

# The Effects of Environmental Stressors on Aggregation in *Parachlorella kessleri*

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## Abstract

The induction of biofilm formation has been explored as a means of harvesting microalgae for bioprocessing applications. Environmental stressors have been implicated in the induction of biofilm formation; however, it is unclear whether all stressors, or a select few, are responsible. This study aimed to investigate the effects of three stressors, nitrogen depletion, reduced or elongated day lengths, and increased culture turbulence on biofilm formation of *Parachlorella kessleri*. We also examined corresponding effects on growth and production of reactive oxygen species. Turbulence induced the greatest response in which a significant decrease in growth plus an increase in superoxide production and flocculation efficiency were seen for the 300-rpm treatment. For varying day lengths, stress response was not observed, however, a significant increase in EPS secretion was measured in both shorter and longer days. Nitrogen depletion induced a low-level stress response, in which superoxide production increased for the highest concentrations, while growth was not impacted. In contrast to previous studies on nitrogen depletion, a significant increase in EPS secretion was not observed. Results indicate that stress response varies according to type and magnitude. EPS production and thus biofilm formation are not linked to the stress indicators investigated. *P. kessleri* uses small quantities of EPS to contribute to cell stickiness, but not necessarily to the full formation of a biofilm; however, cell stickiness served as a mechanism for substrate adherence and cellular aggregation none the less.

## Keywords

*Parachlorella kessleri*, Algal Physiology, Biofilms, Day Length, Microalgae, Nitrogen Deficiency, Turbulence

## 1. Introduction

Newly available environmental substrates are colonized through a successional

pattern of adherence by bacteria, algae, and fungi, with species domination based on nutrient availability and environmental conditions [1] [2]. Their attachment is facilitated by exopolymeric substance (EPS) production [3]. Aggregation into biofilms occurs via cellular encapsulation into EPS matrix [4]. EPS also facilitates beneficial nutrient gradient formation and cellular interactions [5].

Thin, initial layers of EPS reduce static forces between cells, allowing for autoflocculation and increase cell stickiness [1]. At this stage, cells are able to attach to a substrate, particulates, and/or each other into aggregates and, as secretion progresses, EPS can fully encapsulate cells to form the biofilm [6]. Despite the significance of biofilms, research is only beginning to elucidate their formation [7].

Recent research has focused on exploiting biofilms for harvesting microalgae for application in health supplements, water quality management, and biofuel feedstock production [6] [8]. Due to the complexity and specificity of EPS production and biofilm formation, identifying the factors required in the induction of biofilms has proven difficult [1]. In particular, information available for members of division Chlorophyta, which include many of the green, coccoid microalgae that have more recently gained attention in the commercial and industrial applications mentioned above, is notably difficult to find [9]. Factors including irradiance, temperature, nutrients, and species composition have been the major focus of large-scale production in photobioreactors and raceway ponds [9].

Microalgae must adapt to both short and long-term fluctuations of the surrounding environmental conditions [10]. Stress responses appear to be specific to the stressor and the organism, even showing great variance between closely-related species [1] [7]. EPS production has been linked to environmental stressors [7].

Environmental stressors tend to lead to an increase in the intracellular production of reactive oxygen species (ROS) [11]. Antioxidant defense against ROS accumulation has been shown to differ significantly among algal species [12]. Additionally, decreases in growth due to growth arrest, long-term encystment, and changes in the cell morphology have been linked to environmental stress [13] [14].

Nutrient deprivation, high intensity light, salinity, and desiccation stresses have been linked to biofilm formation in microalgae [15] [16] [17]. Although biofilm formation has generally been correlated with stress, it is unclear whether all stressors induce a similar response. Nitrogen limitation has been shown to affect the various stages from initial flocculation to biofilm accumulation. Increases in autoflocculation occur in nitrogen-limited cultures [18] [19], while several studies have made the link between the induction of polysaccharide secretion in nitrogen-limited cultures and the subsequent formation of aggregates [6]. Correspondingly, stimulation of extracellular polysaccharides secretion in response to stress has been noted in a variety of species [20] [21] [22].

Nutrient deprivation often leads to a surplus of carbon within the cell, and thus increasing the C:N ratio. CO<sub>2</sub>-fixation continues unchanged but the carbon cannot be used for protein synthesis and is funneled into carbohydrates [6]. The excess carbohydrate from carbon overflow is stored intracellularly until it exceeds that which the cell can store, and is secreted extracellularly as EPS [22]. Glyoxylate simulates nitrogen deprivation through the acceleration of photosynthesis and the inhibition of photorespiration, without augmenting N-metabolism [20] [23]. Yang *et al.* [6] showed that glyoxylate increased extracellular polysaccharide content and an increase in cellular aggregation and size in *Chlorella sorokiniana* (*pyrenoidosa*).

Microalgal polysaccharide production may be linked to a circadian clock [24]. Cell stickiness of *Chlamydomonas reinhardtii* has been shown to increase at night [25]. Day length changes affect photosynthetic processes and carbon metabolism and intra- and extracellular polysaccharides. Cellular responses to a variation in light intensity or duration show overlap [26].

Grobbelaar [27] showed that turbulence elicits beneficial aspects of mixing, nutrient redistribution, access to higher quality light, and the removal of waste as well as detrimental effects of shear stress, through mechanical disruption of the cellular membrane and/or wall [28] [29]. Mitsuhashi *et al.* [30] found that photosynthesis by *Parachlorella kessleri* is also inhibited by turbulence through hydrodynamic shear stress, a form of disturbance associated with physiological disruption as opposed to mechanical. As is often found for microalgae, the response to turbulence stress is species-specific, where green microalgae have been found to be more tolerant [9] [28].

Decreases in growth in response to turbulence have been observed in *Parachlorella kessleri* and *Scenedesmus quadricauda* [31]. Rodríguez *et al.* [32] showed that turbulence stress led to an increase in lipid peroxidation. Turbulence-induced biofilm and EPS viscoelastic properties have been linked to protection from shear stress [5] [21] [33].

