THE EFFECTS OF ENVIRONMENTAL STRESSORS ON BIOFILM FORMATION OF *CHLORELLA VULGARIS*

A Thesis
by
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Abstract

THE EFFECTS OF ENVIRONMENTAL STRESSORS ON BIOFILM FORMATION OF *CHLORELLA VULGARIS*

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The induction of biofilm formation has been explored as a means of harvesting microalgae for bioprocessing applications. Recently, environmental stressors have been implicated in the induction of biofilm formation; however, it is unclear whether all stressors, or a select few, are responsible for this phenomenon. This study aimed to investigate the effects of three stressors on biofilm formation of *Chlorella vulgaris*. We hypothesized that *C. vulgaris* would experience stress in response to nitrogen depletion (glyoxylate treatment), reduced or elongated day lengths, and with increased culture turbulence. Additionally, we hypothesized that common indicators of stress, such as growth inhibition, cell size changes, and production of reactive oxygen species, would correlate with biofilm formation.

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. Stress response was not found for higher turbulence levels. In addition, stress response was not observed for varying day lengths, although, a significant increase in EPS secretion was measured in both short and long 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, an increase in EPS secretion was not observed. The results indicate that stress response varies according to the applied stress, biofilm formation is not linked to a particular stress indicator, and that *C. vulgaris* uses cell stickiness both as a mechanism for substrate adherence and cellular aggregation.

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Dedication

Thierry and Barbara Olivry: When I was incapable of seeing the forest for the trees, you identified worth and potential; your humble and earnest convictions have guided me both in my studies, but more importantly, in life. Thank you both for so much help and support. To you, I dedicate this thesis.

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Foreword

The research outlined in this thesis will be submitted to the *Journal of Applied Phycology*. This thesis has been prepared according to the citation requirements and formatting for said journal publication.

Introduction

Green algae are a diverse group of photosynthetic eukaryotes, some of which represent the ancestral lineages of modern land plants (Yoshida et al. 2003; Lewis and McCourt 2004). Although most of these organisms inhabit aquatic environments, both marine and freshwater, green algae have been reported to live ubiquitously, inhabiting a wide range of ecosystems and climactic zones (Gustavs et al. 2010; Karsten and Rindi 2010). Within any environment, algae must adapt to both short and long term fluctuations of the surrounding environmental conditions. Common changes include fluctuations in light intensity, pH, temperature, and osmotic and chemical gradients, as well as limitations in nutrient and water availability (Mallick and Mohn 2000; Choo et al. 2004; del Hoyo et al. 2011).

For newly available substrates within the environment, a successional pattern of colonization of bacterial, algal, and fungal species occurs, with species domination based on nutrient availability and the surrounding environmental conditions (Sabater and Romaní 1996; Sekar et al. 2002; Pohlon et al. 2010). These organisms are often found in conjunction with one another; their attachment to the substrate surface is facilitated through the production of exopolymeric substances, or EPS, that are largely composed of polysaccharides. Interestingly, both bacterial and algal cells secrete these high-molecular-weight polysaccharides, although biofilms can be formed by a single species as well (Sabater and Romaní 1996; Sutherland 2001). The polysaccharide matrix eventually encapsulates

individuals into a collective community, called a biofilm. These assemblages are the primary colonizers of new substrates and actively work to alter the surface dynamics and process organic matter, which often allows for the subsequent colonization by plants (Pohlon et al. 2010).

The complexities of biofilm structure and community dynamics have made it difficult to accurately replicate biofilms within a laboratory setting, and thus information on the environmental cues involved and the individual species that produce biofilms is limited. In particular, algal biofilms have received little attention and the mechanisms responsible for formation are not well understood. More recently, research has focused on the adoption of biofilms as a strategy for harvesting algae for commercial and industrial applications, such as health supplements, water quality management, and as a feedstock for biofuel production (Mishra and Jha 2009; Johnson and Wen 2010; Raposo et al. 2013). Environmental stressors have been implicated in the induction of biofilm formation; however, the types of stressors involved have yet to be identified.

EPS secretion is believed to be induced when cells experience stressful conditions, causing an imbalance in cellular homeostasis (Abdullahi et al. 2006). Outside of a laboratory setting this occurs quite frequently, as environmental conditions vary on both short and long time scales. Although high-molecular weight polysaccharides are the main components of biofilm secretions, they can be accompanied by simple sugars, proteins, amino acids, DNA, lipids, and other biopolymers including humic substances from the surrounding environment (Flemming and Wingender 2010; Michel et al. 2011). For eukaryotic organisms, EPS is produced intracellularly, where it is packaged by the Golgi apparatus into mobile secretory

vesicles; vesicle transport is directed towards the periphery of the cytoplasm. These vesicles wait until a supply of EPS is required by the cell, at which point, vesicle fusion to the plasma membrane occurs at specific fusion zones, releasing EPS to the cell wall. These secretions traverse the cell wall and settle onto the cell surface for biofilm formation (Domozych 2007). A proportion of the EPS may be capsular, bound to the cell surface, while the rest is released into the surrounding media; however, the ratio of bound to free EPS is highly dependent upon the species of microalgae and the environment they are currently experiencing (Sutherland 2001; Abdullahi et al. 2006).

Under normal conditions, suspended particles, including cells, hold a negative charge surrounded by a cloud of positive charges, and thus repulsion between cells due to electrostatic forces occurs. However, changes to the cell surface through alterations in the cell wall characteristics, cell surface coatings, or EPS production, can effectively reduce the static forces between cells, allowing for cellular aggregation (Kiørbe et al. 1990; Henderson et al. 2008; Cheng et al. 2011). Cell stickiness is increased by the secretion of organic polymeric substances, which can form thin layers around the cell surface or form the basis for EPS and biofilm formation (Sutherland 2001). Thin layers of polymeric secretions enhance aggregation through polymeric fibril bridges, which loosely connect adjacent cells (Leppard 1995). In contrast, thicker layers in the form of EPS production encapsulate cells to form a protective barrier. Cellular aggregation is enhanced by both an increase in collision frequency of the cells and the probability of cellular adherence due to cell stickiness (coagulation efficiency) (Kiørbe et al. 1990).

Polymeric secretions act as biological glues providing protection and preservation of the encapsulated species through structural architecture and adherence to the substrate (Leppard 1995). However, this infrastructure also provides a microenvironment in which novel nutrient gradients and cellular interactions can occur. Nutrients are provided through three main mechanisms: 1) nutrient flow occurs between the surrounding media and channels/pores within the EPS; 2) nutrients and foreign substances become trapped and sequestered by the EPS; and 3) the EPS components act as a nutrient sink (Sekar et al. 2002; Flemming and Wingender 2010). Proteins within the EPS function as extracellular enzymes to degrade matrix compounds for absorption and utilization as nutrient sources (Pohlon et al. 2010; Di Pippo et al. 2012). Close proximity of neighbors facilitates a physical closeness required for inter- and intraspecies cell-cell interactions (Imase et al. 2008). Biofilms have also been implicated in the protection of cells against desiccation in terrestrial environments by simultaneously decreasing the propensity for water loss and increasing the adsorption of available precipitation. In addition, biofilms have been suggested to protect against UV radiation, heavy metals, oxidizing agents, antibiotic exposure, and grazers (Flemming and Wingender 2010).

Although the links between environmental stressors and biofilm formation are not fully understood, cellular stress is well known to increase the production of reactive oxygen species (ROS) in response to environmental changes (Mallick and Mohn 2000; Choo et al. 2004). ROS are commonly formed during normal metabolic activities; however, in phototrophic organisms, environmental stresses increase ROS by creating an imbalance in the photosynthetic machinery (Kranner et al. 2003; Weissman et al. 2005). This imbalance

causes leakage of excess electrons, or energy, onto molecular oxygen, producing (a) superoxide, hydrogen peroxide, or hydroxyl radicals, and (b) singlet oxygen, respectively (Mallick and Mohn 2000; Apel and Hirt 2004; del Hoyo et al. 2011). Disruption of cellular homeostasis can cause leakage of electrons from electron transport chains of the mitochondria and plasma membrane as well, increasing the sites in which ROS are produced (Mallick and Mohn 2000). ROS have the potential to cause oxidative damage, termed oxidative stress, to proteins, lipids, and nucleic acids, leading to eventual cell death (Dummermuth et al. 2003; Ledford and Niyogi 2005; del Hoyo et al. 2011). In contrast, ROS have been implicated as second messengers, acting as a signal of cellular distress and mediating the response of plants and algae to both abiotic and biotic stresses (Gonzalez et al. 2010; Li et al. 2010; Mittler et al. 2011).

Antioxidant defense mechanisms are pivotal to algal survival under stressful environmental conditions (Li et al. 2010; Piotrowska-Niczyporuk et al. 2012), but have been shown to differ significantly between algal species, especially in reference to the sensitivity of each species to ROS exposure (Choo et al. 2004; Li et al. 2010; Piotrowska-Niczyporuk et al. 2012; Cirulis et al. 2013). Antioxidants work to detoxify ROS by either directly quenching (energy transfer) or enzymatically degrading reactive molecules within the cell; some ROS are associated with specific antioxidants, while others are detoxified through multiple pathways. ROS concentrations are a balance of the rate of production, which increases under environmental stress, to the rate of quenching/degradation by antioxidants, of which production and activity are enhanced in response to increasing ROS concentrations.

Both superoxide (O^-_2) and singlet oxygen ($^1O_2$) can be quenched by low-molecular-weight, non-enzymatic antioxidants such as β-carotene and α-tocopherol, which are

concentrated near the site of production within the thylakoid membranes (Abe et al. 1999). In addition, superoxide is converted into hydrogen peroxide (H_2O_2) by the enzyme superoxide dismutase (SOD), which has several isoforms within plants and algal cells (Aguilera et al. 2002; Dummermuth et al. 2003; Barros et al. 2005). Hydrogen peroxide is the least reactive of the ROS, freely traversing cellular membranes; increases in intracellular H_2O_2 concentration have been shown to activate antioxidant defense enzymes against oxidative stress (Mittler et al. 2011; Holzinger and Karsten 2013). Hydrogen peroxide is detoxified through several pathways: high-molecular-weight, enzymatic catalases (CAT) and peroxidases (POX) reduce H_2O_2 to water molecules, while ascorbate peroxidases (APX) reduce H_2O_2 through the ascorbate-glutathione cycle (Dummermuth et al. 2003; Li et al. 2010; Piotrowska-Niczyporuk et al. 2012). Interestingly, although catalase activity in multicellular green algae is located within microbodies (glyoxysomes and peroxisomes) as seen in higher plants, Kato et al. (1997) demonstrated that CAT activity in the green microalga *Chlamydomonas reinhardtii* was highly associated with the mitochondria. It was suggested that mitochondrial localization of CAT was a reflection of an increase in ROS production within the mitochondria in comparison to plants. As mentioned previously, however, ROS and antioxidant responses to environmental stressors are different between species (Cirulis et al. 2013). In addition, some species of microalgae secrete sulfated polysaccharides into the EPS, which have been shown to neutralize ROS species (Leppard 1995; Raposo et al. 2013).

Decreases in growth and changes in morphology have also been linked to cellular stress in microalgae. Sub-optimal conditions are well known to decrease cellular growth rates, as the energetic needs of the cell shift from growth and reproduction to maintenance of

cellular homoeostasis (Montechiaro et al. 2006). In addition, Rioboo et al. (2009) found that cellular stress decreases the number of daughter cells produced for each reproductively-active mother cell, causing a decline in cell density over time. A decrease in spore germination may also cause population declines, as a review published by Agrawal (2009) indicated that many sub-optimal environmental conditions inhibited spore germination in microalgae. Under higher levels of stress the induction of programmed cell death (PCD), a cell suicide pathway initiated by extreme environmental conditions, inhibits cellular reproduction while enhancing cell mortality and ultimately inducing a net decline in population growth (Zuppini et al. 2010).

Changes in surface-to-volume ratios have been observed in several microalgae as a response to stress. Decreases in surface-to-volume ratios have been shown to occur in response to osmotic and desiccation stresses and high temperatures, as well as UV radiation; a decrease in surface area lessens diffusion of toxins and evaporative processes across the plasma membrane (Gladis et al. 2010). In contrast, high surface-to-volume ratios have been documented for PCD, in which cell shrinkage is a common morphological feature of the cell suicide pathway (Zuppini et al. 2010). Environmental fluctuations associated with seasonal weather changes, such as a decrease in temperature or day length, have been shown to cause a transformation from larger vegetative cells to smaller resting cells (Holzinger and Karsten 2013). Vegetative and reproductively-active mother cells are more susceptible to stress than resting cells; a population shift to resting spore dominance enhances culture survival. In addition to growth inhibition and cell size changes, loss of the photosynthetic pigmentation, termed chlorosis, is commonly observed in cells subjected to environmental stress, a result of oxidative damage to the photosynthetic machinery (Zuppini et al. 2010; Cirulis et al. 2013).

Several studies have found that nutrient deprivation, high light, salinity, and desiccation stresses are linked to biofilm formation in microalgae (Kaplan et al. 1987; Sekar et al. 2002; Mishra and Jha 2009; Holzinger and Karsten 2013). Although the induction of biofilm formation through the production of EPS has generally been correlated to the advent of a stress event, it is unclear whether all stressors induce such an event in microalgae or if a select few are involved. The research reported here investigated whether simulated nutrient stress, photoperiod or turbulence stresses would induce biofilm formation in a common species of green microalgae, *Chlorella vulgaris*. By measuring the flocculation efficiency and polysaccharide content of each culture, the formation of biofilms in response to stress treatment was determined. In addition, the investigation aimed to determine if common stress responses, such as growth inhibition, cell size changes, and ROS production, could be linked to biofilm formation of *C. vulgaris*.

