Growth Rate of *Euglena Gracilis* in Response to Different Incubation Temperatures

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Abstract

Global warming is the world's largest unsolved problem with rising temperatures affecting many organisms. As *Euglena gracilis* is an essential species in the Fraser River of Vancouver, British Columbia, this study aimed to determine if increasing temperatures like those predicted to occur due to climate change, has an effect on the growth rate of *E. gracilis*. Its growth rate was compared in three treatments (representing current temperature conditions (20 ºC), predicted temperature conditions (25 ºC), and a control (30 ºC) based on optimal growth temperatures) with three replicates per treatment. Over the course of 20 days, eight 20 µl samples were obtained from each replicate and cells were counted on a haemocytometer grid. Adjusted results showed that temperature does not affect the growth rate of *E. gracilis*. It also contradicted our prediction that the closer the temperature was to 30 ºC, the higher the growth rate would be. Instead, the highest growth rate was found in the 25 ºC treatment, followed by the 20 ºC treatment, and lastly the 30 ºC control. This discrepancy may be due to study errors as cell concentration was not calculated correctly. Overall, temperature was not found to have a significant effect on the growth rate of *E. gracilis*, which is counter to the broader literature.

Introduction

Euglena gracilis is a common single-celled algae found in both freshwater and saltwater environments (Richter *et al.*, 2003). It plays a critical role as a primary producer in the food webs of many ecosystems (Häder *et al.*, 1998) and these food webs often contain keystone species. An example of an affected keystone species is the pink salmon (*Oncorhynchus gorbuscha*), which relies on *E. gracilis* to increase the populations of the zooplankton that they consume (Shiomoto *et al.*, 1997). As is evident, the growth and population levels of *E. gracilis* has large effects on the overall functioning of many species in their habitats.

In order to maintain adequate population levels of *E. gracilis* for a healthy ecosystem, many factors affecting the growth and survival of *E. gracilis* must be considered. One of these important factors is temperature. This is because temperature strongly influences key cellular processes, like protein synthesis and photosynthesis (Künne & De Groot, 1996) (Zhong *et al.*, 1993). Additionally, the growth rates of *E. gracilis* are also known to be affected by temperature (Buetow, 1962).

An example of a habitat greatly affected by *E. gracilis* is the Fraser River of Vancouver, British Columbia, which has economic, cultural, and ecological significance to the region (Ferrari *et al.*, 2007). Notably, this habitat is also predicted to be significantly impacted by changes in temperature, due to climate change in the coming decades (Ferrari *et al.*, 2007). Thus, due to the temperature sensitivity of *E. gracilis* and its important role in the Fraser River ecosystem, this study aimed to determine how

predicted rises of temperature due to climate change in Vancouver, British Columbia will affect the growth rate of *E. gracilis* populations. We hypothesized that temperature would significantly affect the growth of *E. gracilis* populations in the study.

Previous studies of *E. gracilis* found optimal temperature ranges of highest growth rate for the species; Buetow (1962), found that highest growth occurred between 25 °C - 30 °C, while research conducted by Kitaya *et al.* (2005), identified a similar optimal range of 27 °C - 31 °C. These findings influenced our choice of 30 °C as our control treatment. The first experimental treatment was set at a temperature of 25 °C, as this is similar to the average summer temperatures predicted to occur by climate models in the pacific northwest region in the later decades of the current century (Mote & Salathé, 2010) . The second experimental treatment was set as 20°C as this has been approximately the current average summer temperature between 1981–2010 in the lower mainland of British Columbia (Environment Canada, 2022). Due to the previous results of Buetow (1962) indicating highest growth rate between 25 °C and 30 °C and decreased growth rates outside of this range, we predict that the growth rate of the *E. gracilis* would increase with temperature, resulting in the control (30 °C) having the highest growth rate, followed by Treatment 1 (25 °C), and then finally Treatment 2 (20 °C).

Methods

An approximately three week-long study was conducted from 26 October 2022 – 14 November 2022, involving the growth of nine *E. gracilis* populations in groups of three replicates at all three treatment levels (30 ℃, 25 ℃, 20 ℃).

To prepare these replicates, *E. gracilis* initial stock solution was analyzed using a haemocytometer to determine its starting concentration and then diluted to the start of the growth curve concentration of *E. gracilis* ($1x10^5$ cells/ml). To start, 100 µl from the *E. gracilis* initial stock solution was added to a 500 µl sampling tube, then 20 µl of IKI fixative was added also using a micropipette into the same tube. This initial sample was then resuspended and 20 µl of this sample was added to a haemocytometer slide, which was placed under a microscope. 150 cells were counted and analyzed to determine the concentration of cells in cells/ml depending on the grid squares that the 150 cells were found within. These steps can be seen in Figure 1.

