

THE COMPATIBLE SOLUTE GLYCEROL AS A PHOTOSYNTHETIC SINK AND  
ENERGY CARRIER IN FRESHWATER AND MARINE *CHLAMYDOMONAS*

by

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This thesis entitled:  
The compatible solute glycerol as a photosynthetic sink and energy carrier in freshwater and  
marine *Chlamydomonas*  
written by Tyson Andrew Burch  
has been approved for the Department of Ecology and Evolutionary Biology

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find that both the content and the form meet acceptable presentation standards  
of scholarly work in the above mentioned discipline.

## ABSTRACT

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The compatible solute glycerol as a photosynthetic sink and energy carrier in freshwater and marine *Chlamydomonas*

Thesis directed by Professors Barbara Demmig-Adams and William W. Adams III

The purpose of this thesis was to explore fundamental relationships between the photosynthetic production of energy carriers, the ceiling of photosynthetic capacity, and the regulation of photosynthesis in algae and how this insight may (i) serve to enhance the principal understanding of the limits of primary productivity and (ii) open up novel applications in the context of the generation of biomaterials and bioenergy from renewable resources. Methods were developed or refined to separate algae from their culture medium to study accumulation and release of glycerol from freshwater and marine species of the green algal genus *Chlamydomonas* under various culture conditions (primarily different salinity and light levels). Culture density, chlorophyll content, growth rate, photosynthetic oxygen evolution, respiration, and intracellular and released glycerol were measured. The freshwater species *C. reinhardtii* released considerably more glycerol to the external environment in response to increasing levels of salinity compared to the marine species *C. euryale*, suggesting that limitations to the ability to internally accumulate and retain glycerol as an osmoregulatory compound may be involved in the inability of the freshwater species to grow at elevated salinities. While the freshwater species

exhibited no downregulation of photosynthesis between 0 and 0.2 M NaCl, the marine species exhibited significant upregulation of photosynthetic oxygen evolution between 0.2 and 2.0 M NaCl in the medium, indicating that internal glycerol accumulation, unlike sugar accumulation, does not interact with sugar-signaling networks that induce photosynthetic downregulation upon internal photosynthate accumulation. At elevated salinities, the freshwater species nearly ceased growing yet exhibited no downregulation of photosynthesis, suggesting glycerol may act as a sink for photosynthetic energy. These findings suggest that stimulation of glycerol synthesis and release by *Chlamydomonas* via environmental manipulation is an attractive option for algal energy-carrier production, which could in turn be used as a feedstock for bioenergy and biomaterial production from renewable resources.

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## **CHAPTER 1**

### **INTRODUCTION**

The vast majority of Earth's biosphere relies on the process of photosynthesis and the organisms that are capable of capturing solar energy and converting it to chemical energy to provide the energy and building blocks for life. Likewise, human society relies on photosynthetic organisms to provide food, materials, and energy (from both recent and ancient photosynthetic activity) to fuel human civilization. The goal of this thesis is to elucidate fundamental relationships between the production of energy carriers in photosynthesis, the ceiling of photosynthetic capacity, and the control of photosynthesis by demand in algae and how this insight may (i) serve to enhance the principal understanding of the limits of primary productivity in ecosystems and (ii) open up novel applications in the context of the generation of biomaterials and bioenergy from renewable resources.

The different chapters of this thesis make the following contributions toward these goals:

Chapter 2 provides a detailed description of methodology that was developed to address the questions asked in this thesis. This detail goes beyond the methods described in Chapters 3 and 4.

Chapter 3 focuses on the effect of salinity and light on algal energy-carrier release (see hypotheses at the end of the Introduction, section 1.2.1).

Chapter 4 focuses on energy-carrier production in the context of photosynthetic capacity (see hypotheses at the end of the Introduction, section 1.2.2).

Chapter 5 focuses on potential practical applications of the novel fundamental insight described in Chapters 3 and 4 for the generation of biomaterials and bioenergy from renewable resources.

