A study on the effects of urea concentration on chlorophyll-a and chlorophyll-b content in *Ceratopteris thalictroides* (Water Sprite).

1. Introduction

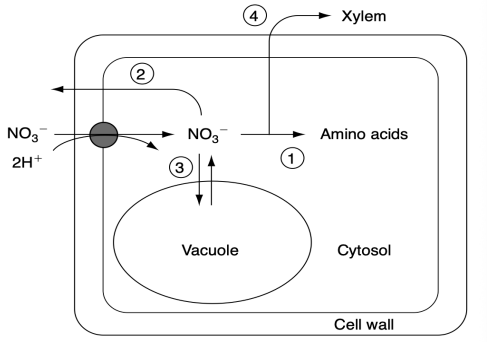
In the past few years, groundwater Nitrate pollution has been a significant problem in my hometown, Xiamen City, Fujian Province. It's been shown that the amount of nitrogen or urea fertilizer applied was positively correlated with nitrate concentration in city groundwater(Rahati et al. 2014). Nitrate toxicity causes the deterioration of water quality, thereby damaging biodiversity through killing aquatic lives as exposure time increases (Li.et al. 2021). The main source of nitrate pollution is urea which contains a high proportion of nitrate and finally flows into the soil and aquatic environment. I found that Water Sprite (Ceratopteris thalictroides, a type of algae) was always used in fish tanks by my grandpa as a bioremediation method to lower the Nitrate content in his fish tank. Therefore, I would like to discuss the relationship between Nitrate concentration and aquatic plants growth in my internal assessment by connecting to Photosynthesis & Molecular aspects of IB Biology. Hence, my research question was:

What is the effect of different dissolved urea concentrations (2.00 ppm, 4.00 ppm, 6.00 ppm, 8.00 ppm, 10.00 ppm) on the chlorophyll-a and chlorophyll-b content in Ceratopteris thalictroides (Water Sprite) measured by the light absorbance by the spectrophotometer upon 9 days of exposure ?

2. Background information

The corresponding independent variable is urea concentration(2.00M, 4.00M, 6.00M, 8.00M, 10.00M) which contains NH_4NO_3. Nitrogen (N) is a major limiting factor of plant growth and crop productivity(Jingestad 1979; Hood 1982; Lee et al. 1983; Agren 1985). In nature, inorganic nitrogen(N) always exists in the form of nitrates(NO_3^-), nitrites(NO_2) and ammonia nitrate (NH_4NO_3), while the last two forms are unstable as they are easily converted to nitrate ions(NO_3^-), which are the major ions in the polluted groundwater in the plain area of Xiamen (Wang.et al. 2020; Li. et al. 2021). The nitrate, as well as the main source of N for plants (Pilbeam & Kirkby, 1990), can induce a wide range of developmental effects that are frequently attributed to the influence of hormonal factors. However, for algae used to reduce nitrate pollution under the steady-state conditions, the degree of N-limitation defines the net photosynthetic rate (Li & Goldman, 1981; Osborne & Geider, 1986).

Fig. 1 "The Fate of Nitrate(NO3-)in the Cell." MCrawford, Nigel M, and Anthony DM Glass. Molecular and Physiological Aspects of Nitrate Uptake in Plants, Science Direct, 1 Oct. 1998, https://sci-hub.se/10.1016/s1360-1385(98)01311. Accessed 25 June 2022.



My experimental subject is *Ceratopteris thalictroides*, commonly known as "Water Sprite" and a fern grown in fish tanks. It always works as a bioremediation by removing combined nitrogen compounds like nitrite from wastewaters (Ahmadzadeh. et al. 2015). Figure 1 shows that nitrate is actively transported across the plasma membrane by both low and high affinity, proton symporters, and NO_3^- involved experiences 4 fates:(1)Reduction to NO_2^- by the cytoplasmic enzyme nitrate reductase; (2)efflux back across the plasma membrane;(3)influx and storage in the vacuole;or (4) transport to the xylem for long-distance translocation to the leaves(Crwarford & Glass,1998). The transport mechanism of nitrate uptakes alter the physiological behaviors of *C. thalictroides*, so I can collect qualitative data of its appearance.

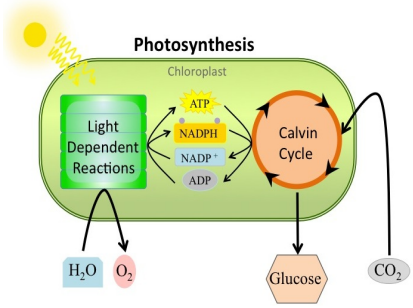


Fig. 2 Photosynthesis process: light -dependent reaction within the chloroplast (https://cnx.org/contents/dEoGMkIy@6/Overview-of-Photosynthesis)

In the **IB Biology photosynthesis chapter**, photosynthesis is a biochemical reaction process to offer energy as a form of ATP that occurs in plants through absorbing light in the chloroplast where photons are captured. In light dependent reaction, the synthetic reaction whereby CO_2 is assimilated and reduced to a number of organic compounds (Glucose) via the photosynthetic carbon reduction cycle, and also produces O_2 as a byproduct (Jensen& Bassham,