The research reported here investigated whether simulated nutrient, photoperiod or turbulence stresses induce aggregation in *Parachlorella kessleri* by measuring the flocculation efficiency and polysaccharide content. This investigation also determined if common stress responses, such as growth inhibition and ROS production, could be linked to cellular aggregation.

## 2. Materials and Methods

### 2.1. Cultivation

Cultures of bacteria-free *Parachlorella kessleri* (*Chlorella vulgaris*) strain UTEX398 were obtained from Carolina Biological Supply (Burlington, NC). Stock cultures were maintained in 2-liter Erlenmeyer flasks using Guillard's F/2 media supplemented with MgSO<sub>4</sub> and CaCl<sub>2</sub> (all chemicals were reagent grade and purchased from Sigma Chemical (St. Louis, MO, USA) unless otherwise stated) at final concentrations of 0.3 mM and 0.17 mM, respectively. A pH be-

tween 7.4 and 7.8 was maintained. Batch cultures were maintained at 23°C and continuously aerated with ambient air. Illumination was provided with a 16:8 h light: dark cycle by 40-watt cool white fluorescent lamps 16 cm from the culture flask. The photosynthetically active radiation (PAR) at the surface of the culture container was 90  $\mu\text{mol photons m}^{-2}\cdot\text{s}^{-1}$  (LI-COR photometric sensor, Lincoln, NE, USA). Cell density was monitored using a Beckman (Irvine, CA) DU 640B spectrophotometer (680 nm) using a standard curve generated using a hemocytometer. Cultures were diluted to an absorbance of 0.75 ( $2.1 \times 10^7$  cells/ml) using distilled water. Experimental subcultures (150 mL) were grown as described above except in 250 mL I-Chem® glass septa jars (5 cm W  $\times$  11 cm H) covered with aluminum foil or as specified below (Section 2.2). All cultures were mixed by swirling the contents of the culture container twice daily.

## 2.2. Stress Treatments.

*Parachlorella kessleri* cultures were subjected to three stress treatments: glyoxylate (to induce nutrient stress), changes in day length, and turbulence. Glyoxylate additions were based on Yang *et al.* [6] but with modifications. Briefly, glyoxylate powder was dissolved directly into culture suspensions (0, 0.001, 0.01, 0.1, and 1 mM final concentration) by stirring briefly. Culture pH was not significantly affected by glyoxylate additions. Day length treatments were performed by adjusting the light: dark cycle from 16 h: 8 h (control) to 8:16, 12:12, 20:4, and 24:0. Culture turbulence was achieved using a 2.5-cm long magnetic stir bar; stir plates were adjusted to 0, 300, 425, 900, or 1100 rpm. The approximate Reynold's number (Re) for a 2.5 cm long magnetic stir bar was calculated using the formula described by Scoma *et al.* [34] as seen below in Equation (1):

$$\text{Re} = \frac{nD_i^2\rho}{\mu} = \frac{300 \times 0.025^2 \times 1000}{9.95 \times 10^{-4}} \quad (1)$$

where  $n$  is the rotations per minute (rpm) of the stir bar,  $D_i$  is the length of the stir bar (m),  $\rho$  is the liquid density of the media ( $\text{kg}\cdot\text{m}^{-3}$ ), and  $\mu$  is the dynamic liquid viscosity at 22.3°C (Pa·s). The Reynold's numbers were 0, 18,844, 26,696, 56,533, and 69,095. Based on these calculations, all turbulence-treated cultures reported here were within the turbulent, rather than laminar flow, range [27].

Experimental treatments were performed in duplicate and monitored for a period of ten days, after which, growth and flocculation efficiency were calculated. In addition, cultures were examined microscopically and tested for superoxide, hydrogen peroxide and polysaccharide content.

## 2.3. Cell Density and Flocculation Efficiency

Experimental cultures were stirred for 60 seconds, and then the OD<sub>680</sub> was measured immediately. Cultures were left undisturbed for a period of 20 minutes to allow for algal aggregates to settle. An aliquot of each sample was then obtained at a depth of 2.5 cm below the surface and the OD<sub>680</sub> was remeasured. Flocculation efficiency was calculated according to the following equation (Equ-

ation (2)) modified from Oh *et al.* [35]:

$$\text{Flocculation efficiency (\%)} = (1 - A/B) \times 100 \quad (2)$$

where *A*: OD<sub>680</sub> of sample, *B*: OD<sub>680</sub> of reference (control prior to settling period).

Once cultures were measured for cell density and flocculation efficiency, each culture was adjusted to pH 7.6 and OD<sub>680</sub> 0.75 (using distilled water) before measurement of superoxide, hydrogen peroxide and carbohydrate concentrations.

**Superoxide Assay.** Superoxide concentrations were measured *in vivo* with nitroblue tetrazolium (NBT) stain. NBT staining produces a water insoluble precipitate, formazan, which was measured with a spectrophotometer at 570 nm. Following techniques from Hückelhoven *et al.* [36], a fresh solution of NBT was made using two stock solutions. A solution of 10% (w/v) NaN<sub>3</sub> and a 70% aqueous solution of dimethylformamide containing 100 mg/ml of NBT were made. Next, 20 µL of the dimethylformamide/NBT solution was added to 1.967 µL of 10 mM potassium phosphate buffer (pH 7.8), followed by the addition of 13 µL of NaN<sub>3</sub>. The final product yielded a clear, light yellow solution, which was shielded from light.

For NBT staining, a 1.5 mL aliquot of each treatment culture was microcentrifuged at 8000 rpm for 8 minutes at 20°C. The supernatant was removed and 200 µL of the NBT stock solution was applied while mixing with a pipette in order to break up the pellet and evenly distribute the NBT. Staining proceeded for 30 - 40 minutes, at which point, samples were recentrifuged. The supernatant was carefully removed and cells were resuspended in 1 mL of clearing solution (methanol: acetone (1:1)) to remove photosynthetic pigments. Samples were incubated for at least 30 minutes or until the solution was deep green in color and vortexed every 10 minutes. Samples were centrifuged for a third time and the clearing solution (supernatant) was removed. The resulting cells were translucent with dark blue dots (formazan product). Samples were resuspended in 1.5 mL of distilled H<sub>2</sub>O and the absorbance (570 nm) was measured using a Molecular Devices plate reader (Sunnyvale, CA, USA).

