Topic: Effect of Nitrogen Limitation on the Lipid Productivity of *Chlorella vulgaris*

Research Question:

This experiment investigated the research question: What is the optimal nitrogen concentration (provided by NO_3^-) for the growth of *Chlorella vulgaris* microalgae to maximize the lipid yield measured by the mass of lipids per liter culture?

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Introduction

Microalgae species such as *Chlorella vulgaris* have become promising carbon-neutral oil sources in the last few years. The harvested lipids from microalgae can be used to make biodiesel. It has been discovered that the lipid productivity of these species is much higher than those of traditionally used vascular plants or oil seeds. Expanding from the notion that fossil fuels are the biggest source of greenhouse gas emissions today and that worldwide fuel demand is projected to increase by 40% in the next 20 years (ExxonMobil, 2013), it can be concluded that an alternative carbon-neutral source of fuel must be investigated. The advantages of algae-based biodiesel lie in the high lipid productivity of microalgae due to their simple unicellular structure. The disadvantage of algae-based biodiesel is that its production is still not economically viable (Tsukahara and Sawayama 2005). With lipid yield optimizations in growth methods and genetic manipulation, the idea of microalgae-based fuels stands firm for the future. The hope to help humanity by improving carbon-neutral fuels has been the main motivation of this investigation.

Microalgae also play an important role in the carbon cycle and oceanic ecosystems as well. They are primary producers and are responsible for approximately half of the oxygen production in the world (Falkowski et al., 2003). They have been fixing carbon from the atmosphere for 2.7 billion years (Falkowski et al., 2004) and the petroleum exploited by us today is the fossilized form of these organisms. We are burning hydrocarbons synthesized

millions of years ago. Today we “borrow land from the past” (Wackernagel & Yount, 1998) by using carbon which was fixed in another era.

The microalgae species *Chlorella vulgaris* accumulates lipids under low nitrogen concentrations at the loss of biomass productivity (Griffiths et al., 2014); (Converti et al., 2009). It has been this tradeoff between lipid accumulation and biomass productivity that has inspired this investigation.

The aim has been to find the optimal nitrogen concentration where total net lipid productivity is at maximum. The key motivation has been that, any knowledge to improve lipid yield from these organisms is a step closer to finding a viable alternative to fossil fuels.

Background Information

Morphology Of *Chlorella vulgaris*

Chlorella vulgaris (Fig. 1.) is a phytoplankton of the kingdom *Plantae*, division *Chlorophyta*, *class Trebouxiophyceae*, order *Chlorellales*, family *Oocystaceae* and genus *Chlorella*. It is a spherical cell with a diameter of 2-10µm and has many similarities to plants.

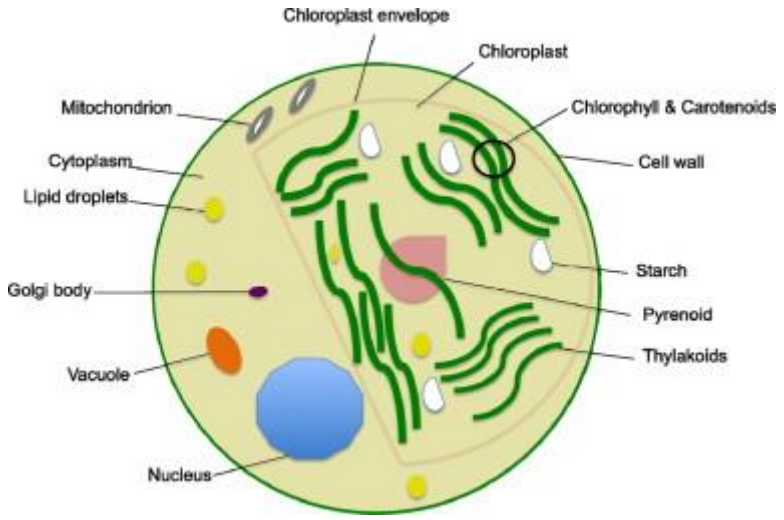


Fig. 1. Schematic structure of *Chlorella vulgaris* representing different organelles (Safi et al., 2014)

The cell wall is rigid which preserves the integrity of the cell and is basically a protection against invaders and harsh environments. This is one of the factors why *C. vulgaris* can live in a great span of environments and is resilient against changes in its environment. The cytoplasm hosts internal organelles of the organism like mitochondria, a small nucleus, vacuoles (Kuchitsu et al., 1987), a single chloroplast, and the Golgi body. *C. vulgaris* has a single chloroplast with a double membrane-bound structure. During nitrogen stress (when there is limited nitrogen in the environment), most of the lipid molecules accumulate at the chloroplast and some in the cytoplasm.

Reproduction of *Chlorella vulgaris*

C. vulgaris is an autospore meaning that the organism is a non-motile cell where the daughter cells are created from the division of the mother cell rapidly. Within 24 hours a cell of *C. vulgaris* grown in optimal conditions reproduces by autosporulation. Thus, 4 new daughter cells with their own cell walls are produced inside the cell wall of the mother cell. Eventually, as the daughter cells mature they rupture the cell wall of the mother cell and are liberated as represented in figure 2 (Safi et al., 2014).

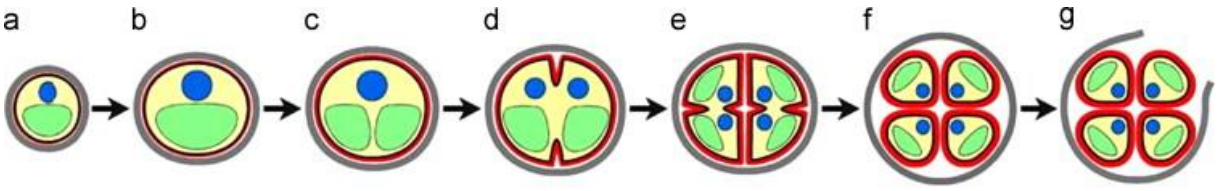


Fig. 2. Drawings showing the different phases of daughter cell-wall formation in Chlorella vulgaris: (a) early cell-growth phase; (b) late cell-growth phase; (c) chloroplast dividing phase; (d) early protoplast dividing phase; (e) late protoplast dividing phase; (f) daughter cells maturation phase and (g) hatching phase (Yamamoto et al., 2005).

