Entry into the Stockholm Junior Water Prize 2019

Business is Blooming:

Optimizing Phosphorus and Carbon Content to Maximize Growth and Lipid Production in an Algal Photo-bioreactor

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Abstract:

Algae is a renewable energy source when lipid and biomass are maximized for biofuel conversion. This experiment was designed to maximize this in *Nannochloropsis oculata* via added nutrients. It was hypothesized that added carbon and phosphate would increase growth and lipid levels. Carbon is involved in photosynthesis and lipid synthesis. Phosphate was hypothesized to increase these more significantly as seen in rapid algal blooms.

Nannochloropsis and growth media were subdivided into four conditions: 100% added carbon, 100% added phosphate, 100% both nutrients, and Control with no additional nutrients. These were grown in a scale-model algal-photobioreactor with optimized lighting, aeration and temperature to mimic an industrial setting. Growth was measured periodically and lipid was measured after around 40 days.

Biomass indicated that each nutrient significantly increased growth, individually and cumulatively. Phosphate increased lipids, whereas carbon's effect was less clear. This suggests that added nutrients, especially Phosphate, can be optimized to maximize algal output industrially. As an extension, a plasmid was designed to compare these improvements genetic alterations and *Chlamydomonas* growth conditions were optimized for this innovation.

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Note: Main sections in bold, subtitles/minor sections not bolded

Key Words/Phrases:

Algae, biofuel, biomass, lipid, optimized growth conditions, algal photo-bioreactor, added carbon, added phosphate, Global Energy Crisis, *Nannochloropsis oculata, Chlamydomonas reinhardtii.*

Abbreviations + Acronyms:

- *C. reinhardtii = Chlamydomonas reinhardtii*
- GGE = Gas Gallon Equivalent
- nm = nanometers
- "P+" = Added Phosphate (100% extra phosphorus) condition
- "C+" = Added Carbon (100 % extra carbon) condition
- "Both+" = Added Carbon and Phosphate (100% extra of each element)
- WRHS = Wachusett Regional High School

Acknowledgements:

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Biography:

Benjamin Dwyer is a Junior at WRHS in Holden, Massachusetts. He has enrolled in Honors Science courses at this school for three consecutive years and completed the required year-long Honors Project for each year. He has participated in the WRHS School Science Fair for the past three years, with projects related to water and aquatic systems and pertaining to Physics and Biology. At the 2019 WRHS Science Fair, Dwyer placed first with his project "Business is Blooming: Optimizing Phosphorus and Carbon Content to Maximize Growth and Lipid Production in an Algal Photo-bioreactor". He then participated in the 2019 Regional Science Fair at Worcester Polytechnic Institute, where he received a second place award as well as the Regional Stockholm Junior Water Prize, and was deemed eligible to participate in the Massachusetts State Stockholm Junior Water Prize Competition as well as the Massachusetts Science & Engineering Fair at the Massachusetts Institute of Technology in May of 2019. Benjamin has always had a passion for scientific research and environmental betterment and plans to continue his research in coming years.

Introduction:

Currently, the world is engaged in a detrimental situation known as the Global Energy Crisis. Modern civilization has come to depend very heavily on industrial techniques that require massive amounts of fuel to operate. Specifically, fossil fuels such as oil, gas, and coal together constitute over 85% of the global supply of harnessed energy. These fuel sources are non-renewable and have detrimental environmental effects. With current usage rates, these fuels are expected to quickly become increasingly expensive and unsustainable. Fossil fuel emissions have been linked to cause environmental harm, like global warming, climate change and pollution. Global warming and pollution most notably harms aquatic, ocean ecosystems. Renewable fuel sources, that are derived from renewable resources on a human timescale, make up less than 15% of the harnessed global energy supply have notably less harmful emissions. By the year 2050, it is estimated that over 75% of the world's energy supply can be constituted from eco-friendly fuels [1]. In order for this industrial transformation to occur, it is imperative that further research be conducted with potential renewable energy sources.

Renewable energy sources are currently under investigation in order to determine the most efficient and effective option among them. Specifically, biofuels, fuels derived directly from living matter, are promising sources of renewable energy that can use biological processes like photosynthesis to benefit the environment and be more sustainable and effective than current fuels when optimized. One of the most heavily scrutinized and potentially beneficial renewable energy biofuels is algae, a photosynthetic, aquatic organism that gives off almost 50% less harmful carbon emissions than current petroleum-based fuel. Efficient algal cultivation could develop a less expensive, more effective, and more eco-friendly fuel alternative [2].

Algal biofuel operates by using photosynthesis to generate algal biomass, composed of lipids, carbohydrates and other nutrients, that can be converted into biodiesels and used as fuel. There are several different strategies behind maximizing biomass production for industrial use. From 2010 to 2014, algal fuel costs were reduced from $100/GGE to $8/GGE. These strategies range from changing light intensity to changing photosynthetic capacity, and recover almost 90% of all carbon in biomass [3].