## **1.1 Background**

### *1.1.1 Solar-energy utilization by living organisms*

Recent attention to the efficiency of biological solar-energy conversion has resulted in the conclusion that the efficiency of biological solar-energy conversion is low compared to non-biological solar-energy conversion efficiency (Blankenship *et al.* 2011; Ort *et al.* 2015). This conclusion has to be viewed in the context of the fact that biological organisms accumulate only a small fraction of the solar energy absorbed over their lifetime in their final biomass, with most of the energy lost as unusable heat during the myriad of metabolic reactions performed by living organisms. Another major source of energy loss as unusable heat is directly in the light-absorbing pigment complexes of photosynthetic organisms (Demmig-Adams & Adams 1996, 2006; Demmig-Adams *et al.* 2012), and it is of interest to understand the factors that impact energy-conversion efficiency within the light-harvesting system of photosynthetic organisms.

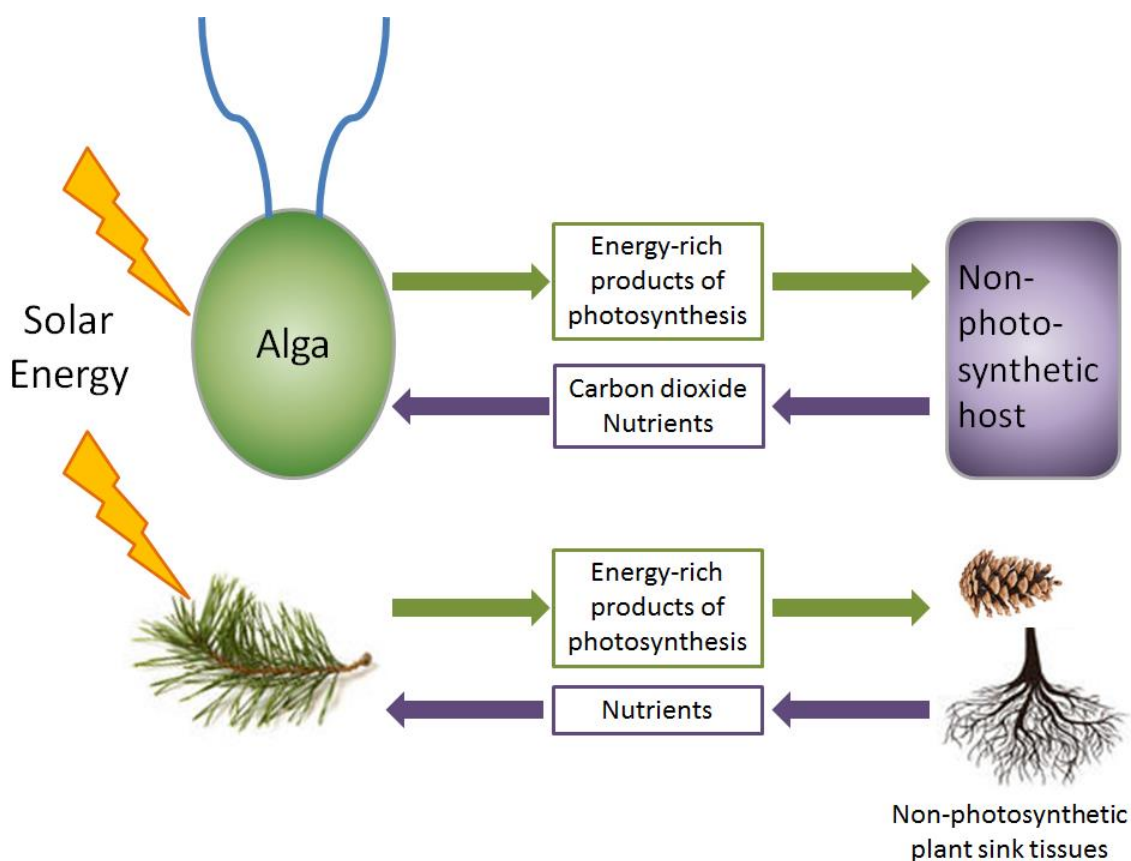
### *1.1.2 Source-sink regulation of photosynthesis*

