The production of hydrogen gas by *Chlamydomonas reinhardtii* in sulfur-deprived conditions under red light and white light.

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Abstract:

As we continue to deplete our limited supply of fossil fuels, finding an alternative energy source is becoming a growing concern. *Chlamydomonas reinhardtii*, a green microalga, utilizes the enzyme hydrogenase to produce hydrogen gas, which has become increasingly considered as a potential fuel source. We conducted a study to determine under what light wavelength conditions *C. reinhardtii* produces the greatest amount of hydrogen gas. Because oxygen inhibits the activity of hydrogenase, we placed *C. reinhardtii* in sulfur-deprived medium to induce anaerobic metabolism, thus promoting hydrogen production. We then tested the effects of light wavelength on the hydrogen metabolism of the organism by comparing hydrogen gas produced by cultures under red light versus cultures exposed to white light. Sulfur-deprived and regular media *C. reinhardtii* cultures were placed under both red and white light separately and a water displacement apparatus was used to measure gaseous volume change due to hydrogen production. Our results demonstrated no statistically significant differences among the treatments exposed to red light and those exposed to white light with regards to hydrogen production, and we therefore failed to reject our null hypothesis. However, we observed trends of sulfur-deprived cultures under red light producing more hydrogen than those under white light. Additionally, existing literature supports these trends in hydrogen production between cultures exposed to different wavelengths of light. Therefore, we discuss that perhaps the discrepancy in our results may be due to the low numbers of replicates utilized and to experimental errors.

Introduction:

As a global society, we depend heavily on our limited supplies of oil, coal, and natural gas. Therefore, efficient use of these resources is of utmost importance. Hydrogen is a clean and high-energy gas that can be used as fuel. Currently, we rely primarily on our limited reserves of fossil fuels for the production of hydrogen gas (Martin del Campo and Rodrigo 2013). The ongoing requirement for alternative energy sources and the discovery (Gaffron 1939) of the hydrogen metabolism of green microalgae led to a spate of scientific research into biological hydrogen production.

Perhaps the best characterized of these hydrogen-producing green microalgae is *Chlamydomonas reinhardtii*; *C. reinhardtii* is a single-celled, motile green alga 10 micrometers

(µm) in diameter that is used as a primary model organism in the study of hydrogen production by green algae (Melis *et al.* 2000). *C. reinhardtii* utilizes a hydrogenase enzyme, as outlined in Figure 1, to catalyze the synthesis of hydrogen from the protons and electrons produced when it splits water molecules (Tysgankov *et al.* 2002). Oxygen inhibits this reaction by interfering with the catalytic capability of hydrogenase (Melis *et al*. 2000). To overcome this limitation and increase hydrogen production, *C. reinhardtii* is placed in a sulfur-deprived medium in order to decrease the level of oxygen.

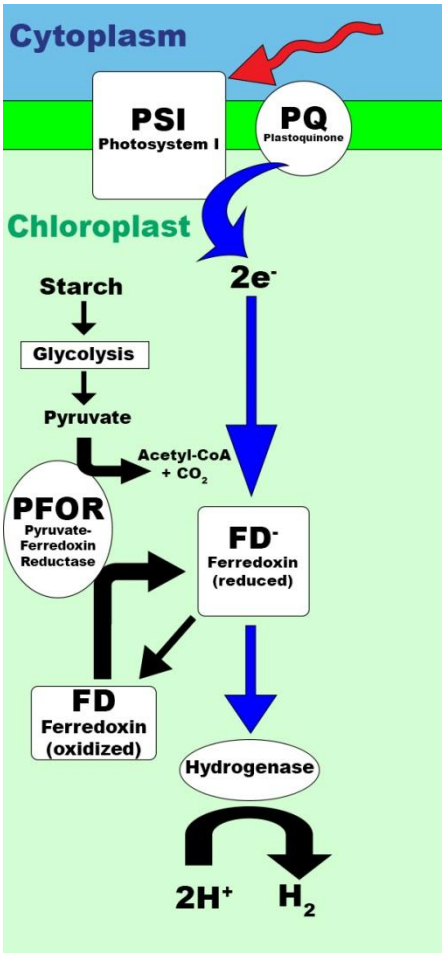

Figure 1- The primary mechanism of hydrogen production via photocatalytic metabolism in *C. reinhardtii.*

When placed in sulfur-deprived conditions, the oxygen evolved by *C. reinhardtii*'s photosystem II activity decreases to the level of oxygen uptake by respiration (Kim *et al.* 2006).

As a result, the environment ultimately shifts from aerobic to anaerobic conditions because of the lack of oxygen. Under such anaerobic conditions, the hydrogenase enzyme is activated, and as previously noted, results in the production of hydrogen gas by utilizing hydrogen ions as terminal electron acceptors as part of photocatalytic metabolism (Melis *et al.* 2000).