Nutrient limitation has been well documented in microalgae. Carbon fixation occurs through the Calvin cycle, the light-independent reaction of photosynthesis. The resulting glyceraldehyde-3-phosphate product is converted into glucose, which is subsequently converted into polysaccharide chains by the linking of consecutive glucose monomers. This process is dependent upon the rate at which the light-dependent reactions take place, and less so upon the rate of nitrogen metabolism. In a nutrient limited scenario, the rate of carbon fixation exceeds the rate of nitrogen metabolism and the cell is subsequently in carbon flux. Without nitrogen metabolism, the cell cannot use excess polysaccharides for growth, and thus they are secreted into the surrounding media as an overflow mechanism. Extracellular polysaccharide content provides the structural components necessary for cellular aggregation and biofilm formation; however, biofilm formation in response to nitrogen limitation is

species dependent. In a study performed by Kiørboe et al. (1990), cell stickiness and flocculation occurred under nitrogen limited conditions for the diatom *Thalassiosira pseudonana*, but not for the diatom *Skeletonema costatum*; the increase in cell stickiness was linked to the cessation of cellular growth and the induction of the stationary phase.

Glyoxylate functions to induce nutrient stress by stimulating carbon metabolism through the acceleration of photosynthesis and the inhibition of photorespiration, without augmentation to other nutrient cycles (Oliver and Zelitch 1977; Bergman 1980; De Philippis et al. 1996; Yang et al. 2010). The inhibition of photorespiration occurs because glyoxylate is a feedback inhibitor of glycolate synthesis, an upstream portion of the photorespiratory process (Oliver and Zelitch 1977; Bergman 1980). In short, the erroneous fixation of oxygen by RUBISCO produces 2-phosphoglycolate, a compound that is toxic for enzyme function within the chloroplast. Hydrolysis of 2-phosphoglycolate produces glycolate, which is shuttled from the chloroplast to the peroxisome and consequently oxidized by glycolate oxidase into glyoxylate. The accumulation of this downstream product causes a down regulation of glycolate synthesis, thereby inhibiting the photorespiratory process.

Since photosynthesis proceeds with greater efficiency while nitrogen assimilation occurs at the same rate, polysaccharides accumulate and the cell perceives this as a nitrogen limitation. Several studies have investigated the effectiveness of glyoxylate in simulating nitrogen deprivation in cyanobacteria, but few have implemented glyoxylate in the nutrient deprivation of microalgae. In a study by Yang et al. (2010), glyoxylate additions were found to increase extracellular polysaccharide content with both increasing time and increasing glyoxylate concentration in the unicellular microalgae *Chlorella pyrenoidosa*. In addition, aggregation occurrence and size increased with increasing polysaccharide presence, thus

forming a positive correlation with glyoxylate concentration. Based on these results, Yang et al. (2010) concluded that glyoxylate was an effective simulator of nitrogen depletion. In the research reported here, the effects of glyoxylate on the closely related microalgae *Chlorella vulgaris* were investigated, in which polysaccharide content was also measured, but flocculation efficiency of the culture was used instead of flocculation occurrence and size. In addition, the links between ROS production and biofilm formation were investigated.

In eukaryotes, the timing of some cellular processes has been linked to an endogenous clock mechanism, showing fluctuations in activity based on circadian rhythms (Barros et al. 2005). Although rhythmicity in the movement of cells towards light (phototaxis) was recognized within *Euglena* in 1948, it was believed that organisms that divide more frequently than 24 hours would not benefit from a circadian rhythm (Suzuki and Johnson 2001); however, further research has linked these rhythms to circadian cycles and indicated their benefits in the increased survival of microorganisms. In particular, microalgae have been shown to sense seasonality by measuring the ratio of day/night, allowing for preparation of reproductively-active, vegetative cells during the warmer summer months and hardier, resting cells for over-wintering or for high stress environments (Suzuki and Johnson 2001; Mittag and Wagner 2003).

Circadian rhythms are endogenously controlled and thought to be regulated by progressive temporal phosphorylation of central oscillator proteins, although the mechanism is not fully understood (Seyfabadi et al. 2011). In *Chlamydomonas reinhardtii*, nutrient acquisition is tightly linked to circadian rhythms. Chemotaxis is used at night to migrate to areas of high nitrogen concentration; however, nutrient acquisition does not begin until dawn (Mittag and Wagner 2003; Cyr and Morton 2006). Cell stickiness of *C. reinhardtii* is well

known to increase at night and is thought to increase adherence for the enhancement of nutrient acquisition as well as reproductive processes (Straley and Bruce 1979; Hama et al. 1988). Visible/UV light sensitive tasks have been shown to occur during the night period, mainly reproductive actions such as DNA and cellular replication. Using phototaxis, cells mobilize to areas of high photosynthetically active radiation (PAR) during the day to increase the photosynthetic efficiency of the cells (Mittag and Wagner 2003). Temporal partitioning of incompatible processes allows the cell to focus energetic needs on critical cellular functions and reduces risks such as UV damage and low resource availability. For instance, in cyanobacteria photosynthesis and nitrogen fixation are partitioned to day and night periods, respectively, since nitrogenase is ineffective in the presence of oxygen (Suzuki and Johnson 2001).

With the exception of the research conducted by Seyfabadi et al. (2011), the effects of circadian rhythms on green microalgae, and especially *Chlorella* species, have not been well studied. The available information is fragmented between highly variable algal groups. As mentioned above, chemo- and phototaxis are rhythmically regulated in the motile organisms *Euglena* and *Chlamydomonas* (protist and green microalga) (Mittag and Wagner 2003; Barros et al. 2005). In the marine picoeukaryote *Ostreococcus,* transcriptional and translational control over gene expression are regulated by circadian rhythms (Monnier et al. 2010). Bioluminescence in the dinoflagellate *Lingulodinium* also follows circadian timing (Suzuki and Johnson 2001). In addition, antioxidant systems have been shown to hold rhythmicity, producing higher activity during day periods when oxidative stress is the highest (Barros et al. 2005; Monnier et al. 2010). Rhythmicity in SOD activity has been observed to continue after being transferred into continuous light in dinoflagellates and red algae (Barros

et al. 2005). ROS and antioxidant fluctuations in several other microalgal clades have also been found (Barros et al. 2005). Intriguingly, in benthic microbial communities isolated from Canadian Shield lakes, circadian rhythms were implicated in the production of loosely connected, colloidal EPS, mainly, increasing with increases in light (Cyr and Morton 2006). However, EPS production was not attributed to a certain species within the isolated biofilm.

In addition to the influence that day length changes have on the regulation of circadian rhythms, changes in the incident light exposure due to elongation or reduction of the photoperiod, affects photosynthetic processes and cellular carbon metabolism. With respect to photosynthetic processes, the responses of cells to variations in light duration and intensity have been shown to overlap (Litchman et al. 2003; Seyfabadi et al. 2011). Both a decrease in photon flux density (μmol photons m^{-2} s^{-1}) and reduction in day length have been observed to increase photosynthetic pigment concentration with a corresponding increase in photosynthetic efficiency; increases in photosynthetic ability compensate for reduced photon loading (Post and Mur 1985; Seyfabadi et al. 2011). In contrast, long day lengths have been shown to induce similar responses to high light; an increase in the photoperiod causes photoinhibition due to oxidative stress (Seyfabadi et al. 2011). Considering that different species of algae differ considerably in their sensitivity to light, changes to the daily incident PAR, corresponding to varying photoperiods, should also affect each species differently.

Photoinhibition can occur as a response to oxidative stress originating in either photosystem I (PSI) or photosystem II (PSII). In high light, increased photon flux disrupts the balance between light harvesting and energy use; as excess energy builds up in PSII, the lifetime of single-state chlorophyll ($^1Chl^*$) molecules are increased, causing the accelerated formation of triplet state chlorophyll ($^3Chl^*$) (Ledford and Niyogi 2005). Consequently, $^3Chl^*$

has the ability to transfer energy to neighboring ground state oxygen molecules, resulting in the accumulation of singlet oxygen within the PSII complex (Ledford et al. 2007). Singlet oxygen is highly reactive, often causing immediate damage to the machinery of PSII, namely, the deterioration of the D1 protein leading to photoinhibition (Trebst et al. 2002; Ledford and Niyogi 2005; Fischer et al. 2007). Trebst et al. (2002) found that in the presence of high light, PSII activity and D1 functionality decreased in the green microalgae *Chlamydomonas reinhardtii* when ROS detoxification processes were inhibited.

In addition, ROS production can be enhanced by high intensity light in PSI. Under equilibrium, reduced ferredoxin reduces $NADP^+$ to NADPH; however, in a high light environment, the over-reduction of electron carriers favors the reduction of O_2 by ferredoxin due to a lack of available $NADP^+$, resulting in the production of superoxide (Niyogi et al. 1997; Aguilera et al. 2002; Fischer et al. 2007). Superoxide molecules can cause damage to thylakoid and chloroplast membranes by direct addition to the double bonds of polyunsaturated fatty acids (PUFA), producing lipid peroxides (LOOH). The peroxidation of polyunsaturated fatty acids causes a chain reaction, as lipid peroxides cause peroxidation in the neighboring PUFA (Barros et al. 2005; Ledford and Niyogi 2005; Fischer et al. 2007). Such reactions radiate out from the site of initial peroxidation, compromising membrane integrity and function. The accumulation of β-carotene and α-tocopherol within thylakoid membranes most likely functions as a preventative mechanism against PUFA formation (Abe et al. 1999; Trebst et al. 2002; Ledford et al. 2004).

Turbulence is a common environmental stressor found in both aquatic and marine ecosystems. Natural disturbances to the water column include tides, waves, currents, bubbling, wind, and other organisms (Thomas and Gibson 1990). Despite the frequency in

which turbulence is encountered, it is unclear exactly how microalgal cells sense turbulent conditions. In addition, the effects of shear stress are experienced differently depending on the species of algae. Thomas and Gibson (1990) noted that various groups of microalgae showed differing sensitivities: dinoflagellates > diatoms > blue-green algae > green algae, with green algae being the least sensitive to turbulence. It has been observed by several authors, including Grobbelaar (1994), that turbulence stress is a balance between the beneficial aspects of mixing and the detrimental effects of shear stress, mainly due to mechanical damage.

Culture turbulence can be beneficial due to the dissipation of nutrient gradients, commonly occurring in stagnant waters (Grobbelaar 1994). Most importantly, light is quickly attenuated due to its reflection and adsorption by floating particles and cells; the process of mixing provides better access to light by reducing settlement of non-motile cells along the habitat substratum (Johnson and Wen 2010). The intermittent light exposure attained through mixing is termed the flashing light effect. This provides optimal light as contrasted with direct, high intensity light exposure. However, the flashing light effect is only beneficial at high cell densities (or high particulate matter), in which light exposure is initially limiting (Marshall and Huang 2010). Secondly, nutritional and gaseous gradients are dissipated, allowing for better access to carbon dioxide and micronutrients, while removing the excess by-product of oxygen (Grobbelaar 1994). In addition, a boundary layer around the cell surface exists in which the friction between the fluid and the cell surface inhibits flow; in this region diffusion is the main pathway for acquisition of nutrients. The faster mixing occurs the smaller the boundary layer around the cell surface becomes; smaller boundary layers allow for increased nutrient accessibility (Marshall and Huang 2010). Lastly, turbulence

prevents cells from sinking to the substratum, where natural accumulation of organic matter and anaerobic digestion by aquatic bacteria produce an inhabitable environment.

Several studies have indicated that cellular stress is experienced by microalgae in response to turbulence. Mitsuhashi et al. (1995) found that unlike species of cyanobacteria, photosynthetic activities of *Chlorella vulgaris* were inhibited by turbulence despite evidence that the smaller cell size of *Chlorella* had a higher hydrodynamic shear stress capacity. In addition, decreases in growth for green microalgae have been observed in response to increasing turbulence, as evidenced in *Chlorella vulgaris* and *Scenedesmus quadricauda* (Hosaka et al. 1995; Hondzo and Lyn 1999). Mechanical damage is believed to occur under high turbulence speeds due to an increase in the collision rate between individuals as well as obstacles within the environment; rupturing of the membrane and subsequent leakage are a consequence of turbulence stress (Honzo and Lyn 1999). Membrane damage due to shear stress is easily visualized under a microscope. However, turbo-hypobiosis has also been observed, in which growth inhibition was noted without apparent physical damage to the cell (Namdev and Dunlop 1995). In these cases, the negative effects are assumed to be physiological in origin.

Namdev and Dunlop (1995) investigated the possible pathways in which unicellular plant and yeast cells in suspension sense turbulence. Although these pathways were not specifically linked to microalgal cells, they provide plausible mechanisms for how microalgae sense turbulence stress. First, when turbulent forces apply pressure to the cell surface, stretch activated ion channels induce calcium influx and subsequent activation of an intracellular signaling cascade. Second, stress proteins may also be involved in turbulence stress; in yeast cells, the activity of stress proteins was observed to increase with increasing

levels of turbulence. In addition, changes in the turgor pressure and cell wall characteristics in response to turbulence and associated collision rates affect the level of osmoregulation required by cells under turbulence stress. Osmoregulation may be linked to changes in cell size and cell wall characteristics, including alterations in the cell wall composition for the enhancement of structural integrity (Namdev and Dunlop 1995). Acclimation to shear stress has been observed in long-term treatments, in which the history of exposure affected the impact of the imparted stress (Bronnenmeier and Märkl 1982; Hosaka et al. 1995; Mitsuhashi et al. 1995).