This rapid asexual reproduction makes *C. vulgaris* a good candidate for industrial purposes because of the high yield per time ratio. One disadvantage could be the lack of diversity within the population due to mitotic division within asexual reproduction.

Primary Composition of *Chlorella vulgaris*

Proteins:

They are a crucial part of this organism's composition because they are involved in vital roles such as the growth, repair, and maintenance of the cell. They also work as chemical messengers, regulators of cellular activities, and defense against foreign invaders. Proteins, make up approximately 42-58% of the biomass dry weight (Servaites et al., 2012). The percentage of protein changes according to growth conditions, especially nitrogen (N) concentration because N is a key element in the structure of amino acids. According to (Griffiths et al., 2014) *C. vulgaris* cells at high levels of N (2500 mg. L^{-1}) compose up to 50% of proteins, and at low levels of N (0 mg. L^{-1}) compose 10 % of proteins. In another aspect, the amino acid profile of the *C. vulgaris* proteins compares positively to a greater extent better with the standard profile for human nutrition proposed by the World Health Organization showing also potential as a candidate for an alternative plant-based food source (Safi et al.,2014).

Lipids:

Lipids are a mixed group of compounds that are soluble in non-polar solvents and relatively insoluble in water. In the cell, the majority of the lipid content acts as energy storage in the form of condensed triacylglycerol (TAG) droplets in the cytoplasm or the chloroplast. Just like protein content the percentage of lipids changes according to growth conditions which is actually the scope of this investigation. As previously mentioned *Chlorella vulgaris* accumulates lipids at nitrogen (N) limitation (Converti et al., 2009). So at lower N concentrations, a high percentage of lipids; and at high N concentrations, a low percentage of lipids can be seen. Lipids extracted from *C. vulgaris* can be used to make biodiesel, giving them importance among the other compounds present in the organism. Aside from biodiesel applications, the lipid profile of *C. vulgaris* is favorable for the human diet according to the World Health Organization (Safi et al.,2014).

Carbohydrates:

Carbohydrates are composed of a group of reducing sugars and polysaccharides like starch and cellulose. Starch is the most common polysaccharide in *C. vulgaris* and is usually situated inside the chloroplast organelle; it is used for energy storage. Cellulose is part of the cell wall just like in many plants and algae and acts as a protective shield for the cell. The number of carbohydrates may vary from 12-55% of the dry weight.

Effect of nitrogen stress on *Chlorella vulgaris*

At nitrogen (N) scarcity in growth conditions, the ability of the organism to synthesize N-containing compounds is compromised. Many of these N-containing compounds are necessary for the growth of the cell and for the organism to reproduce so when the cell can't synthesize these compounds its growth ceases.

Storage lipids which are primarily composed of triacylglycerol (TAGs) provide a dense and effective storage for carbon and energy. Lipids create more energy compared to carbohydrates when oxidized so, lipids present a good stock for biomass production until N becomes available back again (Roessler, 1990). In conclusion the organism starts to accumulate lipids as a survival reaction against N deficient conditions. Lipid accumulation provides a survival storage until N rich conditions are back.

The relation of this knowledge with this investigation is that; the double edged sword, N deficiency ceases growth while increasing lipid percentage. This creates a compromise between organism growth and lipid composition percentage. Thus, as addressed before, the situation poses the question of at which concentration of N the total lipid productivity is at maximum?

Hypothesis

Null Hypothesis: Change in $NaNO_3$ concentration of the growth medium will not affect the lipids per liter culture of *Chlorella vulgaris.*

Alternative Hypothesis: Change in $NaNO_3$ concentration of the growth medium will affect the lipids per liter culture of *Chlorella vulgaris.*

Method

Variables

Independent Variable

The independent variable is the concentration of $NaNO_3$ present in the water in which *C. vulgaris* will be grown in. The values for the different concentrations were decided according to the past studies. According Stephenson et al.,2010 it is stated that the optimal concentration could be around 125-425 mgL^{-1} this range is too small and vague so three values were selected within the range and two were selected to represent the extremes (50 and 600 mgL^{-1}). So the selected $NaNO_3$ concentrations are as follows:

- 50 mgL^{-1}
- 100 mgL^{-1}
- 150 mgL^{-1}
- 300 mgL^{-1}
- 600 mgL^{-1}

Dependent Variable

The dependent variable will be the calculated lipids mass per liter culture. That is, the mass of the lipids contained within a liter of the algae culture. For the calculation of lipids mass per liter culture (volumetric lipid concentration) the dry biomass concentration and lipids per dry biomass concentration is measured.

Controlled Variables

Controlled variable	How the variable affects results	How the variable will be controlled
The concentration of nutrients other than $NaNO_3$ ($CaCl_2$, $MgSO_4$, K_2HPO_4 and NaCl)	Changing the concentration of these nutrients affects the general metabolism and lipid production of *C.vulgaris*	A growth medium called Bold's Basal Medium (BBM) which has become the literary standard for microalgae research was used in every culture (except concentration of $NaNO_3$ because it is the independent variable)
Starting culture population density and culture age	*C.vulgaris* cultures have different growth tendencies according to their culture population density.	The same mother culture was divided equally within all the growth tanks
Light intensity and light exposure time and light wavelength	Light has an effect on photosynthesizing organisms and affects the rate of photosynthesis thus the production of lipids and other compounds	The same led light fixtures were used on every culture (30W led light bulbs with 2500K wavelength) was used and the same light exposure period (12 hours) was applied for every culture so that they get the same amount of light.
Temperature	Temperature affects the enzymatic processes within the cell and thus its metabolism which in turn also affects lipid productivity. Temperature also affects the rate of photosynthesis.	All of the cultures were grown in the same room which provides equal temperature for all the cultures. The temperature of the room was approximately around 25℃.
Quantity of dissolved oxygen and carbon dioxide	Photosynthesizing organisms use carbon dioxide to photosynthesize and oxygen for respiration so a change in these variables affects the metabolism and rate of photosynthesis of the organism thus the number of lipids.	Dissolved gasses were provided by an air pump aerating the cultures constantly. This provides the same quantity of carbon dioxide and oxygen to dissolve from the air into the cultures.
Method for determining the quantity of lipids present in the culture	To find lipids present in organisms a lipid extraction must be done. There are different lipid extraction methods with different chemical processes so the method of lipid extraction changes the yield of lipids from the extraction	A modified lipid extraction method that has become a literature standard for microalgae research called the modified Bligh and Dyer method (Jensen, 2008) was used on every sample.

