The effect of darkness on chloroplast length of *Euglena gracilis* over time

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ABSTRACT

Euglena gracilis is a mixotrophic algae that can gain energy through photosynthesis and phagocytosis. Previous research has extensively studied the effects of light on *E. gracilis* chloroplast length; however, much about the effects of darkness on chloroplast length is still unknown. Therefore, the objective of our study was to investigate whether exposure to darkness will affect chloroplast length of *E. gracilis* over time. We predicted that chloroplast length would decrease after being exposed to darkness with time and that chloroplast length would remain the same when exposed to light over time. To test this prediction, we exposed *E. gracilis* to light and dark conditions and measured the chloroplast length at three different times (0 hr, 48 hr and 72 hr). Our data was analyzed using a 2-way ANOVA test. We found that there was no statistical difference in chloroplast length when considering time independently ($p = 0.1266$). However, there was statistical difference in chloroplast length when exposed to darkness ($p = 0.0011$). We rejected the null hypothesis that the effect of time on the length of chloroplasts is the same in dark and light exposed *E. gracilis* ($p = 0.0387$). These results suggest that darkness limits the growth of chloroplast in *E. gracilis*.

INTRODUCTION

Euglena gracilis are single-celled mixotrophic algae that feed by photosynthesis, converting sunlight to a useable organic carbon source, or by phagocytosis, engulfing other small molecules (Dahoumane et al., 2016). This places *E. gracilis* at the base of the food web for aquatic environments (Lee, Yoon, Shin, & An, 2015) as a microflagellate food source for riverine insects that juvenile salmon prey on (Maier & Simenstad, 2009). *E. gracilis* are freshwater algae and can be commonly found in shallow, low-oxygen waters (Richter et al., 2003), similar to that of many salmon spawning streams (Fellman, Hood, Nagorski, Hudson, & Pyare, 2018). In vitro, *E. gracilis* are cultured in optimal conditions at 20°C, with a 14 hr light and 10 hr dark cycle within an incubator.

The metabolic flexibility of *E. gracilis* populations allows it to thrive in both light and dark conditions because they do not necessarily rely on chloroplasts (Ogbonna, Tomiyamal, & Tanaka, 1998; Richter et al., 2003). However, transitioning between these two conditions can affect the size of their chloroplasts (Scheer and Parthier, 1982). This is because under low light conditions, chloroplast development does not occur in *E. gracilis* (Spano, Ghaus, & Schiff, 1987) and chloroplast size will recover from a reduced state when removed from light-deprived conditions (Stern, Schiff , & Epstein, 1964). The light-induced recovery of bleached cells has been well-studied (Nigon & Heizmann, 1978; Parthier, 1981), however there has been less research on the degradation of chloroplasts in darkness. It has been shown that chloroplast reduction will occur in darkness with an alternative glucose food source (Scheer & Parthier, 1982); however, our objective is to observe the effects of darkness on the effect of chloroplast length in *E. gracilis* over time, in the absence of any additional nutrients.

We consider time and darkness independently and in combination in our tests. *E. gracilis* is unable to produce chlorophyll in the absence of light (Scheer & Parthier 1982). Thus, chloroplast length likely decreases with time in the dark compared to the *E. gracilis* exposed to light for the same amount of time. This is because in the absence of light, *E. gracilis* will favour reducing its fatty acids stores, typically used for chloroplast development, for biofuel instead (Wang, Seppanen-Laakso, Rischer, & Wiebe, 2018). Furthermore, in optimal conditions, chloroplast length of *E. gracilis* will complete development after 3 days (Ben-Shaul, Schiff, & Epstein, 1964). When provided our sample of *E. gracilis,* the population already achieved a plateau stage that occurs after about 1 week (Price & Vallee, 1962), so any further incubation will likely have no effect on chloroplast size. This study is important as the effect of darkness on

chloroplasts in *E. gracilis* has not been explored to the same extent as light exposed *E. gracilis* chloroplasts.

