measured the dissolved CO_2 concentrations with a CO_2 titration kit on the day of the experiment set-up with our leftover diluted solution as our initial CO_2 measurement and on the last day of the experiment as our final CO_2 concentrations. We measured CO_2 concentration three times per sample.

We averaged the three cell counts per sample to obtain the average cell density for each replicate on a specific day. We calculated the instantaneous growth rate by dividing the difference between neighbouring data points by the number of days incubated. We used the average instantaneous growth rate to determine which day the maximum instantaneous growth rate occurred for each treatment group. We performed a one-way ANOVA on the instantaneous growth rates that each respective treatment group achieved its maximum growth rate to determine if the mean maximum growth rates differed. If the data were statistically significant, we would perform a Tukey HSD to determine which groups differed.

We subtracted the initial CO_2 concentration from the average final CO_2 concentration for each sample to obtain the $\Delta[CO_2]$ for each treatment group. Then we performed a one-way ANOVA on $\Delta[CO_2]$. If the data were statistically significant, we would perform a Tukey HSD to determine which treatment groups differed.

Results

Initial cell density was identical for all replicates as they all started from the same diluted solution. Although the 0 μL/control group decreased in cell density initially, it had the highest cell density on the final day and the treatment groups had lower cell density with increasing amounts of mineral oil added (Figure 2). The control group on Nov-06 has a large confidence

interval due to a large variation in cell densities between replicates and no replicate could be excluded as an outlier.

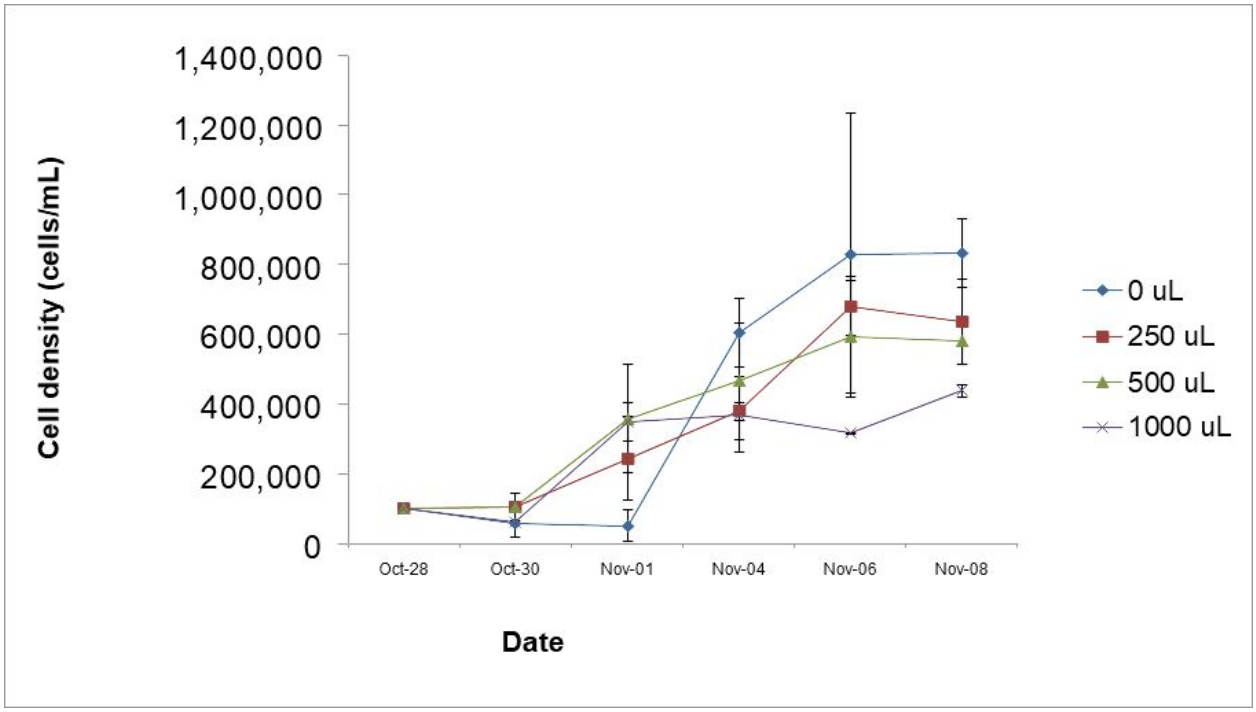

Figure 2. Line graph indicating the mean cell densities each day for each group (0 µL/control, 250 µL, 500 µL, and 1000 µL; n = 3 per group) with 95% confidence intervals.