Previous research has shown that *C. reinhardtii*'s production of hydrogen primarily relies on a photosystem II (PSII)-independent system which derives energy garnered by photosystem I's (PSI) light harvesting complex; the photopigments of PSI are primarily associated with the absorption of light of red wavelengths (Fouchard *et al.* 2005). Given the known capacity for various photosystems to optimize themselves when presented with light wavelengths more closely matching the absorbance of their associated photopigments, it is hypothesized that cultures exposed to red light will produce hydrogen more efficiently, ultimately increasing hydrogen production. Based on these mechanisms, we decided to place *C. reinhardtii* in sulfur-deprived conditions and investigate the effect of light wavelength on its hydrogen metabolism. Our alternate hypothesis (H_A) was that sulfur-deprived *C. reinhardtii* cultures under red light produce a greater amount of hydrogen than those under white light; as such, our null hypothesis (H_0) was that sulfur-deprived *C. reinhardtii* cultures placed under red light produce amounts of hydrogen that are no different, or are less than, the amount produced by cultures under white light.

Knowledge of how wavelength affects hydrogen production by *C. reinhardtii* will bring us closer to understanding the conditions that allow for optimal levels of hydrogen production by photosynthetic microalgae. Furthermore, understanding the effect of light wavelength on hydrogen production by *C. reinhardtii* will provide a foundation of knowledge that may allow us to fully harness the hydrogen-producing abilities of similar green algae. Identifying optimal

conditions for hydrogen production could trigger further inquiry in hydrogen production and hydrogen gas as a possible alternative energy for our future.

Methods:

C. reinhardtii was first grown in two 1000mL flasks both containing 500mL of regular medium for 7 days. We then concentrated the 2x500mL *C. reinhardtii* by centrifugation and used sterile technique to transfer highly concentrated pellets (with negligible amounts of media) into one of two labelled 250mL flasks each containing approximately 50mL of either regular medium or sulfur-deprived medium (see Table 1 and Figure 2). After another day, to ensure sufficient concentration of cells in the two media, a haemocytometer was used to count cells in regular medium and sulfur-deprived medium.

Table 1 - Medium recipe for regular medium and sulfur-deprived medium.

Regular Medium		**Sulfur-deprived Medium**	
Stock Solutions		Stock Solutions	
KH_2PO_4-$7H_2O$	100.0 mg/L	KH_2PO_4-$7H_2O$	100.0 mg/L
K_2HPO_4	130.0 mg/L	K_2HPO_4	130.0 mg/L
$FeCl_3$	12.5 mg/L	$FeCl_3$	012.5 mg/L
$MgSO_4$-$7H_2O$	300.0 mg/L	$MgCl_2$-$6H_2O$	247.0 mg/L
$CaCl_2$	47.5 mg/L	$CaCl_2$	47.5 mg/L
H_3BO_3	4.0 mg/L	H_3BO_3	4.0 mg/L
$ZnSO_4$-$7H_2O$	4.0 mg/L	$ZnCl_2$	0.474 mg/L
$MnSO_4$-$4H_2O$	1.6 mg/L	$MnCl_2$-$4H_2O$	0.266 mg/L
$COCl_2$-$6H_2O$	0.8 mg/L	$COCl_2$-$6H_2O$	0.8 mg/L
$CuSO_4$	0.16 mg/L	$CuCl_2$-$2H_2O$	0.0427 mg/L
NH_4Moltbdate	0.8 mg/L	NH_4Moltbdate	0.8 mg/L
Na_3citrate-$2H_2O$	100.0 mg/L	Na_3citrate-$2H_2O$	100.0 mg/L
NH_4NO_3	300.0 mg/L	NH_4NO_3	300.0 mg/L

Figure 2 – The sulfur-deprived and regular medium *C. reinhardtii* after centrifugation and sterile transfer.

Thereafter, the normal and sulfur-deprived *C. reinhardtii* were divided into six 15mL test tubes (12 total) of which three replicates were wrapped in red film while the other three were wrapped in transparent film; these four treatment groups were then exposed to white light averaging 3133 lux at the test tubes with the classroom lights off (see Figures 3 and 4). Figure 4 also details the setup for hydrogen production measurement by water displacement; the test tubes were filled with glass beads and connected to inverted graduated cylinders filled with water through a series of submerged polyurethane (20cm) and silicon tubing of varying lengths (~15cm) and widths. The extra silicon tubing was necessary to allow a flexible connection between the graduated cylinder and the test tubes. Note that the water level varied in each graduated cylinder but it was always under the terminal portion of the tubing. Also note that the graduated cylinders varied in size (25mL for samples 7 and 9 and 10mL for the rest); this inconsistency was accounted for by using a correction factor requiring the volume displaced per centimetre of tubing and the distance between marked millilitre on each graduated cylinder (see

sample calculations in Results). It was assumed that the system was sealed after detailing with silica gel and capable of accurate gas measurements.