METHODS

Set Up

We were provided with 100 mL of *E. gracilis* cultured in the medium recipe from UTEX Culture Collection of Algae at the University of Texas at Austin in a 250 mL Erlenmeyer flask. To begin our set up, we used clear microcentrifuge (MCF) tubes for the control (light exposure) and dark brown MCF tubes for the treatment (dark exposure). As shown in Figure 1, we labelled three clear MCF tubes and three dark brown MCF tubes for each treatment (0 hr, 48 hr, 72 hr). We pipetted 100 µL of cultured *E. gracilis* into all 18 MCF tubes using a micropipette. The time and date at which each tube received *E. gracilis* were recorded. All of the control MCF tubes were placed in the same vial rack. For the 0 hr dark treatment, we placed three MCF tubes in a separate vial rack and covered it with a black garbage bag to ensure the tubes were not exposed to light. We placed this rack in a cardboard box labelled with the time and date and sealed it to further prevent light exposure. We repeated the procedure for the 0 hr dark treatment for the 48 hr and 72 hr treatments. In total, we had three boxes for each treatment: 0 hr, 48 hr and 72 hr. All three boxes and the control vial rack were placed in the incubator at 20°C with 14 hours of light exposure and 10 hours of dark exposure.

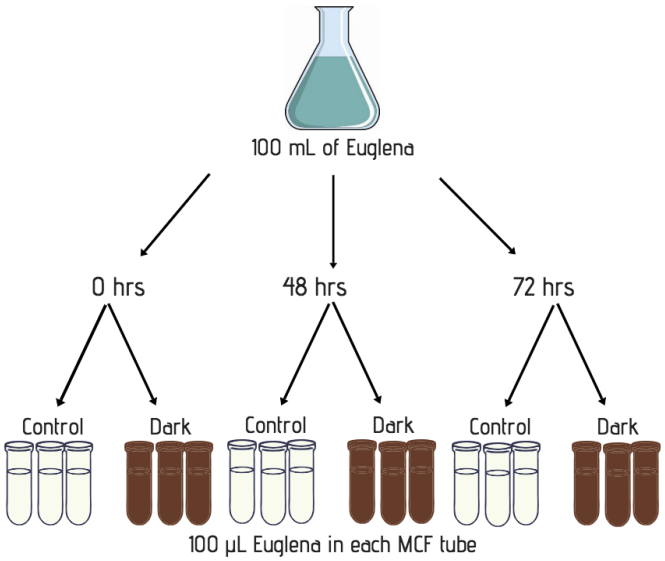

Figure 1. Preparation of MCF tubes for control and treatment.
A total of 18 MCF tubes were used.

At 0 hr

We obtained the 0 hr control and treatment MCF tubes from the incubator. Using a micropipette, we added 10 µL of 3% glutaraldehyde fixative to each of the six tubes. The addition of 3% glutaraldehyde fixed the *E. gracilis* cells at that time. We performed this step under the fume hood to limit the exposure to toxic fumes of the glutaraldehyde. We recorded the time and date at which the fixative was added to the tubes. We then vortexed the six MCF tubes to ensure the *E. gracilis* and glutaraldehyde were mixed thoroughly and placed them in the refrigerator at 4°C until needed for chloroplast length analysis under the microscope.

At 48hr

We repeated the steps performed at 0 hr for the 48 hr control and 48 hr treatment.

At 72h

We repeated the steps performed at 0 hr for the 72 hr control and 72 hr treatment.

Measuring *E. gracilis* Chloroplasts Length

We set up a Zeiss Axiostar compound microscope using Köhler illumination. In order to capture the images of the chloroplast, we used the Dino-Lite digital microscope software. We placed the Dino-Lite digital microscope in the eyepiece of the compound microscope and connected its USB to the laptop. We placed a micrometer slide on the stage in order to calibrate the Dino-Lite program's scale bar to measure the length of the chloroplast. To set the microscope magnification to 1000X, we placed one drop of Zeiss immersol 518N oil where the light shined on the micrometer slide and then moved the 100X lens into place. Using the Dino-Lite software, we created a new calibration profile based on the magnification (1000X) and the size of the micrometer (0.01 mm per division). This allowed us to measure the length of the chloroplast directly on the software. We returned the 10X lens back into position and wiped the 100X lens using a Kimwipe to ensure the immersol oil was removed.