1966).Light is firstly absorbed by chlorophyll which releases energized electrons that are used to produce ATP, and the electrons will be donated to the carrier molecules (NADP+) which is sued in light independent reactions. The electrons lost from the chlorophyll-are replaced by water, which is split(photolysis)to produce oxygen and hydrogen. The following equation of light dependent process is introduced:

$$6CO_2(Carbon\ dioxide) + 12H_2O(water) \xrightarrow[(light\ +\ chlorophyll)]{} C_6H_{12}O_6\ (Glucose) + 6O_2(Oxygen) + 6H_2O(water)$$

Chloroplast are cytoplasmic organelles and the site of photosynthesis apparatus precisely(F. Et al. 2011) that has chlorophyll-a and chlorophyll-b which are essential pigments helping capture photons, thereby reflecting green light(525 nm-625 nm) and absorbing red(625 nm-700 nm) and violet-blue visible lights(400 nm-525 nm) most effectively in the visible light spectrum. The presence of urea exposure allows the nitrate assimilation involving two membrane barriers: plasma and chloroplast membrane. Chloride channels located in the envelope membrane of chloroplast which are permeable to nitrate and nitrite and particularly specific to a passive transport of nitrate and nitrite(Fuks&Hobe, 1999). Thus, once nitrate is reduced to nitrite by the nitrate reductase enzyme in the cytosol, nitrite has to cross the chloroplast envelope membrane for its subsequence reduction to ammonium and incorporation into amino acids (Hoff et al., 1994; Crawford, 1995). *Ceratopteris thalictroides* is a chlorophyllous (green) fern spore which is able to photosynthesis because it appears lush green when examined with either naked eye or light microscope, and has relatively thin perishability and short viability.

Hence, I can determine chlorophyll-a and chlorophyll-b content in *C. thalictroides* as dependent variables through a vernier spectrophotometer to reflect their photosynthesis activity and growth.

3. Hypothesis

3.1. Null hypothesis (H_0): There is no significant change in the chlorophyll content in *C. thalictroides* as urea concentration increases. This might be because urea concentration doesn't affect *C. thalictroides* due to its toxicity intolerance, or there is not sufficient nitrate content in the urea exposed on *C. thalictroides.*

3.2. Alternate Hypothesis (H_1): In a suitable range, when the urea concentration increases, the absorbance of light by the leaf extracts will increase and *C. thalictroides* will grow better, whereas too much nitrate level in urea will inhibit its growth. With greater urea exposure in the acceptable range, nitrate assimilation can promote protein synthesis(enzyme, rubisco, chlorophyll-a/b content) by the permeability of the chloroplast membrane. More enzymes synthesized will promote metabolism and cell respiration, leading to *C.thalictroides*' growth, and more rubisco and chlorophyll-a/b content will increase light absorption in photosynthetic carbon metabolism. However, too high nitrate levels will cause DNA damage, so the toxicity will kill the plants.

4. Variables

4.1. Independent variables

Urea concentration (2.00 ppm, 4.00 ppm, 6.00 ppm, 8.00 ppm, 10.00 ppm)

4.2. Dependent variables

Chlorophyll-a and chlorophyll-b content of *Ceratopteris thalictroides*' leaves (it can be calculated through light absorbance)

4.3. Controlled variables

Control variables	How to control	Effect
Temperature (27°C)	Keep AC(air conditioning) at the same temperature (27°C) in the school lab throughout the whole experiment.	Enzyme activity is greatly dependent on the temperature for the plant's photosynthesis and chlorophyll-activity. The same optimal temperature ensures the enzymes in leaves can support photosynthesis and promote chlorophyll-activities. High temperature might denature the protein and degenerate photosynthesis, leading to a chlorophyll damage.
Amount of water in container	Add 2 L tap water measured by measuring cylinder to the container in each trial.	Providing the same sufficient inorganic products for *C. thalictroides* in each trial can guarantee their basic needs for survival. Therefore, lacking water won't be an issue to judge the case of lower chlorophyll content in the results.
Light condition	Set all the samples beside the glass window to ensure all samples are exposed to the same amount of sunlight.	Same sunlight intensity will provide the same source of light energy for the photosynthesis of *C. thalictroides* used in chloroplast. Higher light intensity will also raise temperature, degenerating enzymes.
Volume of urea	100 mL of urea with concentration of 2 ppm, 4 ppm, 6 ppm, 8 ppm and 10 ppm will be exposed to *C. thalictroides* per trial.	Same volume of urea with different concentrations can give different amount of urea on *C. thalictroides* (Quantity = volume ×concentration). Therefore, the result is directly and solely changed by urea concentration / content.
Quantity of *C. thalictroides* per trial	2g of *C. thalictroides* will be propagated in solution for each trial.	Same quantity of *C. thalictroides* will absorb the same exposure in the same aquatic environment regardless of urea concentration(IV).Too many *C. thalictroides* in one container will compete for the scarce resources (Water, light and urea. etc.), and cause nutrient deficiency.
Exposure time (Three days / 72 hours)	*C. thalictroides* will be propagated under the exposure of urea for three days. After three days, the leaves extract will be used to determine the chlorophyll content.	The exposure time will be limited to 3 days(72 hours) to calculate the chlorophyll content equally. There will be no urea left in the environment for *C. thalictroides* in longer exposure time, resulting in independent variables having no direct effect on the final results.