As a biofuel, algae is a serious contender, especially with recent advancements. Compared to other organisms, algae is a more feasible biofuel due to its high photosynthetic conversion rates, diverse metabolic capacities, superior growth rates, and efficient nutrient storing capacities and

capabilities. With more research, the defining question of many experiments, as well as this one, has come to be which nutritionally optimizing method is superior for algal biomass growth [4].

Additionally, under favorable conditions, algae has been estimated to grow about 5-10 times faster than standard modern crops, like corn and wheat. Microalgal research has been conducted for over 70 years, and its growth rate is steadily improving. With an increased growth and lipid production rate from a favorable species, algae can be a much more reliable fuel standard [5].

Of algal species, *Nannochloropsis oculata* seems to be one of the most potentially feasible. This species is a fast growing algal type with profound biofuel applications due to its highly retained nutrient value. This species contains lipids that have a high concentration of fatty acids and has been heavily tested. It also has possible adaptabilities for genetic testing and is regarded as one of the most effective and flexible algal species [6]. Because of these favorable qualities, *Nannochloropsis oculata* was chosen to be the organism used for main testing trials.

Additionally, there are other considered microorganisms, like *Chlamydomonas reinhardtii,* a green mircoalgal yeast variety. Like regular algae, this species propagates quickly using photosynthesis and is a proven model organism for testing. Genetic and nutrient testing have also been performed on this species and have proven it to be industrially promising. Algae is superior to many bacterial alternatives, and hence many biofuel alternatives, in its resistance to bacteria and other chemicals [7]. Because of its differences to more popular species, this species is often used for more complicated, modern technologies or processes like genetic engineering.

Another beneficial aspect of algal experimentation is its relatively available commercial suppliers as well as its relatively simple experimental abilities. An effective cultivation setting for algal culture is in an algal photobioreactor, which combines aeration, mixture, nutrients and light exposure to cultivate algal growth. These can be fairly simple in design, and made with materials as commonplace as a plastic water bottle. Algae can be purchased in cultures at a relatively low price, from a commercial supplier, or gathered from a natural ecosystem [8]. This experiment involved a homemade algal-photobioreactor using plastic water bottle vessels and optimal temperature, light, and aeration levels to mimic optimal industrial growth.

A potential strategy for biomass optimization, that supposedly increases algal photosynthetic rate, is the addition of nutrient to a culture. As carbon dioxide is one of the reactants of photosynthesis, a greater supply of carbon, which is also an industrial emission in large quantities, has been tested for effectiveness on photosynthesis. However, in many of these studies, these effects have not been perpetual, and wear off after several generations, possibly due to consumption of basic

nutrients and should be researched and regulated to fulfil the potential of this optimization strategy [9].

It is believed that the biomass yield will be increased by increasing photosynthesis. Another element with a significant effect on photosynthetic rate is phosphorus. This element is possibly more efficient in its involvement than carbon. However, its effects on algal lipids are still being quantified. Phosphorus addition and phosphorus deprivation are being tested on algal biomass yield, with no concrete result in terms of industrial implications [10]. This experiment compared the results of added carbon and phosphate, both expected to increase photosynthesis, to determine which more greatly affects lipid and growth rates,.

After recent technological advancement, specifically within genetics, genetic engineering has also been suspected to have experimental potential for algal biofuel. However, the algal genome is very different from plant and mammalian genomes, and attempts to introduce genes into its genome have proven difficult. If possible, genetic manipulation of the algal genome would be easily replicable within that genome, and much longer lasting than with carbon or other elements. Plasmids are genetic carriers and transmitters that can carry inserted genes into cells for lasting genomic effects [11].

The first step towards genetically modifying an algal genome is determining and optimizing an ideal algal species. Research indicates that *Chlamydomonas reinhardtii* is potentially the most promising for this research. However this algae must be optimized to grow as effectively as other species do. Additionally, for biomass testing, absorbance is proportional to biomass [12]. As an extension, *C. reinhardtii* growth conditions were optimized to a degree hopefully suitable for further testing with technologies like genetic engineering and further algal optimization; both a plasmid and a procedure were designed for this aspect.

To test the effects of carbon vs. phosphate addition on biomass and lipid production, this experiment optimized growth of *Nannochloropsis* and *Chlamydomonas* in applicable industrial settings, with the nutrients added in salt form. Absorbance was measured over time and lipids were extracted after around 40 days of testing.

Phosphorus and carbon were both hypothesized to increase the photosynthetic rate to a significant degree, which would consequently increases biomass and lipid yield. However, phosphorus was predicted to increase growth and lipid production more significantly. Carbon itself is a reactant of photosynthesis, and theoretically excess carbon could thus be transferred almost directly to excess lipid. Phosphate has been shown to vastly and rapidly naturally increase algal blooms,

supporting that a similar effect can be visualized with lipid levels in an industrial setting. Ideally, each added nutrient will increase the photosynthetic conversion rates of biomass and lipid, which are each crucial components in biofuel conversion. By creating a more cost effective and efficient biofuel, it becomes more economically viable and increases its chances for large-scale industrial use and hence would decrease harmful industrial emissions significantly. Experimental data largely support this hypothesis, having profound industrial implications towards mitigating the Global Energy Crisis.