We obtained all 18 MCF tubes from the refrigerator to measure the chloroplast sizes under the microscope. First, we prepared a wet mount slide by micropipetting 15 μL of fixed *E. gracilis* culture from the 0 hr control treatment. We placed the slide on the stage and viewed it at 1000X magnification. We measured the first ten chloroplasts from different cells that came into the field of view. Figure 2 shows an image of a chloroplast and its length measured by the Dino-Lite software. The lens was returned to the 10X position and the 100X lens was wiped using a Kimwipe to ensure the immersol oil was removed to view the next slide.

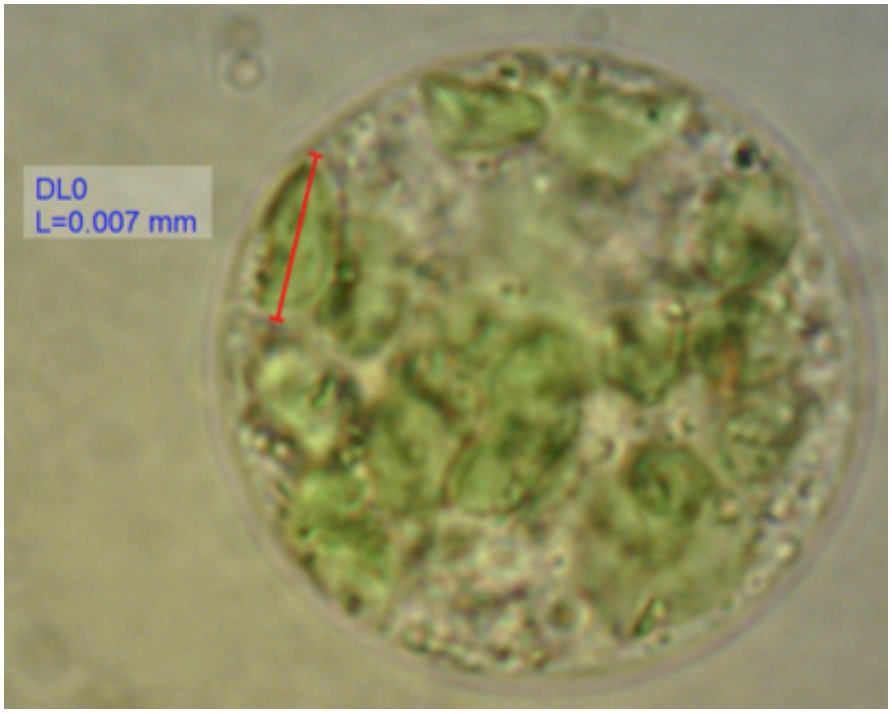

Figure 2. A chloroplast found in the control at 0 hr. The red circle is the chloroplast of interest and red line represents the length of the chloroplast. The length is labelled as L = 0.007 mm.

Statistical Analysis

To determine if there was a statistically significant difference between our variables, we conducted a two-way ANOVA statistical analysis with multiple comparisons and interpreted our results with Tukey's post hoc test. We used the GraphPad Prism 8 software to calculate and analyze statistical data.

RESULTS

The *E. gracilis* chloroplast length was analyzed between two treatment groups, control and dark, at 0 hr, 48 hr and 72 hr and a total of 177 chloroplasts were analyzed. Chloroplast length decreased with time in the dark treatment as shown in Figure 3. In the control, there was

no significant difference in chloroplast length between each time interval. For the dark treatment, there was a significant difference between the dark 0 hr and dark 72 hr ($p = 0.0353$). When comparing the control and dark treatments, there was a statistically significant difference between the control 0 hr and dark 72 hr ($p = 0.0278$), control 48 hr and dark 72 hr ($p = 0.0094$) and control 72 hr and dark 72 hr ($p = 0.0135$). Table 1 show the 95% confidence intervals for each treatment and time.