Mean maximum growth rates were the highest in the control group with lower maximum growth rates with increasing amounts of mineral oil applied in the treatment groups (Figure 3). The 95% confidence intervals were similar between the control group, 250 µL, and 1000 µL groups. The 95% confidence interval for the 500 µL group appears to be much larger compared to the other groups.

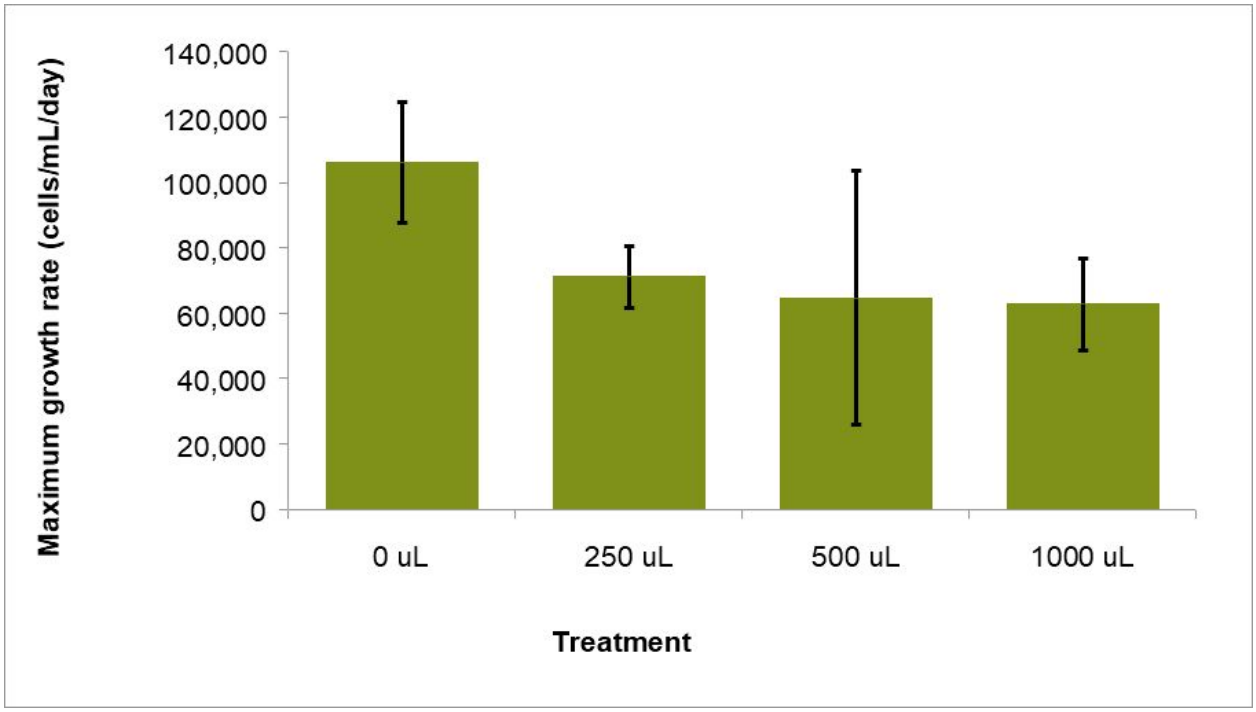

Figure 3. Column graph showing the mean maximum growth rate (cells/mL/day) for each treatment group (0 μL/control, 250 μL, 500 μL, and 1000 μL; n = 3 per group) with 95% confidence intervals. The mean maximum growth rates (cells/mL/day) were 1.1×10^5 (control), 7.1×10^4 (250 μL), 6.5×10^4 (500 μL), 6.3×10^4 (1000 μL). The 95% confidence intervals were 1.8×10^4, 9.4×10^3, 3.9×10^4, and 1.4×10^4, respectively.

$\Delta[CO_2]$ appears to be the lowest in the control group with increasing amounts of CO_2 with increasing amounts of mineral oil added (Figure 4).

