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Article

# Temperatures of 20°C produce increased net primary production in *Chlorella sp.*

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## SUMMARY

***Chlorella sp.*** **are autotrophs that introduce stored energy into biological systems through photosynthesis. Net primary production (NPP) reflects the amount of energy converted to sugar bond energy in photosynthesis minus the amount of energy consumed by cellular respiration. Because carbon dioxide ($CO_2$) acidifies water, the net $CO_2$ production leads to a change in pH that reflects the NPP. Establishing a relationship between temperature and NPP could provide insights into maximizing the biological removal of $CO_2$ from the atmosphere. In this experiment, the effect of temperature on the NPP of *Chlorella sp.* was measured as a function of ΔpH, which was determined using a standard curve of buffers of known pH versus absorbance. The ΔpH increased as the incubation temperatures increased towards 20°C, and then as the temperatures increased after 20°C, the ΔpH decreased, indicating that the NPP of the Chlorella sp. was maximized around 20°C. Around 20°C, the enzymes involved in photosynthesis, such as rubisco, may approach their optimal temperatures and thus be maximally efficient. As global warming is caused by excess $CO_2$ in the atmosphere, *Chlorella* incubated at temperatures around 20°C would be maximally efficient at naturally removing $CO_2$ through photosynthesis and could provide useful insights for new technologies to fight climate change.**

## INTRODUCTION

*Chlorella sp.* are unicellular autotrophs that lives in freshwater, saltwater, and soil (1). *Chlorella sp.* were chosen as a model organism to study photosynthesis due to their relatively easy cultivation. Autotrophic organisms use light energy to drive the synthesis of adenosine triphosphate (ATP) and reduced nicotinamide adenine dinucleotide phosphate (NADPH), which then is used to reduce carbon dioxide ($CO_2$) to glucose. *Chlorella sp.* anabolically produce glucose through photosynthesis, given by the reaction Energy + $6CO_2+6H_2O \rightarrow C_6H_{12}O_6 +6O_2$, and then catabolically break it down through cellular respiration, given by the reaction $C_6H_{12}O_6 +6O_2 \rightarrow 6CO_2+6H_2O$+ATP (2). The process of photosynthesis is carried out by a series of enzymes; for example, enzymes prompt the transfer of energized electrons to $NADP^+$ to form NADPH and attach $CO_2$ to ribulose bisphosphate (RuBP), beginning the process of creating glucose (3). These enzymes are proteins that speed up different photosynthetic reactions by increasing the stress of the bonds of substrates so that the bonds require less energy to be broken, lowering the activation energy (4). Enzymes have active sites where substrates fit in like puzzle pieces (4). These enzymes create microenvironments, which have unique optimal pH and temperatures in which the reaction is carried out (5). Thus, if the temperature of a *Chlorella sp.* solution is manipulated, the microenvironments of enzymes, such as NADP+ reductase and rubisco, will not be the optimal conditions for the enzyme, and thus, the *Chlorella sp.* will not photosynthesize as much. In addition to affecting the functionality of enzymes, increased temperature will also affect the *Chlorella sp.* by manipulating the physics of the solution: according to the kinetic-molecular theory, higher temperatures lead to faster movement of molecules and lower gas solubility (6). While these higher temperatures could cause more intermolecular collisions, they could also remove gas reactants for biochemical reactions, as higher temperatures decrease the solubilities of gases (7). For example, higher temperatures could cause $CO_2$ particles to come out of solution, leaving less substrate for photosynthesis.

The goal of this experiment was to determine the effect of temperature on net primary production (NPP) of *Chlorella sp.* as a function of ΔpH. Given that *Chlorella sp.* grow best in temperatures of 20-30°C (8), we hypothesized that temperatures similar to this environment, such as 20°C, would also maximally stabilize photosynthetic enzymes and produce the highest observed net primary production. Below or above these temperatures, enzymes will not function optimally, and thus pH will decrease steadily as a result of a decreased photosynthetic rate.