Internal accumulation of the products of photosynthesis can exert strong feedback inhibition by downregulating photosynthesis (Krapp & Stitt 1995; Paul & Foyer 2001; Adams *et al.* 2013b; Körner 2013; Fatichi *et al.* 2014) via inhibition, or repression, of the transcription of major photosynthetic genes (Krapp & Stitt 1995). It was recently demonstrated that the apparent

carbon-export capacity of leaves is significantly and positively correlated with these leaves' maximal photosynthetic capacity; growth under some environmental conditions led to a tripling of apparent sugar-export capacity from leaves as well as a tripling of what had been thought of as the maximal photosynthetic capacity in the model plant *Arabidopsis* (Adams *et al.* 2013a; Cohu *et al.* 2013b). Generally, augmentation of carbon export from leaves or algae may lead to hitherto-unknown record rates of photosynthesis. The fundamental tenet of this new paradigm (Demmig-Adams *et al.* 2014b) is that the true ceiling of photosynthesis may lie far higher than currently recognized, and that the massive losses of excitation energy by photosynthetic organisms to heat dissipation are not inevitable and can be dramatically reduced and possibly eliminated. At the same time, enhanced carbon export from algae – compared to enhancing carbon export from leaves – may offer the advantage that the energy carriers released from algae may be more easily harvested in potential industrial applications (see Chapter 5).

The present study focuses on model algal systems with the remarkable capability to *continuously produce* (directly from newly fixed carbon) *and internally accumulate and/or release* (excrete) an energy-rich reduced carbon compound, glycerol. The principle of using algae as highly efficient producers of a continuous stream of energy carriers is used in nature by certain non-photosynthetic organisms that host symbiotic photosynthetic microalgae or cyanobacteria as their “personal” solar cells, prevent growth and cell division of the photosynthetic cells, and maintain those cells immobilized and near-immortal within the host's own tissues (Fig. 1.1; Muscatine, 1967; Davies 1984; Lee 2006; Venn *et al.* 2008; Lee *et al.* 2010; Suescún-Bolívar *et al.* 2012; cf. Usher *et al.* 2007). The genus *Chlamydomonas* includes several species known to be symbionts (partners in symbioses), and one of the species (*C. hedleyi*) evaluated in Chapters 2 and 3 is of symbiotic origin (Lee & Zucker 1969; Lee *et al.*

1974). A long-lived, non-dividing state is also seen in mature leaf or needle cells that no longer divide and churn out sugar for export to the rest of the plant for years in the case of evergreens (Fig. 1.1). One of the goals of this fundamental research was to better understand the feedback-inhibition limitation acting on light harvesting, photochemistry, and carbon fixation, and how this affects the maximal rate and effective quantum yield of photosynthesis (in saturating light) and the high-light tolerance of the organism.

