Utilizing EGCG to Mitigate Heavy Metal Stress Induced *Chlorella vulgaris*

Abstract

The utilization of fossil fuels has led to detrimental effects in the environment as a result of its subsequent stressors. These include heavy metal stress and oxidative stress, such that the latter can develop after exposure to the former. Environmental shifts as a result of heavy metal stress include increased occurrences of acid rain and a reduction in the pH of the soil to 5, which leads to Aluminum exposure. These two instances can lead to increased Aluminum exposure from the soil, which develops the presence of Reactive Oxygen Species (ROS). The antioxidant utilized, epigallocatechin gallate (EGCG), can be found in green tea extract as a method of alleviating the damage induced on *Chlorella vulgaris*. The effects of the EGCG alone, Aluminum alone, and both together were tested in each of the experimental groups and the alleviating effects of EGCG against Aluminum exposure were observed. The population density of the algal cells was quantified through spectrophotometry via transmittance between the wavelengths of 350-650nm to observe the effectiveness of EGCG. These transmittances were then utilized to calculate regressions in population growth through an ANOVA with a significant p-value <0.05. Each of the calculated regressions had a statistical significance of 0.000 with the largest and smallest slopes belonging to the EGCG group and the Aluminum group respectively. Expectedly, the EGCG was able to mitigate the effects of the Aluminum as the slope of the group containing both solutes had population growth between the Control group and the EGCG group.

Introduction

According to the National Oceanic and Atmospheric Administration (NOAA, 2013), approximately half of all photosynthetic processes and subsequent atmospheric oxygen derives from phytoplankton blooms. However, the increased consumption of fossil fuels raises the question of how these phytoplankton will be affected. In 2017, the United States utilized over 27.11 trillion cubic feet of natural gas with consequent fossil fuel emissions, which can negatively affect the ozone layer of the atmosphere and pose a threat to phytoplankton (Chiew-Yen et al., 2015; EIA, 2018). These fossil fuel emissions can also disperse heavy metals such as aluminum, which is the third most common element in the Earth's crust, therefore posing a threat for plants as mutations and other plant damage can occur as a result of over-exposure to aluminum (Jaishankar et al., 2014).

Aluminum over-exposure has detrimental effects on plant life as the aluminum not only leads to plant damage, but it also indicates that the soil pH is at approximately 5 compared to the optimal pH between 6 and 7 for most plants. In fact, deformities such as root growth inhibition, cellular modification in leaves, and leaf discoloration are not uncommon. While the aforementioned changes are more visible changes to the human eye, cellular modifications, especially to the organelles of plant cells, are much more rampant (Bojórquez-Quintal et al., 2017).

For instance, the effect of aluminum exposure to Eucalyptus can lead to the degradation of chloroplast envelopes which consequently leads to reduced chlorophyll content. Therefore, the photosynthetic rates of the Eucalyptus leaves were reduced significantly according to Yang et. al (2015), and even lower photosynthetic efficiency was quantified as Al^{+3} was added to the aluminum acid solution that was initially utilized. An overall decrease in transpiration rates of the Eucalyptus plants were also observed as aluminum concentration increased indicating an inverse relationship between aluminum exposure and transpiration rates. Due to the reduced water use efficiency of the plants which leads to poor distribution, storage, and transport of water throughout the plant. This overall inverse relationship between aluminum and plant processes is a recurring theme and occurs in algae cells as well (Yang et. al, 2015).

The aforementioned effects of aluminum on plants and algae is also evident with human health. For example, the toxic effects of aluminum increase for both humans and other animals as organ and organ system efficiencies decline (Jaishankar et al., 2014). These toxic effects derive from heavy metals, such as aluminum, which can lead to subsequent oxidative stress with reactive oxygen species (ROS) formation. The resulting ROS, however, can be combated with the utilization of antioxidants as antioxidant enzymes stabilize the ROS (Bhaduri et al., 2012). In addition, oxidative stress has its own set of detrimental effects. For instance, excess ROS can lead to carcinogenesis in cells which leads to lipid peroxidation and consequent DNA mutations. These mutations can lead to apoptosis or the rise of

mutations against tumor suppressor genes. An increase in neurodegenerative diseases has also been observed due to a change in the oxygen consumption of the brain when imposed with oxidative damage. These oxidative modifications can lead to mitochondrial dysfunction and/or apoptosis in nerve cells. Organ systems such as the excretory system and the respiratory system can be affected by kidney and lung inflammation, respectively (Mao et al., 2017).