Table 1: showing the controlled variables, how the variables affect the results, and how the variables will be controlled in the experiment

Apparatus

The cultures with 5 differing $NaNO_3$ dilutions were grown in 25 L clear plastic tanks and the experiment was done in triplets thus making a total of 15 tanks (5x3=15). Every culture had the same 30W led lamps and all the cultures were aerated at the same rate 15L per hr. constantly by a central aquarium air pump. Materials used can be listed as:

- 15x clear-plastic rectangular-shaped 25 L containers with lids to avoid evaporation
- 15x 30W and 2500K (daylight color) led light bulbs
- Central air pump with an air filter and a total 250L per hr. airflow
- 25 m of plastic tubing for the transfer of air from the air pump to the cultures
- An electrical timer switch to adjust the light exposure period of the cultures automatically.
- Chemicals needed for standard BBM solution (refer to the appendix for the composition of BBM)

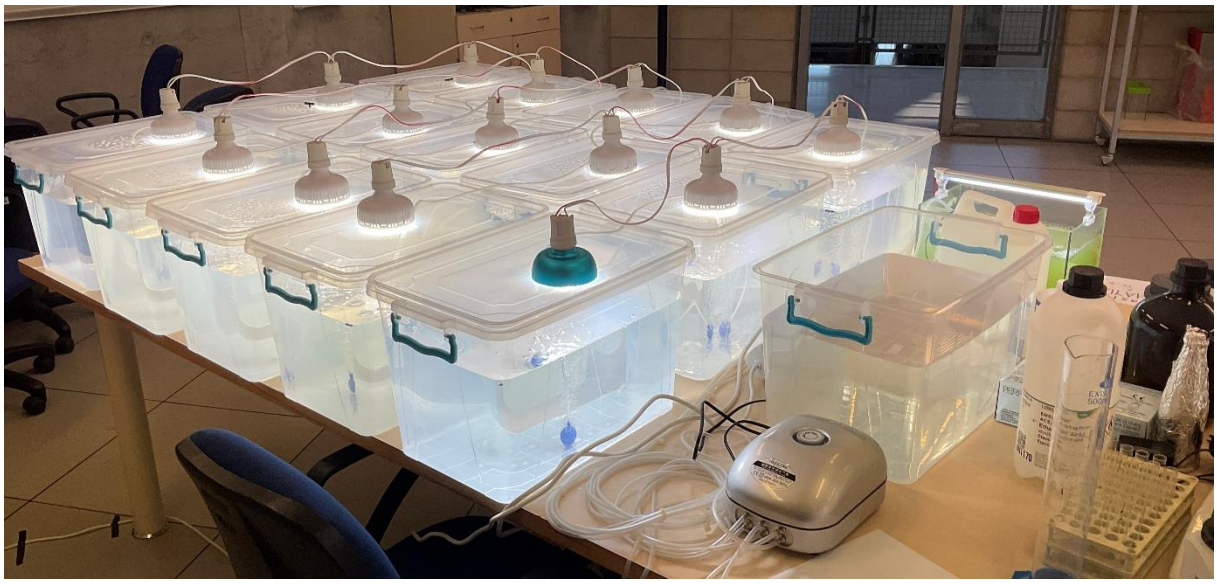


Figure 3: photo of the growth tanks set up, top view

Figure 4: photo of the growth tanks set up, front view

Procedure

The biggest obstacle was designing an experiment in the proportions that high school laboratory-grade equipment could detect. In research, usually smaller samples and higher-grade equipment is used. For example, cultures are grown in 500 ml batches, 10ml samples are taken and analyzed with equipment like photo spectrometers or gas chromatographs. This equipment wasn't available so to compensate for accuracy the scale of the samples was increased and an older chemistry relied method was used for the analysis with simple mass measurement. This posed the difficulty of combining different methods from various articles and creating a plausible method for analysis together with a long phase of preliminary experimentation to develop the method. To sum up, the methods used for growing the starter culture, sampling the culture, analyzing the biomass productivity and lipid productivity of the culture samples is as follows:

1. Growing the cultures

    1.1. All the materials that will be in contact with the cultures are sterilized with ethanol 2 times according to standard sterilization procedure.

    1.2. The growth system is set up with the light bulbs and the air hoses refer to fig.3 and 4 for the layout of the setup.

    1.3. Every growth tank is filled with 25L of fresh distilled water.

    1.4. The chemicals for the BBM solution are added and $NaNO_3$ is added to make differing concentrations of 50, 100, 150, 300 and 600 mgL^{-1}.

    1.5. 2L of the starter culture of *C. vulgaris* is added to every bin.

1.6. The cultures are left to grow 1 week until sampling and an initial sample is taken to give the initial conditions.

2. Sampling the cultures

2.1. 1L water samples are taken from each bin.

3. Analyzing dry biomass of samples

3.1. Mas of a 25-micron filter is measured and noted.

3.2. Samples are vacumm filtrated through the filter paper.

3.3. The filter papers and the alge on them are dried in the oven at 70C for apprx. 30 min or until fully dried.

3.4. The final mass is measured ($M_{final} - M_{filter}$ should give dry biomass of the sample).

4. Analyzing the lipids per dry weight of the cultures

4.1. 100 mg of the dried algae from each sample is taken into 15 ml centrifuge tubes.

