Effect of NaCl and $CuSO_4$ on Chloroplast Length of *Euglena Gracilis*

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ABSTRACT

Copper and sodium chloride are the major contaminants in the water body of cities with large population. In order to study how these contaminants affect the length of chloroplasts in *Euglena gracilis*, which is one crucial primary producer in the salmon food web, we treated *E. gracilis* with culture medium, 100 mM NaCl, 10 µM $CuSO_4$ and 100 mM NaCl + 10 µM $CuSO_4$ after 24 hours of incubation. After fixation, we measured the length of chloroplasts from four treatment groups. One-way ANOVA test showed F statistic of $F_{(3, 8)} = 0.4379$ and a p-value of 0.7321. Therefore, we failed to reject the null hypothesis. This indicates that in our specific experimental setting, the treatments did not have a significant effect on the length of the chloroplasts in *E. gracilis*. In the future studies, we will improve experimental design by eliminating as many as confounding variables and increasing the sample size of our study.

INTRODUCTION

Euglena gracilis, a eukaryotic microalgae species, can be found in various freshwater environments (O'Neill et al., 2015). As an important mixotrophic (Fellman et al., 2018) and a primary producer in salmon ecosystem (Ebenezer et al., 2017), *E. gracilis* converts light energy into chemical energy via photosynthesis (Nisbet, 1984) and can metabolize organic carbon in the environment at the same time even when light is provided (Ogbonna, Tomiyamal, & Tanaka, 1998) *E. gracilis* is then eaten by primary consumers, for example, *Daphnia magna*, which is the main food source for juvenile salmon (Lee et al., 2015) and adult salmon (Tannreuther, 1923). Significant changes in productivity of *E. gracilis* will influence the productivity of the whole aquatic ecosystem, increasing or decreasing salmon population depending on the abundance of the food source (Maier & Simenstad, 2009).

Anthropogenic pollution has become one of the major threats to aquatic environments globally (Siddiqui & Pandey, 2019). *E. gracilis* is highly sensitive to toxic organic and inorganic pollutants including heavy metals (Ullah et al., 2013), which can inhibit photosynthetic efficiency of this organism (Andreas & Ekelund, 2005) and further influence the growth of the salmon population.

Copper is one of the major contaminants in urban runoff released by traffic (Bäckström et al., 2004). Research by McGreer and Belzer (1998) showed that copper was one of the major contaminants in urban runoff in the Fraser basin area. Because the severity of the pollution is proportional to "traffic and the percentage of impervious surface area" (roads, sidewalks, parking lots, and airports) in the watershed (Hall et al., 1999), it is reasonable to predict that copper level in Fraser basin area is now even higher than it was 20 years ago when research by McGreer and Belzer's (1998) was conducted. This coincided with the significant decline of returning salmon population in British Columbia, Canada. Copper is not only toxic to salmon (Mahrosh et al., 2014) but also has a toxic effect on *E. gracilis*. Exposure to Cu^{2+} at 0.5 - 2.0 mg/L for 24 hours can significantly affect the rate of photosynthesis in terms of oxygen production but not the cell shape or motility (Danilov & Ekelund, 2000). Growth and photosynthetic inhibition were observed at the second day upon exposure to as little as 10 µM Cu^{2+} in *E. gracilis* due to damage to photosystem II reaction center (Rocchetta & Küpper, 2009). Study also has shown that 0.02 mM $CuCl_2$ can impair cell division and change ultrastructure of chloroplasts in *E. gracilis* (Einicker-Lamas et al., 2002).

Sodium chloride (NaCl) is another contaminant we concern in this study. NaCl enters the aquatic system via urban runoff as it carries the de-icing road salt into the nearby

streams (Demers & Sage, 1990). According to Gonzalez-Moreno et al. (1997), high salinity (0.1 M and 0.2 M NaCl) was associated with decreased photosynthetic activity in *E. gracilis*. The inhibitory effects of high NaCl concentration acted on several pathways in photosynthesis instead of targeting a specific site or structure, which ultimately change the thylakoid structure and the composition of the chloroplast's membrane. (Gonzalez-Moreno et al., 1997).