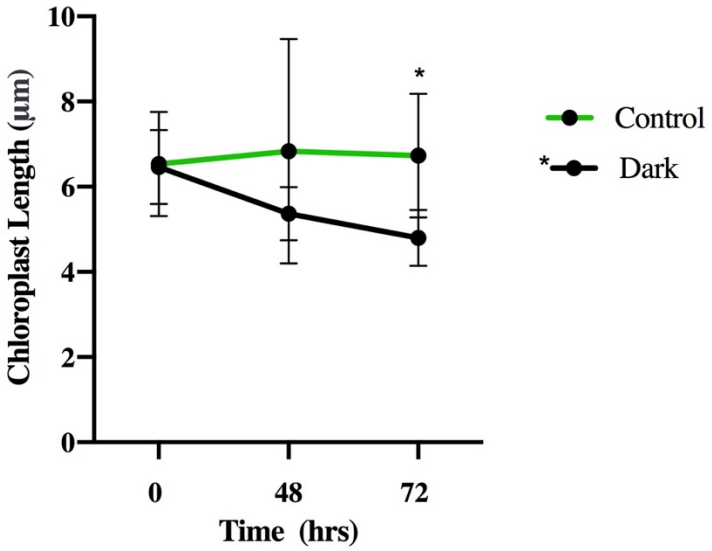

Figure 3. Comparison of the mean length of chloroplast of *E. gracilis* between the control (n=3) and dark (n=3) treatment, error bars indicate the calculated mean ± 95% confidence interval. The * represents significant difference.

Table 1. 95% Confidence Intervals for control and dark treatments

Treatment/Time	95% Confidence Interval
Control 0 hr	6.5333 ± 0.5579
Control 48 hr	6.8333 ± 1.1988
Control 72 hr	6.7333 ± 0.6627
Dark 0 hr	6.4667 ± 0.3972
Dark 48 hr	5.3667 ± 0.28464
Dark 72 hr	4.8000 ± 0.29925

For the null hypothesis that darkness has no effect on chloroplast length in *E. gracilis*, the two-way ANOVA test revealed that there was a significant difference in chloroplast length within the dark treatments ($df = 1$; $p = 0.0011$). For the null hypothesis that time has no effect on the chloroplast length, results showed that there was no significant difference in chloroplast length when considering time independently ($df = 2$, $p = 0.1266$). For the null hypothesis that the effect of time on chloroplast length is the same in the control and dark treatments, results show that there was a significant difference in chloroplast length ($df = 2$, $p = 0.0387$).

DISCUSSION

Data Analysis and Biological Reasoning

According to our results, we reject our null hypotheses that exposure to darkness has no effect on the length of chloroplasts in *E. gracilis* and that the effect of time on the length of chloroplasts is the same in dark and light exposed *E. gracilis*. We failed to reject our null hypothesis that time has no effect on the length of chloroplasts in *E. gracilis*. However, there was a trend in the chloroplast length: in the control, the chloroplast length remains relatively constant but in the dark treatment, the chloroplast length decreases over time (Fig. 3).

The two-way ANOVA test revealed that the dark treatment had an effect on length of the chloroplast as the result was statistically significant with a p-value less than 0.05. Chloroplasts are derived from proplastids and according to Stern et al., the size of dark-grown proplastids increase when exposed to light (1964). Our findings are consistent with the literature as dark exposed chloroplasts were smaller in length compared to light-exposed chloroplasts in the control.

