The Effects of Diluting Growth Media on the Cell Size of *Euglena gracilis*

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Abstract:

With increasing interest in utilizing *Euglena gracilis* bioproducts for cultivation and commercialization, its unique mixotrophic metabolism has been under study for years. The purpose of this experiment is to investigate the effect of nutrient availability on growth measured by cell diameter using a micrometer installed within the microscope's eyepiece. To simulate various brackish conditions where *E. gracilis* are naturally abundant, we devised three replicates (R1, R2, R3) at four treatment levels with decreasing quantity of growth factors by diluting the media with water (0%, 25%, 50%, 75%). Taking into account E. *gracilis'* autotrophic abilities, all samples were kept in an incubator with a fixed light source and temperature (25°C). Observing under 400x magnification at four time points (0 hours, 1 hour, 2 hours, 4 days), fixatives were added to the samples at a 1:10 ratio to preserve *E. gracilis* cells in a circular shape making measuring more feasible. ANOVA & Tukey tests show significant differences ($p=0.0112$) in mean diameter for the 75% dilution treatment for the 2 hour time point; which confirmed our prediction where cell size decreases as nutrients are more depleted. Our results yielded from the 1 hour and 4 day time mark were inconclusive due to inadequate sample size or insignificant differences between data points for statistical analysis. Subsequent studies could look to measure cell volume for better measurement or representation of cell growth in three dimensions.

Avec l'intérêt croissant pour l'utilisation des bioproduits d'Euglena gracilis pour la culture et la commercialisation, son métabolisme mixotrophique unique est étudié depuis des années. Le but de cette expérience est d'étudier l'effet de la disponibilité des nutriments sur la croissance mesurée par le diamètre des cellules à l'aide d'un micromètre installé dans l'oculaire du microscope. Pour simuler diverses conditions saumâtres où E. gracilis est naturellement abondante, nous avons conçu trois répétitions (R1, R2, R3) à quatre niveaux de traitement avec une quantité décroissante de facteurs de croissance en diluant le milieu avec de l'eau (0%, 25%, 50%, 75%). En tenant compte des capacités autotrophes d'E. gracilis, tous les échantillons ont été maintenus dans un incubateur avec une source de lumière et une température fixes (25°C). En observant sous un grossissement de 400x à quatre points de temps (0 heure, 1 heure, 2 heures, 4 jours), des fixatifs ont été ajoutés aux échantillons à un ratio de 1:10 pour préserver les cellules d'E. gracilis dans une forme circulaire pour rendre la mesure plus facile. Les tests ANOVA et Tukey montrent des différences significatives ($p=0.0112$) dans le diamètre moyen pour le traitement de dilution à 75% pour le point de temps de 2 heures ; ce qui a confirmé notre prédiction selon laquelle la taille des cellules diminue au fur et à mesure que les nutriments sont épuisés. Les résultats obtenus aux points de temps d'une heure et de quatre jours n'étaient pas concluants en raison de la taille inadéquate de l'échantillon ou de différences non significatives entre les points de données pour l'analyse statistique. (Translated with DeepL)

Introduction:

Plants have long been in commercial use across several industries for their structural integrity, fragrance, or repurposed bioproducts. In particular, there has been a growing interest in microalgae due to its greater photosynthetic abilities and lower maintenance in comparison to plants. In addition, certain algae contain additive chemicals that are found in supplements as well as makeup depending on the species (Yamada et al., 2016). Classified as a type of microalgae, *Euglena gracilis* is a unique freshwater unicellular protist with both animal and plant cell characteristics utilizing a combination of photosynthesis, phagocytosis, and flagellum-driven motility (Wolken, 1967).

E. gracilis has been readily researched in the pharmaceutical industry due to the increased incentives in harvesting paramylon (crystallized beta-1, 3-glucan), a molecule used to suppress colon cancer (Yamada et al., 2016). Another potential use for *E. gracilis* is the breakdown of paramylon in anoxic conditions, which produces a wax ester viable for conversion into biofuel (Yamada et al., 2016). Consequently, further research on *E. gracilis* is necessary to better understand biotic and abiotic factors that can impact its growth, structure, and composition to optimize its usability.