#### 2.4. Hydrogen Peroxide Assay

Hydrogen peroxide concentrations were determined based on the method described by Li *et al.* [37] for the macroalgae *Corallina officinalis*. First, a 28 mL aliquot was transferred to a 50 mL conical tube and centrifuged at 2500 rpm for 8 minutes to produce a pellet weighing approximately 0.2 g (wet weight). Pellets were resuspended in 3 mL of a 0.1% solution of trichloroacetic acid (TCA) and placed in an ice bath for 3 minutes. Once chilled, a 1 mL sample was placed into a sterile 2 mL Corning® polypropylene cryogenic vial with approximately 1.5 g of 0.5 mm zirconium/silica beads (BioSpec Products, Bartlesville, OK, USA) and bead-beaten for 10 seconds with three repetitions on high speed. Homogenate

was placed on ice for another 3 minutes and then transferred to a 1.5 mL microcentrifuge tube. Samples were centrifuged at 8000 rpm for 8 minutes. A 0.5 mL aliquot of the resulting supernatant was combined with 0.5 mL of a potassium phosphate buffer at pH 7.0 and 1 mL of a 1 M solution of potassium iodide. After a 1 hr incubation in the dark, the absorbance was measured at 390 nm, compared against a standard curve, and expressed as  $\mu\text{mol/g}$  of wet weight.

## 2.5. Polysaccharide Assay

Polysaccharides, soluble extracellular and bound intracellular, were extracted and purified according to Yang *et al.* [6] with modifications. Briefly, a 10 mL aliquot of each sample was removed and centrifuged at  $17,000\times g$  for 20 minutes at  $4^{\circ}\text{C}$ . The supernatant was transferred into a 15 mL conical tube, while the pellet was resuspended in 7 mL of distilled  $\text{H}_2\text{O}$  and placed into a 50 mL conical tube. Both soluble and bound fractions were placed in a  $-20^{\circ}\text{C}$  freezer. After a period of 12 hours, the bound fractions (pellet) were thawed in an  $85^{\circ}\text{C}$  water bath and replaced in the freezer for 12 hours. Samples were again thawed and subjected to a flash-freeze, using liquid nitrogen, then replaced into the  $-20^{\circ}\text{C}$  freezer for 12 hours. For the final thaw, samples were placed into the  $85^{\circ}\text{C}$  water bath for 1 hour to extract the remaining bound polysaccharide then sonicated (Ultrasonic Homogenizer series 4710) for three 8 second repetitions. Soluble fractions were thawed in a water bath at room temperature.

Soluble and bound fractions were centrifuged at  $17,000\times g$  for 20 minutes at  $4^{\circ}\text{C}$  to remove cell fragments and proteins. The supernatant was removed and passed through a 47 mm diameter GFC Whatman membrane filter (GE Healthcare, Chicago, IL, USA) under vacuum filtration. Ions were removed, by dialysis in 3500 MW cut-off Snakeskin tubing (Fisher Scientific, Hampton, NH, USA) in deionized water for 72 hours with five changes of deionized water. Analysis was performed using the phenol-sulfuric acid method [38].

## 2.6. Statistical Analysis

Statistical analyses were performed using SAS/STAT<sup>®</sup> statistical software. A two-way ANOVA with a randomized block design was used to test the effects of stress treatments at a significance level of  $p < 0.05$ . For each of the three stress treatments, two separate trials were completed at different times. Each trial was performed in duplicate. Since stress treatment trials were performed at different time points, a randomized block design was used to account for variations within the data associated with changes experienced between time points, in which stress treatments were considered fixed effects and trials were considered random effects. In addition, a Tukey test ( $p < 0.05$ ) was performed to group homogenous means within each stress treatment, in which letters above bars in graphs represent the results of the Tukey analysis. All bars labelled "A" are statistically similar to each other as are all "B" or all "C". "A" vs. "B", "A" vs. "C", etc. are statistically different.

### 3. Results

#### 3.1. Culture Growth

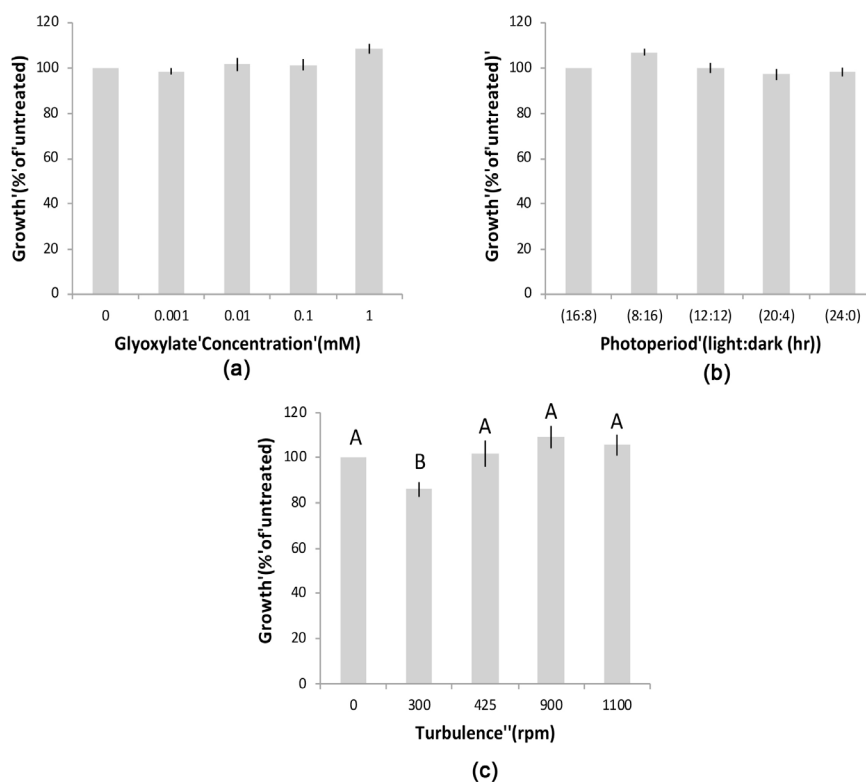
Algae were treated over a broad range (0 - 1 mM) of glyoxylate concentrations in order to simulate nutrient deprivation. As seen in **Figure 1(a)**, at lower concentrations glyoxylate had little effect on growth. A statistically meaningful effect on growth was found in a positive linear correlation ( $R^2 = 0.3755$ ) with glyoxylate concentration and in an 8% increase ( $p = 0.0532$ ) at the highest concentration (1 mM).