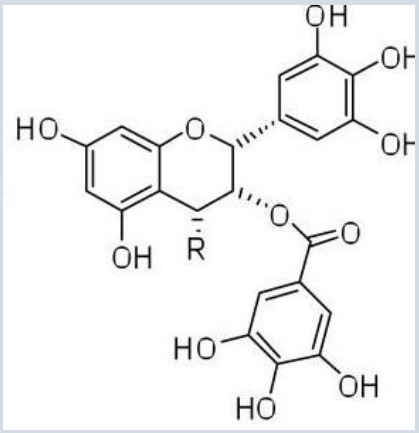

Figure 1 Due to EGCG's classification as a polyphenolic compound, electron delocalization is a property of its molecular structure and its 8 hydroxyl groups give EGCG a higher antioxidant potential (Nagle et. al, 2006; Legeay et. al, 2015).

A prospective solution is a component of green tea extract called epigallocatechin gallate, or EGCG (see Fig. 1), as it can stabilize these ROS by directly searching for ROS and upregulating any antioxidant enzyme activity. The structure of EGCG is imperative as it is an electron donor, which allows for the stabilization of free radicals (Legeay et. al, 2015). These upregulations can be in pathways that diminish ROS interference with cellular processes or inducing oxidative stress relative genes that may inhibit the DNA damage that may have been resulted (Mao et al., 2017). As an antioxidant, EGCG also has other intracellular benefits as it can help repair the cell during instances when the Unfolded Protein Response (UPR) is unable to do so, which is called ER stress. When ER stress is induced by a heavy metal, the cell is unable to refold proteins properly in case they are misfolded or unfolded in general. By fixing these proteins, reduced organelle mutations occur which can stop instances of cancer from forming naturally. In the case of neurodegenerative diseases, EGCG is able to reduce apoptosis rates which may be occurring within the brain. The rampant apoptosis that may occur within the brain may result in Alzheimer's disease, Parkinson's disease, or dementia to name a few (Du et. al, 2018).

Therefore, a procedure was developed in order to examine the mitigation of aluminum-induced heavy metal stress with the utilization of EGCG in *C. vulgaris*. *C. vulgaris* was specifically chosen as it is a subspecies of *Chlorella*, one of the most abundant phytoplankton found in bodies of water. In addition, its ease of acquisition makes it easier to compare to other studies and growth rates determined through other growth mediums to determine optimal growth conditions. The effects of each variable to be tested, EGCG and Aluminum, were utilized in individual groups as well in order to determine if each variable had its intended effects on the *C. vulgaris*.

Materials and Methods

The following procedure was performed in duplicate to attain two complete trials of experimentation to reduce sources of error in statistical analysis.

2.1 Preliminary growth

C. vulgaris was initially grown in a separate flask prior to experimentation in Alga-Gro solution at 25°C in a shaking incubator for a growth period of 3 days. Specifically, 100mL of Alga-Gro solution was utilized in a 125mL flask with three streaks of *C. vulgaris* from the test tube the algae cells were shipped in. Methods of sterilization, including a flaming technique with an inoculating loop, were utilized to ensure that contamination didn't occur. Once the cells were transferred into the flasks, a parafilm seal was punctured to prevent bacterial and fungal contamination, but still allows for proper CO_2 exchange. An LED light source illuminated the flask for a period of 24 hours during each of the three days of incubation.

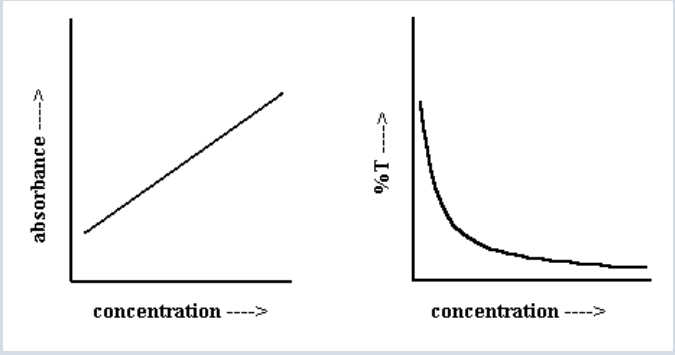

Figure 2 There is an inverse relationship between absorbance and transmittance, thus there is an inverse relationship between transmittance and concentration (Gallik, 2012).