Methodology:

Materials (volumes specify total amounts required between trials):

•2, 50 mL cultures of *Chlamydomonas reinhardtii*

•2, 50 mL culture of *Nannochloropsis oculata*

•40 purified plastic water bottles, 16.9 oz. (Nestle Pure Life™)

•10, 16.9 oz. bottles, spring water (Poland Spring™)

•500 mL of 70% isopropanol

•200 mL Lipid Oil Red dye (Stock Solution: 60 mg Lipid Red O / 20 mL 100% isopropanol)

•5, 8-valve metal manifolds

•5, 40 gallon aquarium air pumps

•150 ft. standard aquarium air tubing

•40 aquarium air stones

•360 g algal growth salt

•Large metal stirring spoon

•50 mL *Nannochloropsis* growth nutrient

•150 g sodium bicarbonate salt

•4 g disodium phosphate salt

•4 ft. full spectrum LED growth light

•1 mL and 5 mL pipettes w/ 200 fitted pipette tips

•1 pair of scissors and 1 permanent marker

•Stainless steel mortar and pestle

•100, 50 mL falcon test tubes and 2 test tube racks

•1, 6 gallon bucket and 7, 2 gallon buckets

•Set of Graduated measuring cups up to 1 L

•80 circle sheets Whatman #1 filter paper

•4 ft., 75 gallon clear, rectangular glass aquarium

•Spectrophotometer and *Sorvall* benchtop centrifuge

•Standardized Freezer @ 0°F

•1 box latex gloves

•150 mL soil water growth media

•5, 3 inch funnels

Procedure:

Creating Standardized Testing Conditions:

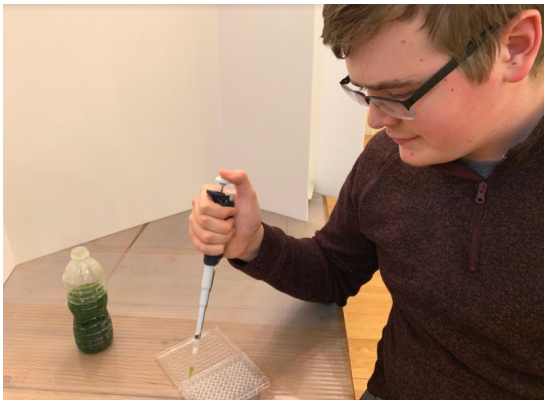
All equipment was sterilized prior to use using soap and warm water. 20 purified water bottles (10 L) were emptied into a 6 gallon bucket, labels were removed and bottles were kept for future experimental use. 180 g of *Nannochloropsis* algal growth salt, 25 mL of liquid growth nutrient and 50 mL of *Nannochloropsis oculata* were mixed into this water using a metal stirring spoon until dissolved to create a standardized growth media for the algae. This stock solution was then subdivided equally into 4 total buckets, each with 2.5 L of the solution. Four testing conditions (levels of independent variable) were created. The **"Control" group** was the first condition, containing no additional nutrients (only has basic industrial nutrients) to serve as a baseline for comparison. The **"C+" condition** was created by mixing 37.5 grams of sodium bicarbonate (calculated to provide 100% extra carbon) to another bucket. The **"P+" condition** was created by mixing 1 gram of disodium phosphate (calculated to provide 100% extra phosphorus) to the third bucket. The **"Both+" condition** was created by mixing 37.5 grams of sodium bicarbonate and 1 gram of disodium phosphate (calculated to provide 100% extra phosphorus and 100% extra carbon) into the final bucket, to test for a cumulative result.

From each group, 5 bottles were refilled with 450 mL of the solution and labeled accordingly, creating 5 replicates of each of the 4 growing conditions.

Establishing Growth in a Photo-bioreactor:

The 5 bottles from each condition in their respective groups, were subdivided from other groups clearly and evenly in the glass aquarium. The testing area was in a room with standardized heating at around 70°F. 1 cm. holes were pierced in each lid using scissors and 3 ft. lengths of air tubing were threaded through each lid so that approximately 6 inches was submerged. For each bottle, airstones were attached to the end of each tube, and the other end was screwed onto a manifold valve. The air pump was connected to power and allowed to run constantly. Then, the growth light was positioned 3 feet above the aquarium on a 16 hour cycle. Purified water was refilled to the top line of each water bottle every week for constancy. This system provided constant levels of light, temperature and aeration, in optimal levels as pictured above.

Taking Absorbance Samples:



Every 4 days, 200 ul samples were taken from each bottle and sealed in a labeled well plate. Each bottle was shaken prior to allow for uniform solutions. Absorbance of these samples was tested at 470 nm (accepted experimental wavelength) in a spectrophotometer to indicate biomass and growth levels. The researcher is shown taking absorbance samples via micropipette.