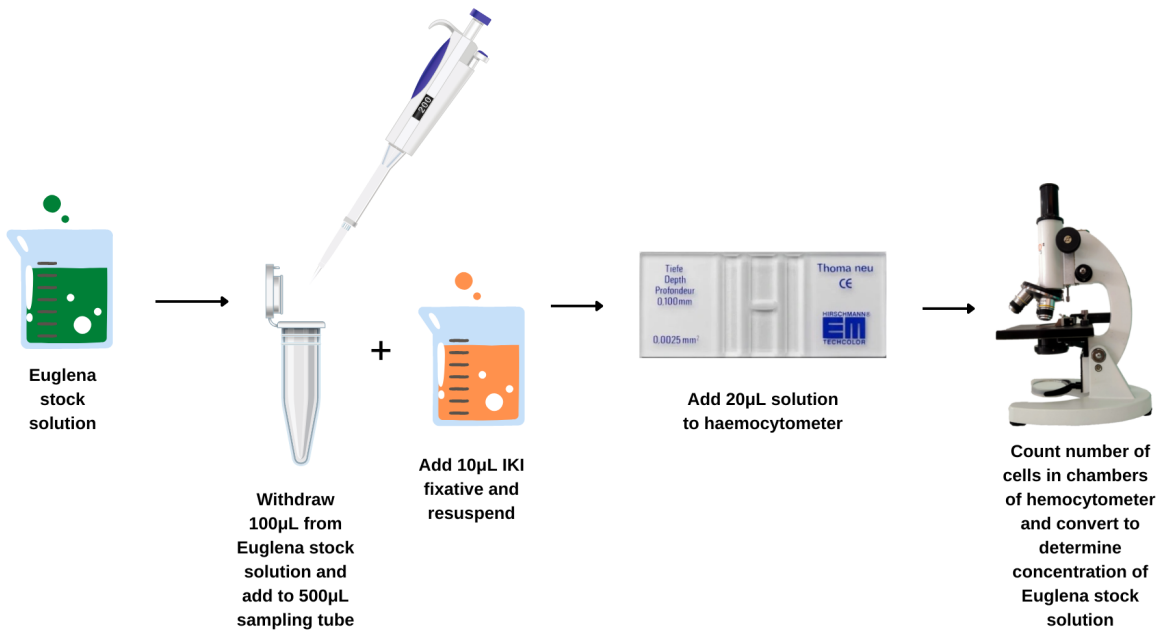

Figure 1: Schematic diagram depicting the process to determine the initial concentration of *E. gracilis* in stock solution, to use in calculating the dilution values for preparing the ideal working solution of concentration of $1x10^5$ cells/ml.

The concentration of the *E. gracilis* initial stock was used in the formula $C_1V_1 = C_2V_2$ to calculate the amount of *E. gracilis* initial stock solution and *E. gracilis* growth medium that would be required to create 100 ml of working solution at an ideal concentration of $1x10^5$ cells/ml. These calculated values were 0.6 ml of *E. gracilis* stock solution and 99.4 ml of growth medium. The 100 ml of growth medium was added to a 250 ml Erlenmeyer flask using a pipette, then 0.6 ml was removed using a micropipette for a final amount of 99.4 ml as calculated previously. Next, 0.6 ml of *E. gracilis* initial stock was added to the Erlenmeyer flask using a micropipette to make the diluted working solution. After the preparation of the working solution was complete, nine replicates were prepared by pipetting 10 ml of the working solution into nine test tubes (three per treatment level). These test tubes were labeled with treatment level (C/1/2), replicate number (1/2/3) and growth temperature (30 ℃/25 ℃/20 ℃). Once labeled, the replicates of each treatment were placed into one of three labeled test tube racks and the racks were placed into an incubator set to the temperature of their assigned experimental treatment. These replicates were then incubated over the following three weeks at these temperatures to allow for growth of the populations to occur. These steps are broadly shown in Figure 2.

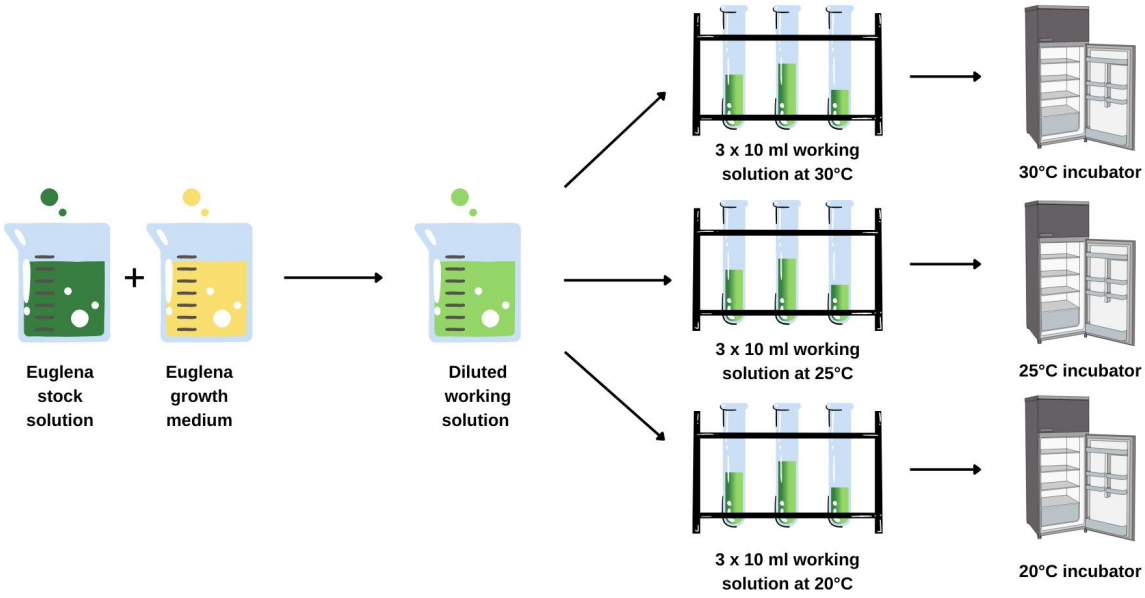

Figure 2: Schematic diagram depicting process of diluting *E. gracilis* stock solution to working solution, separation of replicates for the three experimental treatments (control/30 ℃, Treatment 1/25 ℃, Treatment 2/20 ℃) and storage in incubators.

Between the dates of 26 October 2022 – 14 November 2022, samples were collected from each of the replicates on Mondays, Wednesdays, and Fridays (except Friday November 11). First, one treatment group (a group of three replicates) was removed from its incubator and three 500 µl sampling tubes were labeled with the sample ID (treatment group - growth temperature - replicate number) as well as the date and time of sample withdrawal. After successful labeling of the sampling tubes, 100 µl of the *E. gracilis* solution from a single replicate was added using a micropipette to the corresponding 500µl sampling tube, then 10 µl of IKI fixative was added and this solution was mixed well. The process was repeated for all three replicates in the treatment. Once all three replicates of a treatment were sampled, the replicates would be returned to their corresponding incubator and this entire process would be repeated for the remaining two treatments. All collected samples were then stored in a tube rack

in a refrigerator at approximately 4℃ for the entire duration of the study period. This process is shown in Figure 3.