Methodology

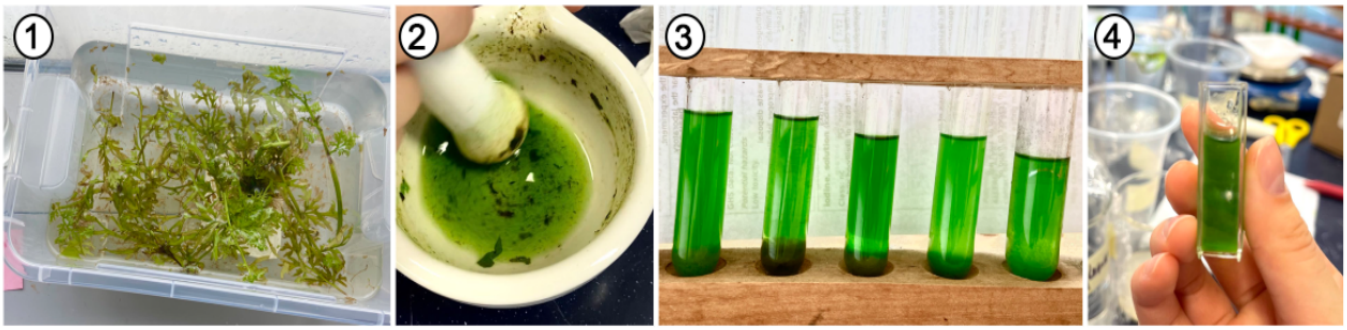
5.1. Materials & Apparatus

Materials	Apparatus
(3) *Ceratopteris thalictroides* (1) 1L Urea (10M) (1) 1 L 80% acetone (1) Distilled water	(1) Spectrophotometer (±0.001) (30) Container (312 cm^3) (1) Electronic scale (±0.5g) (2) 10cm^3 Measuring cylinder (± 0.2 cm^3) (2) 100cm^3 Measuring cylinder (±1 cm^3) (2) 50cm^3 Beaker (5) Test tube (1) Scissor Mortar (1) Funnel (1) Pestle (2) Cuvette

5.2. Procedure:

Figure.3. The experiment was set up.

1. Propagate floating *C. thalictroides* in water for 2 days. (Fig.3 -①)
2. Cut the stem of the floating *C. thalictroides* (2 g) measured by the electronic scale and separate them into 6 groups (Name each group #1 to #6).
3. Before adding the ammonium nitrate, measure the light absorbance of the leaves of *C. thalictroides* by chlorophyll-a/b through a spectrophotometer.
    a. Pick 0.5g leaves off weighted by a weighing scale and grind them with a mortar and pestle for 1 minute.
    b. Add 10mL 80% acetone and keep grinding for 2 minutes. (Fig.3 - ②)
    Transfer the liquid to a test tube and keep shaking the solution for 5 minutes. (Fig.3 - ③)
    c. Transfer the liquid into a cuvette until reaching $\frac{3}{4}$ mark. (Fig.3 −④)
    d. A spectrophotometer is connected to a computer and is calibrated on Vernier spectral analysis (full spectrum).
    e. Measure the absorbance of light by chlorophyll-a/b by using absorbance v.s. wavelength graph in the installation.
    f. Collect the data at OD_{645} and OD_{663}(OD= Optical density).
    g. Calculate the chlorophyll-a/b content according to the law of Lambert-beer.
4. Add 2 L water and 1 L solution of urea with different concentration of 2.00M, 4.00M, 6.00M, 8.00M, 10.00M to sample #1 to #8 respectively.
    On the third day after propagation, measure the light absorbance of the leaf extract form the plant by using a spectrophotometer (Repeat step 3- a to f).
5. Repeat step1 to 6 for 4 trials and collect the data.



a. Figure.3. ①: Step 1,Propagate floating C. thalictroides in water for 2 days; ②: Step 3-a: Grinding the leaves extracts with 10 mL 80% acetone. ③: Step3-c: Transfer the test tube and keep shaking the solution for 5 mins . ④: Step3-d: Transfer the liquid into a cuvette
b. until reaching $\frac{3}{4}$ mark.

5.3. Risk Assessment

Potential hazards	Ethical / Environmental consideration
1. Wear safety goggles, a lab coat and gloves when handling chemical materials. 2. Clean the table after each experiment to avoid ingesting urea accidently, which might cause gastrointestinal irritation with nausea, vomiting and diarrhea. Avoid acetone and 2-propanol contacting eyes, as it will cause eye irritation or drying and redness of skins. Fire should also be banned when using Acetone, as its evaporation might cause burning or explosion. 3. Prevent breaking fragile apparatus such as beakers, measuring cylinders, test tubes. etc. The fragments and pieces might cut our bodies.	1. There are no ethical concerns raised in this investigation, as plants used are non-endangered species. Food waste(of the seeds and leaves) should be minimized. 2. Acetone should be placed in the garbage for disposal. 2-propanol needs to be disposed of diluted with an equal volume of water and poured down the drain. 3. Urea can be directly poured down the drain as it is not considered as a hazardous waste.