## RESULTS

To determine the effect of temperature on NPP of *Chlorella sp.*, we incubated six *Chlorella sp.* balls in cuvettes at temperatures 2°C, 12°C, 20°C, 40°C, and 53°C and, after an 8 hour exposure to light, measured the pH change of the solution as a proxy for NPP (**Figure 1**). We used liquid cultures of *Chlorella sp.* for all the trials. In our experiment, all the $CO_2$ that the *Chlorella sp.* took in or expelled occurred in the liquid medium. When $CO_2$ is placed in water, carbonic acid ($H_2CO_3$) forms and then partially dissociates into a proton ($H^+$) and bicarbonate ($HCO_{3^-}$). Therefore, when cellular respiration is occurring more than photosynthesis, an expected increase in $CO_2$ will render the solution more acidic (-ΔpH), whereas a decrease in $CO_2$ as a result of photosynthesis will render the solution more basic (+ΔpH). Therefore, pH of the solution

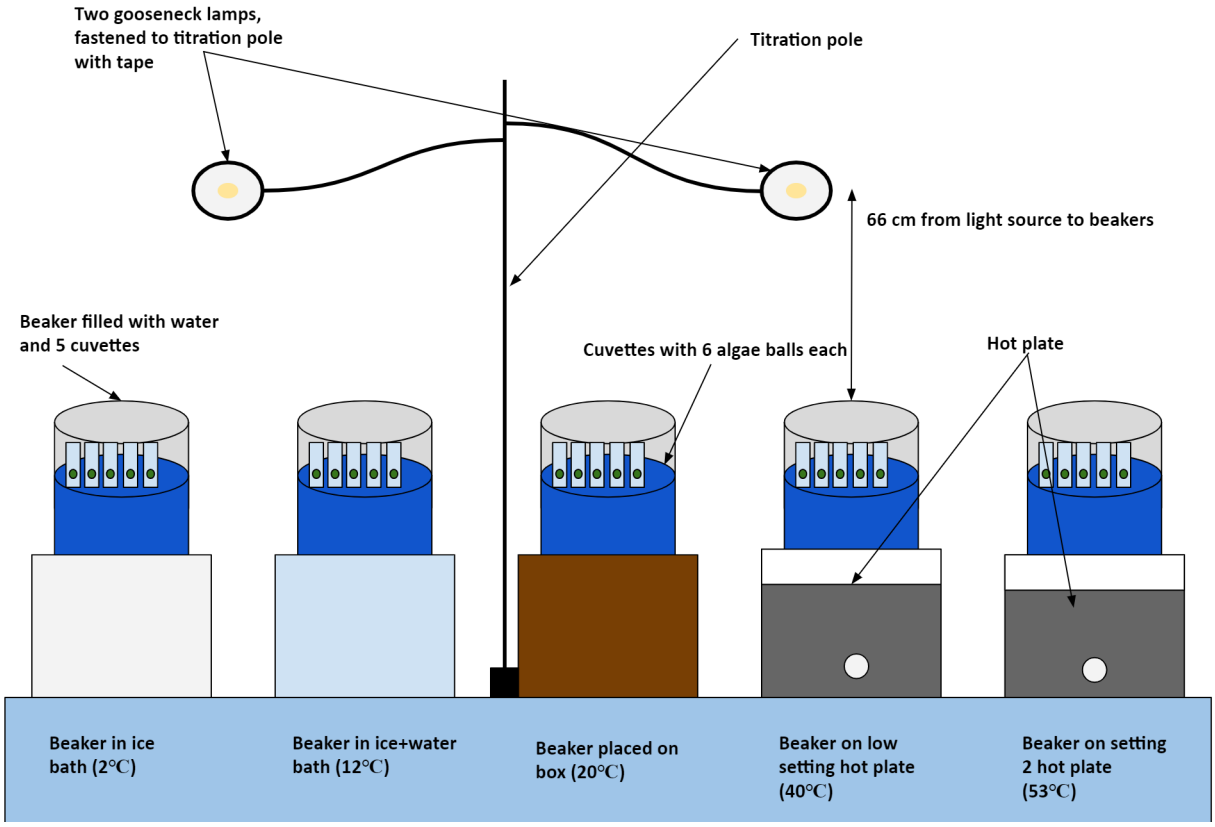


**Figure 1: Experimental design setup**. Pictured are the five water beakers (2ºC, 12ºC, 20ºC, 40ºC, and 53ºC), each containing five cuvettes, thermometers used to take temperature readings, the light source, titration pole, ice baths, and hot plates.

would reflect the NPP of the *Chlorella sp*. pH of the cuvette solution was measured using a spectrometer to measure absorbance before and after 8 hours of exposure to a particular temperature. Since we added hydrogen carbonate indicator to the cuvettes and created a standard curve of known pH versus absorbance, the absorbance measures the pH of the solution, with a higher pH indicating that there is less $CO_2$ and more $O_2$—a greater NPP.