Extracting and Quantifying Lipid Content:

After biomass levels began to level off or decline slightly (~40-50 days), after absorbance sampling, 50 mL aliquots were taken from each bottle into a falcon test tube and sealed and labeled accordingly. These were spun at 3000 Gs in a centrifuge to create a condensed pellet of biomass and lipid at the bottom of each tube. Tubes were frozen overnight (12 hours) to make pellets brittle and were subsequently thawed to begin cellular weakening. The Lipid Oil Red Stock Solution was mixed in a 9:6 ratio with water to create a working solution. 1 mL of 70% isopropanol was mixed to each pellet to break down cellular membranes and 500 uL of the Lipid Oil Red working solution was

mixed to mark the lipid. They were ground for exactly 60 seconds with a mortar and pestle and filtered into a falcon tube, as shown below.

Next, 350 uL samples were taken from each tube and absorbance was tested at 492 nm (accepted experimental lipid absorbance wavelength) to measure marked lipid content. All steps were repeated exactly for a second trial.

Extension Procedure: Optimizing Subculturing of *C. reinhardtii* + Changes in Procedure

The largest change in the original procedure was the transition in culturing of *Chlamydomonas.* Originally, this species was grown alongside *Nannochloropsis,* yet died within weeks of testing. Because of this, *Chlamydomonas* optimization became the focus of the extension rather than main trials. Optimization of this species is important for use in tests with genetic modification. Multiple methods were tested to be optimal including altering nutrient levels, aeration and purified vs. spring water. The optimal variable observed in preliminary testing in which they were applied to a culture indicated nutrient amounts to have the greatest effect.

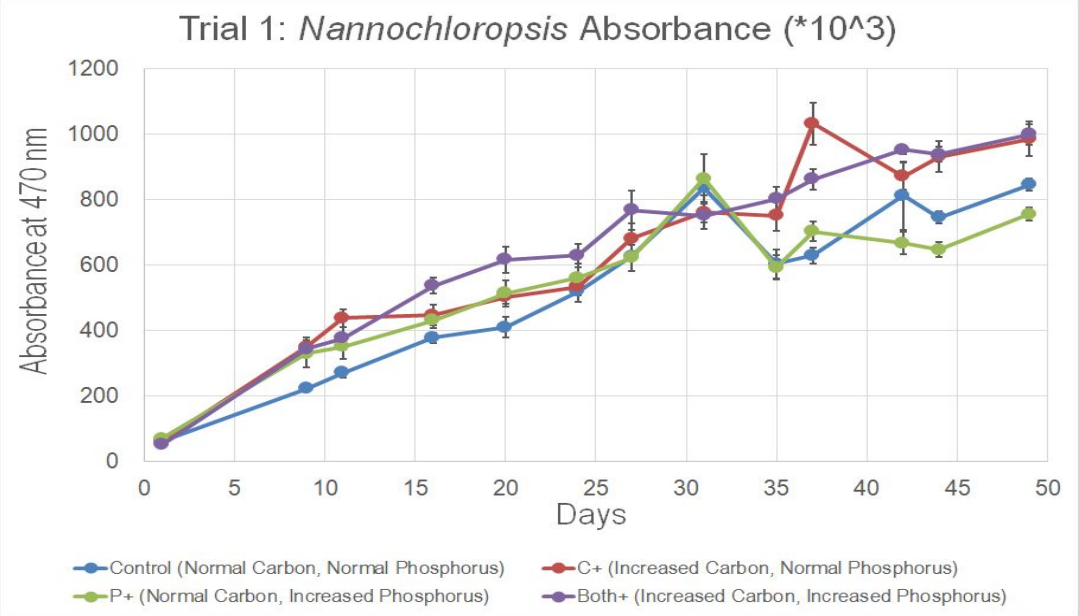
Finalized Extension Procedure-

The independent variable is the amount of nutrient, which is a soil water growth media for this species. 50 mL of soil water was diluted into 950 mL of spring water. 4 mL of soil water was mixed directly to the culture slant. 1 mL of this solution was added to each of 4 empty water bottles and 250 mL of the spring water-soil water solution was added to each bottle. This process was repeated exactly, but using 100 mL of soil water initially. These bottles were grown in the photobioreactor without aeration, as shown below for around 2 months and absorbance was measured at 470 nm.

Results:

Note: Graphs shown were not aggregated due to notable minute differences and points of observation that aggregation would not display. The general trends are similar, however these differences are worth noting.