6. Analysis

6.1. Raw data

Table.1 *The light absorbance of C. thalictroides in wavelengths of 645 nm and 663 nm, after different urea exposure(ppm).*

Urea concentration (ppm)	Light absorbance of *C. thalictroides* (±0.1%)									
	Trial 1		Trial 2		Trial 3		Trial 4		Trial 5	
	OD_{645}	OD_{663}	OD_{645}	OD_{663}	OD_{645}	OD_{663}	OD_{645}	OD_{663}	OD_{645}	OD_{663}
0.00	1.362	1.607	2.003	2.094	2.149	2.249	0.665	0.754	0.848	0.955
2.00	2.032	2.211	1.422	1.740	1.990	2.222	1.548	1.919	2.064	2.287
4.00	2.053	2.210	1.794	2.049	1.563	1.826	1.945	2.148	2.074	2.291
6.00	1.891	2.182	1.777	2.130	1.804	2.127	1.672	2.043	1.931	2.196
8.00	1.745	1.887	1.794	2.005	1.788	1.999	2.015	2.143	1.695	1.946
10.00	1.566	1.878	0.898	1.028	1.945	2.110	1.402	1.459	1.643	1.818

6.2 Data analysis

In the view of the law of Lambert-Beer, the formula to measure the chlorophyll-a/b concentration through a spectrophotometer is given below (see formula (1)). Moreover, at 80% acetone solution, the k constant of the light absorbance will be shown in the table:

Table. 2 *showing the k constant of the light absorbance in chlorophyll a and b.*

Wavelength (nm)	Chlorophyll-a	Chlorophyll-b
663	**82.04**	**9.27**
645	**16.75**	**45.6**

Accordingly, the formula can be conducted:

$$OD_{663} = 82.04 \times C_a + 9.27 \times C_b$$
$$OD_{645} = 16.75 \times C_a + 45.60 \times C_b$$

Where Chl refers to chlorophyll type, and OD refers to the optical density (Light absorbance value). Then the final equation can be conducted to determine the chlorophyll content:

$$Chl_a = (12.7(OD_{663}) - 2.69(OD_{645})) \times V \times W$$
$$Chl_b = (22.9(OD_{645}) - 4.68(OD_{663})) \times V \times W$$

Where V represents the volume of soliton of chlorophyll extract(5mL) in each of the samples used in the spectrophotometer) used in the spectrophotometer. W was the mass of leaf materials used, so W is 0.5g.

Sample of calculation of trial 1 of 0.0ppm urea exposure on *C. thalictroides.*

Chl_a	Chl_b
$Chl_a = (12.7(OD_{663}) - 2.69(OD_{645})) \times V \times W$ $Chl_a = (12.7(1.607) - 2.69(1.362)) \times 5 \times 0.5$ $Chl_a \approx 41.863\ mg/mL$	$Chl_b = (22.9(OD_{645}) - 4.68(OD_{663})) \times V \times W$ $Chl_b = (22.9(1.362) - 4.68(1.607)) \times 5 \times 0.5$ $Chl_b \approx 59.173\ mg/mL$
Calculation of uncertainty	
As the error percentage of uncertainty of light absorbance is $\pm 0.1\%$*, the formula for* Chl_a *is:* $Chl_a = (12.7(OD_{663}) - 2.69(OD_{645})) \times V \times W$ *Therefore, error in absorbance should be multiplied through replacing OD value, where the result can be written as* $(12.7 \times 0.1\% + 2.69 \times 0.1\%) = \pm 0.0153$	*As the error percentage of uncertainty of light absorbance is* $\pm 0.1\%$*, the formula for* Chl_b *is:* $Chl_b = (22.9(OD_{645}) - 4.68(OD_{663})) \times V \times W$ *Therefore, error in absorbance should be multiplied through replacing OD value, where the result can be written as* $(22.9 \times 0.1 + 4.68 \times 0.1\%) = \pm 0.0276$
Standard deviation (See table 3 and 4)	
According to Table 3 and 4(see in the below), the mean of each trials per in one concentration is: *Mean* $= \frac{Sum}{Number} = \frac{41.86 + 53.0 + 56.95 + 19.47 + 25.76}{5} = 39.41$ *Then, the mean can be used to calculate the standard deviation of the data through the formula:* *SD formula :* $\sigma = \sqrt{\frac{1}{N}\sum_{i=1}^{N}(X_i - \mu)^2}$ $= \sqrt{\frac{1}{5}\sum_{i=1}^{5}(X_i - 39.41)^2}$ $= \sqrt{\frac{1}{5}((41.86 - 39.41)^2 + (53 - 39.41)^2 + \ldots + (25.76 - 39.41)^2)}$ $= \sqrt{\frac{1}{5}(2.45^2 + 13.59^2 + 17.54^2 + 19.94^2 + 13.64^2)}$ $=14.71$	*According to Table 3 and 4(see in the below), the mean of each trials per in one concentration is:* *Mean* $= \frac{Sum}{Number} = \frac{59.17 + 90.17 + 96.72 + 29.25 + 37.37}{5} = 62.54$ *Then, the mean can be used to calculate the standard deviation of the data through the formula:* *SD formula :* $\sigma = \sqrt{\frac{1}{N}\sum_{i=1}^{N}(X_i - \mu)^2}$ $= \sqrt{\frac{1}{5}\sum_{i=1}^{5}(X_i - 62.54)^2}$ $= \sqrt{\frac{1}{5}((59.17 - 62.54)^2 + (90.17 - 62.54)^2 + \ldots + (37.37 - 62.54$ $= \sqrt{\frac{1}{5}(2.45^2 + 13.59^2 + 13.59^2 + 34.18^2 + 25.17^2)}$ $=30.35$