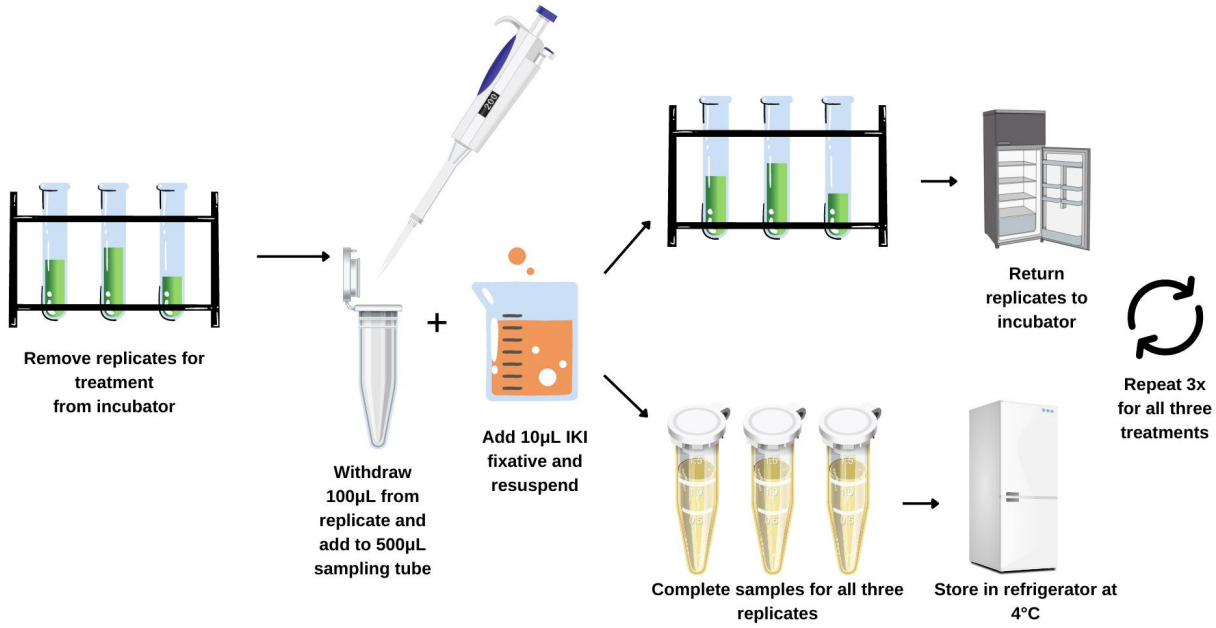

Figure 3: Schematic diagram of *E. gracilis* sample preparation process, which was conducted on Mondays, Wednesdays, and Fridays for approximately 3 weeks. The sample preparation was conducted for all three replicates of all three experimental treatments at each sampling session.

After collection, all samples were counted using a similar procedure to the original stock solution. Each sample was resuspended using a micropipette and 20 µl was added to a haemocytometer slide. The slide was then observed through a microscope and the number of cells in the chambers of the haemocytometer were counted and the count was converted using the appropriate conversion factors to values of cell density (cells/ml). Counts were conducted multiple times (approx. 2-4) for each sample and averaged to help ensure an accurate value for the sample. This process was completed for all samples from all 9 replicates across the entire sampling period. This process is shown in Figure 4.

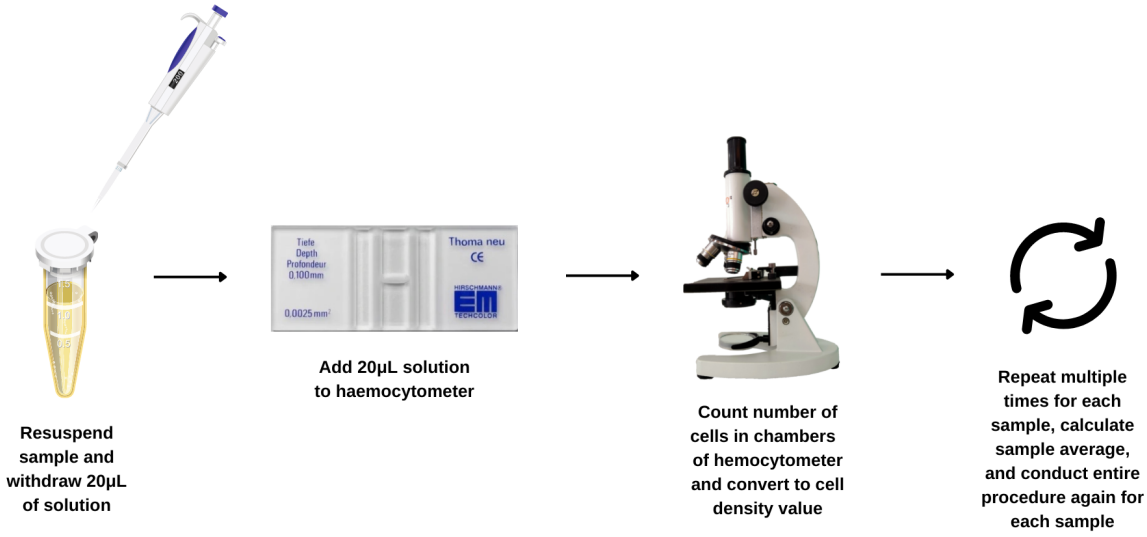

Figure 4: Schematic diagram of process of *E. gracilis* sample counting.

Once all counts were completed and averaged, these values were converted into growth curves for each replicate and compared using a one-way ANOVA test and Tukey HSD test to determine statistical significance of the differences in growth rate. These tests were repeated on adjusted values, where periods of exponential growth were manually determined and only these values were used.

Results

Over the study period, a color change occurred in the replicates of *E. gracilis*. A colour change from clear and colourless to clear and green was first noted on day five of the study period with the control having the darkest green color, followed by Treatment 1 with a less green color, while Treatment 2 remained colourless. On day seven, clumping of *E. gracilis* cells near the bottom of the test tubes was observed in the control and Treatment 1 with a higher amount occurring in the control. This did not

occur in Treatment 2 replicates; however, at this date the Treatment 2 replicates began to exhibit a color change to green. On day nine, the control and Treatment 1 appeared to be slightly opaque. These patterns continued throughout the study period: the green color grew darker and cell clumping increased most notably in the control, followed by Treatment 1, and lowest in Treatment 2. By day 14, Treatment 1 and the control were fairly similar in color and amount of clumping.

It was observed under microscope that the *E. gracilis* cells in the control were almost exclusively small and circular during the latter half of the sampling period, contrasting with the longer and oval-like cells of Treatment 1 and Treatment 2.

For the unadjusted values, the slopes for the control replicates' growth rates were 21732 [cells/ml]/days and, 11549 [cells/ml]/days and 12856 [cells/ml]/days for replicates 1, 2, and 3, respectively (Figure 5). For Treatment 1, they were 33191 [cells/ml]/days, 32677 [cells/ml]/days, and 34899 [cells/ml]/days (Figure 6). For Treatment 2, they were 38026 [cells/ml]/days, 35797 [cells/ml]/days, and 36121 [cells/ml]/days (Figure 7).