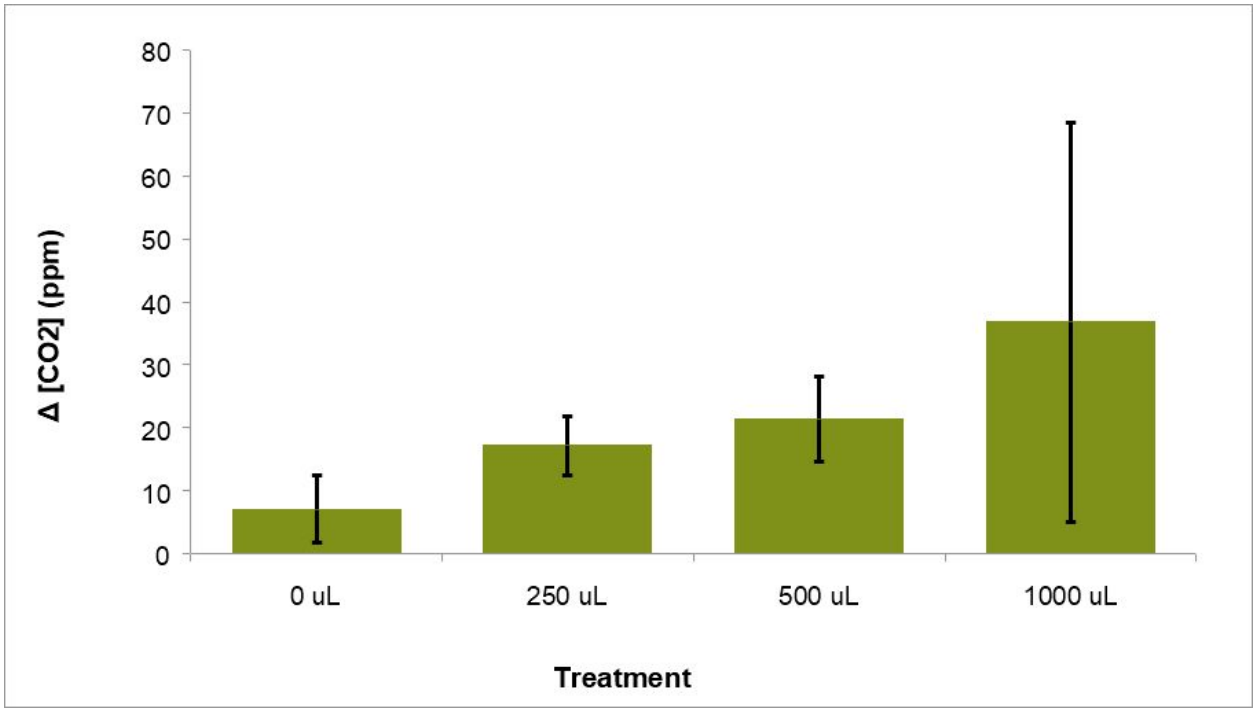

Figure 4. Column graph showing the $\Delta[CO_2]$ (ppm) for each treatment group (7.1, 17.2, 21.3, and 36.8 ppm for 0 µL/control, 250 µL, 500 µL, and 1000 µL, respectively; n = 3 per group) with 95% confidence intervals (5.3, 4.7, 6.8, and 31.7).

Discussion

In this study, we measured growth rates of *C. reinhardtii* and CO_2 concentrations of *C. reinhardtii*'s environment with increasing amounts of mineral oil applied to our cultures.

A one-way ANOVA (d.f. = 3, F = 3.32) on maximum growth rates yields a p = 0.115, thus we fail to reject the null hypothesis ($H_{0,1}$) that there is no difference in the means of *C. reinhardtii* maximum growth rates due to the effect of mineral oil and therefore fail to support the alternative hypothesis ($H_{1,1}$) that the mean maximum growth rates are unequal due to the

effect of mineral oil. Although our results were not significant, the possible trend aligns with our prediction that increasing amounts of mineral oil will decrease the maximum growth rate of *C. reinhardtii* (Figure 3). Similarly, a one-way ANOVA (d.f. = 3, F = 2.13) yields a p = 0.175 for $\Delta[CO_2]$, thus we fail to reject the null hypothesis that there is no difference in the means of $\Delta[CO_2]$ in all groups due to the effect of mineral oil ($H_{0,1}$) and fail to support the alternative hypothesis ($H_{1,2}$) that the means of $\Delta[CO_2]$ in all groups due to the effect of mineral oil are unequal. Even though our results were not significant, the trend contrasts with our prediction that change in CO_2 will decrease with increasing amounts of mineral oil added (Figure 4). Since both one-way ANOVA results for maximum growth rate and $\Delta[CO_2]$ were not statistically significant, a post-hoc test like a Tukey HSD is unnecessary.