4.2. Add 8 ml of chloroform-methanol (2:1) solution to each tube.

4.3. Shake all tubes vigorously at the same time for 2 min. (shaking must be controlled and the same for all samples).

4.4. Centrifuge 15 min 4000rpm.

4.5. Separate the supernatant liquid phase from solid cell debris on the bottom.

4.6. Add 4 ml of 1% NaCl solution and shake.

4.7. Centrifuge at 4000 rpm for 8 min.

4.8. Measure the mass of a test tube.

4.9. Transfer the bottom organic (chloroform layer) to the test tube.

4.10. Place the test tubes in a water bath at 50℃ and wait for the solvent to evaporate

4.11. Measure the final mass of the test tubes, $M_{final} - M_{test\ tube}$ gives the total lipid mass of 100mg algae of that sample.

Results

Raw data

The following Table 2 shows the dry biomasses from 1 L of culture samples over 3 weeks of growth. According to the procedure to find the dry biomasses the masses of the filter papers (Mass of filter paper) and the mass of the filter papers with the algae on them (final mass) are included as well. Dry biomass is the subtraction of filter mass from final mass.

Sample		Week 1			Week 2			Week 3		
$NaNO_3$ concentration (mgL^{-1})	# sampe	Mass of filter paper (mg ±1)	Final mass (mg ±1)	Dry biomass (mg ±1)	Mass of filter paper (mg ±1)	Final mass (mg ±1)	Dry biomass (mg ±1)	Mass of filter paper (mg ±1)	Final mass (mg ±1)	Dry biomass (mg ±1)
50	1	315	791	476	341	826	485	358	843	485
	2	337	780	443	343	843	500	375	863	488
	3	322	801	479	343	841	498	380	864	484
100	1	338	1150	812	323	1323	1000	357	1567	1210
	2	315	1140	825	323	1348	1025	339	1557	1218
	3	304	1127	823	370	1390	1020	362	1575	1213
150	1	328	1195	867	340	1560	1220	382	1857	1475
	2	339	1173	834	370	1630	1260	369	1849	1480
	3	313	1138	825	341	1611	1270	343	1829	1486
300	1	318	1568	1250	353	2078	1725	372	2192	1820
	2	342	1622	1280	361	2161	1800	350	2180	1830
	3	341	1566	1225	376	2156	1780	342	2165	1823
600	1	324	1616	1292	346	2171	1825	370	2195	1825
	2	344	1639	1295	375	2170	1795	345	2178	1833
	3	324	1621	1297	344	2174	1830	368	2196	1828

Table 2: Raw data of the analysis of dry biomass from 1L of culture over a growth period of 3 weeks

Additionally, the dry biomass of the initial mother culture was 150 mg per liter culture.

The following table 3 shows the data from the analysis of the lipids present in 100 mg of the dry *C. vulgaris* biomass. According to the procedure, masses of the test tubes used, the final masses of the test tubes and the mass of lipids are included in the table.

sample		Week 1			Week 2			Week 3		
$NaNO_3$ concentration (mgL^{-1})	# sampe	Mass of test tube (mg ±1)	Final mass (mg ±1)	Lipid mass in 100 mg of algae (mg ±1)	Mass of test tube (mg ±1)	Final mass (mg ±1)	Lipid mass in 100 mg of algae (mg ±1)	Mass of test tube (mg ±1)	Final mass (mg ±1)	Lipid mass in 100 mg of algae (mg ±1)
50	1	9434	9472	38	9392	9442	50	9513	9569	56
	2	9482	9522	40	9340	9398	58	15045	15102	57
	3	9500	9543	43	9512	9564	52	15156	15215	59
100	1	15239	15267	28	9359	9402	43	9341	9393	52
	2	15112	15137	25	15046	15091	45	9359	9409	50
	3	9579	9610	31	9331	9371	40	9330	9384	54
150	1	9483	9508	25	9398	9436	38	9418	9468	50
	2	9371	9394	23	8605	8645	40	8605	8653	48
	3	9687	9711	24	9405	9444	39	9488	9541	53
300	1	9423	9435	12	9493	9511	18	9397	9428	31
	2	9363	9372	9	15160	15176	16	9492	9522	30
	3	9377	9390	13	9440	9456	19	9436	9468	32
600	1	9251	9261	10	9425	9437	12	9433	9442	9
	2	9197	9208	11	9497	9510	13	9406	9413	7
	3	8697	8705	8	9433	9443	10	9432	9442	10

Table 3: Raw data from the analysis of lipids mass present in 100 mg of *C. vulgaris* dry biomass over a growth period of 3 weeks

Additionally, the lipids mass per 100mg of dry biomass of the initial mother culture was 8mg per 100 mg of dry biomass.

Processed data

The experiment was performed in triplets so the average values of the three samples were taken for every concentration through every week.

Example calculation; for 50mg L^{-1} $NaNO_3$ concentration at week 1 the dry biomass average is:

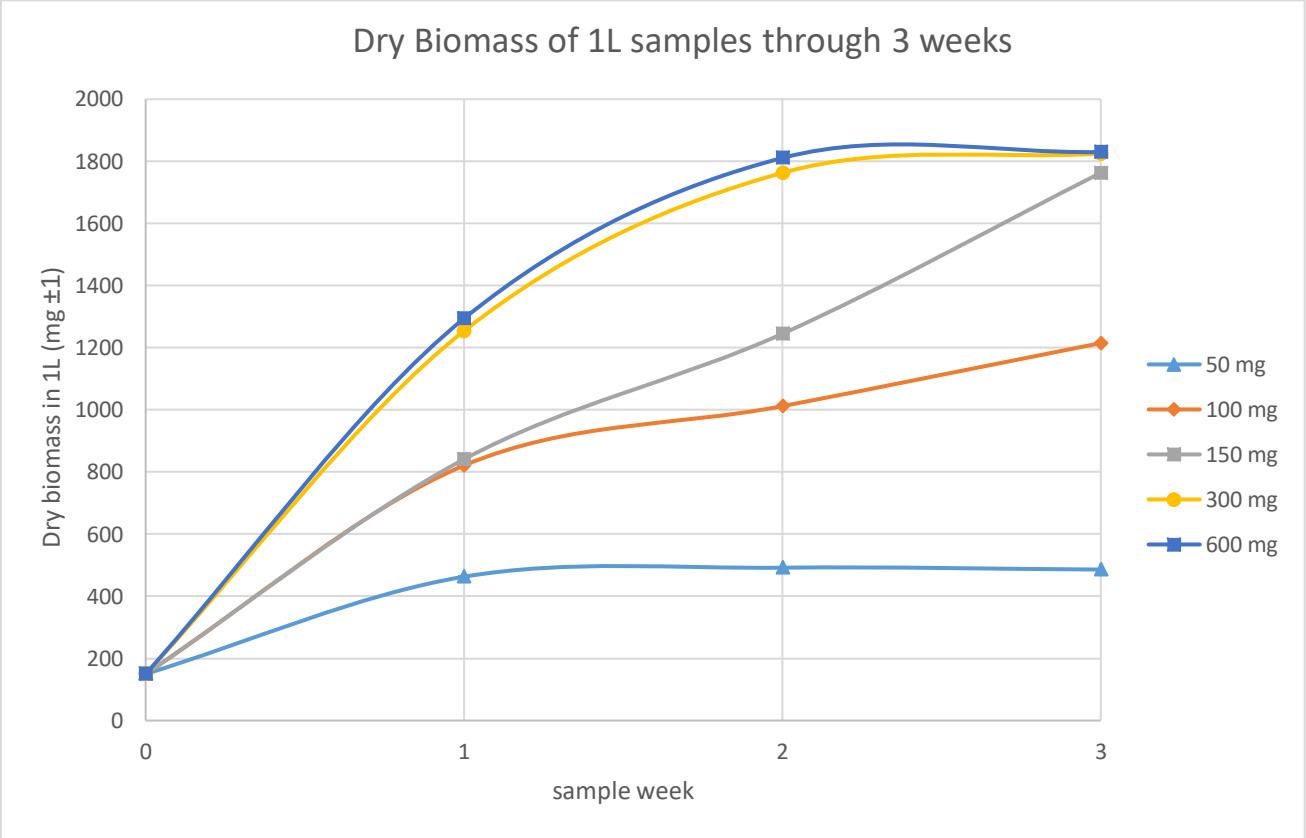
$$\frac{476 + 443 + 479}{3} = 466mg$$

Average values with decimal digits were rounded to integer values. Same process for averages was used to calculate the average values for lipids mass in 100g of dry biomass.

$NaNO_3$ concentration ($mg\ L^{-1}$)	**Week1 Dry biomass (mg ±1)**	**Week2 Dry biomass (mg ±1)**	**Week3 Dry biomass (mg ±1)**
50	**466**	**492**	**486**
100	**821**	**1012**	**1215**
150	**841**	**1245**	**1483**
300	**1254**	**1763**	**1825**
600	**1294**	**1811**	**1830**

Table 4: showing average dry biomass in 1L of algae culture through 3 weeks of growth

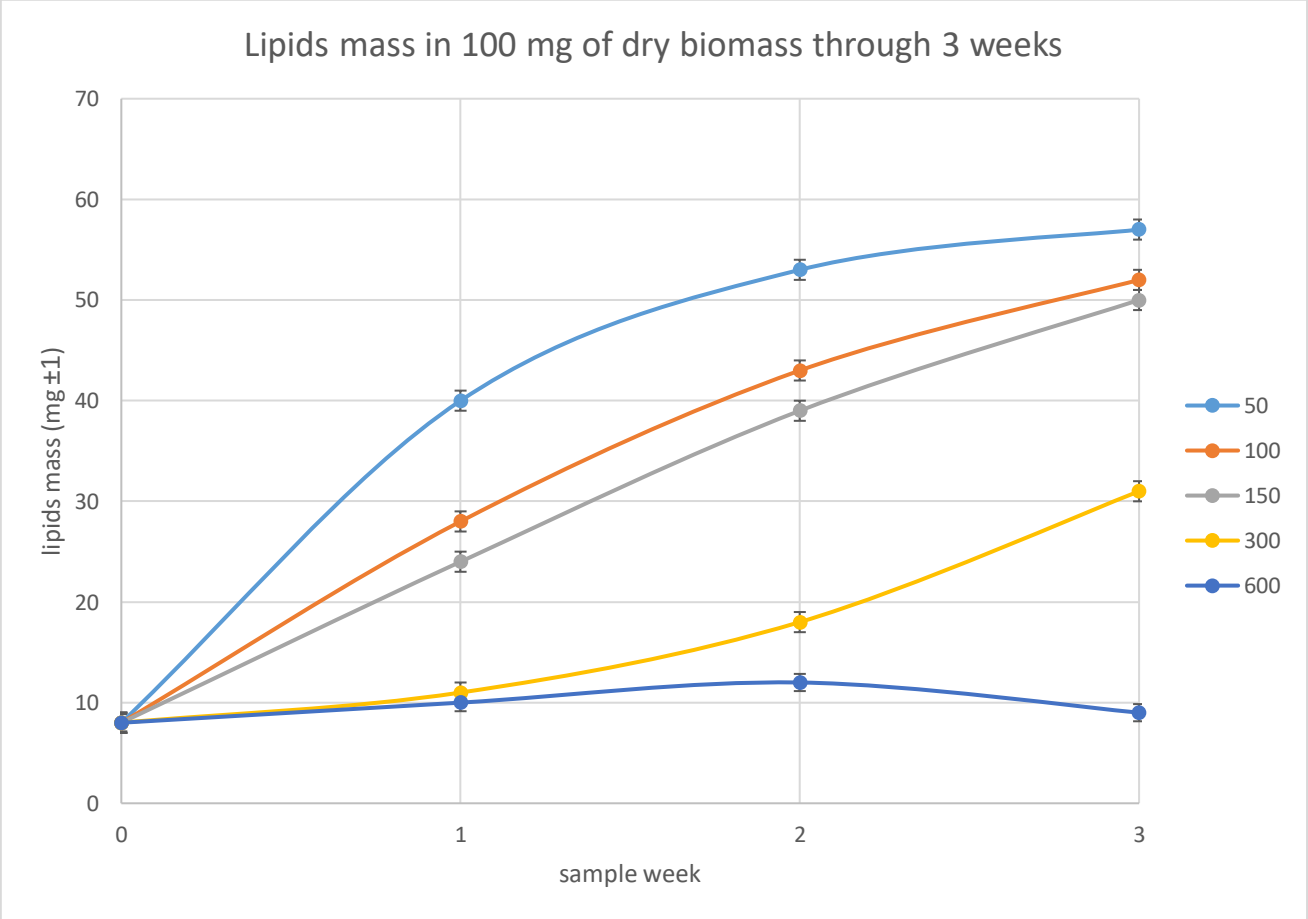
Table 4 was made into a graph (**Graph 1**) to better visualize the progression and trend in the change of biomass through the weeks.