E. gracilis have two main forms characterized by active swimming and inactive sedentary behaviors (Wolken, 1967). Prior studies revealed that *E. gracilis* can undergo changes to their structure and metabolism depending on accessibility to light (Wolken, 1967). Non-motile cells are rounded and can typically be found where *E. gracilis* are cultivated in the absence of

light. Whereas, motile cells take an elongated shape and are usually more abundant when cultured in media and light (Wolken, 1967).

E. gracilis contains chlorophyll in their chloroplast making them appear green under microscopic view, making sampling easier for experiment purposes (Yamada et al., 2016; Wolken, 1967). On average, *E. gracilis* has a cell size of approximately 50 µm long and 15 µm wide (Wolken, 1967). Furthermore, *E. gracilis* cells can also become rounded when exposed to fixative agents which is a technique incorporated in this experiment to make measuring cell size more feasible.

Ogbonna et al. (2002) found that cell growth rate was the sum of the growth rate of cells in purely photoautotrophic and purely heterotrophic conditions. However, if cells are under high light intensity, only photosynthesis occurs, resulting in a lowered growth rate (Ogbanna et al., 2002). When considering the effects of starvation, *E. gracilis* which has been starved for a necessary nutrient in the absence of light has been shown to reduce size of the nuclear and plastid components by 50% (Epstein & Allaway, 1967).

Through this study, we look to examine the impact of altering only the heterotrophic conditions of *E. gracilis* and the effects on its structure. Specifically, this experiment focused on whether diluting growth media would influence cell size in the presence of light. *E. gracilis* were cultured in media to 0%, 25%, 50% or 75% with 0% being undiluted standard growth media. The cell size was measured after 1 hour, 2 hours and 4 days. It was predicted that the cell size would decrease with increasingly dilute media because nutrient starvation has reduced cell size

of *E. gracilis* (Epstein & Allaway, 1967). Therefore, we hypothesize that there will be a decrease in cell diameter for *E. gracilis* at greater dilution of growth media.

Methods:

Beginning our experiment with sterilizing equipment and workstation with ethanol, we then labeled the test tubes that would hold the media and *E. gracilis*. In total, there were 12 test tubes since we devised four different dilution levels (0%, 25%, 50%, 75%) and each treatment was replicated three times (R1, R2, R3). Subsequently, we recorded the cell diameter of several *E. gracilis* for reference purposes.. Using the alcohol lamp to practice sterile technique, 100 µL of *E. gracilis* were transferred from the test tube culture into a counting tube. Then, we added 10 µL of fixative into the counting tube. Using a mixer or scraping the tube rack to mix well, we then prepare a slide with 20µL of the fixed *E. gracilis* mixture. Observing at 400x magnification, we measured the diameters of *E. gracilis* chosen at random with the micrometer (figure 1). In order to obtain a more accurate baseline, five more diameter sizes were recorded at the official start time of the experiment or time 0 (13:18 on March 7th).

Figure 1. **Fixed *E. gracilis* cell diameter measured by the micrometer (reticle)**.

In order to simulate different levels of dilution, we first added 5µL of *E. gracilis* sample into each test tube (12 samples) and varying amounts of media and water depending on the treatment. 5µL of growth media were added into the test tubes labeled “0%” for the control or undiluted samples. For 25% dilution, 3.75µL of growth media and 1.25µL of water were mixed

into test tubes. Then, 2.5μL of growing media and 2.5μL of sterile water into the 50% dilution test tubes. Lastly, 1.25μL of growing media and 3.75μL of sterile water into the 75% dilution test tubes. This aforementioned process was repeated three times for each level of dilution to simulate three replicates. Each sample needed to be mixed well and the test tube rack holding all the cultures was placed into an incubator with fixed light at 25 degree Celsius.

After one hour, we were ready to collect samples for time one. When the test tubes or samples were exposed to air, we always made sure that we had the alcohol lamp lighted to prevent contamination. Labeling 12 counting tubes for each of the test tube samples, 100μL of *E. gracilis* was collected and added into the corresponding counting tubes. The test tube rack was immediately placed back into the incubator and we began the timer again anticipating the second hour mark for sampling. 10μL of fixatives were added into all 12 counting tubes and mixed with the mixer. 12 slides were prepared using 20μL of fixed samples from each counting tube, and the diameters of five random individuals from each slide were measured. All data were recorded digitally on excel and hand written in notebooks independently to minimize human errors during observations. For the purpose of this experiment, we were distinctively looking for fixed *E. gracilis* in a circular shape to allow for more accurate and precise measurements, as shown in figure 1. The sampling process was repeated again for the two hour and four days time point.