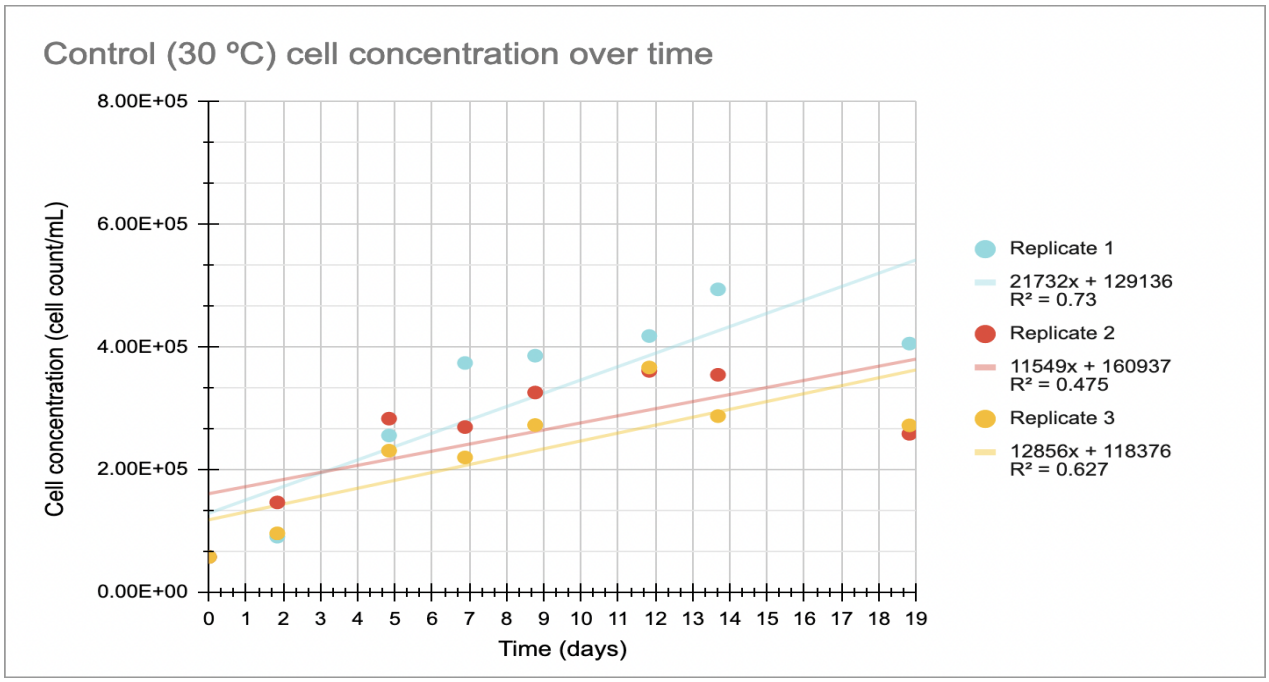

Figure 5: Growth curves for replicates 1, 2, and 3 for the control treatment with time in days on the x-axis and cell concentration in cells per ml on the y-axis. The legend shows the slope equation and R^2 value of each replicate.

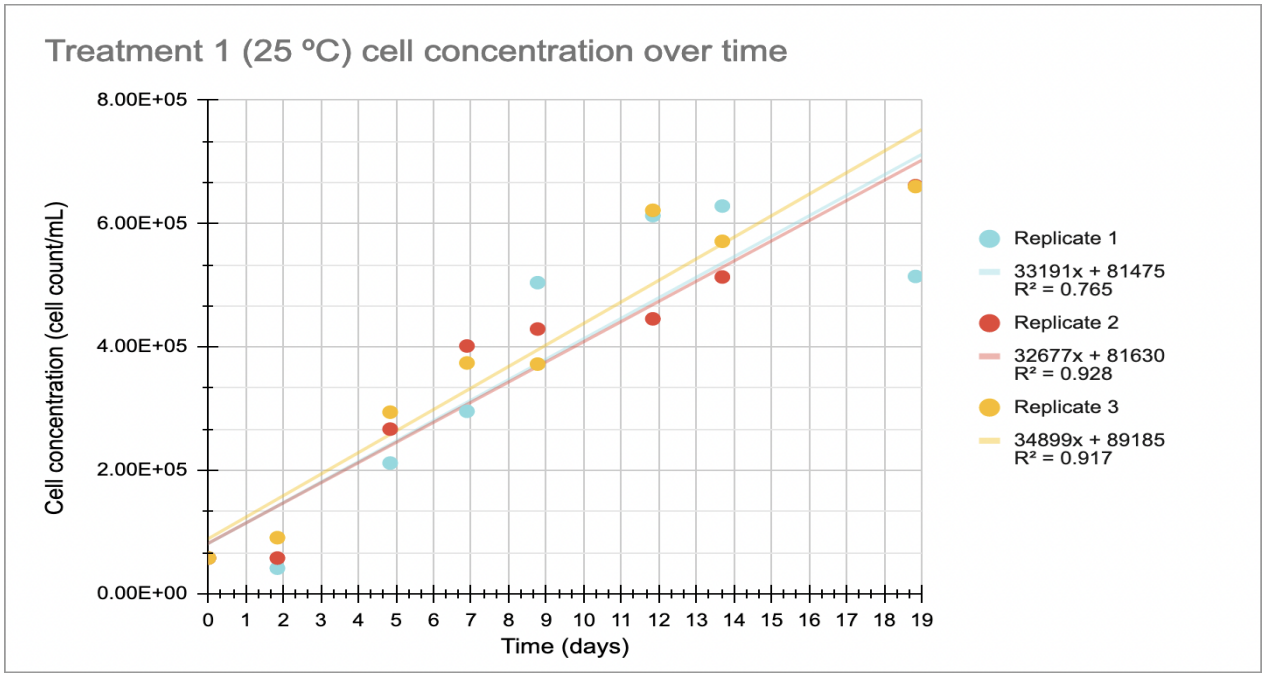

Figure 6: Growth curves for replicates 1, 2, and 3 for Treatment 1 with time in days on the x-axis and cell concentration in cells per ml on the y-axis. The legend shows the slope equation and R^2 value of each replicate.