The effect of time on the length of chloroplasts was statistically insignificant, as the p-value was greater than 0.05. Thus, we can infer that the time independently does not affect the chloroplast length. According to Price and Vallee, *E. gracilis* chloroplast size plateaued after one week of incubation (1962). We can assume that further incubation will likely not affect chloroplast size. This is similar to our results as there was no significant difference in chloroplast length with time in both the control and dark treatments.

The interaction of time and darkness exposure was statistically significant, as the p-value was less than 0.05. This shows that the effect of darkness on chloroplast length changes over

time. This is further supported in Figure 3 which shows that chloroplast length decreases over time when exposed to darkness. According to Scheer and Parthier, the exposure to darkness cause the pre-existing photosynthetic organelles, such as the chloroplast, to decompose and the production of new chloroplast components stops (1982). In addition, light affects the production of carotenoids, polysaccharides and bioactive molecules, the building blocks of chloroplasts, and without light, production of these will stop and the production of chloroplasts will arrest (Wang et al., 2018). Furthermore, it has been found that dark-grown cells have chloroplasts with poorly-developed thylakoid membranes due to having much fewer glycolipids, which are also used to build the thylakoid membranes (Shibata, Arimura, Ishikawa, & Awai, 2018).

The Tukey's multiple comparisons test showed significant results for the following: control treatment at 0 hrs vs. dark treatment at 72 hrs; control treatment at 48 hrs vs. dark treatment at 72 hrs; control treatment at 72 hrs vs. dark treatment at 72 hrs, and; dark treatment at 0 hrs vs. dark treatment at 72 hrs. It can be noted that only the comparisons to dark treatment at 72 hrs were significant.

Sources of Error and Variations

This study was subjected to multiple errors. In the raw data table, it shows that there are only seven different data points for control at 72 hours as we were unable to find ten different chloroplasts to measure. This may be due to inadequate mixing or a contamination in sampling. This affected our results by decreasing the sample size and thereby impacting the mean chloroplast size. Vortexing the MCF tubes before creating a wet slide and using sterile technique

throughout the experiment may help avoid this issue as more chloroplasts can be viewed under the microscope.

In addition, there was an inconsistency in chloroplast selection. This may have introduced sampling bias as we chose the first chloroplast that came into the field of view with proper orientation and clarity. This can be prevented by randomizing the chloroplast selection by dividing the wet slide into quadrants and scanning for chloroplasts.

Future Research

While the chloroplast length remained relatively constant for all the control groups, the length decreased when it was exposed to the darkness, over time. We were able to examine the length of the chloroplast up to 72 hrs. Previously, researchers have performed experiments on photosynthetic capabilities of *E. gracilis* by analyzing the effect of the variables for over 100 hrs (Scheer and Parthier, 1982; Stern et al., 1963). Therefore, our study should be conducted for a longer period of time to confidently draw conclusions on the effect of time and the darkness on the chloroplast length.

In addition, to confirm that photosynthetic function of *E. gracilis* has been completely degraded, the O_2 concentration of the medium can be measured. If the O_2 concentration decreases with the length of the chloroplast, it can be inferred that *E. gracilis*' survival is dependent on phagocytosis.

CONCLUSION

To conclude, our research has found that the darkness affects the length of the chloroplast and the effect of time on the length of chloroplasts is not the same in dark and light exposed *E.*

gracilis. This is consistent with what we predicted; however, we failed to reject our null hypothesis that the time has no effect on the length of chloroplast.

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References

Ben-Shaul, Y., Schiff, J. A., & Epstein, H. T. (1964). Studies of chloroplast development in Euglena. VII. fine structure of the developing plastid. *Plant Physiology, 39*(2), 231-240. doi:10.1104/pp.39.2.231

Dahoumane, S. A., Yéprémian, C., Djédiat, C., Couté, A., Fiévet, F., Coradin, T., & Brayner, R. (2016). Improvement of kinetics, yield, and colloidal stability of biogenic gold nanoparticles using living cells of *Euglena gracilis* microalga. *Journal of Nanoparticle Research, 18*(3), 1-12. doi:10.1007/s11051-016-3378-1