As seen in **Figure 1(b)**, increasing day length had an inhibitory effect on growth, in which growth decreased as day length increased from 8 to 24 hours ( $R^2 = 0.4034$ ). The p-value ( $p = 0.1047$ ) showed no statistical significance.

As seen in **Figure 1(c)**, a significant relationship between turbulence treatment and growth was observed ( $p = 0.0039$ ). Most notably, growth of cultures under a turbulence level of 300 rpm decreased by 15%. Higher turbulence showed little to no effect, which was confirmed by a Tukey test. Linear correlation between growth and increasing turbulence was lacking ( $R^2 = 0.0577$ ).

#### 3.2. Flocculation Efficiency

Cellular flocculation causes aggregations to form, which subsequently fall out of



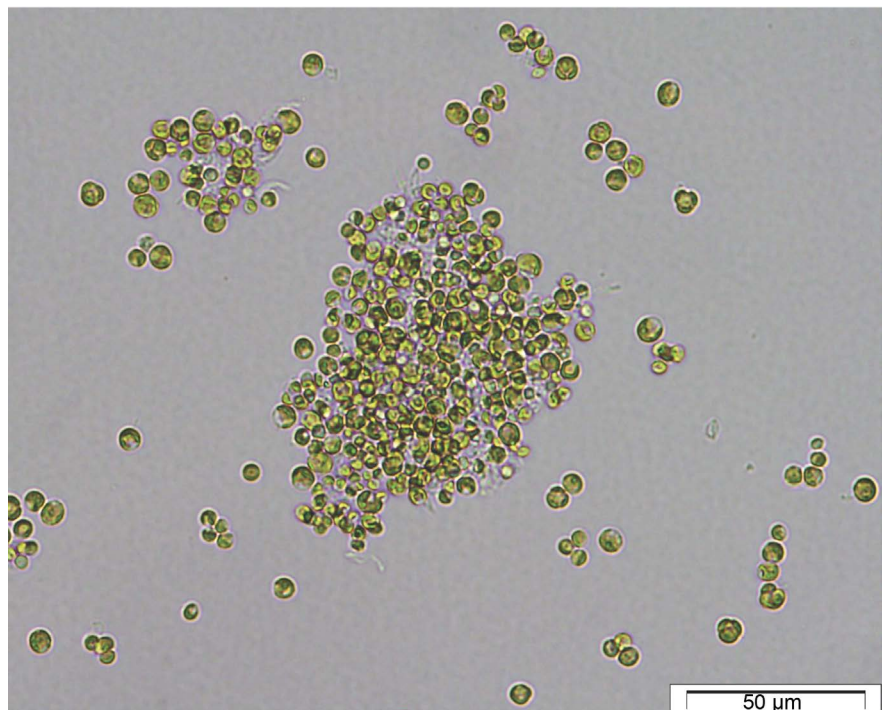
**Figure 1.** Effect of stress on growth. Treatment of cells with glyoxylate (a), photoperiod changes (b), and turbulence (c) was maintained for ten days. Growth values were based on  $OD_{680}$  and normalized as a percentage of the control. Values represent averages ( $n = 4$ )  $\pm$  standard deviation.



suspension due to their increase in weight [39]. Flocculation efficiency measures the proportion of the culture that has aggregated by calculating the difference in optical density pre- and post-settling of flocs. High flocculation efficiency values indicate a flocculation increase whereas zero indicates no effect. Both single cells and cellular aggregates were apparent within each of the cultures examined, as observed under the optical microscope (**Figure 2**); flocculation efficiency was used as quantitative method for measuring relative aggregate frequency in response to stressors.

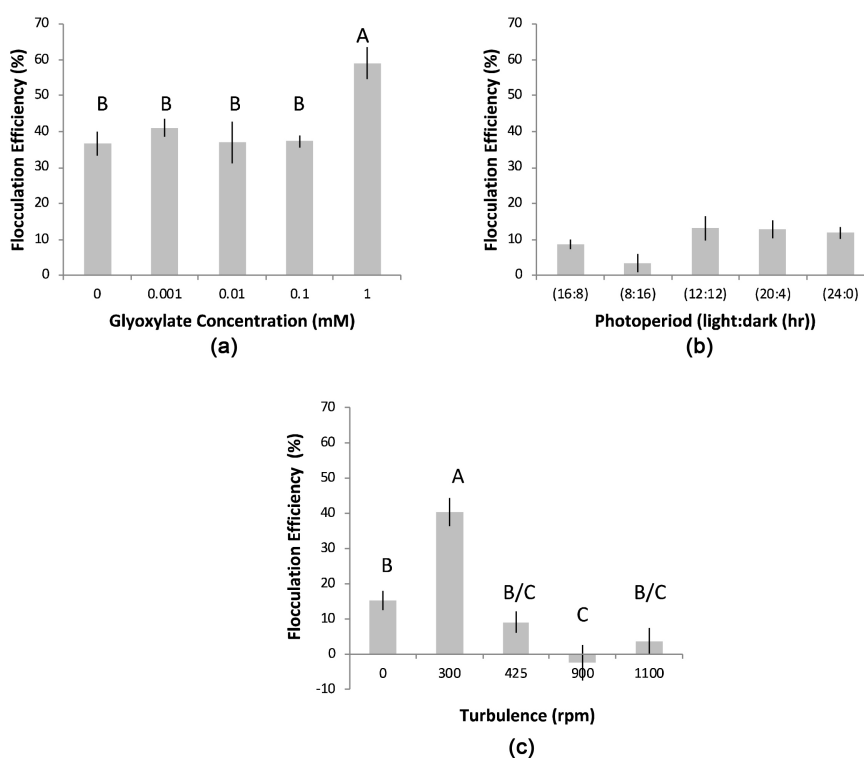
**Figure 3(a)** shows that flocculation efficiency remained relatively unchanged between the control and lower glyoxylate concentrations (0.001, 0.01, 0.1 mM). However, the highest glyoxylate concentration (1 mM) induced a significant increase in flocculation efficiency ( $p = 0.0029$ ). There was a striking visual transition within the water column over the ten-day treatment period from pelagically-dominated cultures to cellular adherence that formed both floating and substrate-adhered flocs, especially with the 1 mM treatment. Flocculation of the 1 mM treatment was supported by the results of differentiation in the Tukey test. The data followed a strong positive linear trend ( $R^2 = 0.5272$ ).