Rodríguez et al. (2009) found that increasing turbulence in cultures of *Protoceratium reticulatum*, a toxic dinoflagellate, induced the release of ROS; however, the correlation between turbulence and ROS production was not as strong as the correlation between turbulence and lipid peroxidation. It was suggested that lipid peroxidation played a key role in cellular damage to the cell in response to turbulence, providing an additional pathway for membrane damage by shear stress. Intracellular hydrogen peroxide concentration was also found to increase for the red-tide dinoflagellate *Ligulodinium polyedrum* exposed to turbulence, but only in cultures experiencing late-exponential growth phase (Juhl and Latz 2002).

Biofilm formation and EPS production were implicated as stress responses to turbulence by Johnson and Wen (2010), who showed that rocker-based turbulence induced firm attachment of *Chlorella* species while stagnant cultures failed to attach to the substrate despite noticeable settlement. In addition, Gasljevic et al. (2008) and Jenkinson and Sun (2013) found that EPS production alters the viscosity and elasticity of the surrounding aquatic (or marine) environment, which may serve two purposes: 1) to reduce the flow by

increasing viscosity, and 2) to decrease the impact of friction by increasing the concentration of drag-reducing compounds (Gasljevic et al. 2008; Jenkinson and Sun 2013). Flemming and Wingender (2010) noted that the viscoelastic properties of EPS provide cells with protection from shear stress by 1) increasing the adhesive and cohesive forces of algal communities, which should reduce collision frequency by adherence to a substrate, and 2) absorbing mechanical "stress energy" through reversible deformation of the biofilm. Intriguingly, a preliminary study performed by Cordoba-Castro et al. (2012) noted that a linear increase between turbulence rate and EPS secretion was observed for the microalga *Scenedesmus obliques*.

For the study reported here, *Chlorella vulgaris* was chosen as the study subject to investigate the effects of stress mechanisms on biofilm formation in microalgae. *Chlorella* is a genus of non-motile, unicellular, green microalgae belonging to the phylum Chlorophyta (Imase et al. 2008). They are found ubiquitously and has been reported in fresh, marine, and terrestrial environments. Cell sizes ranges from 3-10 μm (Scragg et al. 2003). They are non-motile and reproduce asexually through the production of autospores (Rioboo et al. 2009; Zuppini et al. 2010). Although *Chlorella* species have been shown to be involved in symbiotic associations involving a diverse group of symbionts, they are easily grown in laboratory conditions and are considered a model organism (Imase et al. 2008; Widjaja et al. 2009; Yang et al. 2010). Symbiotic associations are facilitated through the EPS sheath produced by *Chlorella*, which has been shown to hold chelating properties, most likely due to the uronic acids within the sulfated polysaccharides in which they are made (Kaplan et al. 1987; Imase et al. 2008; Raposo 2013). Watanabe et al. (2005) determined that Mg^{2+} is an important metal component in the formation and morphological characteristics of *Chlorella*

EPS, while sucrose was found to be the major carbohydrate component; however, the mechanisms behind biofilm formation by *Chlorella* species are not well documented.

Materials and Methods

2.1. Cultivation

Cultures of bacteria-free *Chlorella vulgaris* 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 $MgSO_4$ and $CaCl_2$ (all chemicals were reagent grade and purchased from Sigma Chemical, St. Louis, MO unless otherwise stated) at final concentrations of 0.3 mM and 0.17 mM, respectively. A pH of 7.4 to 7.8 was measured and maintained biweekly. Batch cultures were continuously bubbled with ambient air, at 23°C under a 16:8 h light:dark cycle. Illumination was provided by 40-watt cool white fluorescent lamps, at a distance of 16 cm from the culture flask. The photosynthetically active radiation (PAR) received at the surface of the culture container was measured using a LI-COR photometric sensor, and determined to be 90 μmol photons m^{-2} s^{-1}. Cell density was monitored using a Beckman DU 640B spectrophotometer at an absorbance of 680 nm, where cell concentrations were determined using a standard curve. Briefly, serial dilutions of the batch culture were made in which the optical density (OD_{680}) was measured and cell density was calculated using a hemocytometer. Batch cultures were diluted to a final absorbance of 0.75 using distilled water before being subdivided into 150 mL cultures for experimental treatments. Experimental cultures were grown in 250 mL I-Chem® glass septa jars (5 cm W x 11 cm H) capped with aluminum foil and maintained at a pH of 7.6. Illumination of

experimental cultures was continued in the same manner as batch cultures unless otherwise specified (section 2.2). All cultures were mixed by hand twice daily.

2.2. Stress Treatments

Chlorella vulgaris cultures were subjected to three stress treatments: glyoxylate (to induce nutrient stress), changes in day length, and turbulence. Glyoxylate additions were based on the procedures outlined by Yang et al. (2010) 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 to 16h:8h (control), 8:16, 12:12, 20:4, and 24:0 at the same PAR as provided for batch cultures. Automatic timers were used to regulate the photoperiod of the fluorescent lamps. Culture turbulence was achieved by placing a 2.5-cm long magnetic stir bar into each culturing jar; cultures were placed on stir plates and the rate at which the cultures were stirred was adjusted to 0, 300, 425, 900, and 1100 rpm. Control cultures for the turbulence experiment were mixed as described above (section 2.1); however, treatments undergoing turbulence were not manually mixed. 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. (2012) as seen below:

$$\mathrm{Re} = \frac{nDi^2\rho}{\mu} = \frac{300 \times 0.025^2 \times 1000}{9.95 \times 10^{-4}}$$

where n is the rotations per minute (rpm) of the stir bar, D_i is the length of the stir bar (m), ρ is the liquid density of the media (kg m^{-3}), and μ is the dynamic liquid viscosity at 22.3 °C

(Pa·s). The Reynold's numbers corresponding to the various rates of turbulence (in order) are 0, 18,844, 26,696, 56,533, 69,095. Based on these calculations, all turbulence treated cultures within this report were within the turbulent rather than laminar flow range (Grobbelaar 1994).

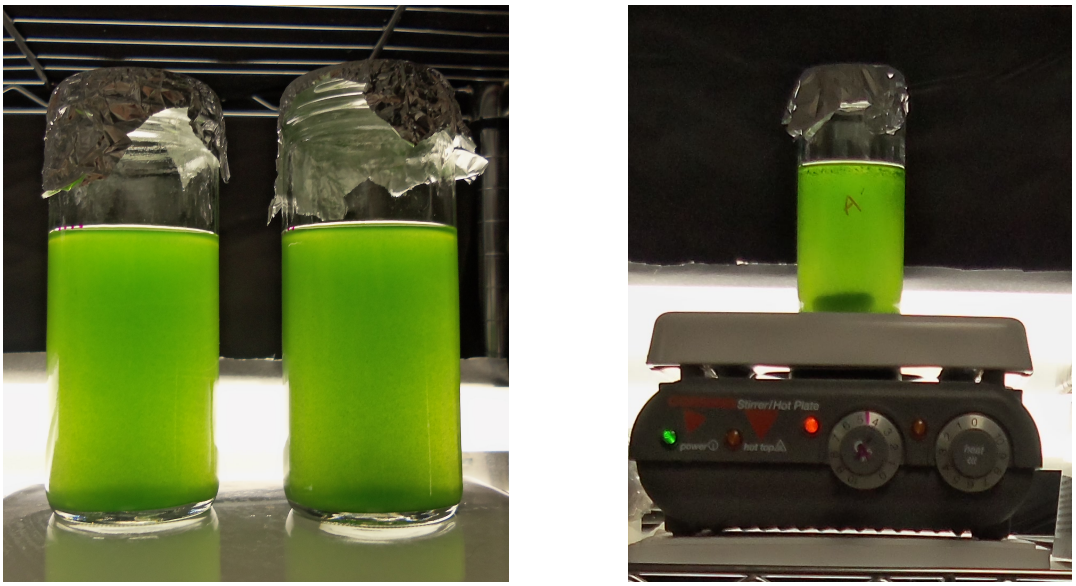

Figure 1. Experimental set up for stress treatment of *Chlorella vulgaris*- At left, general set up for each culture is displayed. At right, set up fro the turbulence treated cultures is displayed, in which the stir bar is evident at the bottom of the culture container. The stir plate is set to a speed of 300 rpm.

Experimental treatments were performed in duplicate and monitored for a period of ten days. An example of the experimental set up, as seen on the first day of the experiment, is provided in Figure 1. After a nine-day stress treatment, growth and flocculation efficiency were calculated. In addition, cultures were examined microscopically and tested for superoxide, hydrogen peroxide and polysaccharide content.

2.3. Microscopy

Cells were analyzed for morphological differences using an Olympus IX81/DP71 optical microscope. For each treatment, pictures were taken using the camera attachment of

the IX81/DP71 optical microscope set up and scale bars (in μm) were applied using Olympus MicroSuite FIVE imaging software. Cell size measurements were performed as follows: For each sample picture, a numbered grid overlay was applied in which grid cells were chosen sequentially using a random number generator. The diameter of each individual within a chosen cell was measured (μm) until a total of 56 individuals per treatment had been measured. Cell size measurements were expressed as the average cell size for each treatment +/- the standard deviation.

2.4. Cell Density and Flocculation Efficiency

Experiment cultures were stirred for 60 seconds then OD_{680} 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_{680} was remeasured. Flocculation efficiency was calculated according to the following equation modified from Oh et al. (2001):

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

where A: OD_{680} of sample, B: OD_{680} of reference (control prior to settling period).

Once cultures were measured for cell density and flocculation efficiency, each culture was adjusted to a pH of 7.6 and OD_{680} of 0.75 (using distilled water) before measurement of superoxide, hydrogen peroxide and carbohydrate concentrations were performed.

2.5. Superoxide Assay

Superoxide concentrations were measured *in vivo* by staining cells with nitroblue tetrazolium (NBT). Staining with NBT produces a water insoluble precipitate, formazan,

which was measured with a spectrophotometer at an absorbance of 570 nm. Following techniques from Hückelhoven et al. (2000), a solution of NBT was made using two stock solutions. A solution of 10% (w/v) NaN_3 and a 70% aqueous solution of dimethylformamide (DMF) containing 100 mg/ml of NBT were made. Next, 20 µL of the DMF/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_3. The final product yielded a clear, light yellow solution, which was shielded from light. New NBT stock solutions were made for each experiment.

For NBT staining, a 1.5 mL aliquot of each treatment culture was removed and placed into a 1.5 mL microcentrifuge tube. Cells were centrifuged in a microcentrifuge at 8,000 rpm for 8 minutes. The resulting supernatant was removed and 200 µL of the NBT stock solution was applied exogenously. As the solution was applied, cells were mixed 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. Stained cells were resuspended in a 1 mL solution of clearing solution (methanol: acetone (1:1)) to remove photosynthetic pigments. Samples were incubated for a total of 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_2O and the absorbance (570 nm) was measured using a Molecular Devices plate reader. Final measurements of formazan product were expressed as OD_{570}.

2.6. Hydrogen Peroxide Assay

Hydrogen peroxide concentrations were determined based on the method described by Li et al. (2010) for the macroalgae *Corallina officinallis*. 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) and bead beat for 10 second 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 8,000 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 1 M solution of potassium iodide (KI). After 1 hr incubation in the dark, the absorbance was measured at 390 nm. The content of hydrogen peroxide was determined using a H_2O_2 standard curve and expressed as μmol/g of wet weight.

2.7. Polysaccharide Assay

Polysaccharides, soluble extracellular and bound intracellular, were extracted and purified according to procedures used by Yang et al. (2010) with modifications. Briefly, a 10 mL aliquot of each sample was removed and centrifuged at 17,000xg for 20 minutes at 4°C. The supernatant was transferred into a 15 mL conical tube, while the pellet was resuspended in 7 mL of distilled H_2O and placed into a 50 mL conical tube. Both soluble and bound fractions were placed in a -20°C freezer.

After a period of 12 hours, the bound fractions (pellet) were thawed in an 85°C water bath and replaced in the freezer for 12 hours. Samples were again thawed and subjected to a flash-freeze using liquid nitrogen. Samples were replaced into the -20°C freezer for 12 hours. For the final thaw, samples were placed into the 85°C water bath for a period of 1 hour to extract the remaining bound polysaccharide and each sample was sonicated (Ultrasonic Homogenizer series 4710) for three 8 second repetitions. Soluble fractions were allowed to thaw in a water bath at room temperature.

Both soluble and bound fractions were centrifuged at 17,000xg for 20 minutes at 4°C to remove cell fragments and proteins. The supernatant was removed and passed through a 47 mm diameter GFC Whatman membrane filter (GE Healthcare) using a vacuum filtration setup. To remove ions, filtered samples were placed into 3500-molecular weight cut-off snakeskin dialysis tubing (Fisher Scientific) and dialyzed against deionized water for a period of 72 hours. During this period, five exchanges of the de-ionized water were performed. Measurement of purified samples was performed by means of the phenol-sulfuric acid method (Dubois et al. 1956). The relative polysaccharide content was determined using a standard curve for glucose and expressed as μg/mL per mL of algae. Total polysaccharide content was determined by adding the bound and soluble fractions. The extracellular carbohydrate ratio was calculated as the fraction of soluble, extracellular polysaccharides compared to the total polysaccharide content.