2.2 Transfer of algae cells and initial population quantification

Twenty-four 60mL test tubes were each filled with aliquots of 42mL of autoclaved Bold's Basal Medium (BBM) and 7.3mL of the preliminary *C. vulgaris* and Alga-Gro solution. The twenty-four test tubes were divided into groups of four with six test tubes per group to reduce sources of error from outliers. An initial population was quantified for each test tube through transmittance with a spectrophotometer with units of wavelength between the interval of 350nm and 650nm. Transmittance was utilized as it quantifies the intensity of light that permeates the test tube which establishes an inverse relationship between transmittance and population (Fig. 2). After transmittances were quantified, the twenty-four test tubes were returned to room temperature conditions and exposed to an LED light source for a period of 24 hours for each of the six days of growth.

2.3 Second day of population testing and addition of solutes

After six days of incubation, the transmittances were re-evaluated and recorded as the second day of population growth with the previously stated method. Then, 4mg and 8mg of EGCG and Aluminum Nitrate, respectively, were added to each test tube of the corresponding experimental groups which were labeled as the EGCG, Aluminum, and Aluminum + EGCG. The test tubes were then left to grow in the same conditions as the previous days of growth at room temperature and with an LED light source for 24 hours for each of the six days.

2.4 Accounting for discrepancies and quantifying final transmittances

Once the previous incubation period was over, population growth was evaluated in the same

manner as the first and second day of transmittance quantification. In addition, discolorations caused by the added solutes were accounted for with the equation:

$$Transmittance_{whole\ solution} - Transmittance_{growth\ medium\ and\ solute} = Transmittance_{Final} \quad [Eq\ 1]$$

2.5 Transmittance analysis

The transmittances quantified for each test tube per group were averaged together to calculate a linear regression of growth with an ANOVA. A statistically significant p-value of <0.05 was utilized to ensure the significance of the calculated regression coefficient with respect to the initial quantified transmittance (Koc et. al, 2017).

Results and Discussion

As stated previously, transmittance and population density have an inverse relationship. Thus, higher transmittances imply lower population and lower transmittances imply higher population. The following transmittances are the averages of the transmittances collected from each of the six test tubes per group for each trial.

3.1 Trial 1 Day 1

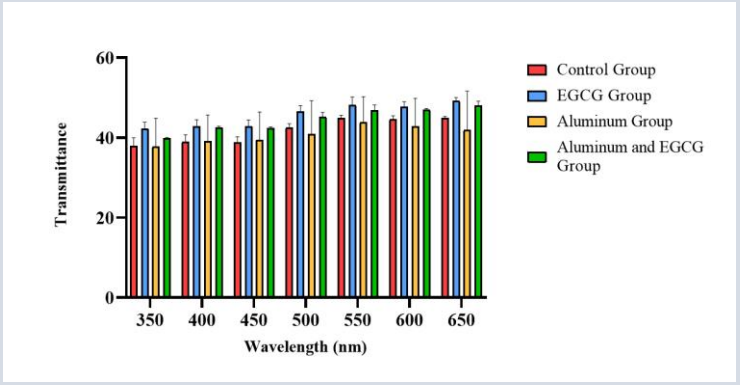

Figure 3 Transmittance levels of the initial test tubes prior to the first growth period as a function of transmittance with error bars denoting standard deviation (created by student).

According to Fig. 3, the EGCG group and the Aluminum group have the highest and lowest transmittances, respectively, for the initial day of population testing. At first, this was believed to possibly skew the incumbent days of population testing, but this was not the case. For instance, if the aluminum group were to have the lowest transmittance, and subsequent highest population density, then the hypothesis would have been nullified due to the initially skewed transmittances. However, the Aluminum group did not have the lowest transmittance for the wavelength of 400nm and 450nm such that the control group had the lowest transmittances for those wavelengths.

On the other hand, the Aluminum and EGCG group had the lowest standard deviation for each of the wavelengths, indicating that the group had the lowest discrepancies between each of the test tubes. Conversely, the Aluminum group had the highest standard deviations for each of the wavelengths due to the high variation between each of the test tubes.