Table.3 *showing chlorophyll-a content (mg/mL) changed by different urea concentration (ppm).*

Urea concentration (ppm)	**chlorophyll-a content** ($mg/mL \pm 0.0153$)						
	Trial 1	**Trial 2**	**Trial 3**	**Trial 4**	**Trial 5**	**Mean**	**SD**
0.00	41.86	53.01	56.95	19.47	25.76	39.41	14.71
2.00	56.53	45.68	57.17	50.52	58.73	53.73	53.73
4.00	56.36	52.99	47.46	55.12	58.79	54.15	54.15
6.00	56.56	55.68	55.40	53.62	56.74	55.60	55.6
8.00	48.18	51.59	51.44	54.49	50.39	51.22	51.22
10.00	49.10	26.56	53.91	36.90	46.67	42.63	42.63

Table.4 *showing chlorophyll-b content (mg/mL) changed by different urea concentration (ppm).*

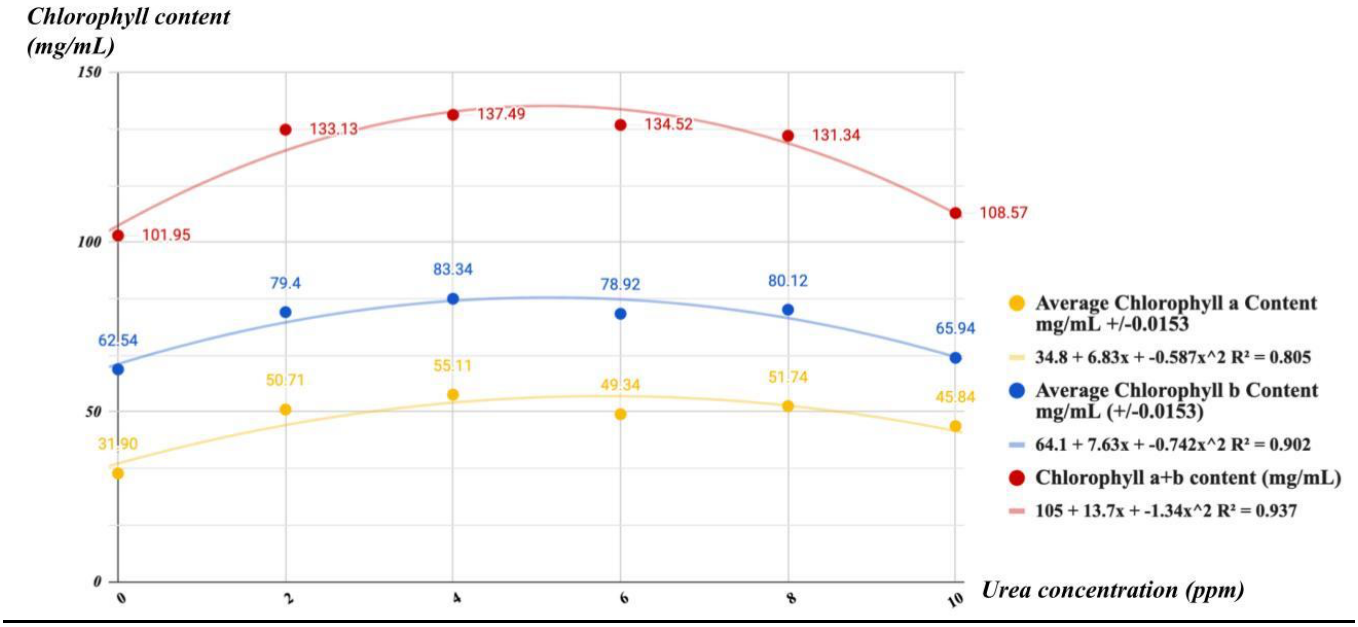
Urea concentration (ppm)	**chlorophyll-b content** ($mg/mL \pm 0.0276$)						
	Trial 1	**Trial 2**	**Trial 3**	**Trial 4**	**Trial 5**	**Mean**	**SD**
0.00	59.17	90.17	96.72	29.25	37.37	62.54	30.35
2.00	90.46	61.05	87.93	66.17	91.41	79.40	14.59
4.00	91.68	78.73	68.12	86.22	91.93	83.34	10.06
6.00	82.73	76.81	78.39	71.82	84.86	78.92	5.12
8.00	77.82	79.25	78.97	90.29	74.27	80.12	6.02
10.00	67.68	39.38	86.66	63.19	72.79	65.94	17.26

Therefore, through calculating all the chlorophyll-a/b content in *C. thalictroides e*xposed with different amounts of urea for each trial, we can get the following results in the above Table 3 and 4. Moreover, I used google sheet to insert charts (Graph 1 and 2) in order to illustrate the relationship between chlorophyll-a and chlorophyll-b content and urea exposure more clearly. However, based on the research question, the chlorophyll content refers to both chlorophyll-a and chlorophyll-b, which is illustrated from Table 5 and Figure 3.