Graph 1: Trial 1 Growth

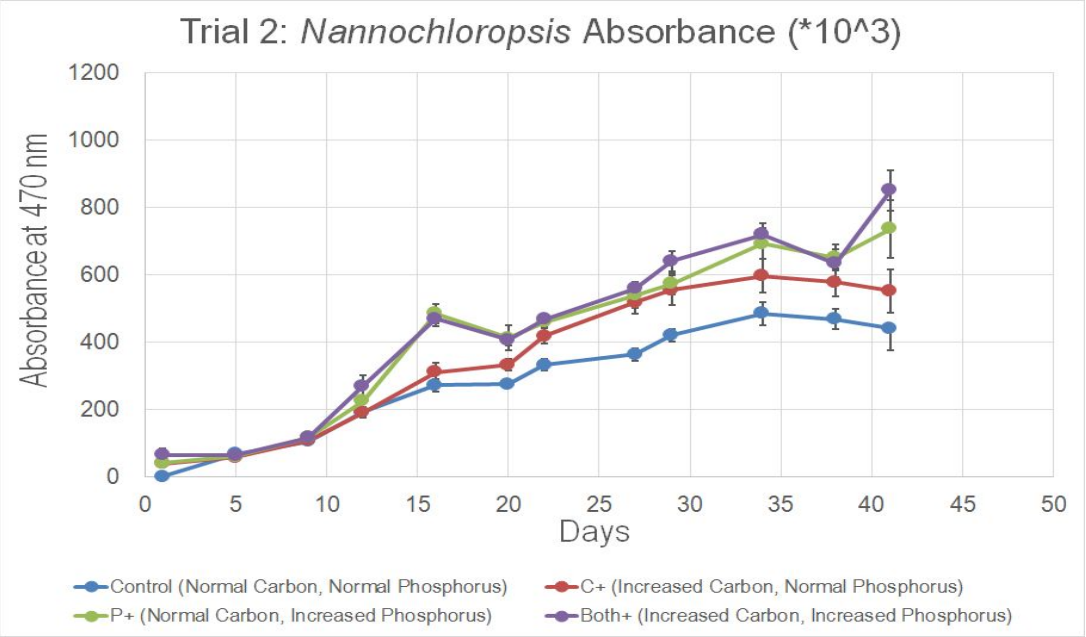


This data shows fairly clear trends until a notable drop off point around 30 days, where values become more sporadic. This was a possible occurrence according to research, which indicated that a given species usually exhibits a drop-off point in growth where they rely on less constant natural functions after nutrients have been consumed. However, for the majority of testing the "Both+" condition was clearly higher in biomass than all other conditions, even reaching over 1,000 OD at the end of testing. Additionally, the control group was steadily lower than all other conditions in biomass levels for the majority of testing. The carbon and phosphate conditions induced a notable result on growth, however, there are only minute differences between them. Because these both increase biomass substantially over time, and the "Both+" condition induced a greater effect than all other conditions, a cumulative effect is suggested to be induced by both nutrients in combination. This suggests that they each induce a unique, positive effect on algal growth. Lastly, the standard deviation, or error bars, of Graph 1's data are fairly small, indicating the robustness the data and its methods of collection. Below is the T-Testing for Day 20 of this trial.

Note: All T-Tests included are based on a maximum significant P Value of .05.

The above T-Test indicates that the "Both+" conditions induced a significant effect compared to not only the Control Group, but the "C+" Group as well. Increased carbon also significantly increased growth compared to the control. Increased phosphate induced a substantial, almost statistically significant effect as well.

Graph 2: Trial 2 Growth



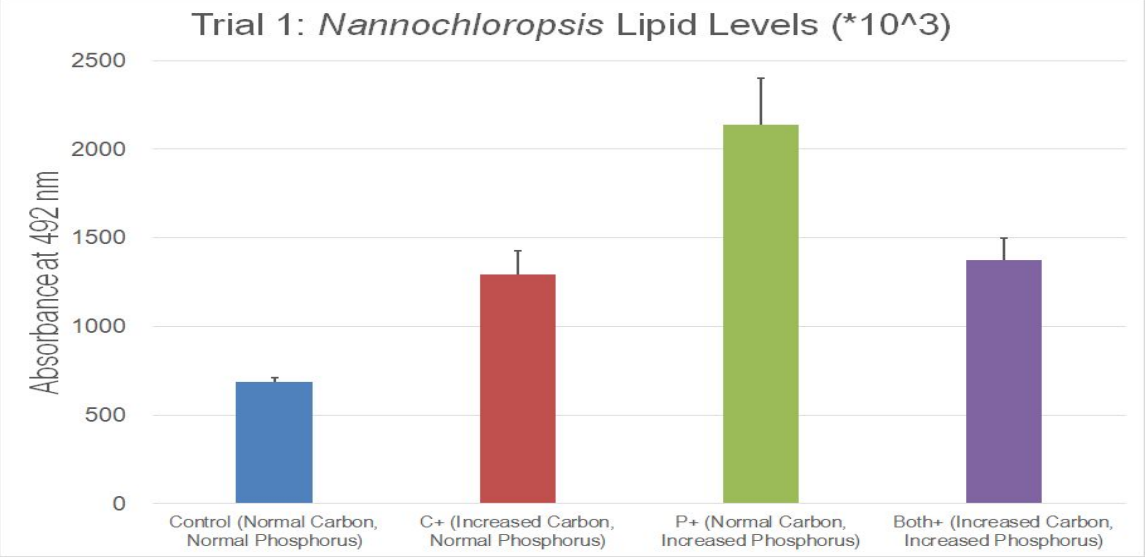
The above graph demonstrates more clear trends among algal growth rates. Similarly to Trial 1, the "Both+" trial remained higher in biomass levels for the majority of testing than any other condition, again suggesting a cumulative result. Additionally, the Control Group was again substantially lower than most other conditions for the majority of the testing period. Most notably, this data more clearly distinguishes the induced effect of carbon vs. phosphate. Phosphate induces algal growth rates comparable to the "Both+" condition for the majority of testing, substantially higher than other conditions. The error bars are even tighter, suggesting that measurement may have become more precise after the first trial, with more experience. Lastly, this data clearly illustrates trends that mimic a normal algal growth cycle. For the first 10-15 days, an acclimation period of slow growth is observed, followed by a growth spike, and finally a plateau of less rapid growth. Testing periods were intentionally ended before the inevitable rapid decline in growth was observed.