A correlation between daylength and flocculation efficiency was not apparent ( $p = 0.3587$ ,  $R^2 = 0.1805$ , **Figure 3(b)**); turbulence, on the other hand, induced a strong effect on flocculation efficiency which was both visually apparent and statistically significant ( $p \leq 0.0001$ , **Figure 3(c)**). Within the lowest turbulence level



**Figure 2.** Micrograph of single cells and aggregates. Cellular aggregation was easily visualized under the optical microscope (Olympus 1X-81, 200X magnification); each aggregate varied widely in size, ranging from two to several hundred cells. Various aggregate sizes are representative.





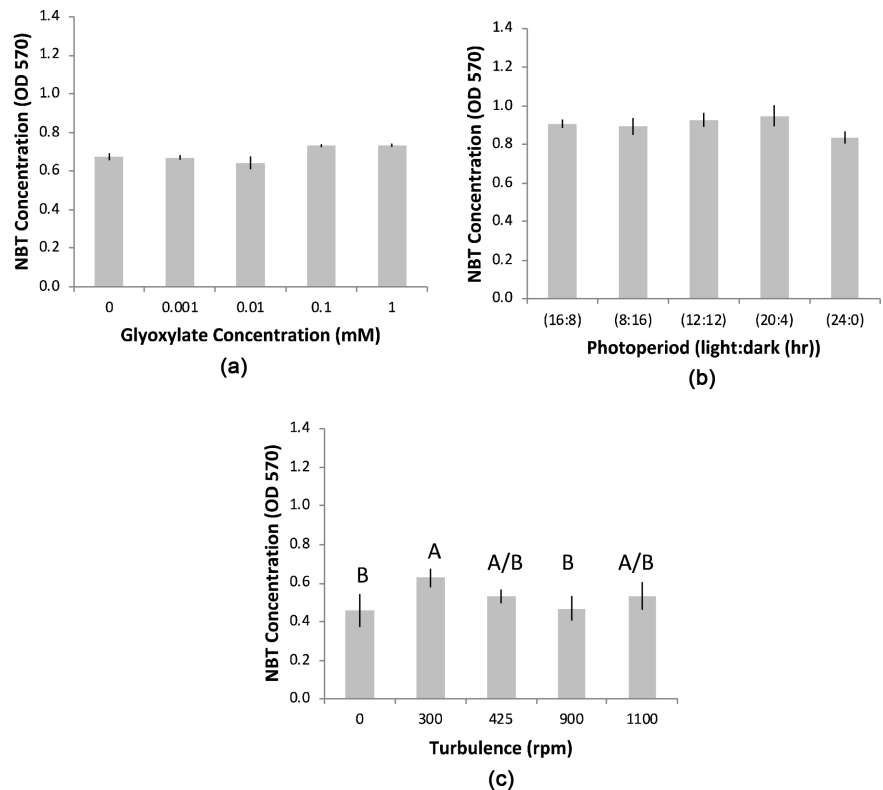
**Figure 3.** Effect of stress on flocculation efficiency. Flocculation efficiency in response to each of three stress treatments: glyoxylate (a), photoperiod changes (b) and turbulence (c) is expressed as a percent change from control  $\pm$  standard deviation with  $n = 4$ .

of 300 rpm the occurrence of flocculation tripled, while cultures subjected to turbulence levels of 900 rpm experienced an unexpected decrease in flocculation efficiency. The 425 and 1100-rpm treatments were statistically similar to each other and the control and 900-rpm treatment (as determined by Tukey's), and thus had much lower values of flocculation efficiency than the 300-rpm treatment.

### 3.3. Superoxide Production

The effect of glyoxylate, photoperiod changes, and turbulence stressors on superoxide and hydrogen peroxide concentrations were measured as indicators of cellular stress. After reduction of NBT by superoxide, a dark blue formazan precipitate was easily visualized within cells as small blue spots and quantitated at  $A_{570}$ .

A small but significant increase in NBT concentration with glyoxylate treatment was observed (Figure 4(a),  $p = 0.0195$ ). Superoxide concentration increased in the two highest concentrations, 0.1 mM and 1 mM, by approximately 9%. A weak positive linear trend was seen ( $R^2 = 0.2522$ ). Interestingly, a significant treatment effect was not observed for changes in daylength (Figure 4(b),  $p = 0.1625$ ,  $R^2 = 0.0277$ ). Turbulence was found to significantly impact the concentration of superoxide concentration within cells ( $p = 0.0232$ ), most notably at 300-rpm (Figure 4(c)). The 300-rpm treatment caused an increase of 36%.



**Figure 4.** Effect of stress on superoxide concentration. Superoxide production in response to each of three stress treatments, glyoxylate (a), photoperiod changes (b) and turbulence (c) was measured as formazan absorbance at 570 nm and NBT concentration was calculated and represented as mean  $\pm$  standard deviation with  $n = 4$ .

Slight increases in superoxide were also noted with the 425 and 1100-rpm treatments. The data did not show a linear correlation ( $R^2 = 0.000$ ).