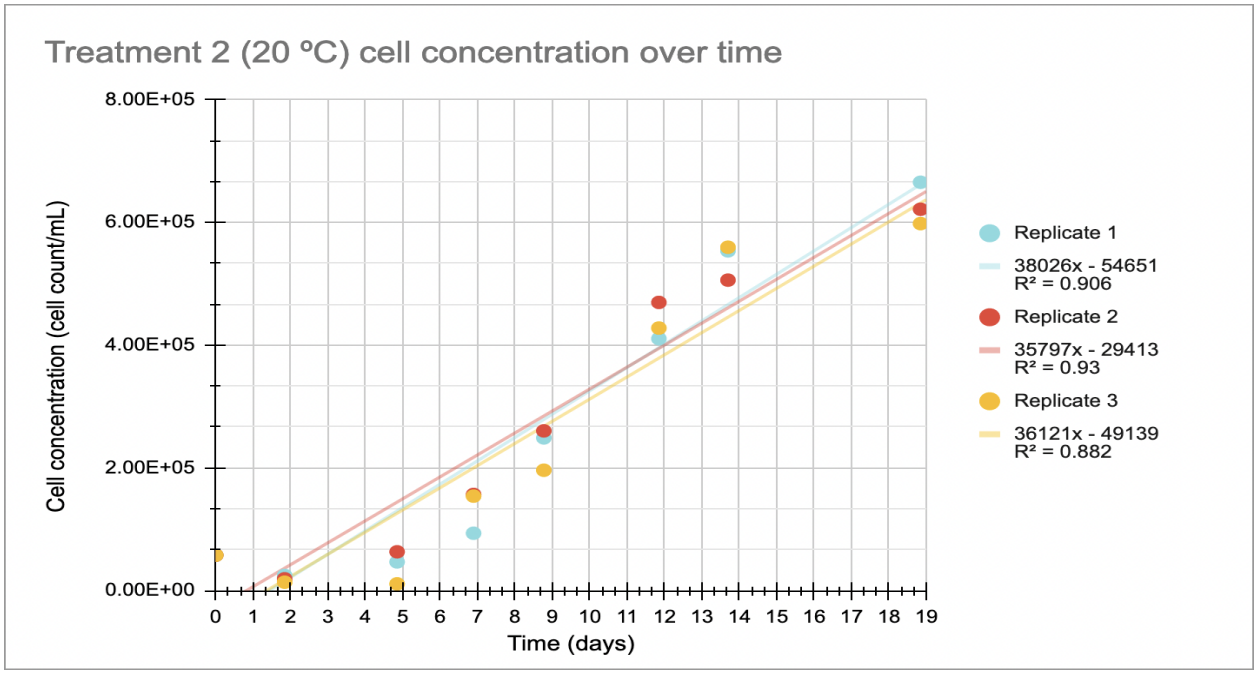

Figure 7: Growth curves for replicates 1, 2, and 3 for Treatment 2 with time in days on the x-axis and cell concentration in cells per ml on the y-axis. The legend shows the slope equation and R² value of each replicate.

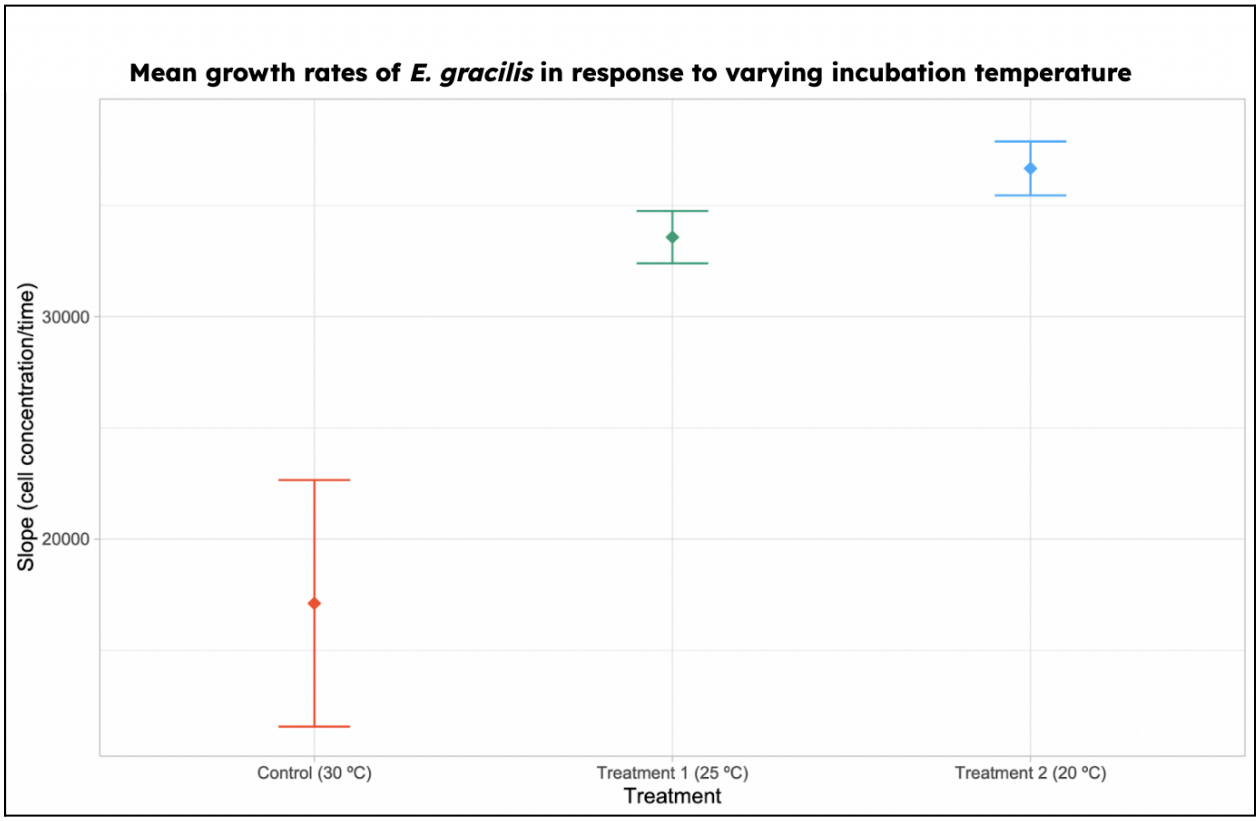

Figure 8: The mean slope of each treatment group calculated from each replicate (slopes as seen in Figures 5, 6, and 7). Error bars show one standard deviation from the mean.