A similar study by Soto et al. (1975) concluded that the addition of naphthalene, another by-product of crude oil refinement, decreased the growth rate and photosynthetic activity of *Chlamydomonas angulosa*. Naphthalene is an aromatic hydrocarbon, and is highly soluble and toxic to marine life including alga (Swigert et al., 2014; O'Brien and Dixon, 1976). Specifically, naphthalene was found to be oxidized into several different metabolites such as 1-naphthol by *C. angulosa* (Cerniglia et al., 1980). Furthermore, petroleum compounds were found to possess considerable toxicity to algae like *C. reinhardtii* (Corner, 1979) as well as other organisms such as larval zebrafish and yellow perch (Scarlett et al., 2013; Peters et al., 2007). This toxicity to algae could explain why our data may indicate that increasing amounts of mineral oil may inhibit the maximum growth rate of *C. reinhardtii*. At the end of the experiment, the control group with 0 μL of mineral appeared to be the darkest green in colour compared to the treatment groups.

This was confirmed by our final cell counts, which showed that the control group had the highest cell concentration compared to the treatment groups.

Another similar study by Brar et al. (2019) which investigated the effect of mineral oil on the CO_2 concentration of *C. reinhardtii*'s environment over time and found that the addition of mineral oil decreased the amount of CO_2 build-up of *C. reinhardtii*'s environment, which contradicts with our finding that the addition of mineral oil increased $\Delta[CO_2]$ of *C. reinhardtii*'s environment. Although CO_2 is used up in photosynthesis by *C. reinhardtii*, it also produced during respiration which is needed for growth and explains why CO_2 concentrations increase even as *C. reinhardtii* photosynthesizes (Babcock and Wikström, 1992). The mineral oil we applied to the surface of the treatment groups may have prevented CO_2 from diffusion from the media and into the atmosphere, which may explain why the $\Delta[CO_2]$ may be greater in the treatment groups than the control.

Although we were gentle during our mixing as to not introduce air into our replicates before sampling, the oil barrier preventing gas diffusion between the atmosphere and *C. reinhardtii* cultures was still broken, which may have caused gas exchange to occur and introducing a potential source of variation by affecting the amount of CO_2 and O_2 available for cell metabolism and growth. Another possible source of variation comes from the observation that while the control group showed little signs of cell clumping, the treatment groups all had significant visible amounts of cell clumping. We attempted to break up the clumps with mixing, but not all clumps may have been broken down sufficiently. Thus, our sampling and cell counts may not have been truly representative of the population.

The endpoints for the titrations we performed for the CO_2 concentration measurements were difficult to assess as the solutions titrated were green in colour, introducing another potential source of variation and affecting our CO_2 concentration measurements. One technique that may mitigate this issue would be to centrifuge our samples and measure the CO_2 concentrations using the supernatant.

Conclusion

Our study aimed to observe the effects of mineral oil on the growth rate of *C. reinhardtii* and the $\Delta[CO_2]$ present in the media in which the cultures grew in. A one-way ANOVA test indicated that no significant differences existed in the growth rate of *C. reinhardtii* as more mineral was added and no significant differences were present in the $\Delta[CO_2]$ as more mineral oil was added. However, the *C. reinhardtii* growth rate may have shown a declining trend and the $\Delta[CO_2]$ may have shown an increasing trend throughout the duration of the experiment as more mineral oil was added.