To analyze our observations, the data was graphed into mean and SEM plots to show standard error of mean for visual comparison between specific dilution levels and time points. Then, we performed one-way ANOVA twice for the findings from the two hour and four days dilutions using the mean diameter of each replica. The parameters of the test had four treatments

or independent groups (k=4) and three replicates or number of observations (n=3). Furthermore, if significant differences were observed in the ANOVA test, a Tukey test would follow to allow further investigation.

Tukey HSD test:

$$Q_{i,j} = \frac{|\bar{x}_i - \bar{x}_j|}{S_{i,j}}$$

$$S_{i,j} = \frac{\hat{\sigma}_\epsilon}{\sqrt{H_{i,j}}} \quad i,j = 1,2,\ldots k;\ i \neq j$$

Figure 2. **Sample calculation for the Tukey HSD test.** Calculation needed to find Q statistics for the Tukey HSD test that would find the true relationships between samples. This test compares means of all samples to means of individual samples.

Results:

The first hour, second hour and day 4 data were presented in figure 3 using mean and SEM plots with the error bars representing the standard error of the mean (SEM).

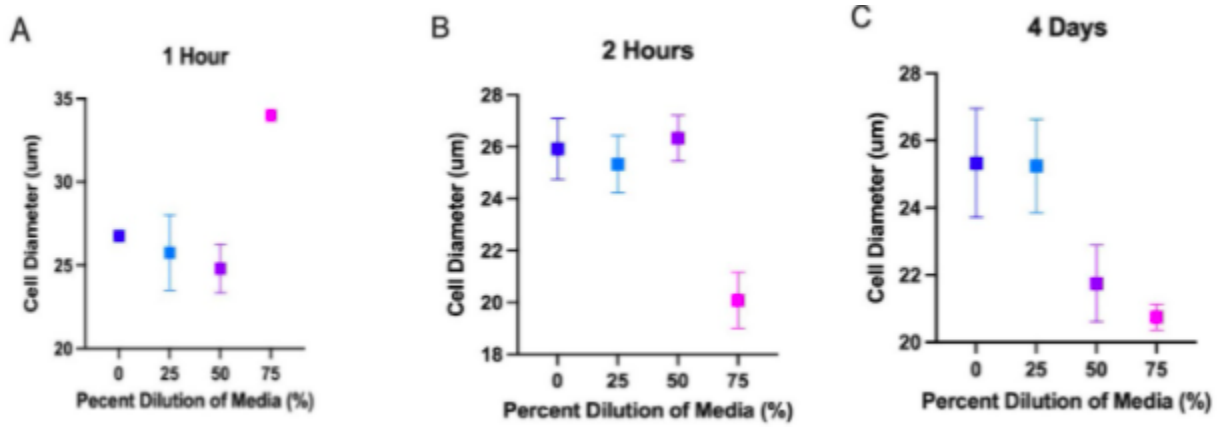

Figure 3. **Changes in Cell Diameter after Different Durations of Time**. (A) Cell Diameter (um) of *E. gracilis* after 1 hour of being in different media solutions (0%, 25%, 50%, 75%), N=<2. (B) Cell diameter (um) of *E. gracilis* after 2 hours of being introduced to different media dilutions (0%, 25%, 50%, 75%), N=3, P<0.05. (C) Cell diameter (um) of *E. gracilis* after 4 days of being introduced to different media dilutions (0%, 25%, 50%, 75%), N=3, P>0.05. The points are the mean and the error bars represent the SEM.

Our data from the first hour was inconclusive since we were not able to find enough individuals in our fixed sample, therefore we were unable to run statistical analysis. We will discuss this issue further in the discussion. However, Figure 3A depicts a decreasing trend in mean cell diameter with increasing media dilution; except for the most dilute media (75%, pink point) which has the largest cell diameters.