Table. 5 *showing total chlorophyll content(chlorophyll a+b) (mg/mL) changed by different urea concentration (ppm).*

Urea concentration (ppm)	**0**	**2**	**4**	**6**	**8**	**10**
chlorophyll-a+b content (mg/mL)	**101.95**	**133.13**	**137.49**	**134.52**	**131.34**	**108.57**

Graph.1. showing the relationship between chlorophyll content and urea exposure



Chlorophyll-a (yellow line)

Specifically, chlorophyll-a is the primary pigment of photosynthesis, trapping the light energy and emitting high energy electrons into the two photosystems P680 and P700 (Lakna, 2017). In Graph 1, when urea concentration increases from 0 ppm to 4 ppm, the chlorophyll concentration also increases from 31.90 mg/mL to 55.11 mg/mL. However, the supposed highest point (6, 49.34)was the outlier which is out of the line of best fit in the graph due to the percentage error. Later, chlorophyll-a content fell from 51.74 mg/mL to 45.84 mg/mL when urea concentration increased from 8 ppm to 10 ppm. Additionally, the function equation: $y= - 0.58 x^2 + 6.83x + 34.8$ can be converted to vertex form: $y = - 0.587(x - 5.818)^2 + 54.67$, so it's obvious that the highest chlorophyll-a concentration will be 54.67 mg/mL when exposed to 5.818 ppm urea. Therefore, when *C. thalictroides* was propagated in an interval of 0 to 5.818 ppm urea, the chlorophyll-a in *C. thalictroides* wase progressively active, and *C. thalictroides* grew better due to more active photosynthetic and metabolic activity (increasing metabolic activities to synthesis protein for photosynthesis).

Therefore, the Alternate Hypothesis (H_1) is accepted in the relationship between chlorophyll-a (mg/mL) and urea concentration (ppm).

Chlorophyll-b (blue line)

Chlorophyll-b in *C. thalictroides* is the accessory pigment that collects the sunlight and then passes into the chlorophyll-a (Lakna, 2017), so *C. thalictroides* having more chlorophyll tend to be more adaptive even without sunlight. With reference to Graph 1, the quadratic relationship between chlorophyll-b content and urea concentration indicates that chlorophyll-b concentration increases from 39.41mg/mL to 55.6 mg/mL when urea concentration increases from 0 ppm to 6 ppm. However, when urea concentration is higher than 6 ppm, increasing urea concentration (from 6 ppm to 10 ppm) will have successive adverse effects on the concentration of chlorophyll-b. With reference to the quadratic equation: $y = - 0.597x^2 + 6.11x + 40.8$, its vertex form can be written as $y = - 0.597(x - 5.117)^2 + 56.43$, which indicates that there will be the highest chlorophyll-b concentration (56.43 mg/mL) when exposed to 5.117 ppm urea. When urea concentration increases from about 5.117 ppm, it will also have a successively adverse effect on chlorophyll-b activity in *C. thalictroides.* Compared with chlorophyll a, it is clear that both functions share almost the same relationship between two variables.

Hence, the Alternate hypothesis(H_1) is accepted in the relationship between chlorophyll-b (mg/mL) and urea concentration (ppm).

R-square Test: In both equations showing chlorophyll a and b, they illustrate a quadratic relationship between chlorophyll-a content and urea concentration where chlorophyll-a content was the highest round 6 ppm concentration. Moreover, R-squared is a statistical measure of fit that indicates how much variation of a dependent variable is explained by the independent variable(s) in a regression model. In other words, r-squared shows how well the data fit the regression model (the goodness of fit) (CFI, 2022). Specifically, R^2 in both equations are 0.805 and 0.983 respectively, and they are both close to 1 (An R^2 =1 indicates a perfect fit). To elaborate, the actual chlorophyll b collected fits the theoretical hypothesis perfectly, as 0.983 can be extremely close to 1. However, the chlorophyll a value collected is less close to its theoretical value as chlorophyll b collected is.

Standard Deviation (SD): With reference to both Table 3 and 4, the standard deviation of the raw data is calculated. As the standard deviation (SD) measures how far the data is from the mean, it gives an insight of whether the data in one condition is spread out or not. In Table 3, the standard deviation is quite higher in a range of 14.71 to 55.6, which means that the data under these conditions were actually remarkably away from the mean. Compared with Table 4, we can see that the standard deviation value is in the range of 5.12 to 30.15, which shows that the data accuracy and precision are higher than Table 5. However, they both have standard deviation values more than 5, which might be caused by uncertainty and error during physical operations (See in 7.2 limitation and improvement.)

Chlorophyll a and b (Red line)

Combining the effect of urea exposure on both chlorophyll a and b in *C. thalictroides*, Graph 1 together, Graph 1 can directly illustrate the relationship between chlorophyll content and urea concentration. It also indicates a quadratic relationship between urea concentration (ppm) and Chlorophyll content (mg/mL). It indicated a positively upward trend in average chlorophyll-a+b content (mg/mL) in *C. thalictroides* as urea concentration increases from 2 to 4 ppm, where the average chlorophyll content was the highest (137.49 mg/mL). However, as urea concentration increased from 6 ppm to 10 ppm, there was a negatively downward trend in average chlorophyll content (mg/mL), where average chlorophyll concentration was the highest (134.52) at 6 ppm urea and was the lowest (108.57) at 10 ppm urea. Based on the research question that aims to investigate the relationship between the urea exposure concentration (ppm) (IV) and change in the chlorophyll content a + b (mg/mL) (DV), we can know that chlorophyll content in *C. thalictroides* will be the highest (140 mg/mL) at (5.112 ppm) urea concentration through converting the equation in Graph 1 to a vertex form: $y = -1.34(x - 5.112)^2 + 140$. At an optimal urea concentration (about 5.112 ppm), chlorophyll content will be the highest (140 mg/mL). Through data interpretation, we can now see that chlorophyll a and b content are the highest at 5.818 ppm and 5.117 ppm urea respectively. The total chlorophyll content will reach the highest when exposed to 5.112 ppm urea, which is close to the optimal urea amount in chlorophyll. Consequently, the optimal urea concentration for photosynthetic activity in *C. thalictroides* is about 5 ppm to 6 ppm.