The mean growth rates for the unadjusted values were 17110 [cells/ml]/days for control, 33574 [cells/ml]/days for Treatment 1, and 36663 [cells/ml]/days for Treatment 2 (Figure 8). The p-value from the ANOVA one way test for these unadjusted mean growth rates was found to be < 0.001. From the Tukey HSD test, the p(F) values were found to be 0.002 between 30 ºC and 25 ºC, and < 0.001 between 30 ºC and 20 ºC, but insignificant between 25 ºC and 20 ºC.

For the adjusted values (only considering the values from portions with close to exponential growth), the slopes for the control replicates' growth rates were 47528 [cells/ml]/days and, 46479 [cells/ml]/days and 36681 [cells/ml]/days for replicates 1, 2, and 3, respectively (Figure 9). For Treatment 1, they were 63554 [cells/ml]/days, 68199 [cells/ml]/days, and 48670 [cells/ml]/days (Figure 10). For Treatment 2, they were 58753 [cells/ml]/days, 53300 [cells/ml]/days, and 60903 [cells/ml]/days (Figure 11).

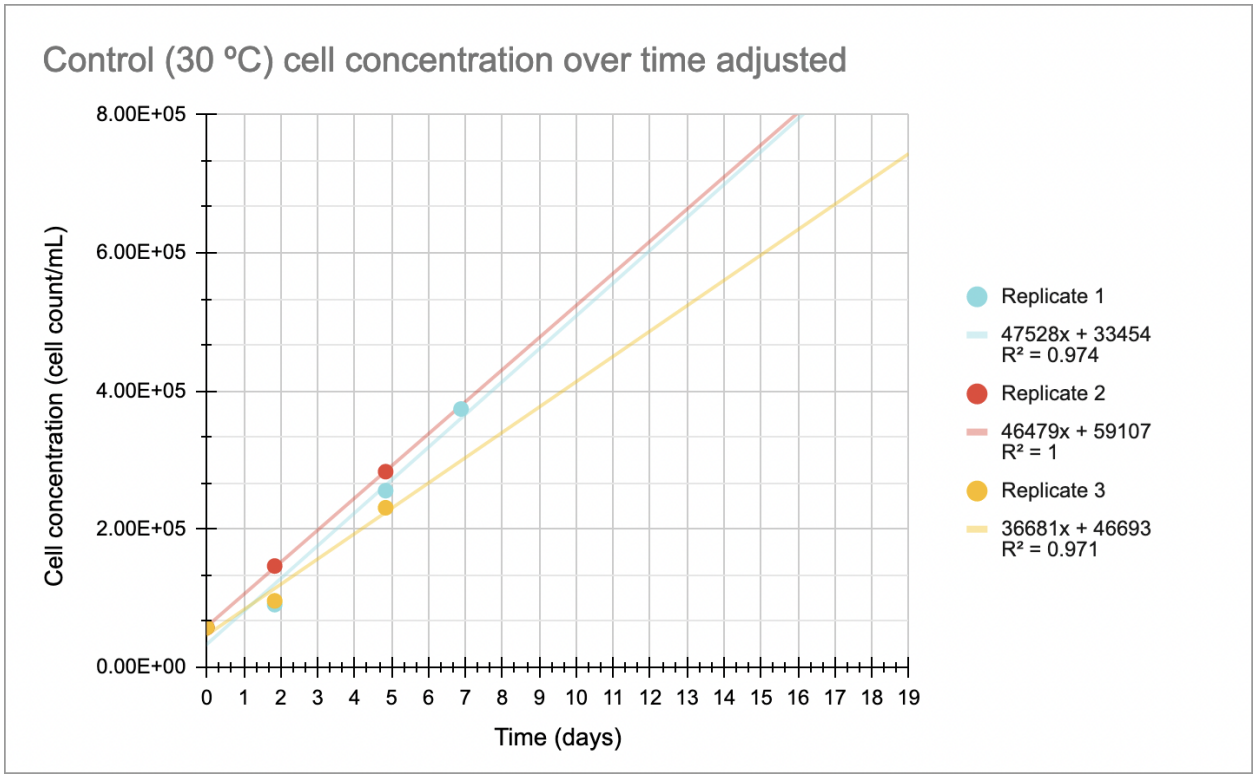

Figure 9: Growth curves of adjusted values for replicates 1, 2, and 3 for the control treatment with time in days on the x-axis and cell concentration in cells per ml on the y-axis. The legend shows the slope equation and R^2 value of each replicate.