Graph 1: showing the change of biomass in 1L samples of cultures grown in 50, 100, 150, 300 and 600 mg L^{-1} concentrations of $NaNO_3$ through 3 weeks of time

$NaNO_3$ concentration ($mg\ L^{-1}$)	Week1 Lipids mass (mg ±1)	Week2 Lipids mass (mg ±1)	Week3 Lipids mass (mg ±1)
50	40	53	57
100	28	43	52
150	24	39	50
300	11	18	31
600	10	12	9

Table 5: showing average lipids mass in 100mg of dry biomass through 3 weeks of growth



Graph 2: showing the change in lipids mass in 100mg of dry biomass of algae cultures grown in 50, 100, 150, 300 and 600 mg L^{-1} $NaNO_3$ concentrations through 3 weeks

Calculation of the volumetric lipid concentration of the cultures

In order to more clearly see the lipid production of cultures the quantity of lipids produced by given culture volume must be calculated. This has been addressed before as when *C. vulgaris* is in nitrogen stress it increases its lipid concentration within the cell but the overall lipid production of the culture decreases because the biomass almost stays constant. Similarly, when in excess nitrogen *C. vulgaris* reproduces more thus increasing biomass but decreases its lipid concentration within the cell overall decreasing the cultures lipid production. Thus the best way to understand the quantity of lipids in the culture is to calculate the whole culture's lipid concentration. That is the mass of lipids in a liter of the given culture.

To calculate the lipid concentration, the mass of lipids present in 100mg of dry biomass (which is known as in table5) should be scaled to the quantity of dry biomass in a liter of the culture (which is also known as in table4)

$$\frac{lipid\ mass}{100mg\ of\ dry\ biomass} \times \frac{dry\ biomass}{1\ liter\ culture} = \frac{lipid\ mass}{1\ liter\ culture}$$

Example calculation; with 50 mg L^{-1} $NaNO_3$ concentration culture at week1 is:

$$\frac{40\ mg\ lipid\ mass}{100\ mg\ of\ dry\ biomass} \times \frac{463\ mg\ dry\ biomass}{1L\ culture} = 185{,}2\ mg \approx 185mg$$

For this example it could also be thought as: If 40% of the biomass is lipids what is the quantity of lipids in 463mg of biomass?

The answer would be: $\frac{40}{100} \times 463 = 185{,}2$

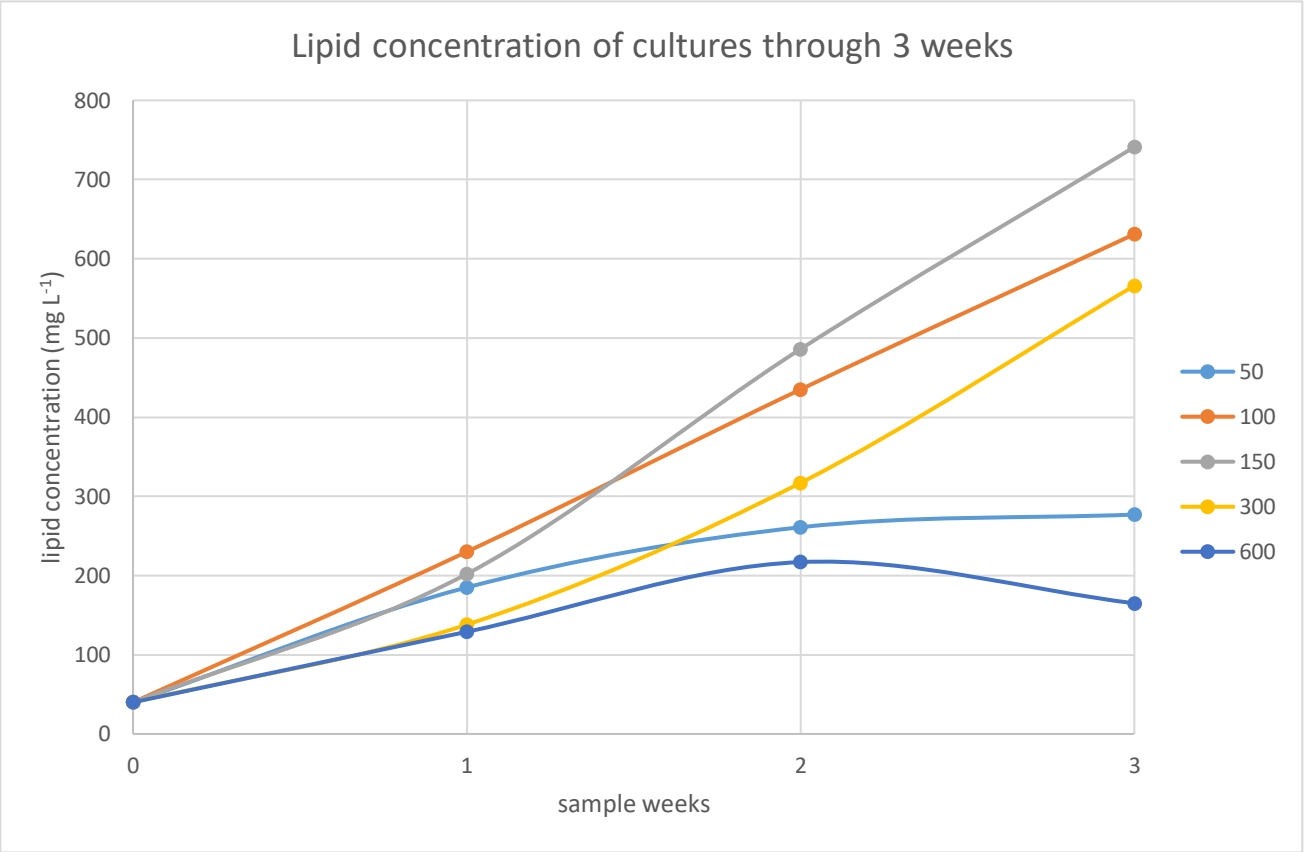
All calculations with decimals were rounded to three significant figures

Table 6 shows the calculated lipid concentrations for the cultures grown in the specified $NaNO_3$ concentrations through 3 weeks.