Room temperature cuvettes were used as a control to allow ΔpH to be more attributable to the changes in temperature rather than the presence of another variable. A blank control group (no *Chlorella sp*. balls) was placed in the grow tower for 8 hours in order to ensure that *Chlorella sp*. balls were producing the changes in color of hydrogen carbonate indicator observed. A t-test showed that pH of blank control after light exposure was not significantly different from pH of black control before light exposure ($p$ = 0.137).

The ANOVA test for variance indicated that the experimental groups were significantly different from one another ($p$<0.0001). A t-test showed that average pH change increased significantly from 0.334 to 0.764 ($p$=0.006) as temperature increased from 2°C to 20°C, but then decreased significantly from 0.764 to -0.059 ($p$=5.74x10-5) as temperature increased from 20°C to 53°C (**Figure 2**). Across the whole dataset, the highest average ΔpH was 0.764 at 20°C, and the lowest average pH change was -0.059 at 53°C. The standard deviations (SD) for each temperature ranged from 0.01 for 40°C to 0.18 for 20°C. At 40°C and 53°C ΔpH falls to close to zero. Therefore, as the temperature increased towards 20°C, the ΔpH increased, but towards 40°C and 53°C, the ΔpH drastically decreased.

## DISCUSSION

The hypothesis that photosynthesis would occur at greater rates in temperatures near *Chlorella sp.* environment, such as temperatures in the 20-30°C range, was supported by the data (**Figure 2**). A two-tailed t-test showed that the change in pH increased significantly between temperatures of 2°C and 20°C ($p$=0.006), and then decreased significantly from 20°C to 40°C ($p$=5.72x10-5). These data indicate that increasing the temperature towards 20°C approached the optimal temperature of the photosynthetic enzymes, such as rubisco. Temperatures around 20°C may have supported the optimal microenvironment for the enzymes involved in photosynthesis because the increased heat stressed the bonds of the substrate in order to require the least amount of energy for the reactions to take place without denaturing the enzyme. For example, the Calvin cycle requires rubisco in order to produce glucose from $CO_2$, and rubisco is most efficient around 22.5-30°C (9). When the temperature was increased to 40°C, the enzymes may have denatured, causing both the 40°C and 53°C replicates to have essentially no NPP (ΔpH = -0.055 and

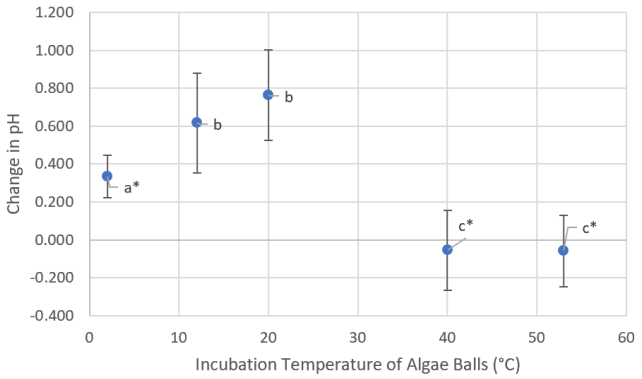


**Figure 2: *Chlorella sp.* NPP, measured through change in pH, is optimized at 20°C**. *Chlorella sp*. balls were incubated for 8 hours at cell density $3.57x10^5$ cells/$mm^3$. Data points indicate the average of 5 independent experiments, and error bars denote standard deviation. Letters denote significant groupings; runs that are not significantly different ($p>0.05$) are annotated with the same letter. Asterisks indicate a significant difference ($p<0.05$) from the control (20°C).

-0.059, respectively). Temperatures above 40°C may provide the energy required to break the hydrogen bonds important for the tertiary structure of the algal photosynthetic enzymes, thus denaturing them (10). Moreover, the *Chlorella sp*. likely photorespired at high temperatures (i.e. temperatures beyond 40°C) because they are C3 plants (11). At high temperatures, C3 plants exhibit photorespiration, a process similar to photosynthesis, but where $O_2$ enters the Calvin cycle instead of $CO_2$ and $CO_2$ is produced instead of glucose. Above 40°C, the *Chlorella sp*. may be producing, rather than consuming $CO_2$, thus producing the negative ΔpH values (**Figure 2**). However, even the photorespiration enzymes likely began to denature, accounting for the small magnitude of the ΔpH. For example, rubisco activase loses 50% efficiency after a 5-minute exposure to temperatures above 33°C (12). Additionally, because the temperature and movement speed of enzymes involved in reactions increased, there were more collisions between the enzymes and the corresponding substrates. As the temperature increased, a greater proportion of substrate molecules involved in photosynthesis may have had the necessary energy to overcome the activation barrier, collide with substrate molecules, and undergo a reaction, thus speeding up each reaction of photosynthesis and allowing the *Chlorella sp*. to photosynthesize more quickly. However, once the temperature exceeded 20°C, the likely denaturation of the enzymes inhibited this effect. Alternatively, at higher temperatures, gas molecules like $CO_2$ can break the intermolecular bonds of a solution (13). Thus, as the temperature increased, there could be less $CO_2$ reactants due to solubility to enter the Calvin cycle and be converted into glucose, causing lowered rates of photosynthesis.