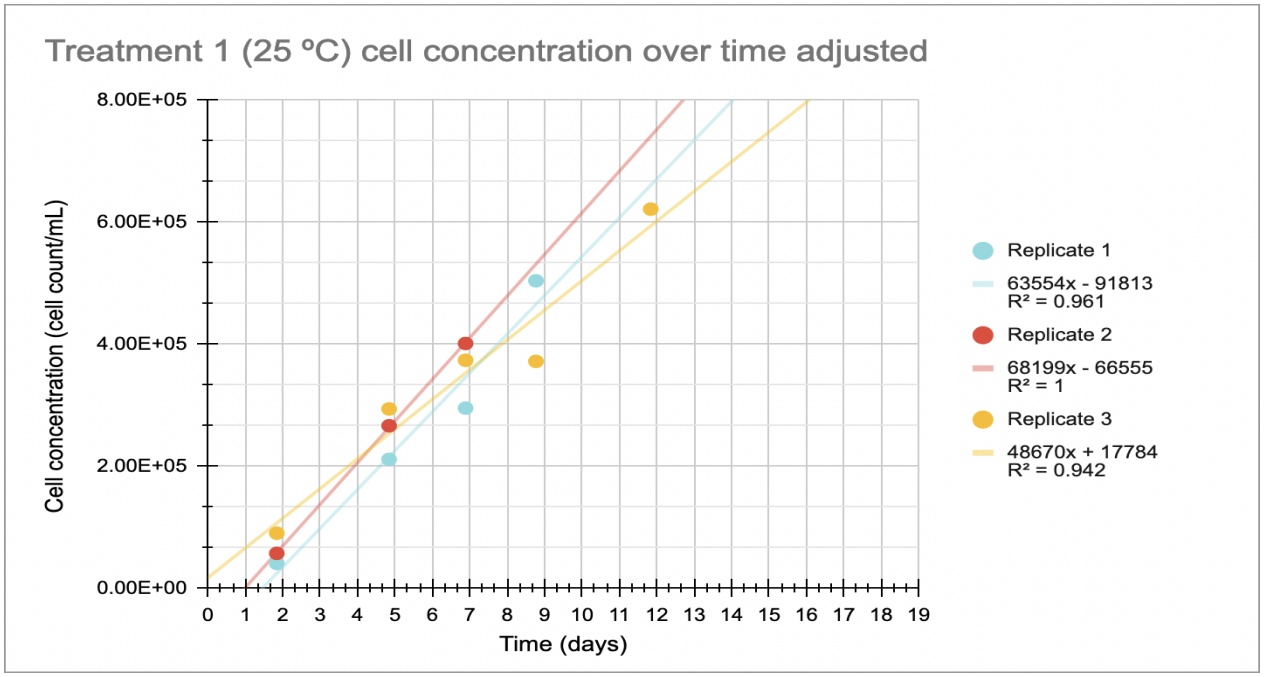

Figure 10: Growth curves of adjusted values for replicates 1, 2, and 3 for Treatment 1 with time in days on the x-axis and cell concentration in cells per ml on the y-axis. The legend shows the slope equation and R^2 value of each replicate.

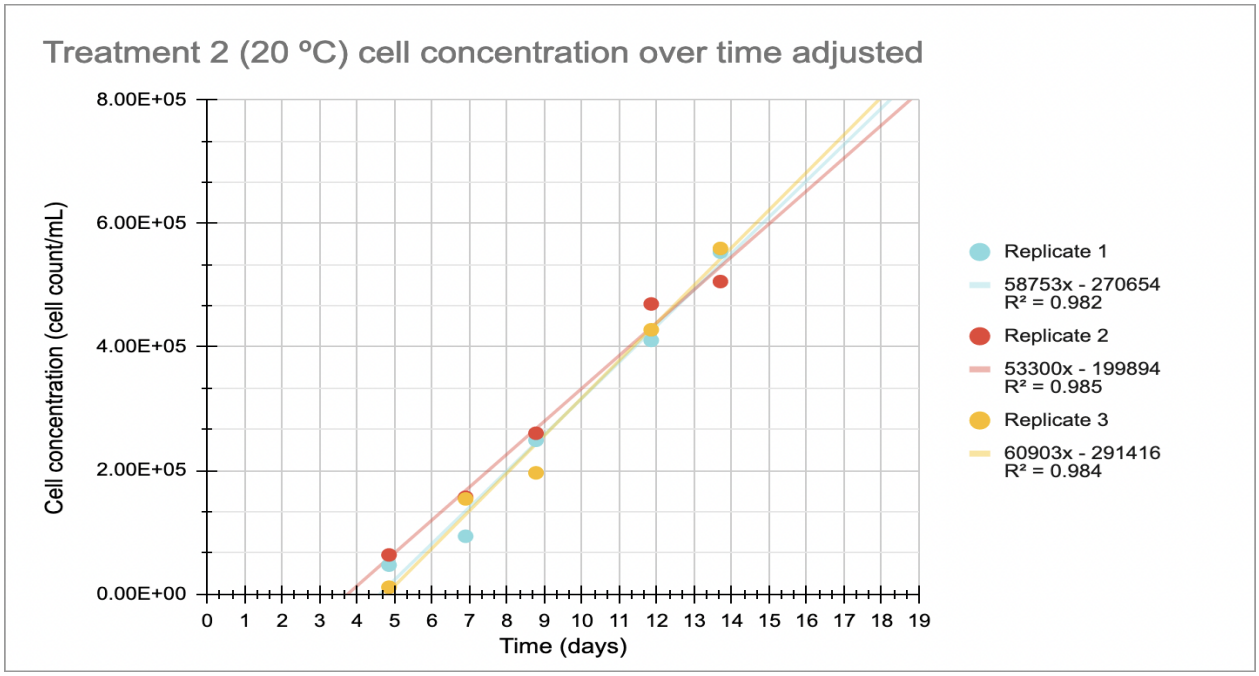

Figure 11: Growth curves of adjusted values for replicates 1, 2, and 3 for Treatment 2 with time in days on the x-axis and cell concentration in cells per ml on the y-axis. The legend shows the slope equation and R^2 value of each replicate.