Fellman, J. B., Hood, E., Nagorski, S., Hudson, J., & Pyare, S. (2018). Interactive physical and biotic factors control dissolved oxygen in salmon spawning streams in coastal Alaska. *Aquatic Sciences, 81*(1), 1-11. doi:10.1007/s00027-018-0597-9

Lee, W., Yoon, S., Shin, Y., & An, Y. (2015). Trophic transfer of gold nanoparticles from *Euglena gracilis* or *Chlamydomonas reinhardtii* to *Daphnia magna*. *Environmental Pollution, 201*, 10-16. doi:10.1016/j.envpol.2015.02.021

Maier, G. O., & Simenstad, C. A. (2009). The role of marsh-derived macrodetritus to the food webs of juvenile chinook salmon in a large altered estuary. *Estuaries and Coasts, 32*(5), 984-998. doi:10.1007/s12237-009-9197-1

Nigon, V., & Heizmann, P. (1978). Morphology, biochemistry, and genetics of plastid development in *Euglena gracilis*. *International Review of Cytology*, *53*, 211-290. doi: 10.1016/s0074-7696(08)62243-3

Ogbonna, J. C., Tomiyamal, S., & Tanaka, H. (1998). Heterotrophic cultivation of *Euglena gracilis* Z for efficient production of α-tocopherol. *Journal of Applied Phycology, 10*(1), 67-74. doi:10.1023/A:1008011201437

Parthier, B. (1981). Chloroplast development in Euglena: regulatory aspects. *Biochemistry and physiology of protozoa*, *4*, 261-300.

Price, C. A., & Vallee, B. L. (1962). Euglena gracilis, a test organism for study of zinc. *Plant physiology*, *37*(3), 428. doi:10.1104/pp.37.3.428

Richter, P., Börnig, A., Streb, C., Ntefidou, M., Lebert, M., & Häder, D. (2003). Effects of increased salinity on gravitaxis in *Euglena gracilis*. *Journal of Plant Physiology, 160*(6), 651-656. doi:10.1078/0176-1617-00828

Scheer, A., & Parthier, B. (1982). Dark-induced chloroplast dedifferentiation in *Euglena gracilis*. *Planta, 156*(3), 274-281. doi:10.1007/BF00393736

Shibata, S., Arimura, S., Ishikawa, T., & Awai, K. (2018). Alterations of membrane lipid content correlated with chloroplast and mitochondria development in *Euglena gracilis*. *Frontiers in Plant Science, 9.* doi:10.3389/fpls.2018.00370

Spano, A., Ghaus, H., & Schiff, J. (1987). Chlorophyll-protein complexes and other thylakoid components at the low intensity threshold in Euglena chloroplast development. *Plant & Cell Physiology, 28*, 1101-1108. doi:10.1093/oxfordjournals.pcp.a077390

Stern, A. I., Schiff, J. A., & Epstein, H. T. (1964). Studies of chloroplast development in Euglena. V. pigment biosynthesis, photosynthetic oxygen evolution and carbon dioxide fixation

during chloroplast development. *Plant Physiology, 39*(2), 220-226. doi:10.1104/pp.39.2.220

Wang, Y., Seppanen-Laakso, T., Rischer, H., & Wiebe, M. (2018). *Euglena gracilis* growth and cell composition under different temperature, light and trophic conditions. *Plos One*, *13*(4), e0195329. doi:10.1371/journal.pone.0195329

APPENDIX

E. gracilis Medium Recipe

1. Sodium acetate 1 g/L
2. Beef extract 1 g/L
3. Tryptone 2 g/L
4. Yeast extract 2 g/L
5. $CaCl_2*2H_2O$ 0.01 g/L

Add dH_2O to 1L and autoclave.

Appendix 1. *E. gracilis* Medium Recipe adapted from UTEX Culture Collection of Algae at the University of Texas.