NPP is important because it allows for natural $CO_2$ removal, which fights off the ever-present threat of climate change. Future projects could attempt to harness the photosynthesizing ability of *Chlorella sp*. and maximize activity in order to naturally remove $CO_2$ from the atmosphere. While there are several factors that affect NPP, such as temperature, precipitation, and soil moisture (14), the findings of this experiment suggest that, to maximize *Chlorella sp*. NPP, the *Chlorella sp*. must be incubated at temperatures around 20°C. Additionally, because *Chlorella sp*. introduce glucose to the ecosystem through photosynthesis, these findings could also be used to maximize *Chlorella sp*. glucose production to be used as a food source.

The experiment had several potential pitfalls. First, when the 2.5 mL semi-micro cuvettes were placed into the beakers of heated or cooled water for incubation, the cuvettes tipped slightly. This tipping caused some of the *Chlorella sp*. balls to overlap, which meant that some of the *Chlorella sp*. balls were blocking others. Consequently, some *Chlorella sp*. balls were receiving more light than others and were likely photosynthesizing at a greater rate. Secondly, because the cuvettes float in water, the cuvettes were only partially submerged in the beakers. Thus, some cuvettes had more exposure to the room temperature air than others, causing slight variance in the internal temperatures of the cuvettes. Both of these uncertainties could have been eliminated by taping the cuvettes horizontally to the side of the beakers so that no *Chlorella sp*. balls were overlapping and so that the cuvettes were fully submerged. Thirdly, for the 2°C and 12°C baths, the ice may have begun to melt during the 8-hour incubation period. This would have resulted in the ice baths being colder in the beginning and the reported temperatures being inaccurately high. Though ice was replenished once after 4 hours, this inaccuracy could have been eliminated by replacing the ice baths every hour with new, fresh ones. In the ice baths, the temperature was observed to be constant over an hour during testing.

This study suggests that, in the presence of light, the optimal incubation temperature for maximizing NPP is around 20°C. However, future experiments could investigate the effect of temperatures in the 20°C to 40°C range in order to find the optimal temperature for producing the highest NPP. Though the graph peaks at 20°C, it appears that the peak is slightly shifted left, and slightly higher temperatures may more accurately represent the optimal temperature. Future experiments could also determine if this high ratio of photosynthesis to cellular respiration was due to an increase in photosynthesis or to a decrease in cellular respiration. This question could be answered by isolating the effects of temperature on cellular respiration by blocking photosynthesis by incubating the *Chlorella sp*. without light. Therefore, the same experiment would be performed, but with no light supplied to the *Chlorella sp*. balls as to prevent them from increasing the pH through photosynthesis.

In summary, as the incubation temperature nears 20°C, the ΔpH of a *Chlorella sp*. ball solution reaches its peak, suggesting that 20°C nears the optimal temperature for *Chlorella sp*. ball photosynthesis and that decreasing or increasing this temperature decreases the *Chlorella sp*.'s ratio of photosynthesis to cellular respiration.

## METHODS

### Creating the *Chlorella sp.* Balls

*Chlorella sp*. balls were created by mixing 5 mL bulked *Chlorella sp*. (Carolina Biological) with 5 mL 2% sodium alginate (Carolina Biological) in a graduated cylinder. Algae was bulked by allowing *Chlorella sp*. to separate in a beaker such that the densest algae were on the bottom. These dense *Chlorella sp*. were extracted using a pipette and used for the algae balls. Cell density was controlled by making *Chlorella sp*. balls from the same batch of bulked *Chlorella sp*.; cell density was standardized using a hemocytometer, with a density of $3.6x10^5$ cells/$mm^3$. A greater cell density would again allow for more cells to absorb photons and thus yeilding a higher chance that $CO_2$ would be used up. This solution was then transferred to a 10 mL syringe, which was dripped into a beaker with 50 mL 2% $CaCl_2$ (Flinn Scientific). $CaCl_2$ was used to harden the outside of the algae into a ball formation. After all of the solution had dripped into the $CaCl_2$, the resulting solution was left for 5 minutes and then placed into a strainer to be rinsed thoroughly with distilled $H_2O$. *Chlorella sp*. balls were then placed into a beaker of 50 mL distilled water, covered with tin foil, and left in a lighted growth tower for storage.