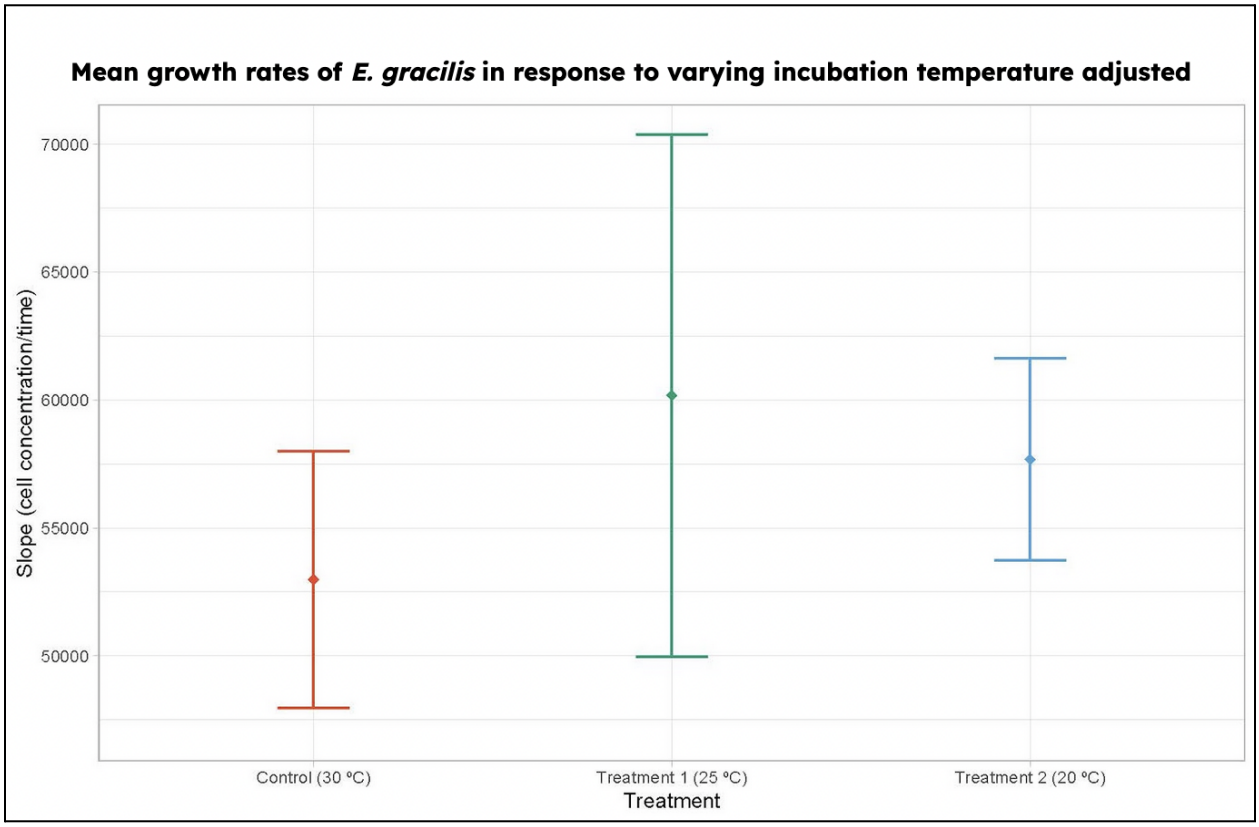

Figure 12: The mean slope of each treatment group calculated from each replicate (slopes as seen in Figures 9, 10, and 11). Error bars show one standard deviation from the mean.

The mean growth rates for the adjusted values were 52981 [cells/ml]/days for control, 60179 [cells/ml]/days for Treatment 1, and 57680 [cells/ml]/days for Treatment 2 (Figure 8). The p-value from the ANOVA for these mean growth rates was found to be 0.481. The Tukey HSD test showed insignificance between all treatment levels.

Discussion

We hypothesized that change in temperature would significantly affect the growth rate of *E. gracilis*. Based on previous studies, we predicted that increased temperature would correspond with an increase in growth rate for *E. gracilis*; thus, the greatest growth rate would be observed in the control (30℃), followed by Treatment 1 (25℃), and lastly Treatment 2 (20℃). However, our results show an unexpected effect of temperature, with the highest growth rates found in Treatment 1 which had the lowest temperature, followed by Treatment 2, and then the lowest growth rates at the control level. Thus, our prediction was incorrect, however the p-value of the differences in mean growth rate was above the alpha of 0.05 (p=0.481) indicating no statistical significance of our values and of the variable of temperature affecting growth. This means that the null hypothesis cannot be rejected, as temperature was found to not affect the growth rate of *E. gracilis*.

These results do somewhat align with the results of previous studies, as Buetow (1962), found a maximized growth rate between 25℃-30℃, so the highest growth rate of our study being observed in Treatment 1 (25℃), which is found within this temperature range does seem consistent. However, this conclusion of this being the ideal temperature cannot be confirmed due to the lack of statistical significance.

Additionally, a similar study of *E. gracilis* that were grown at temperatures of 30℃, 20℃ and 17℃ found the highest growth rate at 30℃ and lowest at 17℃, which greatly contrasts with our results (Kim *et al.*, 2022). Thus, our results seem to not align with the overall consensus of knowledge in the literature regarding growth of *E. gracilis* under different temperature conditions. Due to this lack of similarity and signficance, our results, although observed to occur in this study, likely do not represent an applicable conclusion for all populations of *E. gracilis*.

There are many factors that likely contributed to this surprising result, which likely was influenced by forms of sampling and human error. These potential factors include inaccuracy in sampling counts, the short length of the study, and a lack of true exponential growth in the populations. The accuracy of the sampling was potentially limited by the many steps involved in the procedure, which may have introduced human or sampling error. An example of this potential error was that certain samples that were found to drastically differ in cell density values during the counting process. These differences were likely due to insufficient sample resuspension prior to haemocytometer preparation. However, in these instances of large discrepancies between cell counts, extra counts were conducted of the sample and averaged, as well as removing extraneous outliers, which would have hopefully decreased the influence of this error. However, the risk of these errors impacting the results cannot be fully eliminated. Additionally, it was realized after the counting process was complete, that for some samples the proper counting procedure was not followed, and not enough cells were counted to generate an accurate value. Thus, the cell density values gained from some

of the samples were likely not representative of the true state of the population. Also, when counting, the cells are distributed at random into the grids in haemocytometers which can cause certain grids to potentially receive more cells than the actual average of the sample and be counted, which may have also occurred during this study. However, once again the effect of this was aimed to be reduced by conducting multiple counts of the samples and averaging.

Regarding the limitation of time, a longer duration of study would help our conclusions regarding the growth of the populations likely be a bit stronger, as with greater time, especially for the lower temperature treatments a full lag-growth-plateau pattern of growth could be observed. This ideal growth pattern was not observed for Treatment 1 (25℃) and Treatment 2 (20℃) due to the end of the study occurring when it was still in the growth phase, so a longer study would likely be able to describe the growth rates more accurately.

Exponential growth was also never fully observed in our populations, which may indicate that the conditions of growth (dilution of cells and medium) were prepared improperly and were insufficient to allow for optimal growth to occur in the replicate populations. Thus, the growth values may be inaccurate, as another variable (working solution concentration) was also impacting the growth rates of the samples rather than just the manipulated variable.

Further research could aid in understanding the conclusions that were found in this study and confirming the results being influenced by error. An experiment of larger scale, where greater amounts of temperature treatments could be used to gain greater understanding of the impact of temperature on *E. gracilis* growth, greater number of replicates, and increased sampling to decrease the impact of sampling error on the results would be a great improvement. Another potential study that could be conducted, would be one that uses conditions or manipulates other variables (i.e. light, O_2 concentration) that make the conditions closer to the actual conditions of *E. gracilis* habitat, which could give more practical results.

Conclusion

Ultimately, due to the somewhat anomalous study results and large p-value found, temperature was not proven to be connected with the growth rate of *E. gracilis*. Thus, as temperatures increase due to climate change, based on this study we cannot explicitly predict the potential impact that it will have on *E. gracilis* populations, but greater research on this topic would aid in understanding this problem. Overall, over the coming decades, greater awareness and research of *E. gracilis,* which plays such a significant role in its ecosystem, should be conducted to ensure that this key microorganism can be maintained at proper population levels.

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