R-square Test: As the chart depicts that almost all the data points are located in the quadratic function, it is proved by the high correlation coefficient, which is close to 1. R-square is 0.937, indicating that the actual data is highly correlated to the theoretical one, which proves the accuracy and precision of data collection.

Conclusion:

Alternate hypothesis (H_1) is accepted and the null hypothesis (H_0) is rejected. All values of R squares (>0.8) have shown that there is a strong correlation between independent and dependent variables.

As an autotroph and a fern species, *C. thalictroides* can process nitrate with urease without use of UAL-ase (ATP Urea amidolyase) (Bekheet, I.A.& P.J. Syrett. 1977). Based on the previous surveys, nitrate will effectively increase the crop production, total crop leaf area and the amount of chlorophyll-and protein(including ribulose bisphosphate carboxylase-oxygenase, RuPBc-o) per unit area of leaves and the capacity of photosynthesis, particularly in bright light and with abundant CO_2 (Macnab, 1987). With reference to Graph 1, we can assume that there was a successive adverse effect when exposed to more urea due to the DNA damage brought by high nitrate levels in the urea (a reduction of chlorophyll content after exposure of more than 5.818 ppm urea). It is similar in chlorophyll b. It is acceptable to mention that higher urea concentration might lead to nutritional overload, resulting in a decline in chlorophyll content. Otherwise, according to literature, N-deficiency not only causes change in Chl a/cell and major reduction in phycobilin content (Allen & Smith

1969; Boussiba & Richmond 1980, Yamanaka & Glazer 1980, de Loura et al. 1987) but also affects the enzyme of photosynthetic carbon metabolism and a decline in Rubisco per cell is well established (Kupper & Weidner 1980; Lapointe & Duke 1984; Falkowski et al. 1989; Plumley& Schmidt 1989; Beardall 1991).
Consequently, higher urea concentration in the range of 0 to 5 ppm is acceptable to promote photosynthesis activities in the chlorophyll a/b in *C. thalictroides,* but the excess urea exposure will have adverse effect on plants photosynthesis activities. Therefore, too much nitrate pollution will also harm ecological biodiversity and aquatic organisms.

7. Evaluation

7.1. Strengths:

- **Easy and clear approaches of materials and apparatus**: Procedure is clear, concise and easy to understand due to the simple methodology and uncomplicated materials and chemicals required. Moreover, it's easy to operate due to the availability of apparatus needed and the notion that it can be executed with materials, hence the process of getting the data of chlorophyll content through the law of Lambert-beer is clear and straightforward.
- **Low value of uncertainty and good R-squared test result:** the errors are minimized by repeating the trials 5 times to ensure the validity and reliability of the experiment and increasing the precision to 3 places. We also consider the error and uncertainty of each apparatus which might affect the result. It means that there is a low error and low scope of random uncertainty in the procedure due to multiple steps, conversion and measurements. Moreover, there is a strong correlation between independent and dependent variables as R^2 are all super close to 1(An R^2 =1 indicates a perfect fit), suggesting that the final results support my hypothesis and the data is reliable.
- **Controlled variables are kept constant throughout the experiment:** All the samples are propagated under the same conditions regardless of the amount of urea exposure.
- **High replicability of the experiment:** The experiment is replicable (or repeatable) because when the entire research process is conducted again, using the same methods but new data, it still yields the same results. This will happen because our data are highly consistent shown by the R-squared test and our results are highly accurate due to low value of uncertainty.

7.2. Limitation and improvement:

Limitation	Improvement
The data collected from the experiment relies on a large portion of human input which in turn creates a large capacity such as reading the values and transferring the liquids to different containers during measurement.	We could improve it by adding 10 more trials per urea concentration to make the processed data more accurate.
Rather than 80% acetone, I used Sally Hansen Acetone free Polish Remover, which contains other chemical ingredients (e.g. panthenol, ethoxydiglycol, ppg-12-PEG-50 lanolin, tocopheryl acetate, benzophenone-3, parfum, hydrolyzed wheat protein,etc.) It might affect the extraction of chlorophyll.	80% to 90% acetone is strongly suggested for the extraction of chlorophyll from algae. Tender leaf tissue in 80% buffered acetone at 4°C gives higher yield of pigments compared to other methods. The use of acetone to extract chlorophyll by incubation is found to be superior to methods in extraction of pigments.
Some extracts collected were from different positions of *C. thalictroides* (e.g, stems, leaves and roots), resulting in different initial chlorophyll contents affecting the final results. E.g. There was usually most photosynthesis activities happening in leaves, which contained most chlorophyll content before the propagation in different nitrate exposure.	It could be controlled through only collecting and extracting the chlorophyll from leaves instead of other parts of *C. thalictroides.* In order to achieve it, more *C. thalictroides* can be propagated to extract leaves.