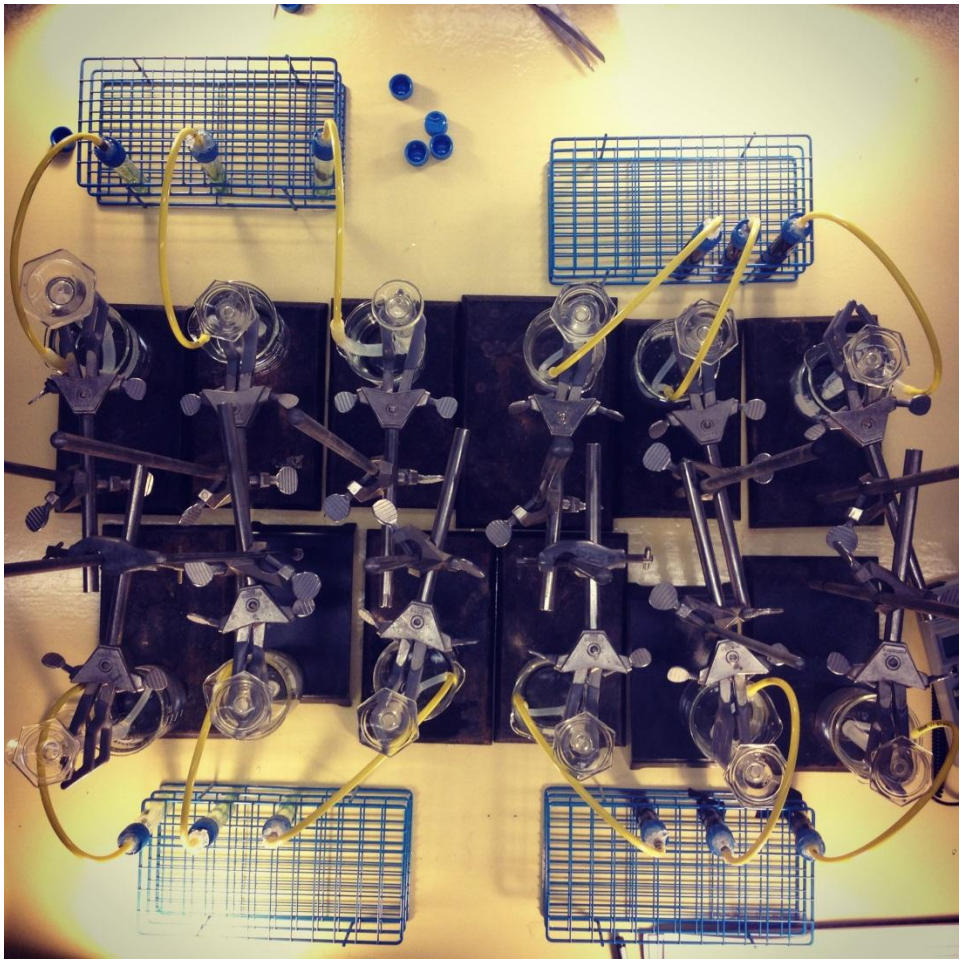

Figure 3 - Regular medium was used as a control for sulfur-deprived treatment and white light was used as a control for the red light treatment. The bottom left (samples 1-3 from left to right) was white light and regular medium. Bottom right (samples 4-6) was red light and regular medium. Top left (samples 7-9) was white light and sulfur-deprived medium. Top right (samples 10-12) was red light and sulfur-deprived medium.