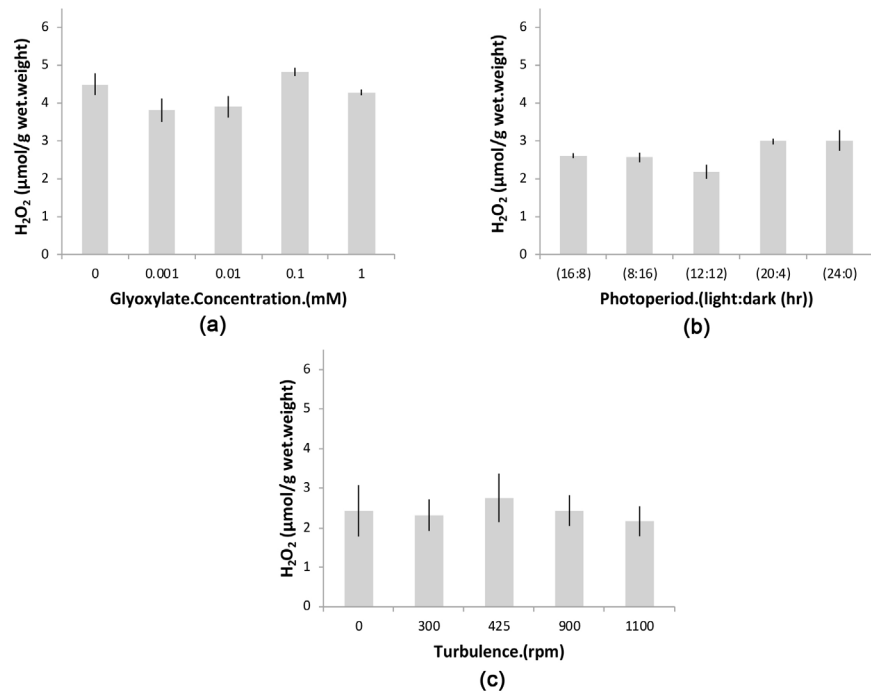
### 3.4. Hydrogen Peroxide Production

Hydrogen peroxide concentration was not affected by any of the three stressors (Figures 5(a)-(c)): glyoxylate ( $p = 0.2555$  and  $R = 0.0046$ ), photoperiod ( $p = 0.1004$ ), where a small increase was observed ( $R^2 = 0.3309$ ), and turbulence ( $p = 0.6144$ ).

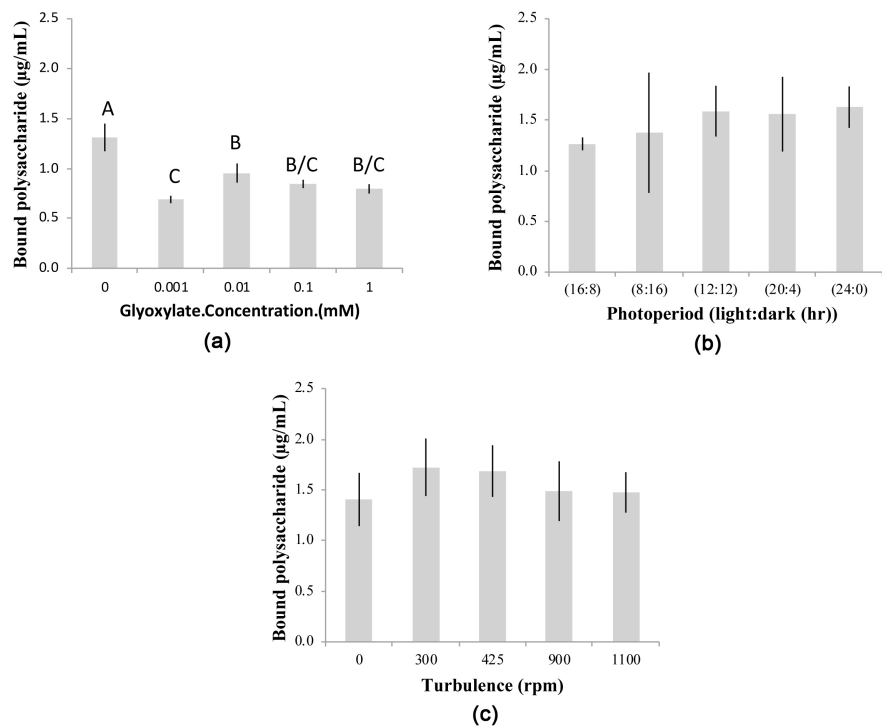
### 3.5. Polysaccharide Content

Figure 6(a) shows a decrease in bound polysaccharides for all of the glyoxylate treated cultures. This trend was supported by a Tukey test, though the ANOVA  $p$ -value was  $p = 0.0620$ . Neither daylength nor turbulence had a significant impact on bound polysaccharide (Figure 6(b) and Figure 6(c),  $p = 0.9591$  and  $p = 0.4381$ , respectively).

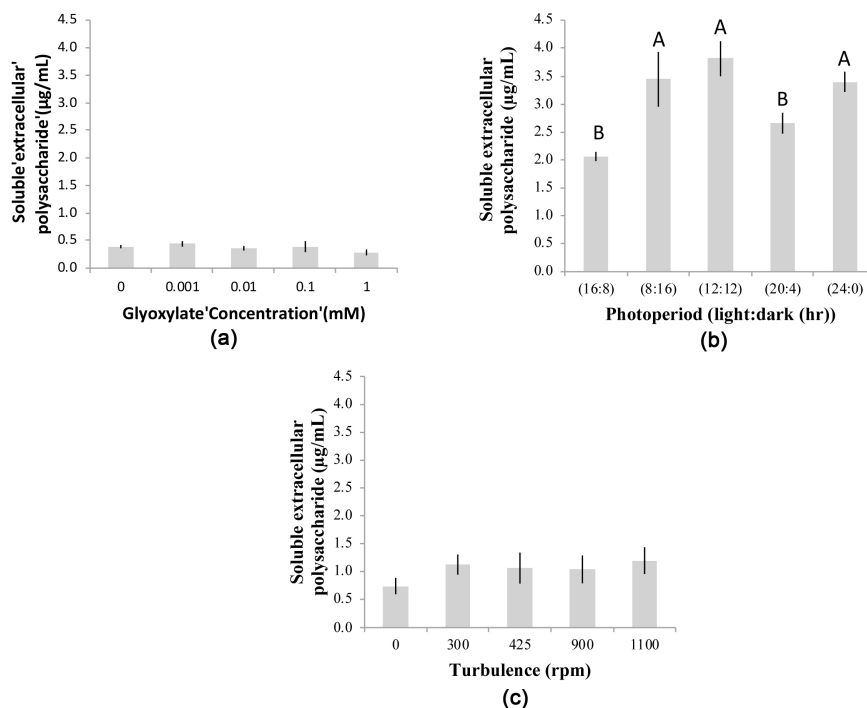
When the effect of stressors on extracellular polysaccharide content was investigated, the biological variability among experiments was extensive (Figure 7). Extracellular polysaccharide content was significantly affected by variations in photoperiod (Figure 7(b),  $p = 0.0110$ ), although not in a linear fashion ( $R^2 =$