2.8. Statistal Analysis

Statistical analyses were performed using SAS/STAT® statistical software. A one-way ANOVA with a randomized block design was used to test the effects of stress treatments

at a significance level of $p < 0.05$. Two replicates for each of the three stress treatments were completed (trials), each of which were 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.

Results

3.1. Culture Growth

Environmental stressors, such as nutrient deprivation, are well known to impair growth as cells divert energy from growth and reproduction into maintenance of cellular homeostasis (Montechiaro et al. 2006). This investigation examined the effect of simulated nutrient deprivation by glyoxylate, changes in day length, and turbulence stresses.

A primary indicator of cellular stress can be inhibition of cellular replication. Algae treated over a broad range (0-1 mM) of glyoxylate concentrations were found to show no significant changes in growth (Figure 2a). However, as indicated by the low p value (p = 0.0532), a trend towards growth enhancement was observed within the highest glyoxylate concentration of 1 mM. Cells were also subjected to severe changes in photoperiod stress. Growth was again found not to be significantly affected (Figure 2b). Although not significant (p = 0.1047), trends witin the photoperiod data were observed, in which long and short day lengths induced constrasting effects: Longer day lengths trended towards growth inhibition, whereas a slight increase was observed for the shortest day length of 8 hours.

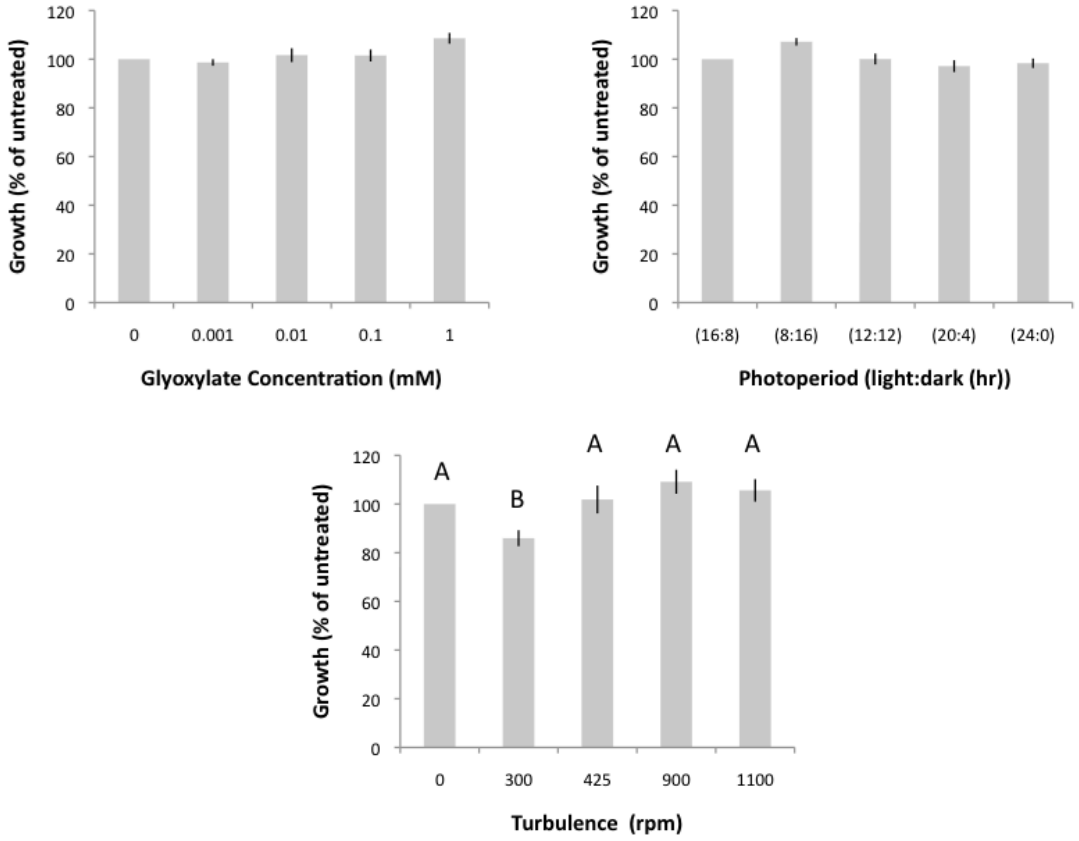

Figure 2. Effect of stress on percent growth of *Chlorella vulgaris*- Treatment of cells with glyoxylate (2a), photoperiod changes (2b), and turbulence (2c) was maintained for nine days, at which point the cell density was measured as optical density at 680 nm. Growth values were relativised as a percentage of the control. Values represent averages +/- standard deviations (error bars) with n =4.

The third stressor, turbulence, was imposed at various levels of increasing agitation. Where as most turbulence levels did not induce a statistically significant impact (Figure 2c), a speed of 300 rpm did significantly reduce growth of *C. vulgaris* (p = 0.0039). Growth declined by 15% for this treatment. Minimal change was observed for growth within the 425 rpm treatment, while turbulence speeds of 900 and 1100 rpm increased growth to a small degree.

3.2. Cell size

Chlorella vulgaris cells have been shown to range from 3-10 µm in diameter (Scragg et al. 2003); the values obtained within this study were consistent with these findings (Table I). Cell size variations occur frequently within natural communities, as the cell changes in diameter over the period of a life cycle; small, condensed resting cells progress to growing vegetative cells, which will eventually enlarge to form autospore mother cells that produce offspring through autosporulation (Rioboo et al. 2009). Although variations in cell diameter were evident, they did not appear to correlate with the applied stress treatments. The cell diameter of *Chlorella vulagris* as measured within this study ranged from 3-6 µm. Figure 3 provides a micrograph of the *C. vulgaris* cells as obtained from the microscopy performed on the tenth day of the experiment.

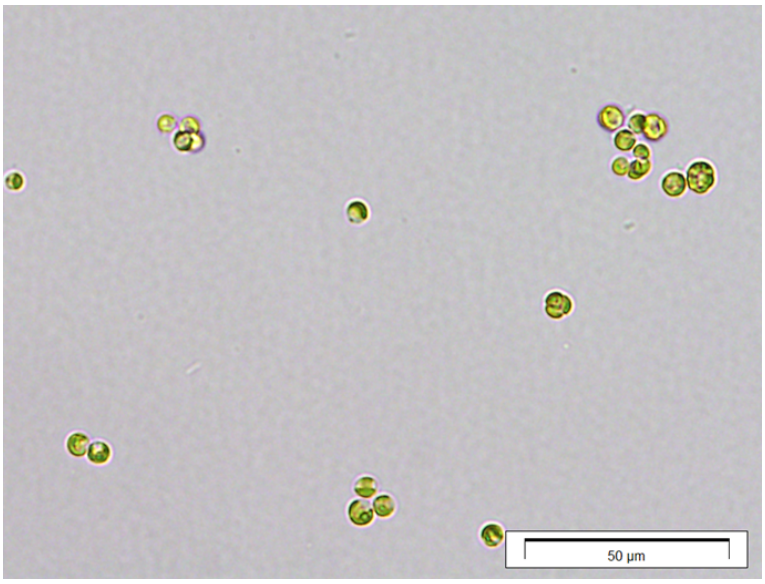

Figure 3. Micrograph of *Chlorella vulgaris* cells - Optical microscope micrograph of *Chlorella vulgaris* cells with applied scale bar for cell diameter measurements.

Table I. Effect of stress on cell size of *Chlorella vulgaris*- Values represent averages for cell diameter measurements +/- standard deviations for each treatment of the three stressors investigated. Units for cell size are in μm. Sample size for each treatment measurement was $n = 56$.

Glyoxylate (mM)	control	0.001	0.01	0.1	1
	4.732 (0.973)	4.830 (0.937)	5.098 (0.804)	4.866 (0.919)	4.786 (0.958)
Day length (hr)	**control**	**8**	**12**	**20**	**24**
	5.327 (1.679)	5.554 (0.871)	5.232 (1.023)	5.429 (1.120)	5.571 (1.028)
Turbulence (rpm)	**control**	**300**	**425**	**900**	**1100**
	4.375 (0.955)	4.357 (0.811)	4.308 (0.658)	4.723 (0.945)	4.259 (0.707)

As indicated by the standard deviations for each data set (Table I), variations in cell size were common within treatment groups. It is possible that cell shrinkage related to a stress-induced cellular response may have occurred, however, these changes were small (if not completely absent) and subsequently hidden by the natural variability between individuals at differing life cycle stages.

3.3. Flocculation Efficiency

Cellular flocculation causes aggregations to form and cells subsequently fall out of the culture solution due to their increase in weight (Grossart and Simon 1998). A decrease in optical absorbance within the water column occurs as the aggregates sink. Flocculation efficiency measures the proportion of the culture that has aggregated by calculating the difference in optical density of the stress treatment to the control prior to the settlement period (preflocculation). Therefore, high flocculation efficiency values indicate a higher flocculation occurrence, a value of zero indicates similarity of the treated culture to the control, and a negative value indicates an increase in single-celled individuals as compared to the control. As seen in Figure 4 below, both single cells and cellular aggregates were

apparent within each of the cultures examined as observed under the optical microscope; flocculation efficiency was used as quantitative method for measuring aggregate frequency in response to each of the three applied stressors.

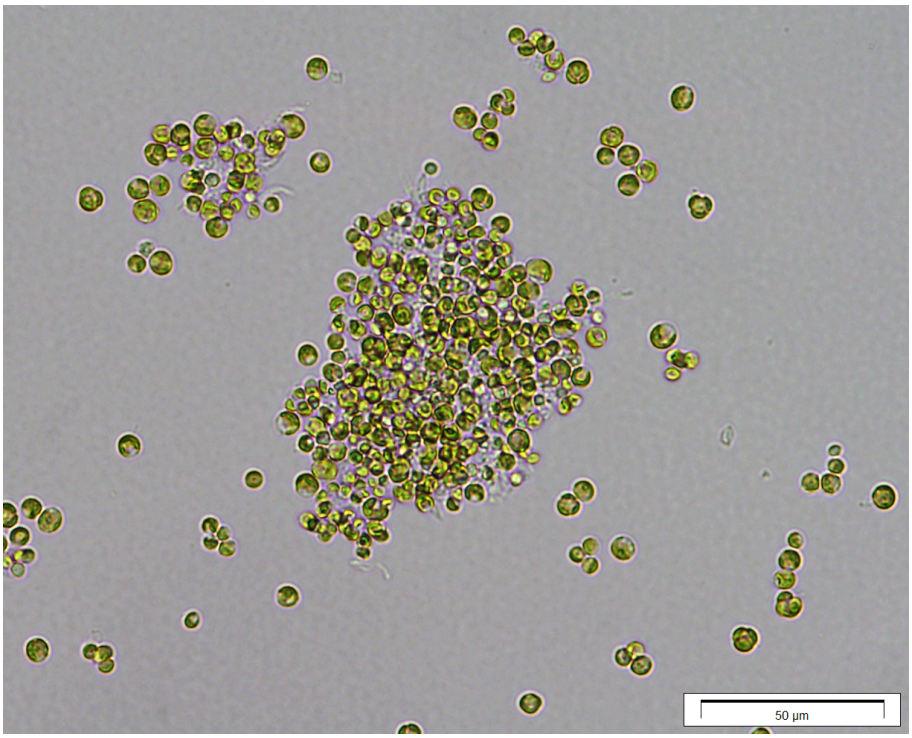

Figure 4. Micrograph of single cells and aggregates of *Chlorella vulgaris*- Cellular aggregation was easily visualized under the optical microscope; each aggregate varied widely in size, ranging from a couple of cells to several hundred cells. Various aggregate sizes are shown in the figure above.