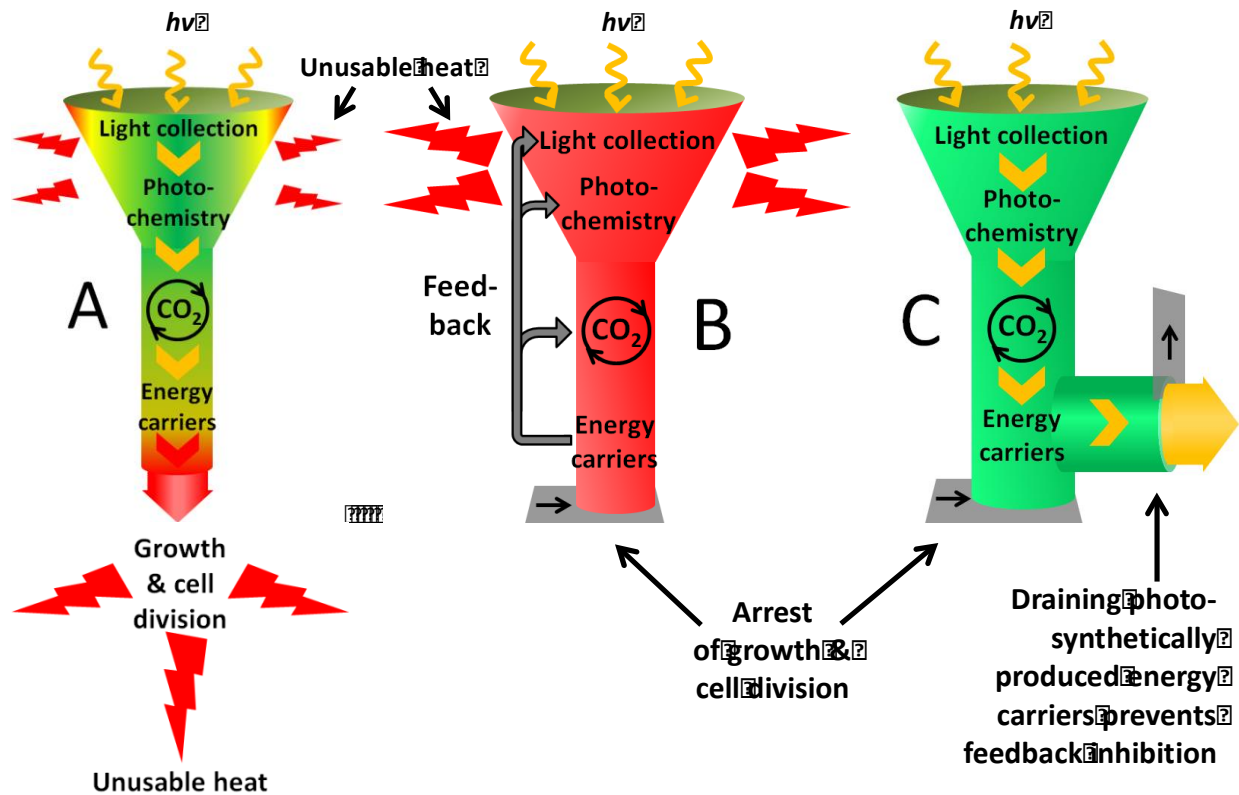


**Figure 1.1:** Schematic depiction of a photosynthetic symbiotic alga excreting energy-rich products of photosynthesis to a non-photosynthetic host in exchange for CO<sub>2</sub> and nutrients, compared to the photosynthetic needles of an evergreen exporting energy-rich products of photosynthesis to non-photosynthetic plant tissues (cones, roots, etc.)



### *1.1.3 Principal impact of energy-carrier removal via various sinks on the efficiency of solar-energy conversion in photosynthetic organisms*

In most natural environments, photosynthetic organisms collect more solar energy than can be utilized because limitations to cell division and growth exist in the form of, e.g., an insufficient level of nutrients (Lin *et al.* 2016). Any potentially damaging unutilized solar energy can be removed harmlessly by effective photoprotective dissipation of excess absorbed energy (Demmig-Adams & Adams 1996, 2006; Demmig-Adams *et al.* 2012, 2014b), and, as stated above, additional energy is lost as heat during the many metabolic reactions that sustain life and contribute to growth (Fig. 1.2 A). Under conditions where cell division and growth are arrested, enhanced dissipation of up to 100% of the absorbed energy occurs (Fig. 1.2 B; Adams *et al.* 2006, 2013b, 2014; Demmig-Adams *et al.* 2014b). Draining of energy carriers, while arresting the photosynthetic organisms' own growth, allows the photosynthetic symbiont to produce energy carriers at a higher efficiency by avoiding feedback inhibition of photosynthesis (Fig. 1.2 C). A better understanding of this principle may open up novel agricultural and industrial opportunities to human society for energy production via photosynthetic organisms (for more detail, see Chapter 5).



**Figure 1.2:** Schematic depiction of solar-energy collection and utilization in photosynthetic organisms. **A**, free-living photosynthetic organisms utilize the vast majority of the energy they collect for their own growth and cell division, while harmlessly dissipating any excess excitation energy; **B**, growth arrest triggers the harmless dissipation of up to 100% of the absorbed energy; **C**, growth arrest coupled with a removal of energy carriers (as in natural symbioses between photosynthetic and non-photosynthetic organisms) has the potential to prevent feedback inhibition of photosynthesis and allow energy-carrier production at a high efficiency of solar-energy conversion. From Demmig-Adams *et al.* (2014b).