Based on previous studies, we predict that heavy metal Cu^{2+} and NaCl treatments may inhibit photosynthesis and cause morphological changes in chloroplasts of *E. gracilis*. More specifically, we predict that the treatments would affect the length of the chloroplasts. Therefore, we planned to treat the *E. gracilis* with: **(1)** culture medium (negative control), **(2)** 100 mM NaCl, **(3)** 10 µM $CuSO_4$, and **(4)** 100 mM NaCl + 10 µM $CuSO_4$ (dual treatment). Given the previous information, it's likely that the chloroplast length in *E. gracilis* will be shortened by NaCl (high salinity) and Cu^{2+} in water. We also predict a synergistic toxic effect on photosynthesis and therefore the average length of the chloroplast of *E. gracilis* is likely to decrease more significantly when treated with both NaCl and Cu^{2+}.

Our null and alternative hypotheses are:

H_0: Different treatments have no effect on the length of the chloroplasts in Euglena gracilis

H_a: Different treatments have an effect on the length of the chloroplasts in Euglena gracilis

METHODS

Set-up

We were provided with 30 mL of *E. gracilis* cultured in medium (recipe from UTEX Culture Collection of Algae at The University of Texas at Austin) in an Erlenmeyer flask (**Appendix 1**). We treated cultured *E. gracilis* with four different solutions: culture medium as negative control, 100 mM NaCl, 10 µM $CuSO_4$, and the dual treatment (100 mM sodium chloride and 10 µM copper (II) sulfate). **Figure 1** is a schematic representation of how we set up our four treatment groups. Each group had 3 replicates, using a total of twelve 6-mL test tubes with caps. Each test tube was clearly labelled with treatment and replicate number. We pipetted 2 mL of *E. gracilis* into each test tubes and sterilized the openings of the test tubes both before and after adding solution to prevent contamination. We added 2 mL of culture medium to all replicates in negative control treatment. In the sodium chloride treatment group, we added 1 mL of 400 mM NaCl and 1 mL of culture medium. In the copper (II) sulfate treatments, we added 1 mL of 40 µM $CuSO_4$ and 1 mL of culture medium. At last, in the dual treatments we added 1 mL of 400 mM NaCl and 1 mL of $CuSO_4$. We placed all the 12 test tubes into the rack and incubated these test tubes for 24 hours at 25°C with light exposure. The time at which the test tubes were placed in the incubator was recorded.