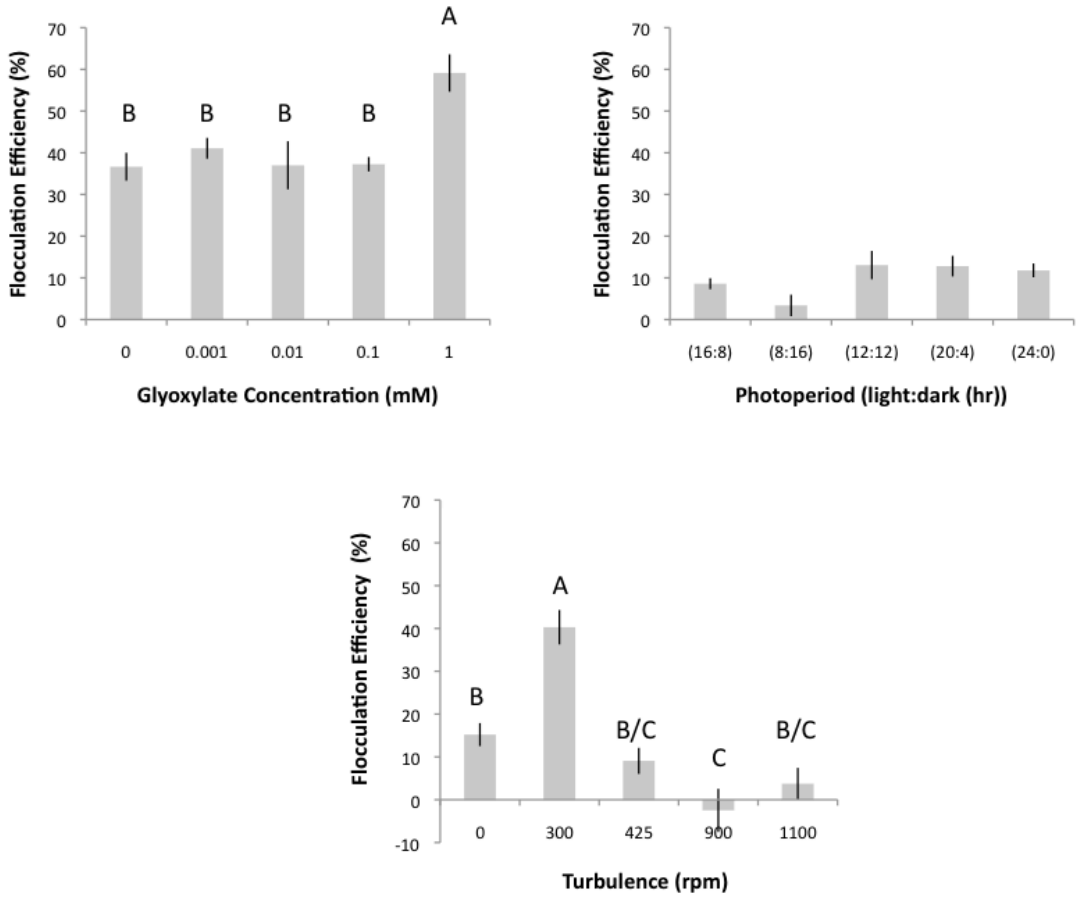

Figure 5. Effect of stress on flocculation efficiency of *Chlorella vulgaris*- Flocculation efficiency in response to each of three stress treatments: glyoxylate (5a), photoperiod changes (5b) and turbulence (5c). Flocculation efficiency is expressed as a percent change from the control. Values within graphs represent averages +/- standard deviations (error bars) with n =4.

As seen in Figure 5a, flocculation efficiency remained consistent between the control and lower glyoxylate concentrations (0.001, 0.01, 0.1 mM). However, cultures exposed the highest glyoxylate concentration (1 mM) induced a significant increase in flocculation efficiency (p = 0.0029). This increase was also evidenced by a striking visual transition within the water column over the nine-day treatment period. In response to changes in photoperiod, a statistically significant effect was not observed (p = 0.2615). Interestingly, despite a lack of significance, a trend towards increased flocculation efficiency was noted for all photoperiod treatments, with the exception of the shortest day. In this treatment, a slight

decrease (5%) was observed instead. Turbulence, on the other hand, induced a strong effect on cellular flocculation ($p = < 0.0001$). The lowest turbulence level of 300 rpm tripled the occurrence of flocculation in comparison to the control. The negative percentage observed for flocculation of the 900 rpm treatment was unexpected; cellular flocculation regressed, producing more single-celled individuals than the preflocculation control. Although the 425 and 1100 rpm treatments were statistically similar to the control, they trended towards a decrease in flocculation efficiency (6 and 12% respectively).

3.4. Superoxide Production

The effect of glyoxylate, photoperiod changes, and turbulence stressors on superoxide and hydrogen peroxide concentration were measured as indicators of cellular stress. After reduction of NBT by superoxide, a dark blue formazan precipitate was easily visualized within *C. vulgaris* cells as small blue spots detected by measuring the optical density at 570 nm. Absorbance of the formazan product provided a relative measure of intracellular superoxide concentration as compared to the control.

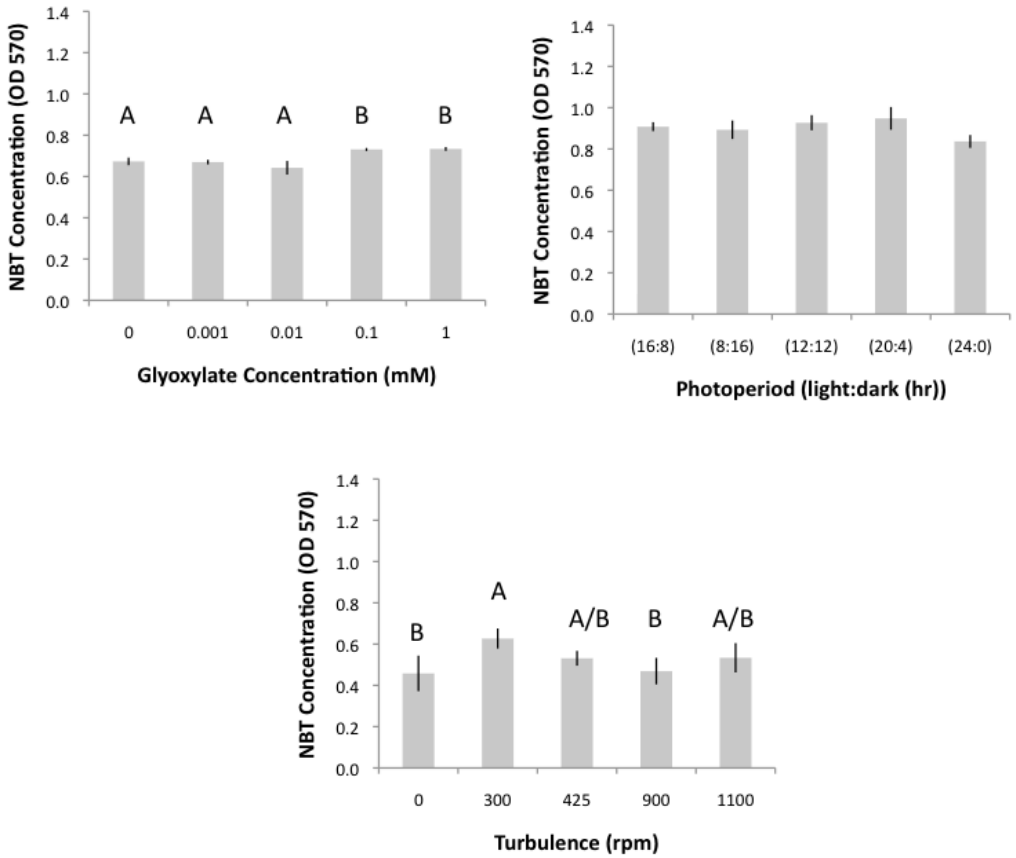

Figure 6. Effect of stress on superoxide concentration of *Chlorella vulgaris*- Absorbance at 570 nm in each of three stress treatments: glyoxylate (6a), photoperiod changes (6b) and turbulence (6c). Formazan product is expressed as the optical density at 570 nm. Values within graphs represent averages +/- standard deviations (error bars) with n =4.

A significant treatment effect was observed for the highest glyoxylate treatments of 0.1 and 1 mM (Figure 6a, $p = 0.0195$). Superoxide concentration increased approximately 9% for both treatments, while lower glyoxylate concentrations were similar to the control. Interestingly, a significant treatment effect was not observed for varying photoperiods (Figure 6b, $p = 0.1625$). In contrast, a significant increase of 36% was observed for superoxide concentration within the 300 rpm turbulence treatment ($p = 0.0232$). Although not statistically significant, a trend towards increasing superoxide concentration was noted for all turbulence-treated cultures (Figure 6c); however, this was quite minimal within the 900 rpm treatment.

3.5. Hydrogen peroxide production

Hydrogen peroxide concentration was determined using a potassium iodide based colorimetric assay as in Alexieva et al. (2001), in which the optical density of the yellow/orange colored product was measured at 390 nm.

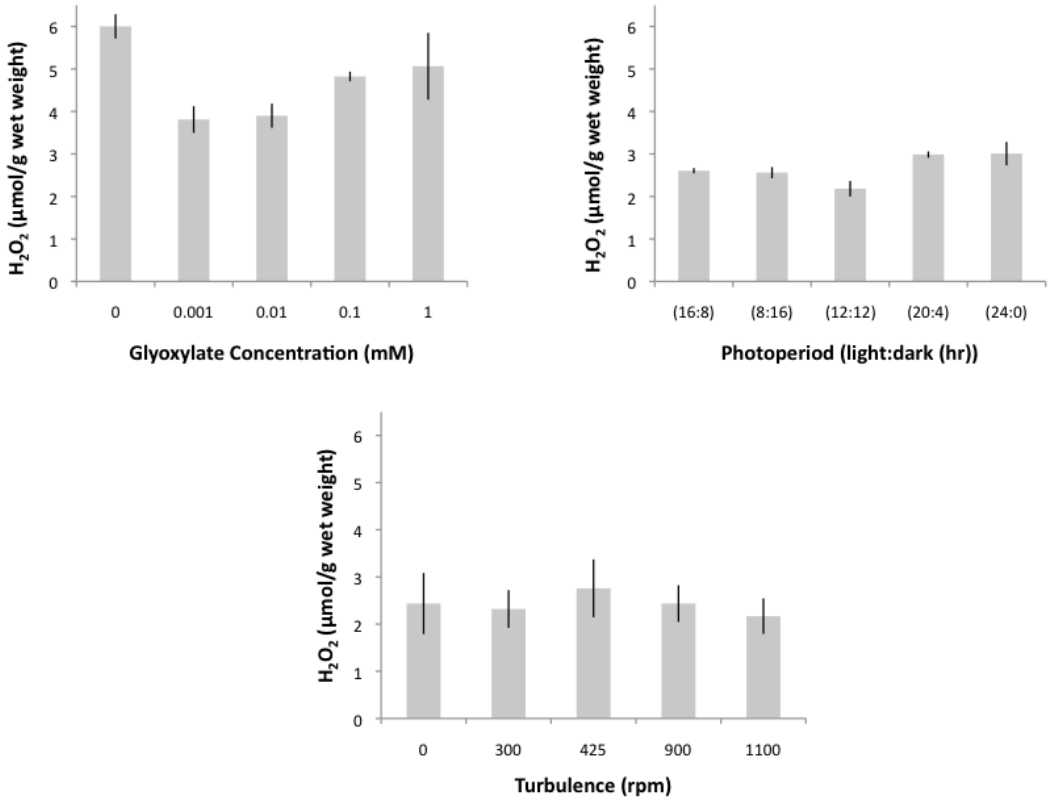

Figure 7. Effect of stress on hydrogen peroxide concentration of *Chlorella vulgaris*- H_2O_2 content was determined for each of three stress treatments: glyoxylate (7a), photoperiod changes (7b) and turbulence (7c). Hydrogen peroxide concentration was standardized to µmol per gram of wet algae. Values within graphs represent averages +/- standard deviations (error bars) with n =4.

Hydrogen peroxide concentration was not statistically different than the control in any of the three stress treatments examined (Figure 7). The 0.1 mM glyoxylate treatment showed a 10% reduction in hydrogen peroxide content, though not significant (p = 0.2555). In addition, the effect of photoperiod on hydrogen peroxide content was not statistically significant (p = 0.1064). However, as seen for the data on growth under photoperiod treatment, a trend towards long and short day grouping was noted, in which longer days

contained slightly higher H_2O_2 concentrations and a 12-hour day produced the least H_2O_2. As seen in Figure 7c, little variation in H_2O_2 concentration was observed between differing levels of turbulence ($p = 0.6144$).

3.6. Polysaccharide Content

Lastly, the effects of stress treatment on polysaccharide concentration and distribution were investigated.

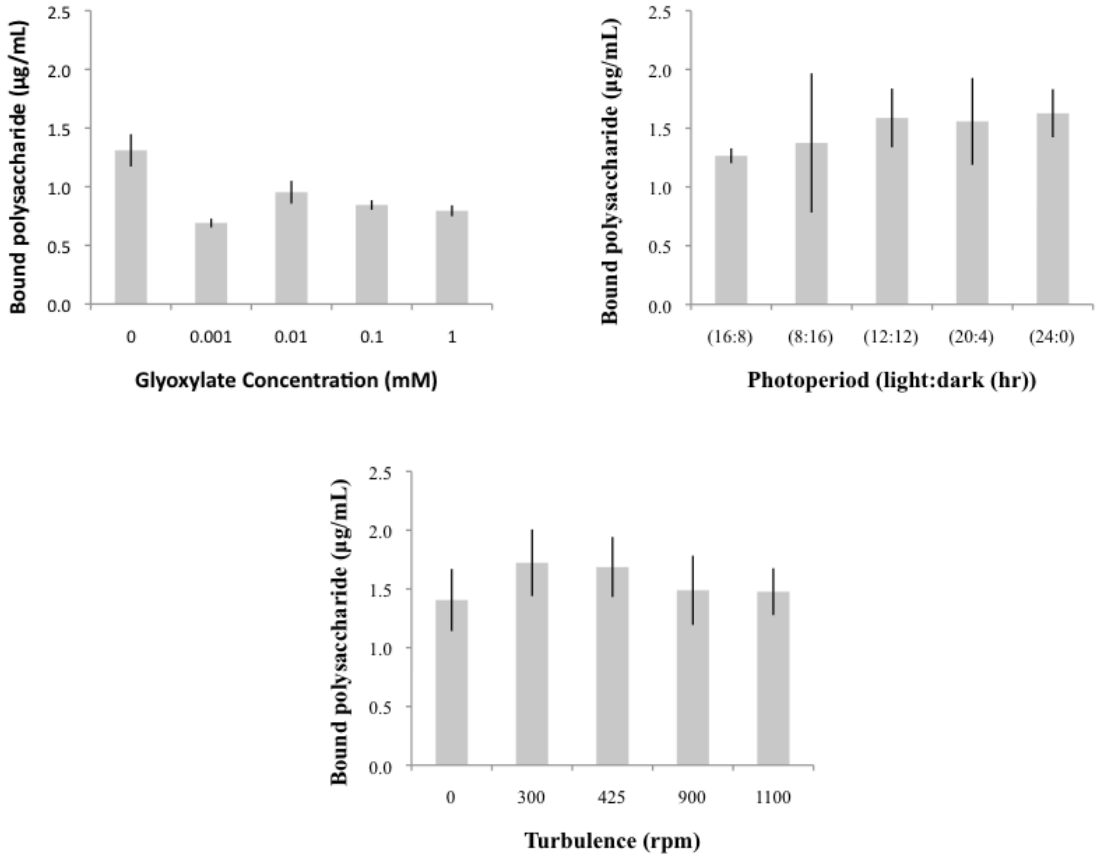

Figure 8. Effect of stress on bound, intracellular polysaccharide concentration of *Chlorella vulgaris*- Polysaccharide content was measured as µg per ml of treated algal culture measured in three stress treatments: glyoxylate (8a), photoperiod changes (8b), and turbulence (8c). Values within graphs represent averages +/- standard deviations (error bars) with n =4.