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Appendix

		Cell count / Number of 1x1 mm grids counted						
Date	**Sample**	**Cell count 1**	**Cell count 2**	**Cell count 3**	**Avg cell count**	**Cell density (cells/mL)**	**Instantaneous growth rate (cells/mL/day)**	**Average instantaneous growth rate**
2019-10-28	Diluted culture	12	10	5	9	99000		
2019-10-30	0 uL #1	5.0	1.0	10.0	5.3	58666.7	-6416.7	-12069.4
	0 uL #2	11.0	7.0	6.0	8.0	88000.0	*231000*	
	0 uL #3	6.0	7.0	3.0	5.3	58666.7	-17722.2	
	250 uL #1	15.0	14.0	7.0	12.0	132000.0	12795.1	21771.4
	250 uL #2	15.0	10.0	7.0	10.7	117333.3	*65236.1*	
	250 uL #3	5.0	7.0	6.0	6.0	66000.0	30747.6	
	500 uL #1	11.0	10.0	7.0	9.3	102666.7	*168666.7*	**64716.7**
	500 uL #2	11.0	11.0	8.0	10.0	110000.0	84638.9	

	500 uL #3	*24.0*	43.0	49.0	46.0	*506000.0*	44794.4	
	1000 uL #1	*8.0*	28.0	14.0	21.0	*231000.0*	76694.4	**62659.3**
	1000 uL #2	2.0	5.0	*45.0*	3.5	38500.0	53579.2	
	1000 uL #3	10.0	6.0	6.0	7.3	80666.7	57704.2	
2019-11-01	0 uL #1	7.7	7.8	4.6	6.7	73333.3	96677.8	**106088.9**
	0 uL #2	131.0	80.0	68.0	93.0	*1023000.0*	115500.0	
	0 uL #3	2.1	2.6	3.0	2.6	28111.1	*30381.0*	
	250 uL #1	15.8	11.6	*76.0*	13.7	150180.6	46811.1	55886.1
	250 uL #2	41.7	33.3	23.2	32.7	359944.4	*127233.3*	
	250 uL #3	15.1	22.0	23.4	20.2	221990.5	64961.1	
	500 uL #1	60.5	69.5	81.0	70.3	*773666.7*	106577.8	45344.4
	500 uL #2	40.3	37.3	41.7	39.8	437555.6	58361.1	
	500 uL #3	36.0	16.7	23.2	25.3	278177.8	-28905.6	
	1000 uL #1	33.7	37.0	40.0	36.9	405777.8	38622.2	42594.4

	1000 uL #2	26.0	22.2	37.3	28.5	313316.7	*151677.8*	
	1000 uL #3	22.2	36.3	31.5	30.0	329816.7	46566.7	
2019-11-04	0 uL #1	56.5	52.0	39.3	49.3	542055.6	*217066.7*	27133.3
	0 uL #2	53.5	53.0	75.0	60.5	665500.0	-28966.7	
	0 uL #3	15.3	17.1	25.0	19.1	210571.4	83233.3	
	250 uL #1	23.5	38.3	38.0	33.3	366055.6	*119563.9*	**71056.5**
	250 uL #2	95.5	50.0	60.0	68.5	753500.0	66244.4	
	250 uL #3	26.3	36.3	44.0	35.5	390805.6	75868.6	
	500 uL #1	65.0	62.0	46.3	57.8	635555.6	*-8800.0*	32987.8
	500 uL #2	28.3	35.0	46.3	36.5	401805.6	32388.9	
	500 uL #3	41.3	32.0	25.3	32.9	361472.2	33586.7	
	1000 uL #1	43.7	33.0	39.0	38.6	424111.1	-17722.2	19865.2
	1000 uL #2	112.0	39.3	66.0	72.4	796888.9	281.1	
	1000 uL #3	19.0	36.3	30.3	28.5	313500.0	77036.7	

2019-11-06	0 uL #1	116.0	107.0	93.0	105.3	1158666.7	59736.1	50604.0
	0 uL #2	88.0	73.5	78.0	79.8	878166.7	54083.3	
	0 uL #3	36.0	34.7	50.5	40.4	444277.8	37992.6	
	250 uL #1	62.5	73.5	*28.3*	68.0	748000.0	61798.6	49881.9
	250 uL #2	65.0	*25.3*	60.7	62.8	691166.7	*0.0*	
	250 uL #3	53.0	53.0	58.0	54.7	601333.3	37965.3	
	500 uL #1	68.0	64.5	66.5	66.3	729666.7	*109236.1*	49576.4
	500 uL #2	53.3	71.5	38.7	54.5	599500.0	53090.3	
	500 uL #3	34.0	53.0	34.7	40.6	446111.1	46062.5	
	1000 uL #1	27.0	31.3	28.3	28.8	317166.7	81888.9	57673.6
	1000 uL #2	19.8	18.7	47.3	28.6	314722.2	*-92277.8*	
	1000 uL #3	58.5	71.5	*37.3*	65.0	*715000.0*	33458.3	
2019-11-08	0 uL #1	67.0	75.0	*28.3*	71.0	781000.0		
	0 uL #2	68.0	55.5	117.0	80.2	881833.3		