#### 1.1.4 Osmoregulation by the compatible solute glycerol

In response to lower water availability, either due to low levels of water or the presence of higher levels of solutes, many organisms synthesize and accumulate organic molecules (Mackay *et al.* 1984; Imhoff 1986; Reed *et al.* 1986; Robertson *et al.* 1990; Roberts 2005). Such molecules are called compatible solutes because they do not interfere with the structure and function of cellular components, such as the functioning of enzymes, transport proteins, and membranes. These compatible solutes are diverse, including amino acids, sugars, larger and

more complex derivatives of amino acids and sugars, and sugar alcohols (Mackay *et al.* 1984; Imhoff 1986; Reed *et al.* 1986; Robertson *et al.* 1990; Roberts 2005). Synthesis of amino acids and their derivatives may, however, come at a considerable cost in nutrient-limiting situations, since nitrogen is a component of these molecules. Sugars and sugar alcohols do not contain nitrogen, but still represent an energetic investment that cannot be used for metabolism and growth. The accumulation of the sugar alcohol glycerol as a compatible solute represents a minimal energetic and structural investment compared to sugars since glycerol has only three carbon atoms per glycerol molecule as opposed to six in a sugar (hexose) or more (e.g., the disaccharide trehalose) and can be synthesized directly from a product of photosynthesis (dihydroxyacetone phosphate = DHAP; Herrera-Valencia *et al.* 2012). In addition to its already established role as a compatible solute in the genus *Chlamydomonas* (León & Galván 1994, 1995; Miyasaka *et al.* 1998), this thesis will explore whether glycerol may also be compatible with continued photosynthetic activity in a way that the accumulation of sugars as compatible solutes are not.

#### *1.1.5 Utilizing fundamental biology as the basis for industrial applications*

Since the productivity photosynthetic organisms is governed by processes such as source-sink regulation and feedback inhibition of photosynthesis, understanding how these principles work in a model system is key to improving yields of such organisms in industrial applications. Chapters 3 and 4 seek to define the fundamental biological responses that control glycerol accumulation and release in a model system in order to allow utilization of these findings in potential industrial applications for glycerol production. Chapter 5 addresses how the findings of

Chapters 3 and 4 can be applied to industrial applications, and how such industrial applications may support the needs of current and future human society.

## **1.2 Research questions addressed in this thesis**

### *1.2.1 Chapter 3*

While previous studies found that both freshwater species of *Chlamydomonas* (León & Galván 1994, 1995, 1997, 1999) and marine species of *Chlamydomonas* (Miyasaka *et al.* 1998) and other green algal genera (such as *Dunaliella*; Ben-Amotz & Avron 1981) release glycerol in response to salinity, no study compared glycerol release in freshwater versus marine species. Chapter 3 addresses the research question of whether or not (i) the response of glycerol release to external salinity differs between a freshwater and a marine species. Freshwater species, presumably adapted to lower salinities, may exhibit glycerol release at low salinities, while marine species, presumably adapted to higher salinities, may exhibit glycerol release at higher salinities than the freshwater species.

Since salinity is presumably the trigger for glycerol production and growth light intensity fuels photosynthesis, Chapter 3 also addresses the research question of whether or not (ii) external salinity and growth light intensity (as the source of energy for photosynthetic electron transport that supports production of glycerol) act synergistically in triggering glycerol release. Specific hypotheses addressing research questions (i) and (ii) are formulated and discussed in Chapter 3.

### *1.2.2 Chapter 4*

Low growth rates in photosynthetic organisms constitute a low demand for photosynthate (a low sink activity; see Fig. 1.2 B). Chapter 4 addresses the question of whether (iii) down-regulation of photosynthesis is avoided under growth-limiting conditions in algal systems with the potential to use glycerol formation as an alternative sink for photosynthesis (see Fig. 1.2 C). Using salinity to induce lower growth rates may not decrease photosynthesis rates if photosynthetic production of glycerol acts as a sink for photosynthetic energy.