Each of the cultures treated with glyoxylate showed a marked decrease in intracellular polysaccharides as compared to the control (Figure 8a), although the trend was just above being significant ($p = 0.0620$). The effects of photoperiod treatment on intracellular

polysaccharides were found to not be statistically significant (Figure 8b, $p = 0.9591$). The trends for turbulence stress on intracellular polysaccharide production were also found to not be statistically significant (Figure 8c, $p = 0.4381$).

When the effect of each stressor on extracellular polysaccharide content was investigated, the biological variability among experiments was extensive (Figure 9). A two-way ANOVA with a block design was used to account for such variation in each of the experiments performed within this investigation, but was especially important for extracellular polysaccharides. Extracellular polysaccharide content was significantly affected by variations in photoperiod (Figure 9b) while glyoxylate (Figure 9a) and turbulence (Figure 9c) did not induce a strong response.

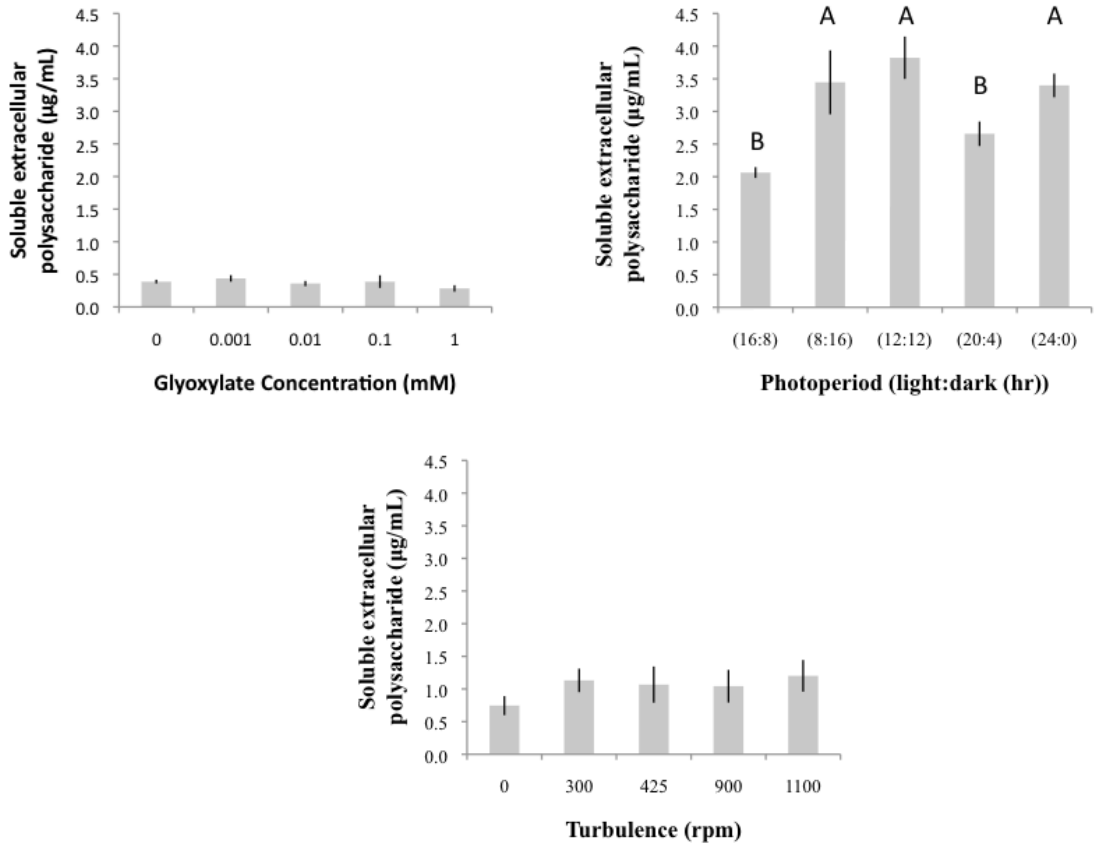

Figure 9. Effect of stress on extracellular polysaccharide content of *Chlorella vulgaris*- Polysaccharide content was measured as µg per ml of treated algal culture measured in three stress treatments: glyoxylate (9a), day length changes (9b), and turbulence (9c). Values within graphs represent averages +/- standard deviations (error bars) with n =4.

A significant treatment effect was not seen for extracellular polysaccharides in glyoxylate-treated cells ($p = 0.2212$). However, a slight increase of 12% was observed in the 0.001 mM treatment, while the lowest concentration was measured within the highest treatment. Contrary to many of the datasets for photoperiod, extracellular polysaccharide content did not seem to group into short and long days. An increase in extracellular polysaccharide production was seen for 8, 12, and 24 hour days, while a 20 hour day length had less of an effect. The effect of photoperiod on extracellular polysaccharide content was significant ($p = 0.0047$). Cultures subjected to turbulence trended toward greater quantities of extracellular polysaccharides than the control; however, it was found not to be statistically significant ($p = 0.4266$).

A relative measure of the total polysaccharide content was determined by the addition of the bound and soluble fractions of each treatment sample.

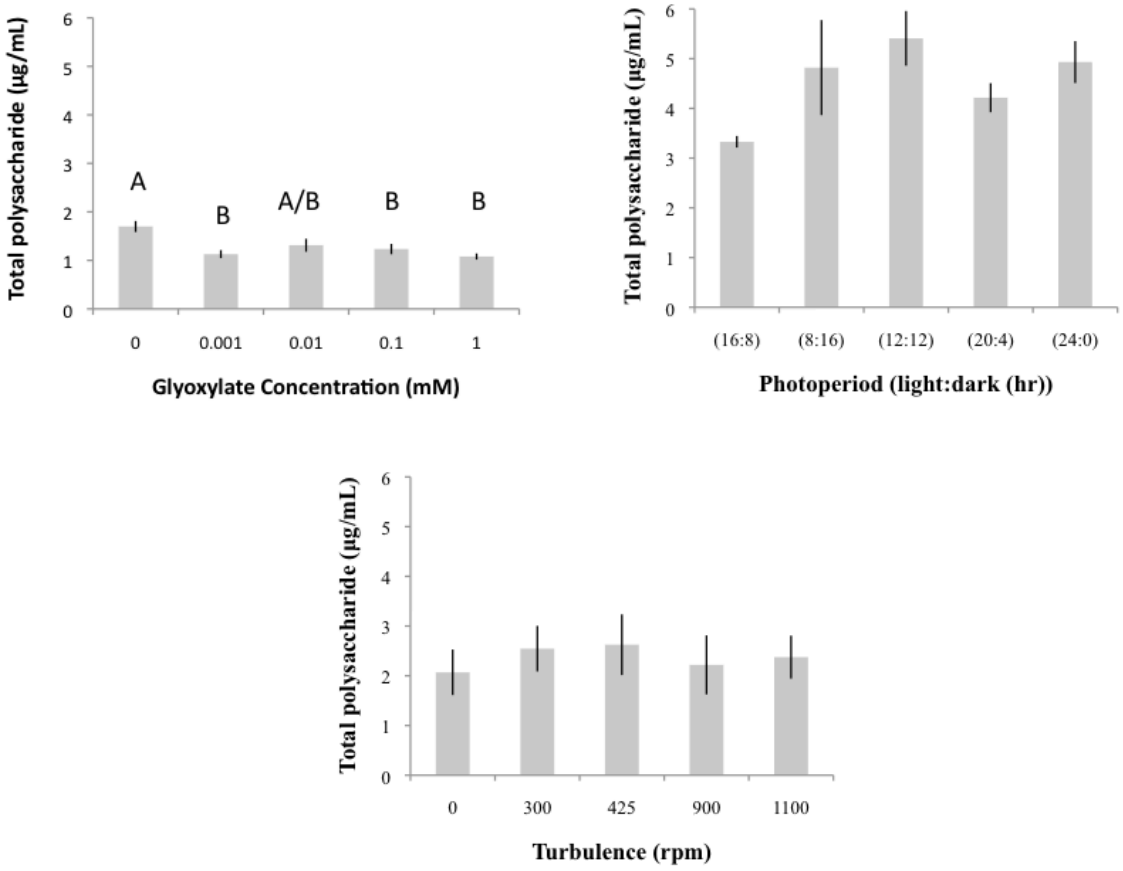

Figure 10. Effect of stress on total polysaccharide content of *Chlorella vulgaris*- Polysaccharide content was measured as µg per ml of treated algal culture determined for three stress treatments: glyoxylate (10a), photoperiod changes (10b), and turbulence (10c). Values within graphs represent averages +/- standard deviations (error bars) with n =4.

Total polysaccharide content was shown to be significantly affected by glyoxylate treatment (Figure 10a), in which a sharp decline in total polysaccharides was seen between treated and untreated cells (p = 0.0480). The low total polysaccharide measurement for glyoxylate-treated cultures was mostly driven by the low intracellular values; however, trends within treated cultures did not seem to correlate with increasing glyoxylate concentrations. For instance, the 0.001 mM treatment was observed to have the lowest intracellular concentration and the highest extracellular, which equaled out to a median value for total polysaccharide. The lowest total polysaccharide concentration was observed within

the highest glyoxylate treatment, a consequence of low values for both intra and extracellular polysaccharides. For varying photoperiods, a statistically significant relationship was not identified ($p = 0.2038$). Changes in the total polysaccharide content were a direct consequence of increased extracellular polysaccharide values for the 8, 12, and 24 hour day length treatments. However, total polysaccharide values were not statistically significant due to attenuation by intracellular polysaccharide content values, which did not correlate with the applied stress treatment. Changes in total polysaccharide content for turbulence-treated cultures were also found to be insignificant, where variations within the data were a consequence of a strong block effect ($p = 0.2626$ and 0.0007, respectively). It should be noted that opposing variations between intracellular and extracellular concentrations were commonly responsible for the attenuation of changes determined for total polysaccharide content.

The ratio of extracellular polysaccharides to total polysaccharide content was used to measure of the proportion of polysaccharides being diverted extracellularly.

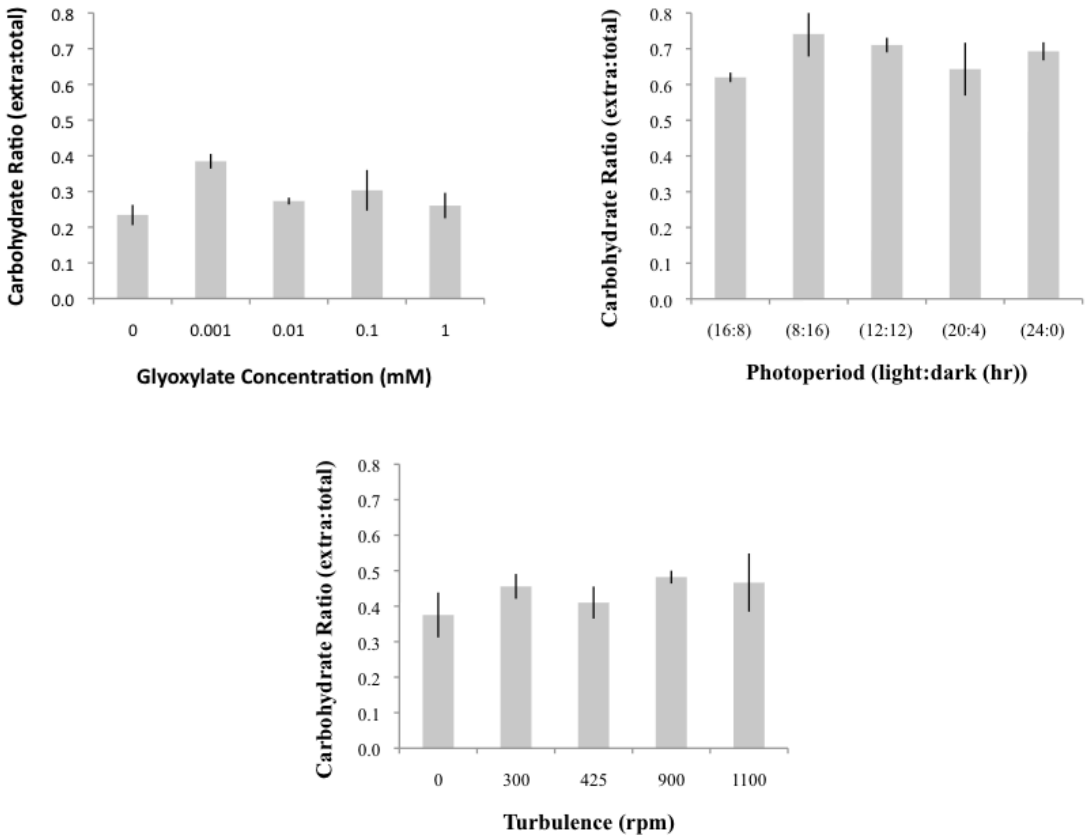

Figure 11. Effect of stress on the proportion of polysaccharide extracellularly located for *Chlorella vulgaris*- Extracellular polysaccharide ratio was determined for three stress treatments: glyoxylate (11a), photoperiod changes (11b), and turbulence (11c). Values are between 0 and 1 in which higher values indicate a larger portion of the total polysaccharide located extracellularly.