**Figure 5.** Effect of stress on hydrogen peroxide concentration.  $H_2O_2$  content was determined following each of three stress treatments: glyoxylate (a), photoperiod changes (b) and turbulence (c).  $H_2O_2$  concentration was standardized to  $\mu\text{mol}$  per gram of wet algae. Values represent mean  $\pm$  standard deviation with  $n = 4$ .



**Figure 6.** Effect of stress on bound, intracellular polysaccharide concentration. Bound, intracellular polysaccharide content was measured following treatment with glyoxylate (a), photoperiod changes (b), and turbulence (c). Values,  $\mu\text{g}$  per ml, represent mean  $\pm$  standard deviation with  $n = 4$ .



**Figure 7.** Effect of stress on extracellular polysaccharide content. Polysaccharide content was measured as  $\mu\text{g}$  per ml in three stress treatments: glyoxylate (a), day length changes (b), and turbulence (c). Values represent mean  $\pm$  standard deviation with  $n = 4$ .

0.0870). Daylengths of 8, 12, and 24 hours produced similarly and optimally, while no difference was noted between the control (16-hour) and 20-hour day lengths. In contrast, glyoxylate (Figure 7(a)) and turbulence treatments (Figure 7(c)) did not have an effect on extracellular polysaccharides ( $p = 0.2212$  and  $p = 0.4266$ ).

#### 4. Discussion

The results shown here demonstrate that there are differences in cellular responses to different stressors and that they must be evaluated on a case-by-case basis. Glyoxylate treatment and nitrogen deprivation have each been shown to inhibit cell growth of microalgae [6] [21] [40]. In the study reported here, however, growth was not inhibited but slightly enhanced. Yang *et al.* [6], found that only the highest glyoxylate concentration of 1.25 mM arrested cell growth. The discrepancy between that study and this is unclear. It is possible that inhibition is masked by early enhancement of growth as a consequence of the increased photosynthetic activity that glyoxylate causes. Bergman [41] did show that glyoxylate stimulated growth in three cyanobacterial species.

We found a significant increase in superoxide at the two highest glyoxylate concentrations. Menon *et al.* [42] found that *Parachlorella kessleri* with higher levels of ROS also had the highest growth rates. Since ROS are notoriously short-lived, measurement of ROS concentrations can only provide a “snap shot” of cell status [42]. The lack of effect that we found for glyoxylate on cellular

growth could be attributed to cellular recovery over the ten days.

Whereas, EPS can act as a carbon overflow mechanism Yang *et al.* [6] noted a step-wise increase in EPS and aggregate occurrence and size with glyoxylate. We only observed an increase in aggregation at the highest glyoxylate treatment (1.0 mM) and extracellular polysaccharides remained largely unchanged. Instead, a decrease in intracellular polysaccharides was noted, and the same was noted for total polysaccharide (data not shown).

The increase in flocculation efficiency without substantial EPS production with glyoxylate was unexpected, since previous reports have shown a link [6]. However, despite the lack of significant extracellular polysaccharide production, significant cellular aggregation did occur. Thin layers of polymeric coatings have been shown to increase cell stickiness and aggregation in microalgae [6] [43].

Since our cultures were supplied solely with atmospheric CO<sub>2</sub>, the increased rate of glyoxylate-induced carbon metabolism could deplete media of available carbon, effectively negating the glyoxylate effect. As such, cellular perception of a nitrogen-deprived state would dissipate.

Studies have indicated that increases in day length produce a proportional increase in growth until reaching the saturation point of the photosynthetic machinery [26] [44]. This would be expected for a culture initially, or as a consequence of high growth, limited by light availability. We report that above 8 hr, light was not limiting since additional light did not appreciably increase growth.

A negative linear trend between growth and daylength was noted with growth maximized in the 8-hour day length. In the diatom *Nitzschia* and green alga *Spaerocystis*, growth rates declined with increasing day length [44]. Krzeminska *et al.* [45] found that 24-hour day lengths stimulated growth of *Botryococcus braunii* and *Scenedesmus obliquus*, but a 12-hour day length was optimal for three *Neochloris* species investigated. With varying light intensities, growth of *Parachlorella kessleri* were all significantly greater under a light regime of 16:8 (light:dark) than 12:12 or 8:16 [26]. Microalgae grown under short day lengths have been shown to adapt by increasing light harvesting pigments [44] which may, in part, explain the ability of *C. kessleri* to grow at an elevated rate when cultured under short days.

Our hydrogen peroxide results suggest that *Parachlorella kessleri* was experiencing light oversaturation at 20 and 24-hour day lengths. Interestingly, superoxide production did not follow the same trend. Hastings *et al.* [46] noted that SOD activity increases during the day in the dinoflagellate *Lingulodinium*. The decrease in growth and increase of hydrogen peroxide suggests that the cells exposed to longer days were under stress.  $\beta$ -carotene and  $\alpha$ -tocopherols, which actively work to dissipate excess energy and directly quench ROS, accumulate in response to UV-B, long day lengths, and high light exposures [11]; these are likely factors in the muted responses we observed after 10 days.