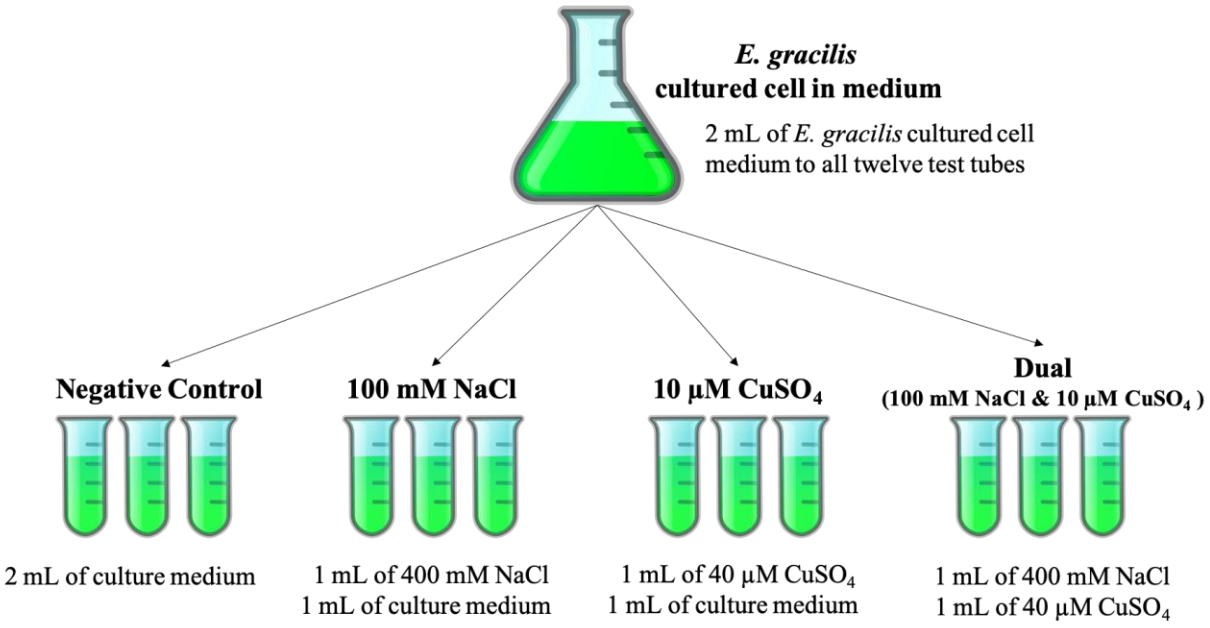

Figure 1. Set-up of the four treatments in the study

MEASUREMENT

- **Slide Preparation**

First, we retrieved the 12 tubes after 24 hours of incubation. Then we took 100 µL of the cultured cells from each tube and added it into a microcentrifuge tube with 100 µL of fixative Iodine Potassium Iodide (IKI). Fixed cells were immediately put into the fridge and chloroplasts' length was measured after 24-hour refrigeration. To prepare the slide, we added 20 µL of fixed cells onto a clean glass slide and carefully put on a coverslip to avoid air bubbles. We only prepared the slides right before we imaged them using the microscope.

- **Oil-immersion Microscope**

Before imaging *E. gracilis*, we set up a Zeiss Axiostar compound microscope (Microscope# R1517-7) using Köhler illumination (Department of Botany, 2019). To calibrate the microscope, we placed a micrometer slide on the stage and measured the length of one ocular unit on the ocular micrometer to be 10.526 μm under 100X objective lens (Department of Botany, 2019).

After setting up the microscope, we placed a prepared slide on the stage and adjusted slide's position until we found one *E. gracilis* cell in the center of the view under 10X objective. Then we switched to the 40X objective and adjusted the focus so that we can see a clear cell image. Lastly, we placed one drop of Zeiss immersol 518N oil onto the slide (where the cell was located) and switched to the 100X objective lens. We tuned the fine focus until we could see a clear image of *E. gracilis* cell and took the picture through the left ocular (with ocular micrometer) with an iPhone X. For each slide, we took six pictures of six different *E. gracilis* cells (pseudo replicates).

- **Chloroplast Length measurement**

ImageJ is the image processing program we used to measure the length of the chloroplasts. We first open the image we took in ImageJ. Then, we set one ocular unit in the image to be 10.526 μm as the scale bar (the yellow line in **Figure 2**) in ImageJ. Then we drew a line (the red line in **Figure 2**) to find the length of a randomly picked chloroplast in the view and clicked the "Measure" operation in ImageJ to get the length of the chloroplast. This procedure was repeated for every chloroplast photo we took to obtain our raw data.

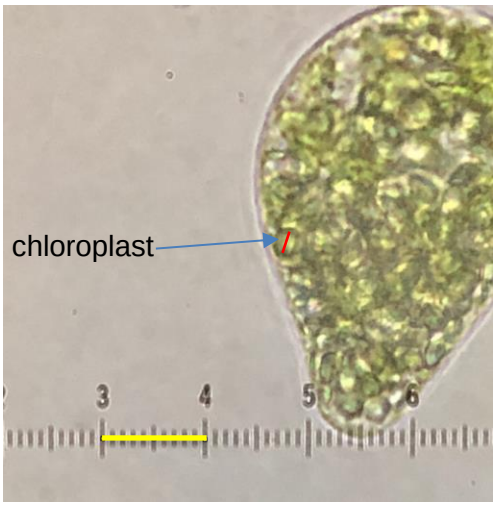

Figure 2. Representative image of *E. gracilis* taken through the ocular of a Zeiss Axiostar compound microscope at 1000X magnification. The scale bar (yellow line) represents 10.526 µm. The red line indicates the length of chloroplast being measured.