Name of Chloroplast (Control 0 hr Replicate 1)	Length (mm)	Name of Chloroplast (Control 0 hr Replicate 2)	Length (mm)	Name of Chloroplast (Control 0 hr Replicate 3)	Length (mm)
C0R1C1	0.007	C0R2C1	0.006	C0R3C1	0.006
C0R1C2	0.007	C0R2C2	0.005	C0R3C2	0.006
C0R1C3	0.007	C0R2C3	0.006	C0R3C3	0.007
C0R1C4	0.006	C0R2C4	0.006	C0R3C4	0.005
C0R1C5	0.006	C0R2C5	0.007	C0R3C5	0.007
C0R1C6	0.006	C0R2C6	0.006	C0R2C6	0.007
C0R1C7	0.008	C0R2C7	0.008	C0R2C7	0.004
C0R1C8	0.008	C0R2C8	0.005	C0R2C8	0.007
C0R1C9	0.009	C0R2C9	0.006	C0R2C9	0.008
C0R1C10	0.007	C0R2C10	0.008	C0R2C10	0.005
Average length	0.0071	**Average length**	0.0063	**Average length**	0.0062

Name of Chloroplast (Control 48 hr Replicate 1)	Length (mm)	Name of Chloroplast (Control 48 hr Replicate 2)	Length (mm)	Name of Chloroplast (Control 48 hr Replicate 3)	Length (mm)
C48R1C1	0.008	C48R2C1	0.005	C48R3C1	0.008
C48R1C2	0.007	C48R2C2	0.005	C48R3C2	0.006
C48R1C3	0.006	C48R2C3	0.006	C48R3C3	0.011
C48R1C4	0.006	C48R2C4	0.006	C48R3C4	0.007
C48R1C5	0.008	C48R2C5	0.005	C48R3C5	0.006
C48R1C6	0.006	C48R2C6	0.007	C48R2C6	0.007
C48R1C7	0.009	C48R2C7	0.006	C48R2C7	0.011
C48R1C8	0.007	C48R2C8	0.006	C48R2C8	0.007
C48R1C9	0.006	C48R2C9	0.006	C48R2C9	0.008
C48R1C10	0.007	C48R2C10	0.005	C48R2C10	0.007
Average length	0.007	**Average length**	0.0057	**Average length**	0.0078

Name of Chloroplast (Control 72 hr Replicate 1)	Length (mm)	Name of Chloroplast (Control 72 hr Replicate 2)	Length (mm)	Name of Chloroplast (Control 72 hr Replicate 3)	Length (mm)
C72R1C1	0.01	C72R2C1	0.006	C72R3C1	0.008
C72R1C2	0.007	C72R2C2	0.006	C72R3C2	0.008
C72R1C3	0.006	C72R2C3	0.007	C72R3C3	0.005
C72R1C4	0.007	C72R2C4	0.006	C72R3C4	0.007
C72R1C5	0.01	C72R2C5	0.006	C72R3C5	0.006
C72R1C6	0.006	C72R2C6	0.005	C72R2C6	0.006
C72R1C7	0.006	C72R2C7	0.006	C72R2C7	0.007
C72R1C8		C72R2C8	0.009	C72R2C8	0.006
C72R1C9		C72R2C9	0.006	C72R2C9	0.006
C72R1C10		C72R2C10	0.006	C72R2C10	0.006
Average length	0.0074	**Average length**	0.0063	**Average length**	0.0065