Flocculation efficiency was not appreciably impacted by variations in day length. In previous studies, we observed that cells grown at high cell densities

with light limitation adhere to the sides of the growth chamber (data not shown). At the much lower cell densities of this report light availability did not appear to be limiting. Therefore, cell adherence due to light limitation was denecessitated.

Di Pippo *et al.* [9] found that higher irradiances of 60 and 120  $\mu\text{mol photons m}^{-2}\cdot\text{s}^{-1}$  are required for biofilm formation including the 90  $\mu\text{mol photons m}^{-2}\cdot\text{s}^{-1}$  used in this study. In addition, EPS has been shown to provide protective functions related to high irradiance, such as quenching ROS and protection against UV-B radiation [5] [8]. It was anticipated that an increase in day length would increase EPS production. An increase in extracellular polysaccharide was observed for a change to a shorter (8 and 12-hr) or longer (24-hr) day length, however, intracellular polysaccharide content did not change.

Wolfstein and Stal [47] found that polysaccharide secretion occurred as an overflow mechanism during low temperature stress, as cells reverted to a resting state. Alternative to the carbon overflow mechanism seen for excess light exposure, reversion to a resting state can cause carbon flux by shutting down the pathways through which carbon is typically funneled for metabolic processes. Thus, carbon builds up within the intracellular spaces and must be diverted extracellularly as EPS. Reduced day lengths, perceived as a seasonal change [44], may also induce EPS production. If shortening the day length were perceived then a reduction in growth would be expected, and thus activation of EPS overflow. Our results confirm that large quantities of EPS are not required for aggregation, and subsequent biofilm formation, in *C. kessleri*.

Despite the frequency in which microalgae naturally experience turbulence, and the importance to environmental studies and commercial algae operations, research on this topic is fragmented [29]. Stirring produced a vortex in cultures subjected to 425, 900, and 1100-rpm, which was not seen in at 300-rpm. Regardless of vortex formation, Reynold's numbers were well above 2000, the laminar flow threshold, and thus produced a more irregular and disordered turbulent flow [27]. In the 300-rpm treatment, the consistency of stirring was compromised over time by a progressive increase in friction between the stir bar and the container bottom; the 300-rpm rate did not prevent settling of cellular debris.

Leupold [29] showed that growth of *Parachlorella kessleri* increases in response to shear stress, although Hosaka *et al.* [30] and Mitsunashi *et al.* [30] noted that growth decreased initially under short-term treatment. Leupold *et al.* [29] also noted that growth rate depended on the magnitude of shear stress applied, increasing with rate but hitting a threshold then decreasing at higher rates. Photosynthetic activity followed a similar trend but recovered after treatment ceased; in agreement with both the results noted by Hosaka *et al.* [48] and Mitsunashi *et al.* [30]. The positive responses to turbulence are true for all species, but detriments are dependent upon the morphological characteristics of the alga [29] [49].

In the 300-rpm treatment cells were sinking to the bottom, and disrupting the



motion of the stir bar. Several studies have noted that microalgae will naturally settle out of solution, increasing aggregation/autoflocculation, but not creating a firm attachment to the surface on which they settle [29] [50]. Any contact, but especially repeated encounters with the stir bar, would greatly increase the likelihood of mechanical damage. Growth was significantly decreased at 300 rpm. With greater turbulence, the positive and negative aspects of stirring appeared to have been balanced, as indicated by growth rates that remained relatively unchanged. At 900 rpm, the balance favored growth.

Flocculation efficiency was significantly increased at 300 rpm. At higher turbulence, flocculation efficiency decreased; in particular, the 900-rpm treatment had a negative % flocculation efficiency. It is worth noting that the treatment with the lowest growth rate had the highest measurement for flocculation efficiency (the 300-rpm treatment). In the 900 and 1100-rpm treatments a ring of cellular aggregation formed on the glassware directly below the water-air interface. This location may have served two purposes: one, this location was furthest from the stir bar, where the cell would experience a reduced turbulence and possibility of mechanical damage, and two, increased access to CO<sub>2</sub>.

Increased flow velocities will decrease the size of the cells' boundary layer, increasing cell: cell and cell: surface collisions [1]. Aggregation will occur until the shear becomes great enough to overcome cellular attachment [1]. Johnson and Wen [50] noted that microalgae will form a firm attachment, only when subjected to some turbulence.

Extracellular polysaccharide concentrations were elevated by turbulence. Dayananda *et al.* [51] found that *Botryococcus* had greater EPS production with turbulence treatment, while Cordoba-Castro *et al.* [52] noted a linear relationship between turbulence rate and EPS secretion in *Scenedesmus obliquus*. Although the mechanism has not been resolved, several studies have noted that EPS increases viscosity and thus decreases the impact of turbulence [21].

It would be expected that with similar levels of EPS, that aggregation in lower turbulence treatment would be less. Di Pippo *et al.* [1] noted that biofilm formations were disrupted by the detachment of flocs, or aggregates, by increasing circulation rate. This agrees with our results in that despite the higher collision frequency in treatments with higher turbulence flocculation efficiency was low. The finding that extracellular polysaccharide concentrations remained consistent shows that increased polysaccharide secretion was not the only factor in the mechanism for attachment.

Rodríguez *et al.* [32] reported that oxidative stress increased during turbulence. Interestingly, ROS production depended greatly on the level of shear stress. Superoxide was significantly increased by the 300-rpm treatment, correlating with the decreased growth and increased flocculation efficiency. In contrast, at 900-rpm ROS was similar to that of the control. Our results suggest that cells undergoing mechanical stress, at 300-rpm do not acclimate.

Overall, the effect of the stressors investigated elicited strikingly varied res-

ponses in EPS production and biofilm formation. The response of *P. kessleri* showed variation among the environmental stressors tested. These results were unexpected in that we set out to confirm what was reported in the literature then develop an effective harvesting method for algae grown for biofuels but we found a much more complex system. This body of research demonstrates the complexity of stress detection and response in these organisms.

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## Conflicts of Interest

The authors declare no conflict of financial interest.

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### Abbreviations

EPS, extracellular polymeric substances;

ROS, reactive oxygen species;

NBT, nitroblue tetrazolium.