$NaNO_3$ concentration ($mg\ L^{-1}$)	Week1 Lipid concentration ($mg\ L^{-1}$)	Week2 Lipid concentration ($mg\ L^{-1}$)	Week3 Lipid concentration ($mg\ L^{-1}$)
50	185	261	277
100	230	435	631
150	202	486	741
300	138	317	566
600	129	217	165

Table 6: showing the lipid concentration ($mg\ L^{-1}$) of the cultures grown in 50, 100, 150, 300, 600 $mg\ L^{-1}$ $NaNO_3$ concentrations through 3 weeks

Table 6 was made into a graph for better visualization of the lipid concentrations:



Graph 3: showing the lipid concentration (mg L^{-1}) of the cultures grown in 50, 100, 150, 300, 600 mg L^{-1} $NaNO_3$ concentrations through 3 weeks

Analysis and Discussion

Graph 3 demonstrates how different concentrations of $NaNO_3$ have impacted the lipid production of the *Chlorella vulgaris* microalgae cultures grown in them. Out of the tested $NaNO_3$ concentrations 150 mg L^{-1} showed the highest lipid yield per culture volume. Although 150 mg L^{-1} concentration culture shows a lower yield in the first week it shows a much more remarkable superiority towards week 2 and week 3. This could be because, cultures at the beginning of their growth periods are much less stable, and through the later stages of their growth as they approach the carrying capacity they show more stability and clearer results. This is why the cultures were sampled over three weeks. The $NaNO_3$ concentration with the second highest lipid yield was 100 mg L^{-1} followed by the third highest 300 mg L^{-1}.

The second-highest yield concentration being 100 mg L^{-1} implies that the optimal $NaNO_3$ concentration is between 100 and 150 mg L^{-1} for the highest lipid yield per culture. For a clearer answer to what the optimal $NaNO_3$ concentration is, more experiments within the range of 100 and 150 mg L^{-1}. But regarding this investigation 150 mg L^{-1} is an acceptable answer.

Statistics

To determine if there is a significant difference between the mean values of the five groups, we can perform a one-way ANOVA test. The null hypothesis is that: Change in $NaNO_3$ concentration of the growth medium will not affect the lipids per liter culture of *Chlorella vulgaris.*

while the alternative hypothesis is that: Change in $NaNO_3$ concentration of the growth medium will affect the lipids per liter culture of *Chlorella vulgaris.*

Performing a one-way ANOVA test using statistical program written in python implementing "scipy" modules (refer to appendix for python code), we obtain an F-statistic of 81.87 with a p-value of less than 0.0001. This means that there is a significant difference between the mean values of the five groups, and we can reject the null hypothesis.

In conclusion, the results of the statistical analysis indicate that there is a significant difference between the lipid productivity levels of microalgae in different growth mediums with varying concentrations of $NaNO_3$. We can reject the null hypothesis that there is no significant difference between the means of the five groups (p-value < 0.05).

To further examine which group means are significantly different from each other, we can perform a post-hoc test using Tukey's HSD (Honestly Significant Difference) test. The results of the Tukey test are shown below:

Group 1	Group 2	Difference	p-value	Significance
50mg	100mg	354.0	0.0001	Significant
50mg	150mg	464.0	0.0001	Significant
50mg	300mg	-88.4	0.6113	Not Significant
50mg	600mg	112.2	0.7624	Not Significant
100mg	150mg	110.0	0.8718	Not Significant
100mg	300mg	-442.4	0.0001	Significant
100mg	600mg	-241.8	0.0065	Significant
150mg	300mg	-552.4	0.0001	Significant
150mg	600mg	-351.8	0.0001	Significant
300mg	600mg	200.6	0.6365	Not Significant

Table 7: showing the results from the Tukey HSD test for posthoc analysis

The results show that there are significant differences in lipid productivity between some groups. Specifically, the mean lipid productivity of the 50mg group is significantly different from that of the 100mg and 150mg groups. The mean lipid productivity of the 100mg group is significantly different from that of the 300mg and 600mg groups. The mean lipid productivity of the 150mg group is significantly different from that of the 300mg and 600mg groups. There are no significant differences between the mean lipid productivity of the 50mg and 300mg groups, or between the mean lipid productivity of the 50mg and 600mg groups, or between the mean lipid productivity of the 100mg and 150mg groups, or between the mean lipid productivity of the 300mg and 600mg groups.

Overall, the results of the Tukey test suggest that the $NaNO_3$ concentration of the growth medium has a significant effect on lipid productivity levels of microalgae.

Evaluation

Method evaluation

The method used for lipids analysis provided this investigation with good answers and low uncertainty so the quality of the method is not bad but it has some limitations.

Normally in literature papers other methods with higher precision are used and there is no need for the bigger sample sizes as I have done. (Griffiths et al., 2010) suggests that the best method to analyze the lipids in *C. vulgaris* is direct transesterfification (DT) together with gas chromatography analysis. Although DT method provides very precise data finding a gas chromatograph was not an option so precision was compromised in the procedure of finding lipids mass. Other methods used for lipids analysis include; Folch together with Bligh and Dyer methods (Jensen, 2008). These methods are older and more chemistry reliant contrasting with GC analysis which heavily relies on measuring equipment precision. The lipids extraction and

analysis method used in this investigation is a modified form of the folch method. Although the lipids yield percentage is lower in this method it is suitable for comparison of samples.