The effects of glyoxylate treatment on the extracellular polysaccharide ratio were not found to be statistically significant ($p = 0.1271$). However, the effect of glyoxylate treatment trended towards increasing the extracellular polysaccharide ratio, as seen by a slight increase in all treated cultures as compared to the control, most notably within the lowest glyoxylate treatment of 0.001 mM. The effect of varying photoperiod on the extracellular polysaccharide ratio was also statistically insignificant ($p = 0.5335$), despite the slight increase observed for treated cultures. Similarly to both glyoxylate and photoperiod treatments, the effect of turbulence treatment was insignificant ($p = 0.3816$), although a trend towards increasing the ratio of extracellular carbohydrates was noted for treated cultures.

Discussion

To investigate plausible links between stress exposure and biofilm formation in microalgae, we employed three environmentally based stressors: simulated nitrogen deprivation through glyoxylate addition, changes in the photoperiod, and increases in flow regime corresponding to increasing levels of turbulence. Common indicators of stress were examined including growth, cell size, flocculation efficiency, production of intracellular ROS (superoxide and hydrogen peroxide), and the concentration and location of polysaccharides. The combination of flocculation efficiency and secretion of extracellularly-located polysaccharides was used to provide a relative indication of biofilm formation through EPS production.

The first stressor investigated was nitrogen deprivation, as simulated by glyoxylate addition. Several studies have indicated the difficulty in monitoring and adjusting nutrient levels during nutrient deprivation of microalgae; the use of glyoxylate was chosen to provide a consistent increase in nitrogen deprivation over time (Yang et al. 2010). The mechanism for glyoxylate-induced nitrogen deprivation and nitrogen deprivation itself do not work through the same mechanism. Under nitrogen limiting conditions within the environment, carbon metabolism and thus carbohydrate synthesis continue at a constant rate, while nitrogenous sources become increasingly limited; as a result, the carbon to nitrogen ratio increases, producing carbon flux within the cell. In contrast, glyoxylate works to increase photosynthetic efficiency, thereby increasing carbon metabolism, while nitrogen metabolism

proceeds at the same rate. This action also causes an increase in the carbon to nitrogen ratio, which is perceived by the cell as nitrogen deprivation.

Inhibition of cellular growth is a characteristic of nutrient deprivation as nitrogenous sources required for both amino and nucleic acid synthesis during cell growth and division become limited (Arad et al. 1988; Widjaja et al. 2009). Stress induced by nitrogen deprivation has been implicated in the transition of cells from an exponential growth phase to the stationary phase, as noted in *Porphyridium*, *Chlamydomonas*, and *Chlorella* species (Arad et al. 1988; Menon et al. 2013). In the study reported here, neither growth nor cell size were affected by glyoxylate addition. This apparent lack of growth inhibition may be attributed to a lack of cellular stress induced by glyoxylate, or by low-level cellular stress which has yet to impede growth. Similar results were noted by Yang et al. (2010), in which lower concentrations of glyoxylate increased cell density similarly to the control, while the highest concentration of 1.25 mM arrested cellular growth. Mortality was not implicated in either of these investigations, as indicated by a net gain in the percent growth. Yang et al. (2010) attributed the lack of growth inhibition to the fact that stress can be perceived by the cell on several levels, in which high stress induces mortality and low stress causes changes within the physiology of an individual. The change in cell growth between 1.0 mM (highest treatment in our study) and 1.25 mM (the highest glyoxylate concentration used by Yang et al. (2010)) could be extrapolated as a threshold between low and high stress perception by *Chlorella* species, as indicated by the transition from growth to growth arrestment. Additionally, Bergman (1980) noted that glyoxylate addition stimulated growth in three cyanobacterial species, as a consequence of the enhancement of photosynthetic processes. It

may be that an overall nullification of growth inhibition occurs relative to the prior enhancement of growth by increased photosynthetic ability.

As a consequence of growth inhibition by nutrient stress, the transition of the cell cycle from large, reproductively-active, vegetative cells, to smaller, hardier, resting cells was expected to induce a reduction in cell size (Kiørbe et al. 1990). In addition, nitrogen depletion has been shown to induce chlorosis in the cyanobacteria *Synechococcus* and *Oscillatoria* as well as the green alga *Botryococcus* (Lupi et al. 1994; Sauer at al. 2001; Kumar et al. 2003). Chlorosis occurs as a response to oxidative stress induced under severe nitrogen deprivation; chlorophyll molecules are damaged by reactive oxygen species over time, causing an overall loss in cellular pigmentation. However, the cell size of *Chlorella vulgaris* was not significantly impacted by glyoxylate additions, while pigmentation remained relatively constant as determined visually over the course of the experiment. These data further indicate that the cells exposed to glyoxylate with our study were not experiencing a high level of perceived stress.

Flocculation efficiency was observed to drastically increase in cultures exposed to a glyoxylate concentration of 1.0 mM. Visually, cells exposed to lower glyoxylate concentrations did not show signs of cellular aggregation, which was confirmed by the low flocculation efficiency values measured for these cultures. The results of our study supported the data reported by Yang et al. (2010), in which aggregate occurrence and size increased with increasing glyoxylate concentration. However, a step-wise progression was not observed within our study. The measurement techniques utilized by Yang et al. (2010) were more precise, as measuring the aggregate frequency and size allowed for small changes in aggregation to be discriminated. However, measurement of the aggregation efficiency proved

relatively easy to perform and produced consistent results; this technique provides a more time-efficient method for the measurement of aggregation occurrence.

Increased superoxide concentration was measured within cells of the highest glyoxylate treatments. Superoxide content is a balance between production and depletion, the latter of which can occur through non-enzymatic quenching mechanisms or by enzymatic degradation by SOD. Since growth and morphological characteristics did not indicate cellular stress, an increase in ROS could indicate: one, that ROS production is initiated toward the end of the treatment period and the negative impacts on cellular growth and function are not yet apparent, or two, the defense systems responsible for detoxification of superoxide were working effectively to inhibit cellular damage, and thus cellular growth was not impaired. Menon at al. (2013) found that nitrogen deprivation in *Chlorella vulgaris* induced both hydroxide radical and superoxide production, in which the highest concentrations were observed once the cultures reached the stationary growth phase. The results of our study indicate that a similar response is seen in *C. vulgaris* in reaction to glyoxylate-simulated nitrogen deficiencies.

A linear increase in total and extracellular polysaccharide content in response to increasing glyoxylate concentration was not observed in our study, in sharp contrast to the data obtained by Yang et al. (2010). Rather, extracellular polysaccharides remained consistent between treated and untreated cultures, while a decrease in total polysaccharide occurred in treated cultures as a consequence of reduced intracellular polysaccharide concentrations. The increase in flocculation efficiency without substantial EPS production was unexpected, since glyoxylate additions thus far have been documented to increase the

extracellular polysaccharide content of cyanobacteria and microalgae (Bergman 1980; De Philippis et al. 1996; Yang et al. 2010). Two tentative explanations are proposed.

One, cells may be newly experiencing nitrogen deprivation stress and beginning to secrete polymeric substances extracellularly. Thin layers of polymeric coatings have been shown to increase cell stickiness and subsequently cellular aggregation (Leppard 1995). For changes in the cell coating, only small quantities of exopolysaccharide would be detected. Although species dependent, Kiørbe et al. (1990) noted that cell stickiness changes in response to the nutritional state of the algae. In the diatom *Thalassiosira pseudonona*, cell stickiness was significantly increased by nitrogen depletion, while for the diatom *Skeletonema costatum*, stickiness was consistent despite changes in nitrogen availability. With respect to glyoxylate-induced nitrogen deprivation, the increases in polysaccharide secretion in response to glyoxylate treatment observed by Yang et al. (2010) may be accompanied by increases in the cell stickiness of *Chlorella* species.

Alternately, carbon limitation within the culture media may have altered glyoxylate action, causing the unexpected lack of extracellular polysaccharide. An elevated rate of carbon metabolism in response to glyoxylate simultaneously increases the rate at which dissolved CO_2 is utilized by the cell while inducing a carbon flux intracellularly, which is shunted extracellularly as EPS; however, since cultures were not supplemented with CO_2, the increased rate of carbon metabolism could effectively deplete surrounding media of available carbon over the nine day treatment period, inducing carbon deprivation as well. In this case, the carbon to nitrogen ratio would be reduced and thus cells would no longer perceive the environment as nitrogen limited. Over the course of the experiment, a loss of previously

excreted EPS would occur due to degradation over time, reducing extracellular polysaccharide.

Our results appear to be more consistent with the second explanation. Intracellular polysaccharide content showed a measureable decline in treated cultures within our study. Under a carbon-limited scenario, the use of intracellular carbon stores for carbon metabolism would occur, effectively reducing the intracellular polysaccharide content of the cell. In addition, extracellular polysaccharides increased slightly for treated cultures, indicating that glyoxylate-induced polysaccharide secretion occurred, at least to some extent. In addition, this would explain why a change in the total polysaccharide content was not observed, as a decrease in intracellular polysaccharide content combined with an increase in extracellular polysaccharide would negate an overall change. It should be noted that although carbon flux from glyoxylate addition has solely been implicated in the process of EPS secretion by microalgae, nitrogen deprivation of *Chlorella vulgaris* has also been linked to intracellular starch and lipid accumulation (Lupi et al. 1994; Widjaja et al. 2009; Brányiková et al. 2011; Dragone et al. 2011; Menon et al. 2013). Based on the results obtained within our study, one cannot discount the possibility of an alternative carbon flux pathway.

The majority of the data available for changes in day length are not applicable to *Chlorella* species, since they are non-motile organisms, which do not possess the ability for fluorescence; therefore, a need to investigate the effects of photoperiod on a non-motile, green microalgae was of high interest. Growth was not significantly affected by changes in photoperiod. Several other studies have indicated that increases in day length produce a proportional increase in growth until reaching the saturation point of the photosynthetic machinery (Lupi et al. 1994; Litchman et al. 2003; Seyfabadi et al. 2011). This is typically a

result observed for cultures initially limited by light availability (Lupi et al. 1994; Seyfabadi et al. 2011). The data we obtained from this experiment indicated that light was not a limiting factor, since increases in light availability did not appreciably increase growth. In fact, the shortest day length induced the maximal growth, although only slightly better than other photoperiods. In the cyanobacteria *Oscillatoria* and *Phormidium*, as well as the diatom *Nitzschia* and green alga *Spaerocystis*, growth rates normalized to day length showed a decline with increasing photoperiod, although overall growth showed an increase over the duration of the treatment period (Post et al. 1986; Litchman et al. 2003). For microalgae grown under short day lengths, acclimation to inadequate light availability is accomplished by increasing the concentration of light harvesting pigments, which subsequently increase the photosynthetic ability of the cell (Post and Mur 1985; Litchman et al. 2003). Falkowski and LaRoche (1991) noted that after the transition of microalgae to subsaturating light intensities, overcompensation of chlorophyll enhancement may occur, providing the cell with higher photosynthetic capacity than beforehand. Increased chlorophyll concentration may in part explain the ability of *C. vulgaris* cultured under short days to grow at a similar rate as cultures exposed to longer day lengths.

Longer day lengths, as well as high intensity light exposure, have been shown to induce photoinhibition and subsequent cellular damage (Seyfabadi et al. 2011). In our study however, longer photoperiods did not significantly impact the growth of *C. vulgaris*. In intense light, microalgae are known to possess mechanisms for dissipating excess light energy, including excitation of xanthophyll cycle pigments, direct antioxidant quenching of excited chlorophylls and/or ROS, and a reduction in antennae size and number per cell (Falkowki and LaRoche 1991; Niyogi et al. 1997; Trebst et al. 2002; Seyfabadi et al. 2011).

Chlorella, *Dunaliella*, and *Chlamydomonas* species have been shown to possess unusually plastic chlorophyll antennae, in which antennae sizes shrink under higher irradiance, reducing the adsorption of excess photons (Neidhardt et al. 1998) In addition, chlorophyll pigments have been shown to oscillate based on the average irradiance received during a photoperiod, adjusting in response to the needs of the cell (Falkowski and LaRoche 1991). Green microalgae are well known to show high capacity for light acclimation; the findings of this study are consistent with this phenomenon (Richardson et al. 1983; Neidhardt et al. 1998; Falkowski and LaRoche, 1991). In addition, it was thought that cell size may also change in response to variations in day length, as cell cycle changes have been shown to occur as an acclimation response to seasonal changes in microalgae (Suzuki and Johnson 2001). In our study, cell size remained constant between treatments. Such changes may require a longer period of time, or multiple environmental cues, such as a decrease in day length and temperature, to induce a shift to resting cell abundance.