3.2 Trial 1 Day 2

Unlike the linear regression that could be applied to the groups for the initial qualifications of

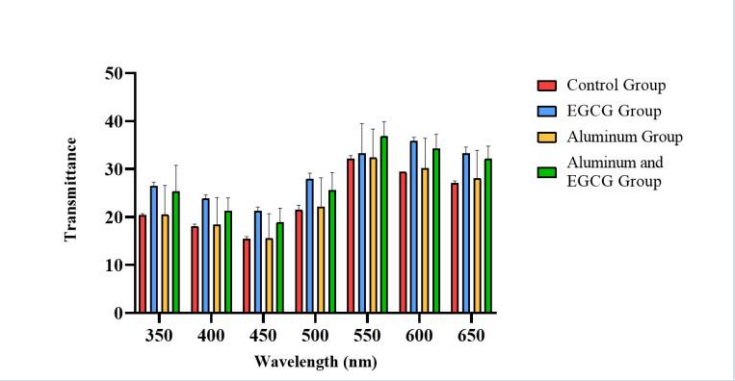

Figure 4 Quantified population density after a six-day growth period with twenty-four hours of light exposure and no solutes. Spectrophotometry was reutilized and results were calculated as transmittance ± standard deviation (created by student).

transmittance, the second day of testing showed a sinusoidal trend for each of the groups. For instance, each of the groups had their lowest transmittance for the 450nm wavelength and their highest transmittance for the 550nm wavelength. This trend was not expected, however, especially considering the shifts in the highest and lowest transmittances across each wavelength. The EGCG group had the highest transmittance for each of the transmittances except for 550nm where the Aluminum and EGCG group had a higher transmittance of 5.8 compared to the EGCG group. The lowest transmittances, however, are fought for between the Control group and the Aluminum group across each wavelength.

Unlike the initial day of population testing, there is no singular group with the highest and lowest standard deviation, indicating that there was a test tube in each group that influenced the averages out of their norm (Fig. 4).

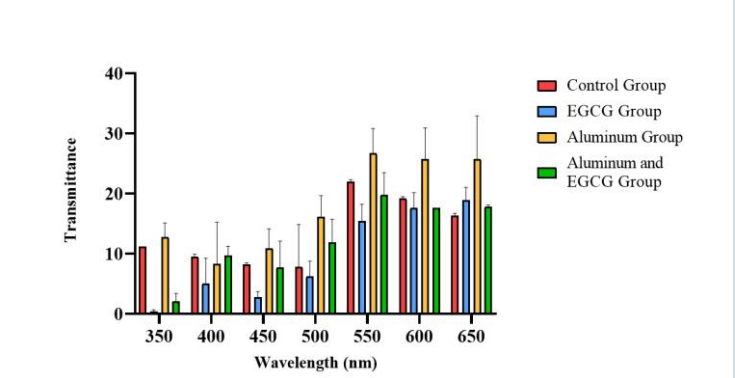

Figure 5 Population density after six days of exposure to respective solutes and a total growth period of twelve days from day zero under the same conditions. The error bars denote standard deviation (created by student).

3.3 Trial 1 Day 3

The third day population testing also shows a sinusoidal trend for each group, but the wavelength of the lowest transmittance for each group varied. For instance, the EGCG group and the Aluminum and EGCG group had their lowest transmittances at 350nm of 0.4 and 2.1, respectively. Contrary to the previous days of quantification, the Aluminum group had the highest transmittance for each wavelength except for 400nm which indicates that the Aluminum group did end up with the lowest population density after exposure to Aluminum as expected. This also does not support any hesitation about a possible skew for the third day of population testing considering the Aluminum group had the lowest transmittance during each of the past two days of testing. The EGCG group had the lowest transmittance for each of the wavelengths followed by the Aluminum and EGCG

group for the second lowest transmittance for all of the wavelengths tested except for 400nm. This shows that the EGCG did have a positive effect on the population density of *C. vulgaris* after exposure to Aluminum (Fig. 5).