In figure 3B, we observed a similar diameter among 0%, 25%, and 50% dilution samples while 75% dilution had shown a smaller diameter compared to the others. Using the one-way ANOVA test, we were able to determine the *p*-value which stands at 0.0112 (shown in figure 4, left). This indicated a statistical significant difference among the samples. To investigate which treatment had the significant difference, we performed the Tukey HSD test (shown in figure 4, right). We found that 75% dilution, in this case treatment D, had a significant difference from the other dilution levels.

source	sum of squares SS	degrees of freedom ν	mean square MS	F statistic	p-value
treatment	67.3073	3	22.4358	7.2888	0.0112
error	24.6250	8	3.0781		
total	91.9323	11			

treatments pair	Tukey HSD Q statistic	Tukey HSD p-value	Tukey HSD inferfence
A vs B	0.5759	0.8999947	insignificant
A vs C	0.3291	0.8999947	insignificant
A vs D	5.4298	0.0206897	* p<0.05
B vs C	0.9050	0.8999947	insignificant
B vs D	4.8539	0.0362471	* p<0.05
C vs D	5.7588	0.0151268	* p<0.05

Figure 4. **One-way ANOVA statistics (left) , and Tukey HSD test statistics for time 2 (right)**. Treatment A: 0% dilution; treatment B: 25% dilution; treatment C: 50% dilution; treatment D: 75% dilution.

As for day 4 (figure 3C), we could observe a smaller cell diameter for 50% and 75% dilution samples when compared to 0% and 25% dilution samples. It is visible that the cell

diameters are progressively decreasing for the increasingly dilute media. However, after performing the one-way ANOVA test, we found a *p*-value of 0.0608. Since the results indicated that the difference is statistically insignificant, we did not perform a Tukey test for this set of data.

Discussion:

The result of our one hour data is inconclusive due to a small sample size. We were not able to observe at least 5 individuals in each fixed sample from each of the three replicates because we failed to mix the test tubes and most of the *E. gracilis* were stuck at the bottom of the tube. Therefore, we did not have a sample size of at least 2 for all of the media dilution treatments (0%, 25%, 50%, 75%) which is necessary to run statistical analyses like a one way ANOVA.

In hour 2, we found a significant difference in mean diameter for 75% dilution sample when compared to mean diameter of the other dilutions. Based on the statistical analysis performed, we were able to reject the null hypothesis at a 95% confidence level. Before the experiment, we predicted that the increase in media dilution would cause a decrease in cell diameter. While we were able to observe such a phenomenon with statistical significance in the 75% dilution sample, our results only provided the information that this one treatment is different but not the overall trend. For day 4 data analysis, we failed to reject the null hypothesis. While the distribution difference could be observed via the box plot in figure 2C, we did not find any difference that is statistically significant. Since we only found one sample to be significantly different at one single time point, we found our results to be inconclusive. The uncertainty could

be the result of many reasons.

One uncertainty in our experiment could be the way our data was measured. In Sheshata and Kempner's study (1977), *E. gracilis* cell volumes were recorded electronically instead which allowed accurate measurements in three dimensions. While we attempted our best to get accurate readings in two dimensions, our measurements were limited by sampling errors since not all *E. gracilis* were fixed in our samples. Due to the pursuit of conducting observations as close to the planned time points as possible, we may have rushed the sampling process which may have prevented full fixative effects. Nonetheless, we tried our best to locate *E. gracilis* cells that are the most circular since several cells remained in the motile ellipse shape.

In the other studies of *E. gracilis*, samples were often tested in controlled conditions with constant light sources, temperature, and nutrient availability (Wang et al., 2018). While we tried our best to keep the environment sterilized and stable, contaminations likely still occurred somewhere along the way. This may affect the quality of media used in our samples and introduce deviations in nutrient availability. Ultimately, these uncertainties could influence the growth rate of *E. gracilis* and lead to variations in our data. In general, we had a relatively small data set which is prone to biases stemming from variability affecting reliability of the survey. Since we only took five measurements in each replica and only considered the mean of each replica for ANOVA analysis, we not only had a high variation for the mean (n=5), but also a small sample size for each treatment (n=3). These factors could all raise the uncertainty in our analysis.