Chapter 4 furthermore addresses the question of whether (iv) freshwater and marine species differ in their relative proportions of internal glycerol accumulation versus glycerol release to the external medium. Freshwater species may lose more glycerol into the medium, while marine species may accumulate very high glycerol levels internally. Specific hypotheses addressing research questions (iii) and (iv) are formulated and discussed in Chapter 4.

## CHAPTER 2

### METHODOLOGY DEVELOPMENT

This chapter presents a detailed description of the methods used to generate the data presented in Chapters 3 and 4 (more detailed than what is presented in these chapters themselves) as well as a description of the development of additional methodological detail used for preliminary inquiry leading to the hypotheses tested in Chapters 3 and 4 or for the pre-culturing of algal cultures used for data generation but not featured in detail in those chapters.

#### 2.1 Experimental vessels

Three types of photobioreactors were employed for microalgal cultivation; agitated flasks of up to several hundred mL for stock cultures, stirred 500 mL bottles continuously bubbled with 5% CO<sub>2</sub> (in ambient air) for pre-culture prior to experiments, and 50 mL tubes continuously stirred and bubbled with 5% CO<sub>2</sub> (in ambient air) for experimentation.

##### 2.1.1 *Flasks for stock cultures*

Simple Erlenmeyer flasks (Fig. 2.1) were used for maintaining stock cultures of algae at volumes of several hundred mL on a shaker table set to 100 revolutions per minute (rpm) in a temperature-controlled growth chamber at 20°C under a light intensity of no more than 30  $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$  provided by cool white fluorescent tubes, which resulted in slow, continuous

algal growth and high stock culture density. Culture pH was maintained between 7.0 and 7.9 by pH buffers (see section 2.3 below on buffer systems) in the medium. Algae were harvested by centrifugation, resuspended in fresh culture medium, and used to inoculate pre-experimentation cultures in Roux bottles.



**Figure 2.1:** Erlenmeyer flasks on a shaker table inside a growth chamber for stock culture maintenance.

### 2.1.2 *Roux bottles for pre-culture prior to experiments*

1 L Roux bottles (flasks with strictly parallel surfaces and a depth of 5.7 cm; Pyrex, Czech Republic, Fig. 2.2) were used to maximize light exposure and reach sufficient algal biomass concentrations for subsequent experimentation. Roux bottles were fitted with two-hole #4 rubber stoppers, through which 1 mL glass pipettes (VWR, USA) were inserted, with gas exhaust sterilizing filters (Aervent 50, 0.2  $\mu\text{m}$  pore size, Millipore, Billerica, MA, USA) secured

to the outlet hole. The tops of the pipettes were connected to T joints, with one end of each T connected to a clamped tube covered in aluminum foil and the other end connected to an inlet gas-sterilizing filter. The Roux bottles were autoclaved and, after removing the aluminum foil, medium-sterilization filters (50 mm diameter Millex-GP, 0.22  $\mu\text{m}$  pore filters, Millipore, Billerica, MA, USA) were inserted into the side tubes in the laminar flow cabinet. Medium was pumped into each bottle through the sterilizing filter using a peristaltic pump (Sci-Q400, Watson Marlow, Wilmington, MA, USA). Culture volume was 500 mL. Mixing occurred by a combination of bubbling with air containing 5%  $\text{CO}_2$  and a magnetic stirrer. The pH was set to 7.9 by buffering with 60 mM  $\text{NaHCO}_3$  (see section 2.3). Roux bottle cultures were grown in a small temperature- and light-controlled room. The temperature of the cultures was controlled by a portable air-conditioning unit (Frigidaire, Dublin, OH, USA) set to 20°C, with a 0.2 meter box fan continuously circulating air across the surface of the Roux bottles. Continuous illumination with 150  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$  at the surface of the Roux bottles was provided by 8 cool white fluorescent bulbs (Philips, The Netherlands).