7.3. Extensions

My research subject is algae, but I only chose *C. thalictroides*, a fern species that can grow with urea as a nitrogen source containing urease but not UAL-ase (ATP Urea amidolyase). I dismissed the effect of urea exposure on other members of class that can utilize nitrogen also with UAL-ase (ATP Urea amidolyase). This essay can be further extended to a wider range of microalgae organisms that utilize UAL-ase (ATP Urea amidolyase) to possess nitrogen. E.g.Volvocales, Chlorococcales and ChaetophoralesVolvocales, Chlorococcales and Chaetophorales.

Reference

Allen, John F., et al. "A Structural Phylogenetic Map for Chloroplast Photosynthesis." Trends in Plant Science, vol. 16, no. 12, 2011, pp. 645–655., https://doi.org/10.1016/j.tplants.2011.10.004.

Bekheet, I.A., and P.J. Syrett. "Urea-Degrading Enzymes in Algae." *British Phycological Journal*, vol. 12, no. 2, 1977, pp. 137–143., https://doi.org/10.1080/00071617700650151.

Camargo, Julio A., et al. "Nitrate Toxicity to Aquatic Animals: A Review with New Data for Freshwater Invertebrates." Chemosphere, vol. 58, no. 9, 2005, pp. 1255–1267., https://doi.org/10.1016/j.chemosphere.2004.10.044.

"Ceratopteris Thalictroides." *Wikipedia*, Wikimedia Foundation, 14 Feb. 2022, https://en.wikipedia.org/wiki/Ceratopteris_thalictroides.

Douglas. E Greer & Charles D. Ziebell. "Biological Removal of Phosphates from Water ". *Water Pollution Control Federation,* Vol. 44, No. 12 (Dec., 1972), pp. 2342-2348 2015. http://www.jstor.org/stable/25037688

Flores, Enrique, et al. "Photosynthetic Nitrate Assimilation in Cyanobacteria." Photosynthesis Research, vol. 83, no. 2, 2005, pp. 117–133., https://doi.org/10.1007/s11120-004-5830-9.

Galván, Aurora, et al. "Nitrite Transport to the Chloroplast in Chlamydomonas Reinhardtii: Molecular Evidence for a Regulated Process." Journal of Experimental Botany, vol. 53, no. 370, 2002, pp. 845–853., https://doi.org/10.1093/jexbot/53.370.845.

Gupta, Manisha, et al. "Effects of Long-Term Low-Dose Exposure to Cadmium during the Entire Life Cycle of Ceratopteris Thalictroides, a Water Fern." Archives of Environmental Contamination and Toxicology, vol. 23, no. 2, 1992, pp. 184–189., https://doi.org/10.1007/bf00212273.

Lakna, "Difference between Chlorophyll a and b" 2017, https://www.researchgate.net/profile/Lakna-Panawala/publication/316584030_Home_Science_Biology_Cell_Biology_Difference_Between_Chlorophyll_A_and_B_Difference_Between_Chlorophyll_A_and_B_Main_Difference_-_Chlorophyll_A_vs_Chlorophyll_B/links/59054d0b0f7e9bc0d58fb91b/Home-Science-Biology-Cell-Biology-Difference-Between-Chlorophyll-A-and-B-Difference-Between-Chlorophyll-A-and-B-Main-Difference-Chlorophyll-A-vs-Chlorophyll-B.pdf

Li, Zhenghong, et al. "Characteristics, genesis and treatment measures of NO3 type groundwater in Xiamen, Fujian Province" 2021. http://geochina.cgs.gov.cn/html/2021/5/20210510.htm

Martin Lorin, "What are the roles of chlorophyll a and b?", Sciencing, 2019 https://sciencing.com/what-are-the-roles-of-chlorophyll-a-b-12526386.html

Matthew, Tracy, et al. "The Effect of Nitrates on Algae Growth" n.d https://undergradsciencejournals.okstate.edu/index.php/jibi/article/view/2396

McIntyre, G. I. "The Role of Nitrate in the Osmotic and Nutritional Control of Plant Development." *Functional Plant Biology*, vol. 24, no. 2, 1997, p. 103., https://doi.org/10.1071/pp96064.

"Nitrate Assimilation: Nitrate and Nitrite Reductases." Nitrogen Assimilation by Plants, 2001, pp. 55–74., https://doi.org/10.1201/9781482279849-11.

Turpin, David H. "Effects of Inorganic n Availability on Algal Photosynthesis and Carbon Metabolism." Journal of Phycology, vol. 27, no. 1, 1991, pp. 14–20., https://doi.org/10.1111/j.0022-3646.1991.00014.x.

Tseng, Mei-Hwei, et al. “Detection of Chlorophylls in Spores of Seven Ferns.” Journal of Plant Research, vol. 130, no. 2, 2017, pp. 407–416., https://doi.org/10.1007/s10265-016-0901-5.

Yu, Guimei, et al. “The Analysis of Groundwater Nitrate Pollution and Health Risk Assessment in Rural Areas of Yantai,China.” 2020, https://doi.org/10.21203/rs.2.20392/v2.