RESULTS

To test our hypotheses, we used the one-way ANOVA. Since we had three replicates (n = 3) in each treatment group and six pseudo-replicates per replicate (Raw data in Appendix 2 and 3), we first calculated the average length of chloroplasts for each replicate by averaging the 6 measurements from the pseudo-replicates. Then we imported the data to GraphPad 8.3.0 to perform the statistical test. One-way ANOVA test returned an F statistic of $F_{(3, 8)} = 0.4379$, which confirmed that the between group variation is smaller than the within group variation, and a p-value of 0.7321 (ANOVA table in Appendix 4). Based on the results, we failed to reject the null hypothesis, which means there is no difference in the mean chloroplast length between different treatment groups.

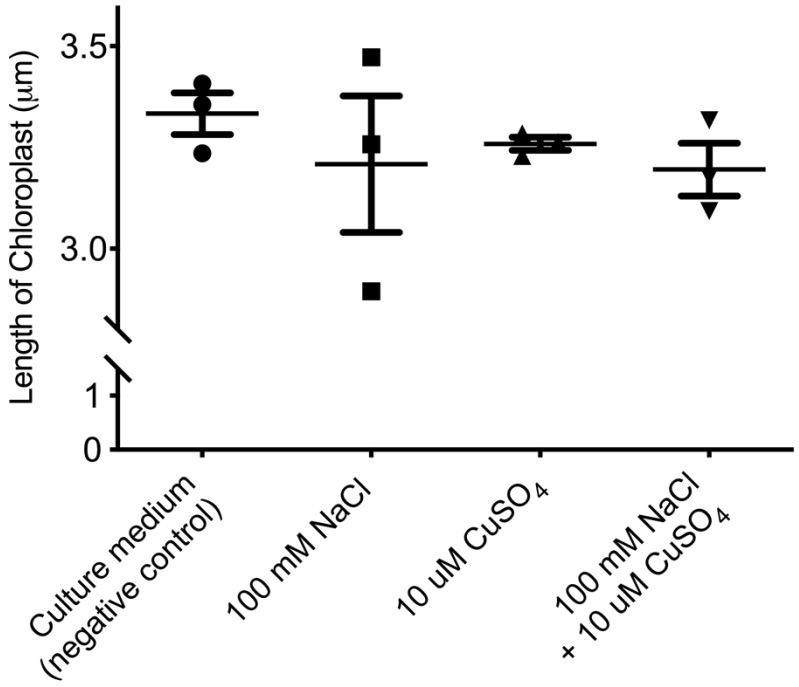

Figure 3. Length of chloroplasts for four different treatment groups. Error bar represents the mean chloroplast length (µm) ± standard error of the mean (µm). Solid circle represents three replicates (n=3) in medium treated group (3.333 µm ± 0.05093 µm). Solid square represents three replicates (n=3) in 100 mM NaCl treated group (3.209 µm ± 0.1687 µm). Solid triangle represents three replicated (n=3) in 10 µM $CuSO_4$ treated group (3.259 µm ± 0.01629 µm). Inverted solid triangle represents three replicates (n=3) in (100 mM NaCl + 10 µM $CuSO_4$) treated group (3.169 µm ± 0.06533 µm).

DISCUSSION

The main purpose of this experiment was to determine whether inorganic salt solution such as NaCl and $CuSO_4$ affect chloroplast length in *E. gracilis.* Contrary to our initial prediction, we fail to reject the null hypothesis, which means there is no difference in the chloroplast length across four treatment groups.

We predicted that the chloroplast length in *E. gracilis* would more likely decrease when exposed to the different treatments (NaCl, $CuSO_4$). This prediction was based on the

detrimental effects that high concentrations of NaCl and Cu^{2+} have on the photosynthetic activity of *E. gracilis* (Danilov & Ekelund, 2000). According to Rocchetta and Küpper (2009), exposure of *E. gracilis* to Cu^{2+} ions leads to the inhibition of the photosynthetic activity due to the formation of heavy metal chlorophylls, Cu as the central atom of chlorophyll instead of Mg, this substitution causes the breakdown of photosynthesis (Küpper, Küpper, & Spiller, 1998). However, the chloroplast length alone may not be a sufficient indicator of photosynthesis efficiency. Xiong et al. (2017) found that a small number of larger chloroplasts did not carry out as efficient photosynthesis when compared to a large population of smaller chloroplasts in *Arabidopsis thaliana.* In our case, it might be necessary to count the number of chloroplasts to better evaluate the effect of different treatments. However, since Xiong et al. (2017) studied the *A. thaliana*, a higher plant rather than species closely related to *E. gracilis*, we should be cautious about the species differences when extrapolate their findings.