	0 uL #3	30.3	35.7	17.5	33.0	*362541.7*		
	250 uL #1	58.5	53.0	*29.0*	55.8	613250.0		
	250 uL #2	*142.0*	73.0	64.0	68.5	753500.0		
	250 uL #3	57.5	52.5	38.0	49.3	542666.7		
	500 uL #1	112.0	111.0	69.5	97.5	*1072500.0*		
	500 uL #2	54.0	55.5	58.0	55.8	614166.7		
	500 uL #3	56.5	59.0	33.3	49.6	545722.2		
	1000 uL #1	69.0	74.0	62.0	68.3	*751666.7*		
	1000 uL #2	35.0	46.7	35.0	38.9	427777.8		
	1000 uL #3	37.3	46.3	38.3	40.7	447333.3		

Raw data table 1. Cell count and cell density data. Potential outliers are highlighted in yellow. Maximum instantaneous growth rates are bolded.

		CO2 ppm 1	CO2 ppm 2	CO2 ppm 3	Average CO_2 ppm	**CO_2 difference**	**Average difference**
Initial CO2		53.5	48.5	49.0	50.3		
Final	0 uL #1	53.0	56.0	49.0	52.7	52.7	57.4

CO2							
	0 uL #2	59.0	58.0	56.0	57.7	57.7	
	0 uL #3	64.0	50.0	71.9	62.0	62.0	
	250 uL #1	68.0	66.0	65.6	66.5	66.5	67.5
	250 uL #2	63.0	64.0	64.9	64.0	64.0	
	250 uL #3	72.0	72.0	72.0	72.0	72.0	
	500 uL #1	80.0	71.8	83.8	78.5	78.5	71.6
	500 uL #2	70.0	56.0	77.1	67.7	67.7	
	500 uL #3	67.0	68.0	71.0	68.7	68.7	
	1000 uL #1	100.0	92.0	153.3	115.1	115.1	87.2
	1000 uL #2	138.2	42.0	81.6	87.3	87.3	
	1000 uL #3	55.0	62.0	60.2	59.1	59.1	

Raw data table 2. CO_2 data

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
maxgrowth$Treatment	3	2.631e+09	877102626	3.319	0.115
Residuals	5	1.321e+09	264245894		

Analysis summary 1. Maximum growth rate one-way ANOVA ($p = 0.115$)

	diff	lwr	upr	p adj
1000 uL-0 uL	-43429.630	-98185.37	11326.11	0.1098493
250 uL-0 uL	-35032.381	-95014.28	24949.52	0.2541466
500 uL-0 uL	-41372.239	-101354.14	18609.66	0.1660076
250 uL-1000 uL	8397.249	-46358.49	63152.98	0.9381207
500 uL-1000 uL	2057.391	-52698.34	56813.13	0.9989195
500 uL-250 uL	-6339.858	-66321.76	53642.04	0.9777459

Analysis summary 2. Maximum growth rate Tukey HSD

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
co2data$Treatment	3	1374	457.9	2.126	0.175
Residuals	8	1723	215.4		

Analysis summary 3. CO_2 One-way ANOVA (p = 0.175)

	diff	lwr	upr	p adj
1000 uL-0 uL	29.733333	-8.638957	68.10562	0.1379404
250 uL-0 uL	10.100000	-28.272290	48.47229	0.8329881
500 uL-0 uL	14.233333	-24.138957	52.60562	0.6504523
250 uL-1000 uL	-19.633333	-58.005624	18.73896	0.4114784
500 uL-1000 uL	-15.500000	-53.872290	22.87229	0.5913192
500 uL-250 uL	4.133333	-34.238957	42.50562	0.9848685

Analysis summary 4. CO_2 Tukey HSD