3.4 Trial 2 Day 1

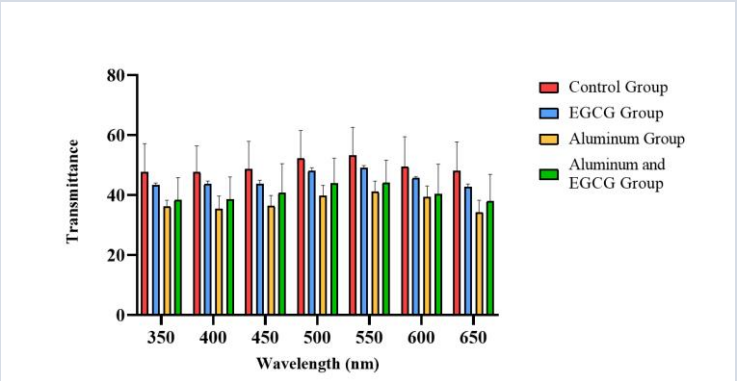

Figure 6 *C. vulgaris* from a different initial flask was distributed to each test tube and tested for population density. The concluded results had higher error bars, and thus standard deviation, compared to the first trial (created by student).

Based on Fig. 6, the control group had both the highest transmittance across the 350-650nm interval of wavelength and standard deviation. Thus, the control group had both the lowest population density and a test tube which was skewing the transmittances higher compared to Trial 1. Similar to Trial 1, the Aluminum group had the lowest transmittance and subsequent higher population density for each wavelength with the smallest standard deviation. In fact, the groups with the highest to lowest transmittances were in the order of the Aluminum group, the Aluminum and EGCG group, the EGCG group, and the Control group for each wavelength tested. So, while there may not have been a specific trend in terms of gradual increase and decrease as in Trial 1, there was a consistent trend between the groups.

3.5 Trial 2 Day 2

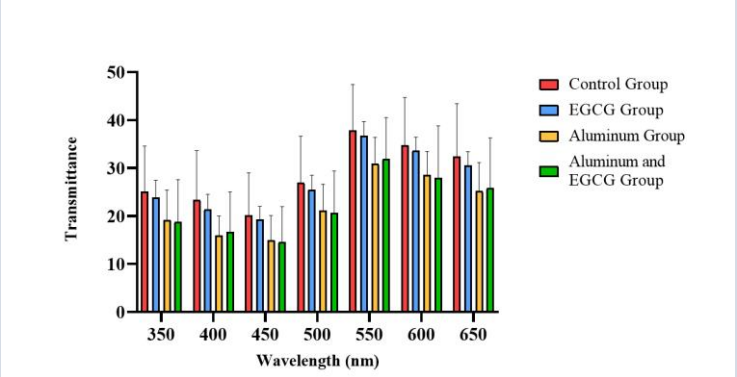

Figure 7 Transmittances were quantified once more after a growth period of six days with no added solutes. The error bars increased drastically compared to the first day and the first trial (created by student).

Fig. 7 reintroduced the sinusoidal model that Fig. 4 had exemplified in Trial 1, indicating that there is a growth trend between the initial day and the sixth day of testing which leads to a sinusoidal trend between the wavelengths. Similar to the first day of testing, the Control group remains as the group with the highest transmittance and standard deviation for each of the wavelengths tested with a maximum average transmittance of 37.8 for the 550nm wavelength. The lowest transmittance was the Aluminum and EGCG

group with a transmittance of 14.6 which just barely edged out the Aluminum group with a difference of 0.4 in the 450nm wavelength.

These drastic changes in population density for the second day of testing in both Trial 1 and Trial 2 were unexpected because no solutes had been added yet. Therefore, it was inferred that there would have been no shift in transmittance across the wavelengths. This indicates that the population growth model would have a steep slope across each day rather than a plateau for the first and second day of quantification followed by a steep slope between the second and third day of testing.

3.6 Trial 2 Day 3

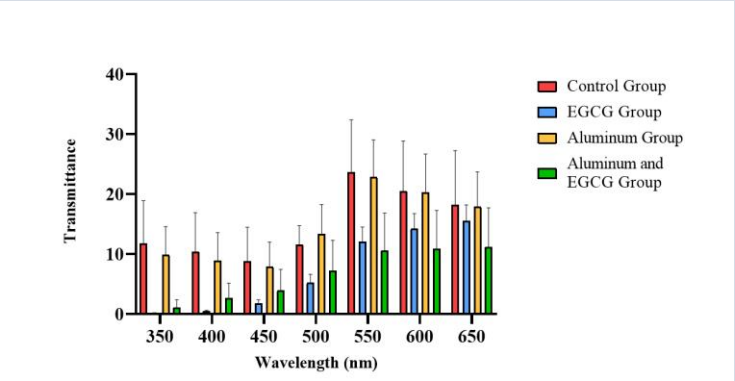

Figure 8 After another growth period of six days, for a total period of twelve days, population density was quantified after each group was exposed to its corresponding solute. Error bars denote standard deviation (created by student).