Significant changes in flocculation efficiency were not observed for variations in day length. Based on the data from growth and morphological characteristics, the perception of cellular stress was not apparent, and therefore the response of biofilm formation was not induced. Under laboratory conditions, *C. vulgaris* cells have been observed adhering to the sides of the culturing container under high cell densities in which light limitation was experienced (data not shown). The low cell densities used in our study may have allowed for full light penetration throughout the culture and thus adherence of cells to highly lit substrates was not required. In agreement with flocculation efficiency results, changes in the superoxide and hydrogen peroxide concentrations were also not evident, further indicating

that treated cells were not experiencing photoperiod-induced stress on the tenth day of the experiment.

Notably, the longest day length of 24 hours induced a slight decline in superoxide production, although not significant. Microalgae have been shown to accumulate high levels of both β-carotene and α-tocopherols in response to a variety of stressors, including UV-B, long day lengths, and high light exposures, which actively work to dissipate excess energy and directly quench ROS (Malanga and Puntarulo 1995; Abe et al. 1999; Mallick and Mohn 2000). In addition, Hastings et al. (1991) noted that SOD activity increases during the day in the dinoflagellate *Lingulodinium*, to accommodate for increases in accrued ROS. The minor increase in hydrogen peroxide observed for the 20 and 24 hour treatments may reflect increased SOD activity during longer photoperiods, in which SOD conversion of superoxide into hydrogen peroxide is upregulated. In a study performed by Malanga et al. (1997) on *C. vulgaris*, the activity of SOD decreased with increases in UV-B exposure, while APX activity and β-carotene concentration did not change. Instead, antioxidant detoxification was attributed to drastic increases in the α-tocopherol concentrations within chloroplasts. As indicated by the findings of our study, more research on the ROS and antioxidant mechanisms within *Chlorella vulgaris* exposed to varying day lengths is needed.

Changes in the intracellular polysaccharide content in response to photoperiod were not seen, while a drastic increase in extracellular polysaccharide was observed for shorter (8 and 12 hr), as well as longer (24 hr) day lengths. Wolfstein and Stal (2002) found that polysaccharide secretion occurred as an overflow mechanism during low temperature stress, as cells reverted into a resting cell state and reduced growth processes. Carbon that would normally be fixed for growth was diverted into EPS secretion instead. In the case of a

reduced photoperiod, the perception of a seasonal change from summer to winter could induce resting cell formation or simply reduce growth, activating a subsequent EPS overflow mechanism. In addition, Perkins et al. (2001) found that an increase in loosely connected colloidal EPS occurred when light was limited by 50% in intertidal biofilms, although the microorganisms and mechanisms responsible for this phenomenon were not identified. With respect to longer days, extracellular polysaccharides have been shown to provide protective functions related to high irradiance, such as quenching of ROS species and protection against UV-B radiation (Flemming and Wingender 2010; Raposo et al. 2013). In our study, these benefits would not actively work to benefit treated cells, as UV radiation was not applied and the elevation of intracellular ROS was not observed. Although the mechanisms behind EPS production in response to photoperiod were not fully elucidated, increases in extracellular polysaccharides did not affect the flocculation efficiency of the culture, indicating that an increase in polysaccharide secretion is not the only factor required for biofilm formation in *C. vulgaris*.

The effects of continuous turbulence at varying levels were also investigated. Despite the frequency in which microalgae experience turbulence within the environment, the body of research on this topic is limited. The effects of turbulence on green, unicellular algae are highly relevant to environmental studies as well as large-scale culturing of commercially valuable algal products. Growth was significantly impacted by turbulence speed, in which growth of cultures exposed to turbulence levels of 300 rpm were significantly decreased. Interestingly, the growth of cultures exposed to higher rates was not impaired. Visually, a full vortex within the water column was not apparent within 300 rpm treated cultures. Flow regime within these cultures proceeded at a reduced rate. It should be noted that at Reynold's

numbers well above 2000, all turbulence-treated cultures were above the laminar flow threshold. Instead, these levels would be classified as turbulent flow, which is characterized by a more irregular and disordered flow regime (Grobbelaar 1994). The consistency in which the stirring occurred was compromised over time by a progressive increase in friction between the stir bar and the bottom of the culture container. Collection of organic debris (cells, cell fragments, EPS, etc.) was implicated, since an agitation rate of 300 rpm did not prevent the settlement of particulate matter. Fully formed vortices occurred for agitation rates above 300 rpm and stir bar rotations were highly consistent.

For slower rates of turbulence, stirring rates may not have been high enough to dismantle nutrient gradients, which would reduce nutrient availability and inhibit cellular growth. In addition, cell settlement occurred over time, increasing the likelihood of cellular contact with the stir bar, and subsequent mechanical damage to the cell. For faster rates of turbulence, a balance between cellular damage and increased access to light and nutrients was reached, as indicated by similarity of growth to the control values. Of particular interest was the 900 rpm treatment, in which growth was enhanced in comparison to the control; the beneficial aspects of turbulence at 900 rpm were greater than the shear stress experienced within this treatment. Cell size was not significantly impacted by turbulence treatment. These results are consistent with the data obtained by Hondzo and Lyn (1999), in which the cell size of the green microalga *Scenedesmus quadricauda* remained constant despite turbulence exposure.

Flocculation efficiency was also significantly affected by turbulence stress, in which an increase in flocculation was observed within the 300 rpm treatment, while flocculation efficiency decreased in comparison to autoflocculation observed within the control for higher

turbulence treatments. Visually, it was noted that the two highest treatments (900 and 1100 rpm) had a thick ring of cellular aggregation, which had adhered to the glassware directly below the water-air interface. Since diffusion of carbon dioxide occurs optimally at the water's surface, cellular aggregation along this border would provide increased access to carbon substrates. In addition, the strength of the water current is lowest at the furthest point from the origin of rotation, which, for these experiments, was the magnetic stir bar. Since collision frequency is lower in lower turbulence levels, cell stickiness appeared to be a dominant factor in cellular aggregation of slowly agitated cultures. In highly turbulent cultures, the collision frequency was high but cellular aggregation was low. Based on the data collected for EPS, in which concentrations of extracellular polysaccharide remained consistent between treated cultures, enhancement of polysaccharide secretion for substrate attachment was not utilized as an acclamatory mechanism under turbulence stress. Instead, cellular aggregation decreased due to fragmentation of cellular flocculants. It is unclear as to why fragmentation processes occurred more frequently within the 900 rpm treatment than in the 1100 rpm treatment.

The increased production of superoxide in response to turbulence stress was observed within the 300 rpm treatment. No change was seen in hydrogen peroxide concentration for cells experiencing turbulence stress. In a study performed by Rodríguez et al. (2009), lipid peroxidation sharply increased during the first two days, then decreased over time reaching near-normal levels by the eighth day. Although ROS concentrations were measured, the authors did not find these data to correlate to shearing stress. It is reasonable to conclude that lipid peroxides are a proxy for ROS production, since they are a product of oxidative damage.

Based on these assumptions, ROS production during turbulence stress may be ameliorated by acclimation processes over time, reducing the detrimental effects of turbulence on cellular functions. Several studies have noted the ability of turbulence stressed cultures to acclimate to shear stress over the course of long-term experiments, regaining normal growth rates over time under continued stress (Bronnenmeier and Märkl 1982; Hosaka et al. 1995). After acclimation, the production of ROS would cease and eventually be dissipated by the defense system of the cell. It is unclear from the results obtained from our study whether ROS production was absent in higher turbulence levels or if acclamatory processes resolved accrued ROS; more research is needed to elucidate the nuances of ROS production under turbulence stress.

Polysaccharide content and location were not significantly affected by turbulence treatment, although a slight increase in extracellular polysaccharide was observed for all treated cultures. These findings were highly unexpected, since cellular aggregation was visually apparent within each of the treated cultures. As seen for glyoxylate-treated cultures, increases in the flocculation efficiency are more likely a consequence of cell stickiness than to the production of biological glues, such as EPS. In addition, the slight increases in extracellularly located polysaccharides observed in each of the treated cultures may represent a general response to turbulence, in which secreted materials are produced as either drag reducing or viscosity increasing compounds, used to decrease cellular damage irrespective of turbulence levels (Gasljevic et al. 2008; Jenkinson and Sun 2013).

Hydrogen peroxide concentrations did not fluctuate in response to stress for *Chlorella vulgaris* within this study. Hydrogen peroxide is less reactive than superoxide radicals and can traverse cellular membranes, moving both throughout the cell and extracellularly

(Velikova et al. 2000; Dummermuth et al. 2003; Choo et al. 2004). As a signaling molecule, hydrogen peroxide mediates the response of cells to both biotic and abiotic stresses (Li et al. 2010; Mittler et al. 2011). Signaling responses occur as an immediate response to stress exposure, and thus measurement on the tenth day of exposure would not be expected to exhibit increases. Little information is available on the processes involved in ROS production and detoxification within microalgae in response to environmental stressors; the majority of studies available are based on high light, metal, and chemical toxicity stressors. In addition, microalgae have been shown to exhibit species-specific ROS production and antioxidant response to varying stressors, of which also differ based on the stressor applied (Gao and Tam 2011; Cirulis et al. 2013). Therefore, ROS production should be analyzed on a species basis, while considering the source of the stress responsible for ROS induction.

Studies on the effects of toxin and heavy metal exposures on *Chlorella vulgaris* have been studied most extensively. The activity of SOD, CAT, and POD have been shown to increase under applied chemical stress, as seen for the allelochemical N-phenyl-2-napthylamine, herbicide glufosinate, and nonyphenol (Qian et al., 2008; Qian et al. 2009; Gao and Tam 2011). Copper metal stress was shown to induce an increase in SOD activity in *C. vulgaris*, while a lack of activity was observed for *C. kessleri* in response to the same stressor (Mallick 2004; Sabatini et al. 2009). Although chlorosis commonly occurs in response to oxidative stress, increases in the concentration of secondary pigments, such as carotenoids, have been implicated as a general response to environmental stress in green microalgae; however, these responses are not well understood (Circulis et al. 2013). In a study performed by Li et al. (2010), constitutive action of catalase and peroxidase activity was proposed as an explanation for why hydrogen peroxide concentrations remained

consistent between UV-B treated and untreated cells in the green macroalgae *Corallina officinalis*. Although a similar mechanism has not been shown for *Chlorella* species, constitutive action of either enzymatic or non-enzymatic antioxidants may explain the ability of *Chlorella* species to tolerate a wide range of environments and climatic conditions and is a likely cause for the lack of response observed for H_2O_2 concentrations.

The response of *C. vulgaris* to environmental stress showed variations between stressors. Nutrient stress, as simulated by glyoxylate, induced the production of superoxide and flocculation efficiency, but did not affect cell growth or morphology during the treatment period. As noted previously, the concentration of glyoxylate needed to inhibit growth and induce a strong stress response must be above a 1.0 mM threshold, under which low-level stress is experienced. In addition, flocculation of cultures in response to glyoxylate was not induced by an EPS matrix, which may implicate the use of cellular coatings and/or changes to the cell wall components, although EPS production was shown to account for aggregation formation in Yang et al. (2010). Therefore, *C. vulgaris* may utilize a variety of mechanisms to induce cellular aggregation under nutrient stress. Variations in the photoperiod did not induce stress realization within *C. vulgaris* as indicated by the common indicators of stress measured within our study. It was unclear whether these results indicated a lack of stress in response to short or long day lengths, or the ability of *Chlorella* to effectively photoacclimate under varying light regimes. In either case, the results suggest that *Chlorella vulgaris* is highly tolerant to changes in the incident photon flux, which most certainly contributes to the ability of this species to live ubiquitously. Unexpectedly, high levels of extracellular polysaccharides were measured within both short and long day treatments, despite the lack of

evidence for stress realization. This may indicate that common indicators of stress in microalgae are not solely responsible for EPS secretion mechanisms within *C. vulgaris*.

Lastly, turbulence stress induced the greatest stress response of the three tested stressors, indicating its importance in the population dynamics of microalgae within aquatic ecosystems. Turbulence represents a complex stress mechanism, since turbulence rates affect the collisions rates between suspended particles, diffusion rates of limiting nutrients, as well as exposure to light. As such, turbulence must be understood as a dynamic stressor in which the contributions of the negative and positives qualities are considered. Lower levels of turbulence provide less mechanical damage due to shear stress, but reduce nutrient dispersal and light availability. In contrast, high levels of turbulence increase mechanical damage by increasing collision rates, but provide high nutrient distribution and optimal light conditions. As proposed for nutrient deprivation, cellular aggregations are attributed to changes in the cell coating or cell wall components. As a fully formed biofilm was not identified for any of the three stressors investigated within the time-frame of our experiment, the mechanisms behind biofilm formation in *Chlorella vulgaris* remain to be identified. Collectively, this body of research implicates the complexity of aggregation and biofilm mechanisms with *Chlorella vulgaris*.

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Vita

Carra Leigh Parker was born in 1986 in Raleigh, North Carolina, to Carol Leigh Parker and Michael Leslie Parker as the older of two children. Carra has younger sister, Sarah Kathryn Parker, who graduated with a degree in Fine Arts in December 2013. Carra was awarded a Bachelor of Science in Biology with a minor in Chemistry and City/Regional Planning in 2008. She received her Master of Science in December 2013. She is currently working as a veterinary assistant for Blue Ridge Veterinary Clinic with aspirations of going into environmental conservation research.