Another confounding factor would be the culture medium we used. *E. gracilis* is a mixotrophic species, therefore providing carbon source in the culture medium and treating them with metal ions that have a toxic effect on photosynthesis at the same time might favor the heterotrophic pathway even when the light source was provided (Ogbonna et al., 1998).

Source of Error and Variation

This study was subjected to multiple errors throughout our procedure. One reason for the lack of significance in our experiment may be due to the relatively short incubation given to our organisms. This short period potentially led us to insignificant measurements

of chloroplast length as it is likely that the *E. gracilis* were not given enough time to adjust to their designated treatment environments and ceased to grow normally.

In our study, we only had three replicates for each treatment group. Such small sample size could potentially lower the statistical power of our results. As seen in **Figure 3**, the standard error in NaCl treatment samples were fairly large. By increasing the sample size, we might have a better idea of the true distribution of the population and therefore choose statistical testing method that fits better to the population which allows us to compare our results more accurately.

Another source of error may be the fixation process. After incubating for 24 hours, we fixed each *E. gracilis* samples with IKI. However, we were unable to immediately measure the lengths after they were fixed. The fixed samples were stored in the refrigerator and measured 24 hours later. Because IKI is a strong oxidative reagent, we believe this may not only kill the *E. gracilis* but also might have an effect on chloroplast with prolonged treatment. Since the effect of fixative IKI on the chloroplasts are unknown, we do not know if the lengths would have given different results if we have measured them immediately after fixation.

Due to the inherent limitations of the microscope resolution and how the images were taken, we were unable to accurately define the measurements we took as the "true" length of the chloroplasts. During the measuring process of the chloroplast, we took photos with an iPhone X and measured the chloroplast length using those photos with ImageJ. By this way, we can measure the chloroplast length with less human error. However, the three-dimensional structure of the chloroplasts prevented ideal focus of the image, causing difficulty in the identification of the boundary of the chloroplast. Furthermore, this made

us realize that "length" may not be an appropriate way to measure the effects of different treatments on the *E. gracilis.* Due to the fact that the compound microscope only gives images from one direction while the chloroplast is three-dimensional, it is not clear whether we are measuring the length or the diameter of the chloroplast.

Lastly, there might be inconsistency in chloroplast selection. We measured the first six chloroplast samples, pseudo-replicates for each treatment, that we have encountered in each wet mount slide. This could have been avoided by dividing the slide into sections and choosing one chloroplast sample from each section.

Further Research

Since the research on the association between chloroplasts length and efficiency of photosynthesis are limited, we need more direct evidence to indicate photosynthetic efficiency in *E. gracilis* upon the treatments of NaCl and $CuSO_4$. Previous studies (Danilov & Ekelund, 2000) (Rocchetta & Küpper, 2009) have used oxygen production to assess the efficiency of photosynthesis. We believe oxygen production level can be a feasible parameter to measure in our current lab setting but we need to be cautious about how we interpret the results as oxygen is also consumed during the respiration process.

Additionally, we should conduct a more thorough study on how confounding variables, such as fixative, light exposure regime, and incubation time, can influence photosynthesis and potentially alter our observations before we carry out any further experiments.

Last but not least, we could improve the reliability of the result by carefully plan our experiments, including **(1)** writing more detailed protocols, **(2)** conducting pilot

studies, and **(3)** increasing the sample size that we can perform an appropriate statistical analysis on them.

CONCLUSION

Freshwater sources such as the Fraser river in BC face both heavy metal pollutants as well as the impact of high salinity mostly due to urban road runoff. Since those pollutants has been observed to have detrimental effects on photosynthesis in *E. gracilis, they might indirectly impact the growth of salmon population in BC.* Our research aimed to link *E. gracilis* chloroplasts size with its photosynthesis, by adding photosynthesis inhibitory solution. Although our observations were not in line with our prediction that exposure to NaCl and $CuSO_4$ treatments for 24 hours with light exposures would affect the length of chloroplast in *E. gracilis*, the lessons we learned from the experiments have led us to generate ideas for future studies.