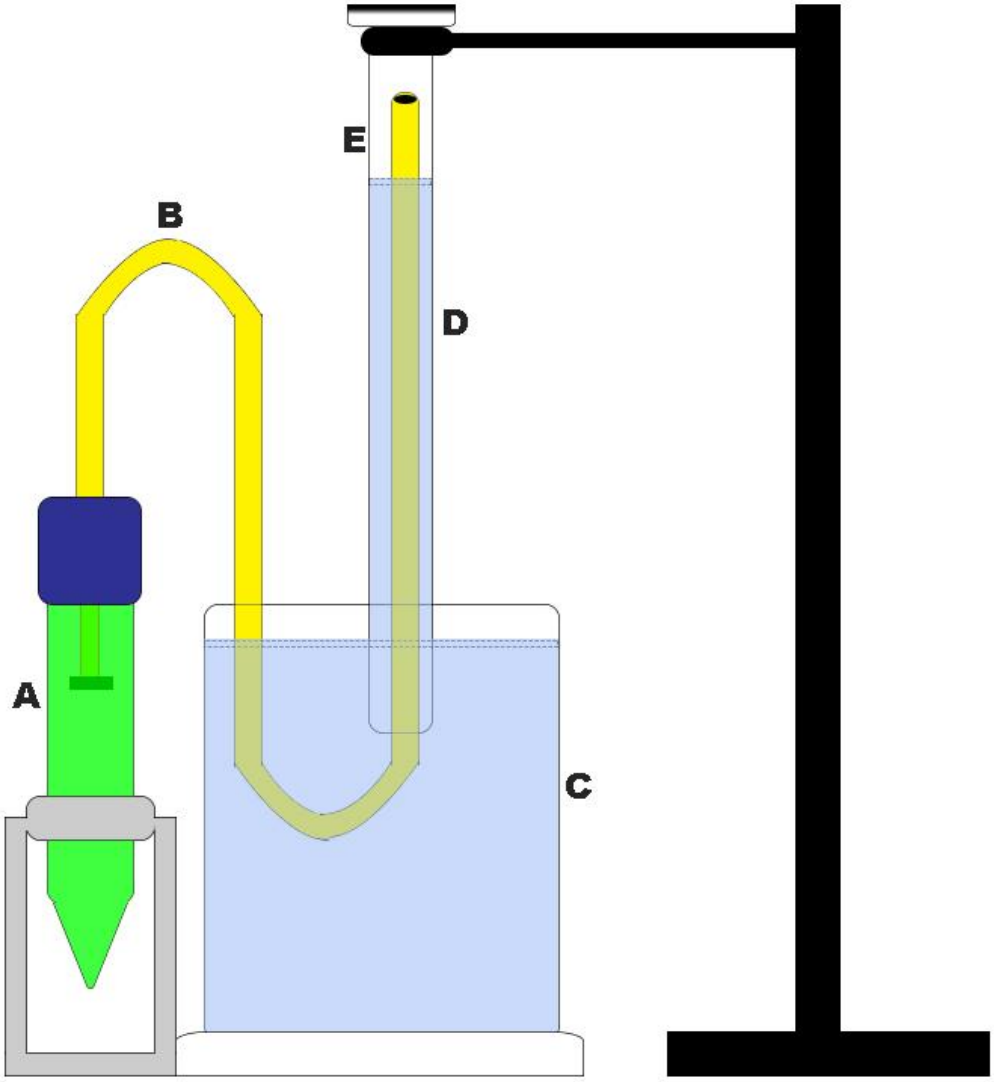

Figure 4 - The basic setup for measuring gas production through water displacement. **A.** 15mL test tube filled with sulfur-deprived and regular *C. reinhardtii* and glass beads. Note: tubes were wrapped in either red or transparent film **B.** Tubing connected by a syringe and sealed silicone glue **C.** Beaker filled with water **D.** Inverted graduated cylinder **E.** Gas-collecting chamber

We immediately measured and recorded the initial water level on the graduated cylinder. Previous literature states that exchange between oxygen and carbon dioxide due to photosynthesis and respiration would be insignificant at such low volume levels (Thongbai *et al.* 2011). Theoretically, the sulfur-deprived *C. reinhardtii* could not photosynthesize (produce

oxygen) and any carbon dioxide produced by respiration was insignificant; thus another key assumption was that all the displacement of water was due to hydrogen production. We measured the volumes the day after the initial reading and twice two days after the initial reading to determine if any water had been displaced by hydrogen (or another gas) production. Furthermore, using the same experimental setup but without *C. reinhardtii*, a post-experimental negative control was setup using purely regular and sulfur-deprived medium to test if there was significant gas production without *C. reinhardtii* present.

We also measured temperature at the test tubes (25.5°C) to verify consistency among controls and experimental groups. In order to ensure that the light intensities were not affecting the growth of *C. reinhardtii*, light intensity was measured half a meter from a light source with the individual films in the middle; the measured light intensities were applied to a logarithmically-derived scale of literary values of hydrogen production specific to *C. reinhardtii* and their associated light intensity (Gfeller and Gibbs 1984). Once the light intensity and tubing was accounted for, to give a volume of hydrogen produced, we compared 95% confidence intervals of the means to determine if the amount of hydrogen produced by sulfur-deprived *C. reinhardtii* under red light was statistically different from regular medium *C. reinhardtii* under red light. We applied the same statistical analysis considering sulfur-deprived/white light versus regular medium/white light, regular medium/red light versus regular medium/white light and finally sulfur-deprived/red light versus sulfur-deprived/white light.

Results:

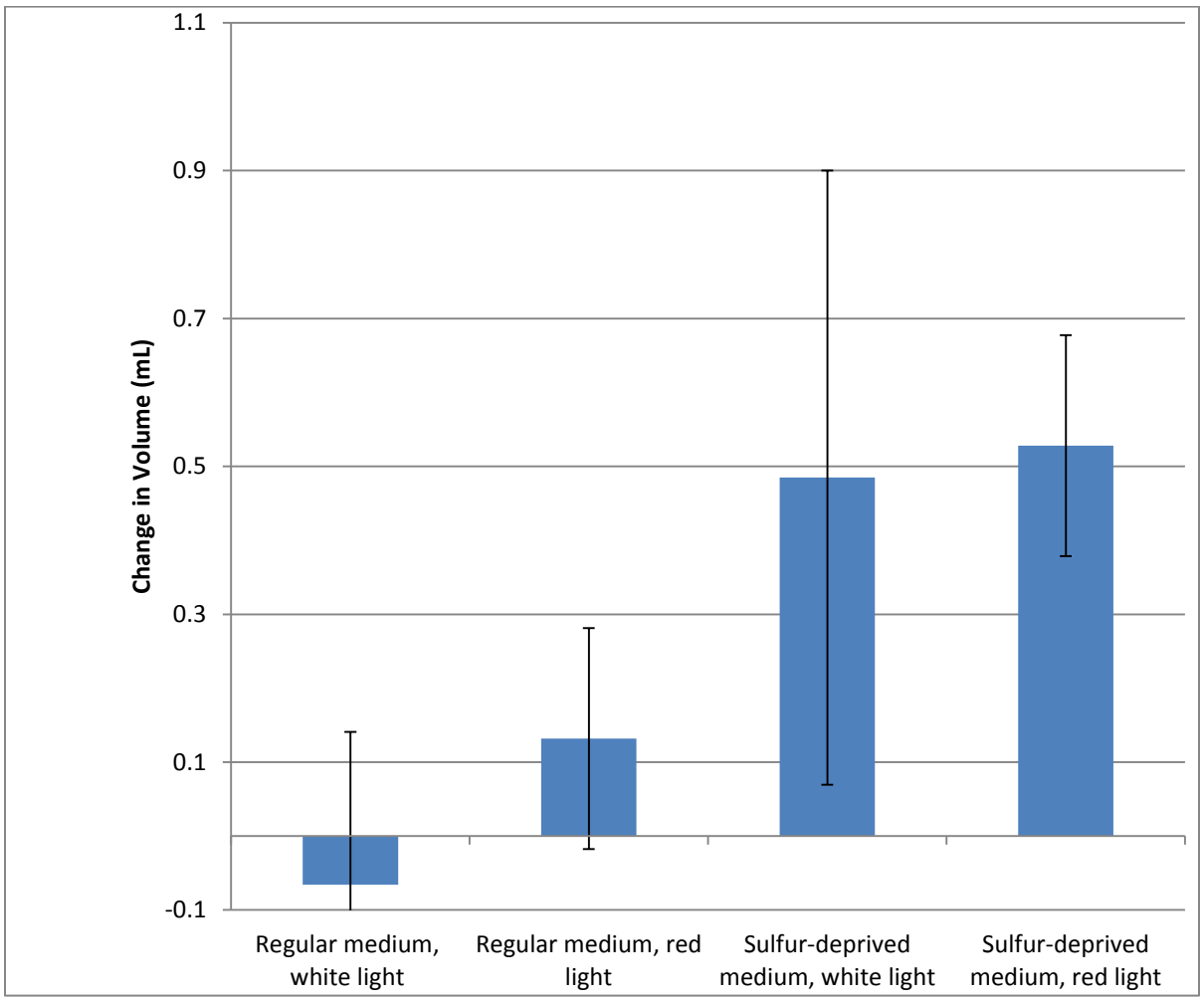

Figure 5 – Mean volume displacement in graduated cylinder in different media exposed to different qualities of light. n=3 for each treatment. Displacement measured 44 hours after start of experiment. Error bars represent 95% confidence intervals.

As shown in Figure 5, sulfur-deprived treatments exhibited a larger volume of gas displacement than treatments in the regular medium. Under red light, the confidence intervals of the sulfur-deprived medium group and regular medium do not overlap, thus these two groups show a significant difference. Sulfur-deprived replicates under red light also displaced significantly more water than regular medium *C. reinhardtii* under white light. However, under

white light the confidence intervals of the sulfur-deprived group and regular medium overlap, therefore these two groups do not show a significant difference.

We observed a slight trend for increased hydrogen production under red light (compared to white) in sulfur-deprived medium. However, the confidence intervals of the white light group and the red light group overlap in the sulfur-deprived medium, thus there is no significant difference in the hydrogen output for these two groups. The same is true for the white light and red light groups in the regular medium.