E. gracilis could be considered as both a plant cell and an animal cell with the mixotrophic ability to photosynthesize like a plant cell, and also behaves like an animal cell (Wolken, 1967). While we aimed to decrease nutrient availability in the treatments, *E. gracilis* cells were still able to metabolize through photosynthesize with a fixed light source. We predict that this autotrophic ability potentially mitigated the intended starvation effects, resulting in significant diameter difference from only the most diluted sample at one time point. In reference to background research, future starvation studies could look to compare diameter size between *E. gracilis* cultured in purely autotrophic and purely heterotrophic conditions for a prolonged amount of time (Ogbanna et al., 2002; Epstein & Allaway, 1967).

Conclusion:

In alignment with current findings, the results suggested a decreasing trend in cell diameter for *E. gracilis* exposed to nutrient depleted condition; confirming our expectation of smaller sizes at greater dilutions of growth media. In terms of statistical significance, only one time point and treatment level gave grounds to reject the null hypothesis. Findings from this study could contribute to future application research on possible profit-oriented or industry-related problems with regards to culturing *E. gracilis* bioproducts under stressed conditions. Subsequently, future observations and findings from similar experiments may be extrapolated for possible industrial uses facilitating cultivation, conversion, and commercialization. Specifically, in exploiting feasible cultivation, low maintenance cost, high productivity, heightened adaptability, and resilience in extreme environments for microalgae alike.

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Appendix:

A. Raw data from hour 1 (In ocular units: 1 Ocular Unit=25µM).

Hour1	0%	25%	50%	75%
R1	0.9	1.1	1.1	
R1	1	1.3	0.9	
R1	1	1.2	0.8	
R1	1.5	0.9		
R1	0.95	1.1		
R2			1.1	1.5
R2			1.05	1.2
R2			1.1	1.4
R2			1	1.5
R2			1	1.2
R3		1.1		
R3		0.9		
R3		0.8		
R3		1		
R3		0.9		

B. Raw data from hour2 (In ocular units: 1 Ocular Unit=25µM).

HOUR 2	0%	average	25%	average	50%	average	75%	average
R1	1		1		1.2		0.8	
R1	1.2		1.1		1		0.7	
R1	1		1		1.3		0.8	
R1	1.3		0.8		1.1		0.8	
R1	1.15	1.13	0.9	0.96	1	1.12	1	0.82
R2	0.9		0.9		0.8		0.8	
R2	1		1.1		0.8		0.9	
R2	1		1		1.4		0.8	
R2	1.1		1		1.2		0.7	
R2	1	1	0.9	0.98	1	1.04	0.6	0.76
R3	1		1.1		0.85		0.85	
R3	0.9		1		1.1		0.9	
R3	1.1		1.1		0.9		1	
R3	1		1.2		1.1		0.8	
R3	0.9	0.98	1.1	1.1	1	0.99	0.8	0.87

C. Raw data from day 4 (In ocular units: 1 Ocular Unit=25µM).

DAY 4	0%	average	25%	average	50%	average	75%	average
R1	0.9		1.05		1		0.8	
R1	0.9		1.1		0.9		1	
R1	1.15		1		0.8		0.8	
R1	1.3		1		0.9		0.9	
R1	1.3	1.11	1.1	1.05	1.2	0.96	0.8	0.86
R2	0.85		1		0.8		0.7	
R2	0.8		1.1		0.8		0.75	
R2	0.8		1.2		0.75		0.9	
R2	1.1		1		0.9		0.9	
R2	0.9	0.89	1.1	1.08	0.8	0.81	0.8	0.81
R3	1.15		0.9		0.8		0.9	
R3	1		1.1		0.9		0.9	
R3	0.9		1		0.8		0.7	
R3	1.1		0.8		0.8		0.8	
R3	1.05	1.04	0.7	0.9	0.9	0.84	0.8	0.82

D. Raw data from time 0 (In ocular units: 1 Ocular Unit=25µM).

Time 0	
slide1	1
slide1	1
slide1	1
slide1	0.8
slide1	0.8
slide2	1
slide2	0.9
slide2	0.8
slide2	0.8
slide2	1
	0.91