### Initial pH Measurement

25 glass cuvettes were prepared with 1 mL distilled water, 1 mL hydrogen carbonate indicator, and 6 *Chlorella sp*. balls each in order to test 5 temperatures, each with 5 replicates. The number of *Chlorella sp*. balls was also controlled by placing six equally sized *Chlorella sp*. balls into each cuvette. It was important to control for the number of *Chlorella sp*. balls because more *Chlorella sp*. balls would result in more photosystems to harness the energy of photons and thus a greater ability to use up $CO_2$, increasing the pH of the cuvette. A Vernier spectrophotometer was calibrated using a blank cuvette containing 1 mL of distilled water and 1 mL of hydrogen carbonate indicator. Once a cuvette was prepared, it was immediately placed under a sheet of tinfoil so as to not expose one cuvette to more light than the other. Each cuvette was then removed, one at a time, and the initial absorbance at 550.1 nm was taken using a spectrophotometer before placing it back under the tinfoil.

### Set-up of temperature-controlled water and ice baths

Two hot plates were used to heat beakers of water to 40°C and 53°C, settings that preliminary testing revealed were the maximum settings before water in the cuvettes would begin to evaporate. Five beakers, each containing 300 mL of water, were filled and thermometers were placed inside. Two ice baths were then drawn, one containing 4000 grams ice (2°C) and the other containing 2000 grams ice and 2000 mL water (12°C). One 300 mL beaker was placed in the 4000 gram ice bucket (2°C), one was placed in the 2000 grams ice and 2000 mL water bucket (12°C), one was kept at room temperature (20°C), one was placed on the low-setting hotplate (40°C), and one was placed on the high-setting hotplate (53°C). The ice buckets as well as the room temperature beaker were placed on boxes to be at even heights with the beakers on hot plates (**Figure 1**). The beakers were then left for 30 minutes to change temperature, which is the time it took for temperature changes to plateau in preliminary testing.

### *Chlorella sp.* Cultivation

While the beakers were acclimating to the changing temperatures, two flexible gooseneck lamps were clipped and taped onto a titration tower at a height of 66 cm above the 5 beakers (**Figure 1**) to ensure stability and equal access to light. Boxes were used to level beakers with the height of the hot plates. A closer distance would decrease the distance photons had to travel, thus speeding up the rate of reaction. After beakers had been set up for 30 minutes, 5 capped cuvettes were floated in each beaker of distilled $H_2O$. Once the cuvettes were placed in beakers, a timer was started and was stopped after 8 hours, an interval that preliminary testing had indicated would yield some change in pH. The amount of time that each cuvette was exposed to light was also controlled because a greater exposure to light would mean more time for energy from photons to fuel the anabolic reaction of photosynthesis. Time of exposure was controlled by keeping cuvettes under tinfoil and only removing them to take preliminary absorbance readings. All cuvettes were placed into water beakers at the same time and removed at the same time. Beakers were left for four hours; after four hours, 1000 grams more of ice was added to the two ice baths to ensure consistently low temperatures (i.e. 2°C and 12°C) for four more hours.

### Measuring post-incubation pH and calculating ΔpH

After four more hours, the temperature was recorded by reading the thermometer, yielding the temperatures of 2°C, 12°C, 20°C, 40°C, and 53°C. Each cuvette was removed, placed in the previously calibrated spectrophotometer, and the absorbance at 550.1 nm was recorded. A standard pH versus absorbance curve was used to calculate the pH of each cuvette before and after the incubation. This curve was previously determined by placing 1 mL buffer solution of known pH and 1 mL of hydrogen carbonate indicator, which changes color to indicate pH of the solution, inside a cuvette and obtaining the absorbance using a spectrophotometer. The pH was calculated according to the equation, $a=0.4757 x p-3.721$, where p represents pH and a represents absorbance. Single-factor ANOVA was used to determine if the tested temperatures significantly altered the ΔpH of the solution. To further investigate the trends in the data, a t-test was performed between every combination of two temperatures from the five recorded in order to determine whether or not differences in ΔpH were statistically significant from one another.

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