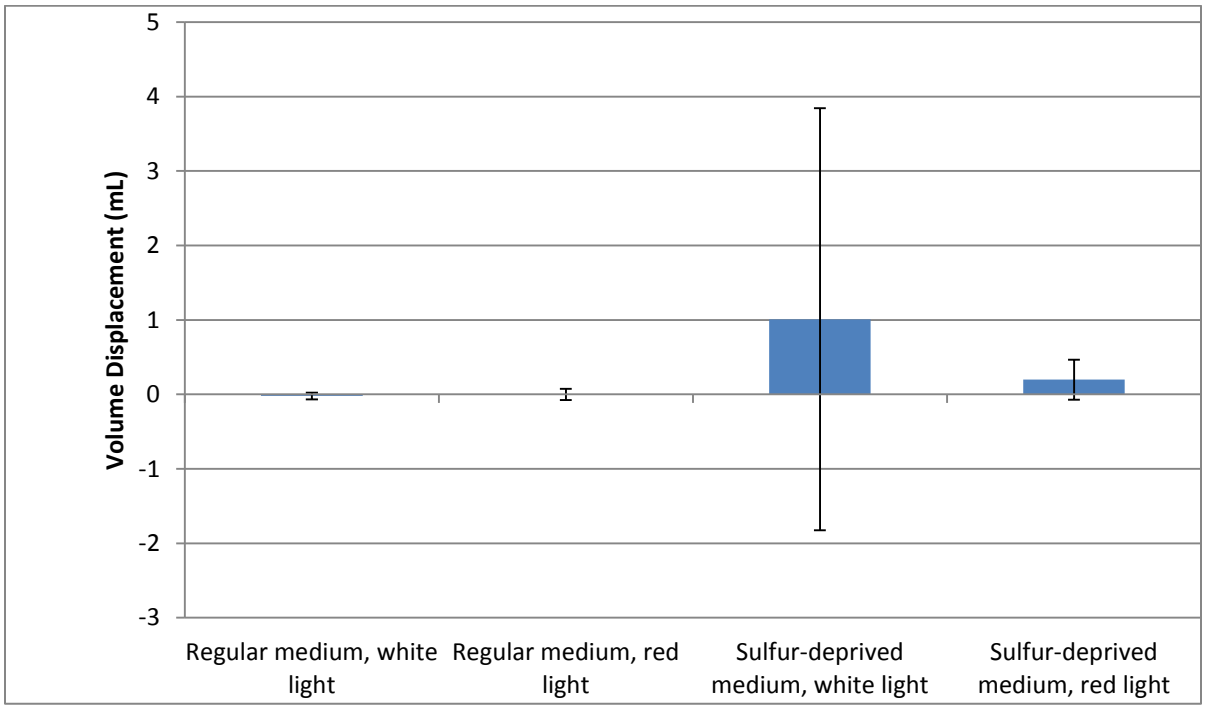

Figure 6 – Mean volume displacement in graduated cylinder vs. treatment type for medium only control. 3 replicates for each treatment. Displacement measured approximately 44 hours after start of experiment. Error bars represent 95% confidence intervals.

The confidence intervals overlap for all treatments in the medium only control (see Figure 6). Furthermore, the confidence intervals all overlap with zero, thus the volume displacement for all treatments in the medium-only control do not significantly differ from zero.

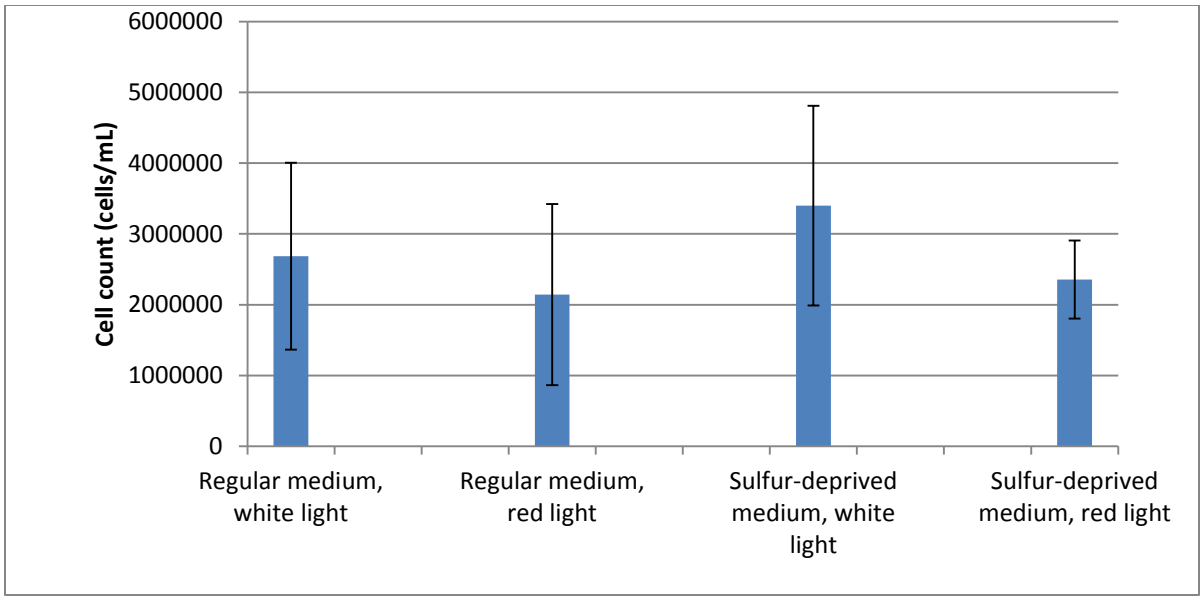

Figure 7 – Mean final cell count for each treatment. Counts measured approximately 6 days after start of experiment. n=3 for each treatment.. Error bars represent 95% confidence intervals.

Figure 7 shows that the confidence intervals for the cell counts overlap for each treatment. Therefore, the cell counts do not significantly differ at the 95% confidence level.

Table 2 – Mean temperature and light intensity with lights on and off for each treatment. Each treatment contained 3 replicates. Intensity was measured at 3 different times for each replicate.