	Control	C+	P+	Both +
Control	X	0.2799365	0.0002294	0.0001501
C+	X	X	0.0027676	0.0028211
P+	x	X	X	0.6917563
Both +	X	X	x	X

Above is the T-Test from Day 16 of Biomass Levels in Trial 2. Increased phosphorus and increased Both+ nutrients produced a strongly significant effect on not only the control, but also on the increased carbon values, further distinguishing that phosphorus increases growth more than increased carbon.

Between both biomass graphs (Graphs 1 + 2), clear trends are shown that both carbon and phosphate can increase algal growth individually and cumulatively. Both datasets correspond with normal algal growth conditions, indicating that algae were at relatively low stress levels to allow for significant growth. Specifically, Graph 2 indicates that Phosphate may be more effective than Carbon.

Graph 3: Trial 1 Lipid Levels

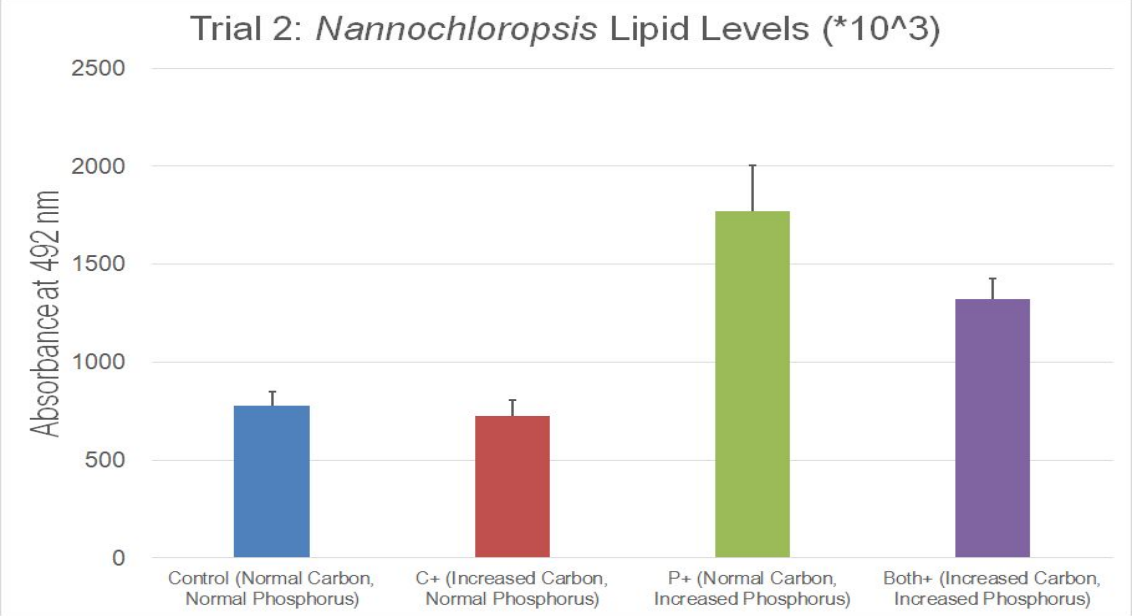


Graph 3 data indicates trends similar to patterns seen with Biomass Levels, which are theoretically proportional to a certain degree with Lipid Levels. The Control Group produced the lowest lipid levels whereas "Both+" substantially increased lipids compared to the control group. However, this data clearly indicates that increased phosphate greatly increases lipid levels, compared to all other conditions. Phosphate absorbance levels even breached 2,000 OD, which is hugely significant compared to the control group and other conditions (See below T-Test of Trial 1 Lipid Levels).

	Control	C+	P+	Both +
Control	X	0.0022618	0.0005937	0.0005468
C+	X	X	0.0216175	0.6583576
P+	x	X	X	0.030873
Both +	X	X	x	X

Every increased nutrient condition produced a significant effect on lipid levels compared to the control group. Phosphate had the most significant effect (P=.00059).