Following the sinusoidal trend of the second day of testing, Fig. 8 presents an even more extreme trend compared to Fig. 5 which exemplified the third day of quantification for the first trial. Similar to the third day of the first trial, the EGCG group and the Aluminum and EGCG group have the lowest transmittances, but the Control group has maintained its status with the highest transmittance for each wavelength. This contradicts the previous model presented by the first trial and of what was expected considering the predicted effects of Aluminum on *C. vulgaris*. The Control group did, however, continue to have extremely high standard deviations indicating that the average transmittances may have been skewed.

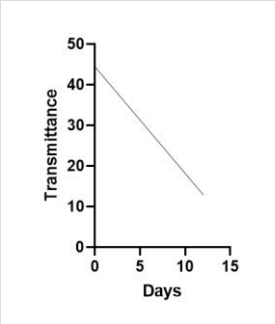

Figure 9 The linear regression of the Control group for the total twelve days of population testing. R^2 value of the regression was 0.844 with a y-intercept of 45.841 (created by student).

As aforementioned, EGCG and the Aluminum with EGCG group had the lowest transmittance with the latter and former having transmittances of 0.15 and 1.1, respectively, for the 350nm wavelength. With a transmittance so close to 0, the EGCG group had the highest population density calculated throughout the experiment. On the other hand, there were instances such that the Aluminum with EGCG group had highest transmittances as can be seen in the interval of 550-650nm.

Control group population change

The linear regression calculated for the Control group had a slope of -2.622 which was inferred to come out negative as transmittance decreased

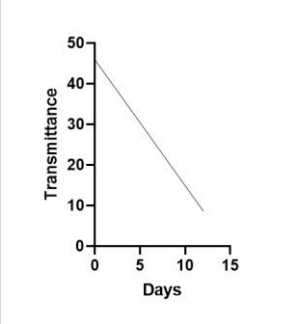

Figure 10 The decrease in population density as a function of time for the EGCG group with a R^2 value of the regression was 0.990 (created by student).

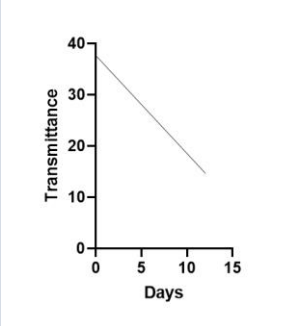

Figure 11 The quantified regression of the Aluminum group with a R^2 value of the regression was 0.809 (created by student).

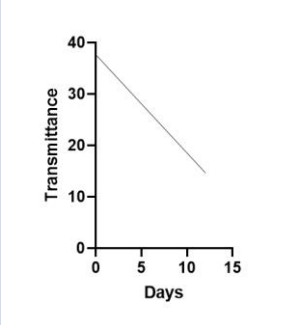

Figure 12 The calculated slope of the Aluminum and EGCG group with a y-intercept of 42.218 and R^2 value of 0.890 (created by student).

for all of the groups as time went on. This regression is expected to not be the fastest growing slope as the EGCG transmittance levels have been the lowest for the last day of both trials, indicating a greater skew for a faster slope. The p-value of the slope was 0.000, exemplifying high significance such that the points which the linear regression passes are viable for future calculations (see Fig. 9).

EGCG group population change

The linear regression of the EGCG group is the steepest with a regression coefficient of -3.098 which was expected due to the sharp decline in transmittance between the second and third day in both trials. In addition, by reducing sources of error through the aforementioned formula utilized, the slope is comparable to the other groups such that the regression coefficient could have been more negative resulting in a drastically steeper slope compared to the other groups. The p-value of the regression was 0.000 showing high significance for both the days tested and predicted values (see Fig. 10).

Aluminum group population change

The calculated regression coefficient of the Aluminum group was -1.911 with a y-intercept of 37.620. The y-intercept of the calculated regression was the smallest of the four groups indicating that the small y-intercept coupled with the low population growth may have affected the steepness of the slope. The slope also shows that there was the least growth as expected with a decrease in transmittance of less than 2 each day. This gentler slope also shows how there was much less growth compared to the control group which had a regression almost 1.5 times steeper. Like the previous regressions, the p-value was significant with a value of .000 for both the slope and y-intercept (see Fig. 11).