Treatment	**Average light intensity (lights on)**	**Average light intensity (lights off)**	**Average Temperature**
Regular medium, white light	2697 Lux	2550 Lux	25.5°C
Regular medium, red light	2730 Lux	2534 Lux	25.5°C
Sulfur-deprived medium, white light	2470 Lux	2254 Lux	25.5°C
Sulfur-deprived medium, red light	2310 Lux	2177 Lux	25.5°C

Sample Calculations:

a) Volume displaced by tubing per marked mL (samples 1-6, 10-12):

$$Volume\ displaced = \frac{distance}{marked\ mL\ on\ graduated\ cylinder} \times \frac{volume\ displaced\ by\ tubing}{cm\ of\ tubing}$$

$$= \frac{1.1\ cm}{marked\ mL\ on\ graduated\ cylinder} \times \frac{0.4 mL}{cm\ of\ tubing} = 0.44\ \frac{mL}{marked\ mL}$$

b) Tubing correction factor/mL (samples 1-6, 10-12):

$$Correction\ Factor = 1 - volume\ displaced\ by\ tubing\ per\ marked\ mL$$
$$= 1 - 0.44 = 0.66$$

c) Sample Calculation for 2 Particular Wavelengths (contributes to light intensity correction factor):

$$Iv = 683 \int_{700.1}^{700.0} \bar{Y}(\lambda) \frac{dIe(\lambda)}{d\lambda} d\lambda = 683 \left(0.000682 \frac{lm}{sr} + 0.000684 \frac{lm}{sr}\right) = 0.9331146 \frac{lm}{sr}$$

d) Final volume displacement with corrections (Sample 10):

$$= (Final\ Volume - Initial\ Volume) \times Tubing\ Correction\ Factor \times Light\ Intesnity\ Correction\ Factor$$
$$= (2.3mL - 1.5\ mL) \times 0.66 \times 1 = 0.528$$

e) 95% Confidence Intervals (for regular medium, white light):

$$= 1.96 \times \sqrt{\frac{s^2}{n}}$$

$$= 1.96 \times \sqrt{\frac{0.0334}{3}}$$

$$= 0.207$$

Discussion:

Analysis of the data failed to indicate that *C. reinhardtii* cultures exposed only to red light produced significantly more hydrogen than those under white light (Figure 5). Given this, we fail to reject the null hypothesis that *C. reinhardtii* cultures produce reduced to no change in hydrogen production under red light when compared to white light and we fail to support the alternative hypothesis that *C. reinhardtii* yields a greater amount of hydrogen whilst in sulfur-deprived medium under red light (as opposed to white light). . Such a result was not unexpected, given that resource constraints (temporal and material) required the utilization of a minimal

number of replicates for each treatment, such that only extreme shifts could possibly register as significant. However, this is not to say that the experiment yielded no new information, quite the opposite, as it provided a wealth of information, explorative and procedural.

Whilst the data do not support the original supposition that the utilization of solely red light increases the extent of hydrogen production, the statistically significant amount of volume displacement in treatment D, sulfur-deprived medium in red light, as well as the overall trends in the data, indicate the production and capture of hydrogen gas by the apparatus; this, in turn, indicates the system design itself was successful. That the difference between the sulfur-deprived media (treatments C & D) was negligible is telling in its own right; it indicates that, despite restriction of incoming light to that falling within the red spectrum of visible light, there appeared to be no significant decrease in hydrogen production. This supports past assessments which hold that the primary mechanism of hydrogen production in *C. reinhardtii* is via PSII independent energetic pathways that utilize red wavelength associated photopigments in the anoxic catabolism of starch (for which hydrogen is a by-product) (Fouchard *et al.* 2005, Hemschemeir *et al.* 2008, Jo *et al.* 2006, Melis and Happe 2001).

A peculiarity noted amongst the experimental results is the significantly increased variance of treatment C (white light, sulfur-deprived medium) compared to each of the other results, as indicated by its much larger 95% confidence interval in Figure 5; this is further made apparent given the relative uniformity of the other treatment's 95% confidence intervals. A possible explanation for this aberrance, apart from experimental errors, is the energetic and biochemical nature of *C. reinhardtii*'s photosystems. As previously noted, *C. reinhardtii* primarily utilizes PS-II independent energetic pathways in the production of hydrogen, however, it also may utilize a much less efficient (from a hydrogen production standpoint) PS-II dependent

pathway (Hemschemeir *et al.* 2008). This secondary pathway serves as an alternative source of energy for *C. reinhardtii* (by introducing a secondary source of energized electrons apart from those obtained in the catabolism of starch), allowing it to survive longer in situations where it cannot engage in photosynthesis (i.e., a sulfur-deprived medium) (Melis and Happe 2001). Utilization of this pathway, however, prevents *C. reinhardtii* from specializing in PSII-independent pathways, the more efficient effector of hydrogenase activity, thus decreasing overall hydrogen production (Hemschemeir *et al.* 2008, Melis and Happe 2001, Tamburic *et al.* 2008). Notably, not every *C. reinhardtii* specimen may utilize PSII-dependent pathways to the same extent as another; pigment composition and oxidative stress accumulated prior to engagement of hydrogen producing metabolism limits the degree to which PSII-dependent pathways may be used, such that a rather variable gradient of how much each system is utilized forms across populations (Yildiz *et al.* 1994).