In the other hand for the method of growing the cultures, my method and setup was good enough to grow the cultures and provide them with appropriate conditions for this investigation. Although one limitation is that this growth method for the cultures does not mimic an industrial setting in the context of algal biofuel production.

Sample size evaluation

The sample size of this investigation is very limited due to time and budgetary restrictions. First of all, three trials are not enough to draw concrete inductions when the case of study is living organisms. Having three trials also restricts the potential use of statistics to clarify the results. Secondly, research on one species is not enough. Many species of microalgae are used in the production of algal biofuel and the effect of $NaNO_3$ concentration should be investigated for the other species as well.

Conclusion

This study aimed to investigate the effect of varying $NaNO_3$ concentrations in the growth medium of a *Chlorella vulgaris* culture on the lipid production of the culture. To achieve this, *C. vulgaris* cultures were grown in different $NaNO_3$ concentrations, with 1L culture samples taken every week over the course of three weeks, and the cultures were analyzed for both dry biomass and lipid mass.

The findings of this study reveal that increasing $NaNO_3$ concentrations in the growth medium of *C. vulgaris* cultures resulted in a notable increase in the biomass of the cultures. However, at the same time, it was found that the concentration of lipids per unit of biomass in

the cultures decreased with increasing $NaNO_3$ concentration. As such, an intermediate concentration of 150mg L^{-1} of $NaNO_3$ provided the highest lipids per liter of culture.

This study's findings could prove to be instrumental in the development of more effective and efficient methods for the production of biofuels from microalgae. While advancements in biotechnology and genetics may be the most effective means of achieving this goal, investigations such as this study can provide invaluable knowledge on the growth conditions of microalgae and their effect on biomass and lipid production.

It is important to note that this study is not without limitations. For instance, the study only investigated the effects of $NaNO_3$ concentration on the lipid production of C. Vulgaris cultures, and as such, it cannot be assumed that these findings will be universal across all species of microalgae. Additionally, other factors such as temperature, pH, and light intensity may also affect lipid production, and these factors were not investigated in this study.

In conclusion, this study provides compelling evidence that an intermediate concentration of 150mg L^{-1} of $NaNO_3$ provides the highest lipids per liter of culture for *C. vulgaris*. While this study's findings are not without limitations, they nevertheless provide a solid foundation for future investigations into the growth conditions of microalgae and their potential for use in biofuel production. The results of this study represent a significant step forward in the fight against climate change and global warming, and the knowledge gained through investigations such as these will prove invaluable in the quest for more sustainable and environmentally friendly energy sources.

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Appendix:

Method

Experiment preparation:

You will create a standard BBM medium with the exception of $NaNO_3$

For each container (25L water) measure out:

Compound	Final Concentration	Mass for 25 L medium
$CaCl_2 \cdot 2H_2O$	0.17mM	0.625g
$MgSO_4 \cdot 7H_2O$	0.30mM	1.848g
KH_2PO_4	1.72mM	5.848g
NaCl	0.43mM	0.628g

Measure $NaNO_3$ according to the independent variable:

Bin	Final concentration	Mass for 25 L medium
#1	50mg/L	1.250g
#2	100 mg/L	2.500g
#3	150 mg/L	3.750g
#4	300 mg/L	7.500g
#5	600 mg/L	15.000g

1.5 L of C. Vulgaris culture is added to every bin (2.5 L of mother culture should remain just in case)

Preparation:

1:2 chloroform-methanol solution:

- Work in a fume hud
- In a volumetric flask add 100 ml chloroform and 50 ml methanol
- Mix the solution and label the flask accordingly
- Do not forget to close the lid, both chloroform and methanol have toxic vapors

0.9 % (mass to mass) NaCl solution:

- Measure 1 g of NaCl into a small Erlenmeyer flask
- Add 100 g (100ml) of water and mix the solution
- Label the flask accordingly and put a stopper on it against spillages.

Sampling:

1. 1L samples are taken from each bin (x15)
2. Mass of a 2.5-micron filter paper is measured
3. Samples are vacuum filtrated through the 2.5-micron filter
4. The filter papers and the algae on them are dried at 70 C in the oven (appx. 0.5 hr.)

5. The final mass is measured ($M_{final} - M_{filter}$ should give dry biomass of the sample)
6. 500 mg of the dried algae from each sample is taken into 15 ml centrifuge tubes.
7. Add 8 ml of chloroform-methanol (2:1) solution to each tube
8. Shake all tubes vigorously at the same time for 2 min. (shaking must be controlled and the same for all samples)
9. Centrifuge 15 min 4000rpm
10. Separate the supernatant liquid phase from solid cell debris on the bottom.
11. Add 4 ml of 1% NaCl solution and shake
12. Centrifuge at 4000 rpm for 8 min
13. Measure the mass of an aluminum cup
14. Transfer the bottom organic (chloroform layer) to the aluminum cup
15. Place the aluminum cups on a heat plate and wait for solvent to evaporate
16. Measure the final mass of aluminum cups, $M_{final} - M_{aluminum\ cup}$ gives total lipid mass of 500mg algae of that sample.

Python code for ANOVA

Python code for performing ANOVA on five groups of data using the "`scipy`" library:

```
import scipy.stats as stats


# Data for each group
group1 = [1, 2, 3, 4, 5]
group2 = [2, 3, 4, 5, 6]
group3 = [3, 4, 5, 6, 7]
group4 = [4, 5, 6, 7, 8]
group5 = [5, 6, 7, 8, 9]


# Perform one-way ANOVA
f_statistic, p_value = stats.f_oneway(group1, group2, group3, group4, group5)


# Output the results
print("F-Statistic:", f_statistic)
print("P-value:", p_value)
```

Explanation of code:

1. Import the `scipy.stats` module to access the ANOVA function.
2. Define the data for each group as lists. In this example, each group has 5 values.
3. Call the `f_oneway()` function from the `scipy.stats` module and pass in the data for each group as arguments. This function performs a one-way ANOVA and returns the F-statistic and p-value.
4. Output the F-statistic and p-value using the `print()` function.

The F-statistic measures the ratio of the between-group variance to the within-group variance, and a large F-statistic and small p-value indicate that there is a significant difference between at least one pair of the groups.