Graph 4: Trial 2 Lipid Levels



The above data exhibits largely similar trends to those from Trial 1 (Graph 3). The "P+" condition most substantially increased lipids, whereas the "Both+" increased lipid levels noticeably. However, the increased carbon ("C+") condition resulted in reduced lipid only comparable to the Control Group. Below is the T-Test for Trial 2 Lipid Levels.

	Control	C+	P+	Both +
Control	X	0.6429783	0.0036157	0.0026313
C+	X	X	0.0029267	0.0020585
P+	x	X	X	0.1191195
Both +	X	X	x	X

The "C+" condition did not significantly increase lipids compared to the control group, however the "P+" and "Both+" again resulted in significant lipid levels..

Graphs 3 and 4 demonstrate generally similar trends, however "C+" results are noticeably different between trials. Additionally, because of the discrepancy with this condition and because the "Both+" condition was not higher in lipids than all other conditions as it was in Biomass testing, a cumulative result was not observed.

Extension Results:

Trial 2 Chlamydomonas Reinhardtii 470 nM								
1000								Days
1/6/2019	1	2	3	4	5	Average	SEM	1
100 mL	43.7	37.3	42.4	40.3	44.9	41.7	1.34	
50 mL	40.1	39.8	39.4	42.9	40.1	40.5	0.62	
3/19/2019	1	2	3	4	5	Average	SEM	73
100 mL	542.9	893.8	1080.6	1085.4	1037.5	928	102.35	
50 mL	501.8	462.7	425.1	303.2	830.5	504.7	87.99	

After around 2 months, *Chlamydomonas* biomass levels grew about twice as much when the nutrient amount was doubled, suggesting that growth conditions were optimized compared to original testing and that amount of nutrient is proportional to growth in *Chlamydomonas* subculturing.

Error Analysis:

This experiment contained noticeable errors that were notably improved on with more experience. Trial 1 lipid levels were even remeasured to combat outliers. There are several possible sources for this error within experimentation. Firstly, there could have been error in measurement. There is human error present in the use of pipettes and aliquoting liquids, in which liquid can be lost in the process, causing data to be skewed. During absorbance sampling or lipid extraction, some wells may have mixed slightly as they were filled at maximum capacity. Also, there may have been errors in the machine measurement process, where the spectrophotometer may have misread a certain value, and produced slightly inaccurate data. Additionally, contamination may have caused error within experimentation. During testing, a rust-colored powder was observed to collect on the edges of many of the water bottles, which may have been a bacteria or a yeast. This may have skewed absorbance and lipid level readings to account not only for algae. Lastly, although standardized heating was applied, the temperatures may have varied slightly as experimentation took place during the coldest months of winter. These are all potential sources of error in the conduction of this experiment. To attempt to circumvent these sources of error, measurements were taken more carefully, machines were calibrated in between testing, a thermometer was installed in the aquarium, and the outsides of the bottles were scrubbed with antibiotic wipes to prevent bacterial contamination.

Discussion:

The results of this experiment indicated that both added carbon and phosphate can potentially increase algal growth and lipid levels and that especially with lipid content, and in biomass levels to a certain extent, phosphate increases these endpoints more significantly. In both lipid trials, phosphate increased lipid levels more significantly than any other condition. Additionally, carbon substantially increased lipid levels in the first trial, whereas not in the second.

These results call for a third trial with carbon lipid testing to produce a more conclusive result, however clear trends exhibit phosphate to increase these endpoints more significantly. It is possible that due to the basic pH of sodium bicarbonate, the "C+" algae experienced stress and lipid production was stunted. This experiment had other limitations as well. The testing period, although relatively long, could be extended to observe an entire algal industrial growth cycle. Additionally, this experiment was only a scale model of an industrial photo-bioreactor and is in much lesser concentrations and conditions compared to industrial ones. Lastly, more trials in cases like the carbon lipid trials and the biomass trials between carbon and phosphate could provide more conclusive and applicable results.

Overall, these results indicate that not only may phosphate and carbon be potentially promising industrial biofuel nutrients, but that addition of nutrients in general to algal photo-bioreactors could significantly increase industrial production of algal biofuel. Modern biofuel optimization does not focus on added nutrients as its most favorable option, rather it is transitioning towards other more complicated innovations, like nutrient deprivation. This experiment indicates that a relatively simple and inexpensive addition to an industrial growth media may be economically viable.

These results were similar to those of Metzger, who was able to rapidly increase algal blooms of the *Botryococcus* species, essentially due to an increased photosynthetic conversion rate [13]. In correspondence with increased growth and lipid levels, a similar effect was observed with the addition of phosphorus to *Nannochloropsis* in this experiment. Additionally, this experiment created a successful homemade algal photo-bioreactor using water bottles and inexpensive materials, like the work of Luleva, who made a similarly inexpensive reactor [8].

This project has many profound implications on industrial renewable energy research. It elucidates the many significant effects that carbon and phosphate have on algal growth and lipid production in addition to their low costs, perhaps as a viable industrial nutrient. It additionally

suggests that researching nutrients outside of algal photosynthetic conversion, like phosphorus, may be useful subjects of research. Lastly, its main trials not only demonstrated the optimization of *Nannochloropsis* as a biofuel, but the optimization of *Chlamydomonas,* based on the results of the extension.