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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	$CaCl_2 \cdot 2H_2O$	0.01 g/L

Add dH_20 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 (Medium Replicate 1)	Length (µm)	Name of Chloroplast (Medium Replicate 2)	Length (µm)	Name of Chloroplast (Medium Replicate 3)	Length (µm)
MER01C1	2.453	MER02C1	3.498	MER03C1	3.562
MER01C2	3.437	MER02C2	3.002	MER02C2	3.363
MER01C3	3.158	MER02C3	3.580	MER02C3	3.621
MER01C4	2.505	MER02C4	3.236	MER02C4	3.216
MER01C5	4.273	MER02C5	3.780	MER02C5	2.829
MER01C6	3.588	MER02C6	3.354	MER02C6	3.545
Average Length (µm)	3.236	Average Length (µm)	3.408	Average Length (µm)	3.356

Name of Chloroplast (100 mM NaCl Replicate 1)	Length (µm)	Name of Chloroplast (100 mM NaCl Replicate 2)	Length (µm)	Name of Chloroplast (100 mM NaCl Replicate 3)	Length (µm)
NAR01C1	3.292	NAR02C1	2.809	NAR03C1	4.071
NAR01C2	3.445	NAR02C1	2.430	NAR03C2	2.758
NAR01C3	3.815	NAR02C3	3.392	NAR03C3	2.706
NAR01C4	3.526	NAR02C4	2.960	NAR03C4	3.298
NAR01C5	3.519	NAR02C5	3.137	NAR03C5	3.619
NAR01C6	3.239	NAR02C6	2.642	NAR03C6	3.094
Average Length (µm)	3.473	Average Length (µm)	2.895	Average Length (µm)	3.258

Appendix 2. *E. gracilis*. chloroplast length measurements for culture medium treated group and 100 mM NaCl treated group.

Name of Chloroplast (10 µM $CuSO_4$ Replicate 1)	Length (µm)	Name of Chloroplast (10 µM $CuSO_4$ Replicate 2)	Length (µm)	Name of Chloroplast (10 µM $CuSO_4$ Replicate 3)	Length (µm)
CUR01C1	3.368	CUR02C1	3.266	CUR03C1	3.199
CUR01C2	4.086	CUR02C2	3.476	CUR02C2	3.327
CUR01C3	3.349	CUR02C3	2.851	CUR02C3	3.412
CUR01C4	2.815	CUR02C4	3.336	CUR02C4	2.949
CUR01C5	3.144	CUR02C5	3.133	CUR02C5	3.194
CUR01C6	2.814	CUR02C6	3.312	CUR02C6	3.630
Average Length (µm)	3.263	Average Length (µm)	3.229	Average Length (µm)	3.285

Name of Chloroplast (100 mM NaCl + 10 µM $CuSO_4$ Replicate 1)	Length (µm)	Name of Chloroplast (100 mM NaCl + 10 µM $CuSO_4$ Replicate 2)	Length (µm)	Name of Chloroplast (100 mM NaCl + 10 µM $CuSO_4$ Replicate 3)	Length (µm)
NCR01C1	3.283	NCR02C1	4.274	NCR03C1	2.779
NCR01C2	3.209	NCR02C2	2.566	NCR03C2	3.711
NCR01C3	2.900	NCR02C3	3.338	NCR03C3	3.312
NCR01C4	2.741	NCR02C4	2.821	NCR03C4	3.454
NCR01C5	3.408	NCR02C5	3.112	NCR03C5	2.854
NCR01C6	3.017	NCR02C6	3.793	NCR03C6	2.951
Average Length (µm)	3.093	Average Length (µm)	3.317	Average Length (µm)	3.177

Appendix 3. *E. gracilis* chloroplast length measurements for 10 µM $CuSO_4$ treated group and (100 mM NaCl + 10 µM $CuSO_4$) treated group.

ANOVA table	**SS**	**DF**	**MS**	**F (DF_n, DF_d)**	**P value**
Treatment (between columns)	0.03505	3	0.01168	$F_{(3, 8)} = 0.4379$	P=0.7321
Residual (within columns)	0.2135	8	0.02668		
Total	0.2485	11			

Appendix 4. One-way ANOVA table.

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