Such variability among specimens is important as, given hydrogen production can be seen as a function of how much the individual *C. reinhardtii* specimens rely on PSII-independent pathways, the more a population varies in its ability to utilize PSII-dependent pathways (and thus specialize less with PSII-independent ones), the more variable its hydrogen production will be (Tsygankov *et al.* 2002). While such variance certainly explains why treatment C experienced such vastly increased variance in our experiment, it then begs the question why treatment D failed to also have such an increased variance. The answer to this is relatively simple: *C. reinhardtii*'s PSII is dependent on photopigments associated with blue light such that, if no blue light is available, *C. reinhardtii* must rely on PSII-independent metabolism (Hemschemeir *et al.* 2008, Melis and Happe 2001, Tsygankov *et al.* 2002); given the cultures in treatment D were only exposed to red light, they were forced to rely entirely on PSII-independent metabolism,

meaning each culture was producing approximately the same amount of hydrogen by virtue of relying on PSII-independent metabolism to the same extent (that is, 100%). Treatment C, however, also had access to blue light; as such, an additional variable was introduced in the form of to what extent each population could utilize PSII-dependent pathways. Ultimately this means that, while treatment D's variance in hydrogen production would not vary especially much between cultures (as each is utilizing hydrogen production mechanisms in the same manner), the variability between cultures in treatment C in their utilization of the two photosystems would result in an increased overall variance.

The experiment was host to a series of issues, some of which were overcome via the flexibility of the experimental design and others which may represent lurking variables in the study. Foremost of these issues was the matter of light intensity difference between samples located underneath the red wrap and those located under the clear wrap. As further testing using the two wrappings indicated, the red wrapping decreased the intensity of lighting (unsurprising, given total intensity is a function of the integral of the sum of each wavelength's light intensity) (Van Derlofske *et al.* 2000). Given that changes in light intensity have been correlated with shifts in hydrogen production for *C. reinhardtii* (Gfeller and Gibbs 1983, Jo *et al.* 2006, Tamburic *et al.* 2008, Tsygankov *et al.* 2002), this was invariably a concern in examining the data. The solution, however, was quite simple; with the knowledge that *C. reinhardtii* primarily utilizes red light during hydrogen production, and using information garnered by Van Derlofske *et al.* (2000) relative contributions of red light to the overall intensity of a standard fluorescent bulb were found and utilized to produce the red light intensity of the light passing through the clear film. With this in hand, the intensity values were compared against that for the light passing through the red film to determine the intensity differential of red light between the two films, which was

then compared against the recorded values associating light intensity with hydrogen production (Gfeller and Gibbs 1984).

Two additional issues raised were the lack of systemic controls in the experiment (i.e. seeing whether pure medium produced changes in gas volume) and gas volume change due to standard photosynthesis (as would be carried out by the samples in regular medium). However, these issues were determined to be non-factors via both literary and experimental means. The matter of systemic controls was solved serviceably by doing a second test to see whether pure medium produced volume changes for which the results were emphatically negative and, furthermore, all literature on the subject agreed pure medium in such a set-up would not produce changes in gas volume (Datsenko *et al.* 2012, Melis and Happe 2001). Similarly, no significant changes were noted in the gas volume associated with cultures in regular medium and the literature indicated only extremely large volumes of gas over extended periods of time could produce discernible variations in volume due to photosynthetic and other associated metabolic processes by the cultures (Thongbai *et al.* 2010).

One final factor which, unfortunately, represents a possible unresolved lurking variable is variations between group members in the measurement protocol of gas levels in the graduated cylinder as well as the generally fallible nature of human perception. While little can be done at this juncture to identify and resolve any possible issues of this sort, future experiments in this vein would be recommended to utilize the same group of people (if possible) for measurements to eliminate variation among individuals and to utilize a reference alongside the tubes to ensure precision in measurement (as was used in the first set of measurements). Similarly, physical disturbances to the apparatus (likely during measurement) may have resulted in undesired

displacements of volume; this is likely the cause of the (statistically insignificant) drop in gas volume for treatment A, regular medium, white light (Figure 5).

Conclusion:

We failed to reject our null hypothesis that the hydrogen production of sulfur-deprived *Chlamydomonas reinhardtii* under red light is less than or the same as the hydrogen production of sulfur-deprived *Chlamydomonas reinhardtii* under white light. We did observe a trend for increased hydrogen production under a red filter compared to under a clear filter, however this trend was not statistically significant. As a pilot study, we found various areas to address and improve upon for subsequent experimentation in this field.

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