Considering how added nutrient, specifically phosphate, was able to significantly increase crucial elements of biofuel conversion, biomass and lipid, algal biofuel may become a viable energy source. Implementing algae as a biofuel would reduce greenhouse gases and the usage of fossil fuels, and thus help purify environmental systems. This transition, having less emissions, would result in significantly less pollution in aquatic ecosystems like oceans and lakes. Additionally, algae in itself is not toxic or harmful to aquatic biology. Different forms of algae can grow in saltwater, freshwater, or brackish water. As an organism, it is incredibly versatile in many aquatic systems and will not decrease drinking water availability significantly, as it can be grown in these other settings. The ocean and other aquatic systems are among the most harmfully affected by fossil fuel emissions, and algal biofuel would pollute these waters significantly less, due to its composition as well as its reduced emissions in an industrial setting.

In future trials, if this experiment were to be continued, several different areas could be investigated. Firstly, methods besides added nutrient such as deprivation could be further tested. Also, tests adding additional nutrients to a photo-bioreactor could be performed measuring how much nutrient is added, what nutrient is added, and when it is added. Nutrients could also be re-dosed to a culture periodically to see if a more lasting effect is observed. Lastly, because algal growth in this experiment degraded after over 30 days, more promising scientific innovations, like genetic engineering may be considered. This experiment optimized *Chlamydomonas* growth conditions, and plasmid-transferal methods and components have been thoroughly researched for future innovation.

These results indicate that added nutrients, especially phosphate or carbon, can be economically viable and biologically preferable solutions to the Global Energy Crisis.

Conclusions:

1. The hypothesis was supported: For both tests, phosphate "P+" resulted in greater lipid increase and biomass at each endpoint compared to "Control" or "C+" groups. Carbon raised lipid and biomass levels higher than control for majority of testing.
2. Added carbon and added phosphate demonstrated a cumulative result in growth, rather than lipid levels. The biomass absorbance levels in the "Both+" group were higher than phosphate and carbon groups individually.
3. Increased carbon, and to a greater extent, increased phosphate, will yield significantly enhanced algal growth in biofuel production. Added nutrients in general may be a favorable area of investigation for further industrial algal growth.
4. Algal growth and lipid levels can be optimized to be significantly greater and thus be possibly viable levels for industry and be favorable for retained purity of environmental systems, such as aquatic ecosystems.
5. The stage has been set for further experimental investigations to prolong a beneficial effect, involving scientific innovations like genetic engineering or other processes with nutrient addition, such as re-dosing, adding more initial nutrient, or a better nutrient in general.
6. Additional soil water in growth media further optimizes *Chlamydomonas reinhardtii* growth conditions to be more favorable for genetic research.

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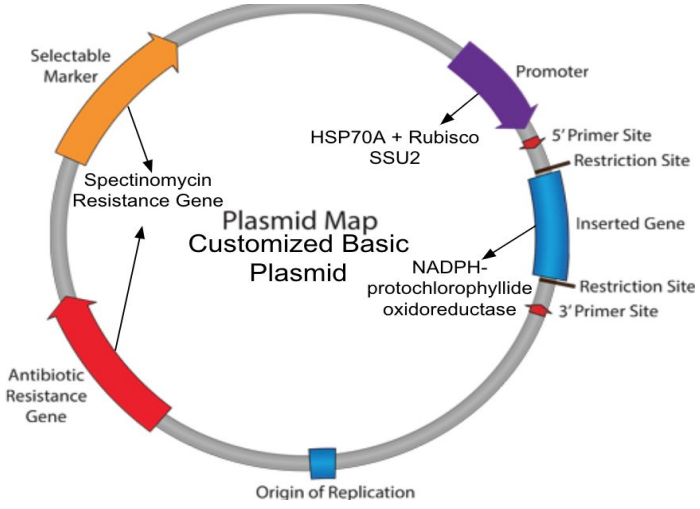
Annex: Plasmid Research - Extension Background/ Application



The extension of this experiment was to optimize *C. reinhardtii* growing conditions so that concentrations could be significant enough to be affected by genetic transfection methods, like electroporation. After achieving these conditions, further research was conducted and a plan was formed to initiate transfection of a selected gene that could increase photosynthesis for a longer time period. The custom-designed plasmid, designed by this researcher for this experimental setting, is pictured above. Firstly, it contains Spectinomycin resistance as a selectable marker and resistance gene. Spectinomycin is a deadly toxin to *C. reinhardtii* and with this resistance successfully transfected, this can be visualized by its survival upon exposure [12]. These promoters were chosen as research indicates they increase genetic expression as well as stabilize transfection further [14]. Lastly, NADPH- POR is the main gene in light-dependent reactions in plants, and was hypothesized to have the same effect in algae, which is photosynthetic, and thus increase biomass and lipid from increased chlorophyll in algal samples, which would be a favorable long term effect.