Appendix 2. Raw data for control at time 0 hr, 48 hr, and 72 hr

Name of Chloroplast (Dark 0 hr Replicate 1)	Length (mm)	Name of Chloroplast (Dark 0 hr Replicate 2)	Length (mm)	Name of Chloroplast (Dark 0 hr Replicate 3)	Length (mm)
D0R1C1	0.006	D0R2C1	0.006	D0R3C1	0.006
D0R1C2	0.005	D0R2C2	0.009	D0R3C2	0.005
D0R1C3	0.008	D0R2C3	0.006	D0R3C3	0.006
D0R1C4	0.007	D0R2C4	0.006	D0R3C4	0.007
D0R1C5	0.008	D0R2C5	0.006	D0R3C5	0.008
D0R1C6	0.006	D0R2C6	0.007	D0R2C6	0.005
D0R1C7	0.004	D0R2C7	0.007	D0R2C7	0.007
D0R1C8	0.006	D0R2C8	0.007	D0R2C8	0.006
D0R1C9	0.005	D0R2C9	0.006	D0R2C9	0.006
D0R1C10	0.01	D0R2C10	0.008	D0R2C10	0.005
Average length	**0.0065**	**Average length**	**0.0068**	**Average length**	**0.0061**

Name of Chloroplast (Dark 48 hr Replicate 1)	Length (mm)	Name of Chloroplast (Dark 48 hr Replicate 2)	Length (mm)	Name of Chloroplast (Dark 48 hr Replicate 3)	Length (mm)
D48R1C1	0.004	D48R2C1	0.006	D48R3C1	0.005
D48R1C2	0.004	D48R2C2	0.005	D48R3C2	0.006
D48R1C3	0.007	D48R2C3	0.006	D48R3C3	0.005
D48R1C4	0.006	D48R2C4	0.005	D48R3C4	0.005
D48R1C5	0.006	D48R2C5	0.006	D48R3C5	0.005
D48R1C6	0.004	D48R2C6	0.007	D48R2C6	0.006
D48R1C7	0.005	D48R2C7	0.006	D48R2C7	0.006
D48R1C8	0.006	D48R2C8	0.005	D48R2C8	0.007
D48R1C9	0.005	D48R2C9	0.004	D48R2C9	0.006
D48R1C10	0.004	D48R2C10	0.004	D48R2C10	0.005
Average length	**0.0051**	**Average length**	**0.0054**	**Average length**	**0.0056**

Name of Chloroplast (Dark 72 hr Replicate 1)	Length (mm)	Name of Chloroplast (Dark 72 hr Replicate 2)	Length (mm)	Name of Chloroplast (Dark 72 hr Replicate 3)	Length (mm)
D72R1C1	0.005	D72R2C1	0.005	D72R3C1	0.005
D72R1C2	0.006	D72R2C2	0.005	D72R3C2	0.004
D72R1C3	0.004	D72R2C3	0.005	D72R3C3	0.004
D72R1C4	0.007	D72R2C4	0.004	D72R3C4	0.005
D72R1C5	0.005	D72R2C5	0.006	D72R3C5	0.005
D72R1C6	0.005	D72R2C6	0.005	D72R2C6	0.004
D72R1C7	0.005	D72R2C7	0.005	D72R2C7	0.004
D72R1C8	0.006	D72R2C8	0.006	D72R2C8	0.005
D72R1C9	0.003	D72R2C9	0.005	D72R2C9	0.004
D72R1C10	0.003	D72R2C10	0.004	D72R2C10	0.005
Average length	**0.0049**	**Average length**	**0.005**	**Average length**	**0.0045**

Appendix 3. Raw data for dark treatment at time 0 hr, 48 hr, and 72 hr

1. Calculation for mean chloroplast length

$$\bar{x} = \frac{x1+x2+x3}{3} = \frac{7.100+6.300+6.200}{3} = 6.5333\ \mu m$$

2. Calculation for standard deviation of chloroplast length

$$S = \sqrt{\frac{\sum(x_1 - \bar{x})^2}{n-1}} = \sqrt{\frac{(7.100-6.533)^2 + (6.300-6.5333)^2 + (6.200-6.5333)^2}{3-1}} = 0.493288$$

3. Calculation for 95% confidence interval of chloroplast length

$$6.5333 \pm 1.96 \times s/\sqrt{\eta} = 1.96 \times \frac{0.493288}{\sqrt{3}} = 6.5333 \pm 0.5579$$

Appendix 4. 95% confidence interval sample calculation for control 0 hr