Aluminum and EGCG group population change

The Aluminum and EGCG group had a calculated regression coefficient of -2.748 and y-intercept of 42.218 which is between the Control group and EGCG group. With a slope between the aforementioned groups, there is an indication the EGCG was able to mitigate the effects of the Aluminum as population growth was faster than both the Control group and the Aluminum group, but slower than

the EGCG group as expected. In addition, with a p-value of .000, the calculated regression is highly significant and comparable to the other regressions (see Fig. 12).

Conclusion and Future Work

The data presented based on growth rates were consistent with the results of prior literature. According to Koc et. al (2017), *Chlorella vulgaris* experiences exponential growths in population until the fifth day of growth after which the growth rate plateaus. This was evident with the *Chlorella* in the initial flasks before experimentation, as the coloration of the beakers changed from clear to green within 5 days. This is due to the fact that similar techniques were utilized in the study within the journal and the currently performed procedure.

The reduction in population coincided with the other inverse correlations that were found within the study of Yang et. al (2015). For instance, the Aluminum group showed the least population growth compared to the other groups which showed faster growth in population. While a reduction in growth was visible in the current study, a diminished total population was not quantified as population still grew.

However, the discoloration which was apparent with the groups with the EGCG was not mentioned within any of the previously read literature. This discoloration lead to a slight skew in the data which had to be accounted for and by subtracting the difference between the transmittance of water and the transmittance of the EGCG and BBM.

The apparent discoloration that resulted from the EGCG can be combated by utilizing the same calculation technique as before or by utilizing other nutrients. In particular, Glycyrrhiza Glabra extract (Shetty et al., 2002) and Melatonin (see Fig. 13) can be utilized as they possess radio protective properties as they protect dividing cells from chromosomal injury and inhibit lipid peroxidation from ROS exposure. Utilizing antioxidants which protect against radiation and ROS can ensure the protection of the algae cells under most conditions of growth as they are placed under lighting for 24 hours.

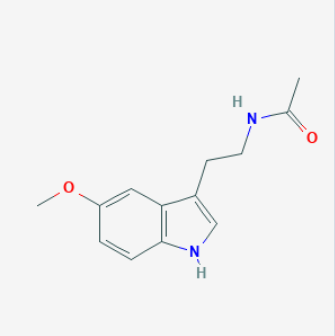

Figure 13 Melatonin's scavenging tendencies for hydroxyl molecules due to its molecular structure reduces the risk of over exposure of hydroxyl to nearby critical molecules (NCBI, n.d.; Tahamtan et. al, 2015).

Melatonin is generally known as a sleep aid as it is the main hormone produced by the brain to induce sleep, but its free radical fighting nature is imperative for future research. In regards to lipid peroxidation, it was observed that ROS accumulation and lipid peroxidation had a positive correlation. The utilization of Melatonin, however, could subdue the lipid peroxidation, which resulted from ROS exposure. This recovery in the cells after exposure to ROS also led to long term restoration in cellular growth rates which contrasted with the significantly fewer growth rates after ROS exposure without Melatonin

exposure (Vázquez et. al, 2018). This is similar to the growth trends which were found with the EGCG and Aluminum Nitrate samples.

The test tubes can also be looked at as a microbiome. For instance, the algae cells could be BBM in such a way that byproducts that may occur as a result of their photosynthesis and other chemical reactions could affect the short-term and/or long-term sustainability of *C. vulgaris*. For instance, testing for nitrogen levels is essential, as the concentration of nitric oxide can allow for an increased number of cellular processes to occur. According to Henard et. al (2017), more processes occur in *C. vulgaris* as there is an abundant exposure to nitrate compared to instances in which there is a depleted concentration of nitrate.

Examining the growth rates of phytoplankton and discovering methods of multiplying their growth rates in spite of exposure to detriments such as heavy metals are imperative if our current fossil fuel emissions and exorbitant exploitation of the environment continue in the coming years. By utilizing antioxidants, especially EGCG, some of these detriments can be alleviated and phytoplankton blooms can be artificially created in areas where trees and other photosynthetic organisms are disappearing. Phytoplankton also play roles in medicine and in the biofuel industry with better results compared to sunflower oil and soybeans which have been the standard for the past few years for the latter.

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