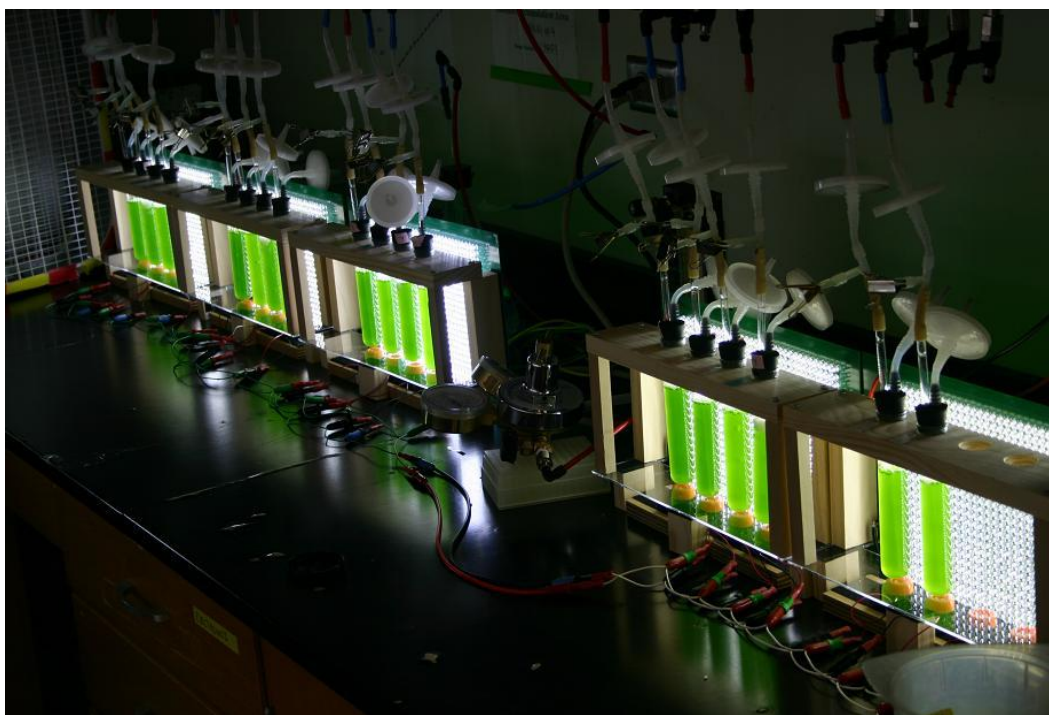


**Figure 2.2:** 1 L Roux bottles used to acclimatize *Chlamydomonas* species to different salinities and develop sufficient quantities of algae for experimentation.

### 2.1.3 Experimental liquid cultures in 50 mL glass tubes

50 mL test tubes were fitted with two-hole #4 rubber stoppers, through which nine-inch Pasteur pipettes were inserted, with gas exhaust sterilizing filters secured to the second hole. The top of the pipettes were connected to T joints, with one end of each T connected to a short tube (1.5 cm) clamped at the end, and the other connected to an inlet gas sterilizing filter. A magnetic stir flea was added to each tube, and the tubes were autoclave sterilized. Throughout the experiments, the cultures were slowly magnetically stirred to keep algae from settling to the bottom, with the majority of mixing being accomplished by continuous bubbling with air containing 5% (v/v) CO<sub>2</sub>. Small 4 cm computer chip fans (Sunon, Taiwan, China) were used to construct a custom-built device for stirring by gluing a plastic Falcon tube cap, inside which two rare-earth magnets were inserted, to the fan. The custom stirring devices were then linked in

parallel to a 32-volt DC power supply (HY3003D, Mastech, Pittsburgh, PA, USA) and stir speed was set by adjusting the output voltage. The test tubes were held above the stirring devices in custom-built holders (Fig. 2.3) made from wood and clear Plexiglas to position the tubes a precise distance above the stirring devices and next to light-emitting diode (LED) arrays (see section 2.2 below). The 50 mL test tubes were maintained in the same small room as the Roux bottles above, and the temperature was controlled by the air conditioning unit, large box fans, and small 12 cm fans close to the surface of each custom-built holder/LED array set-up.



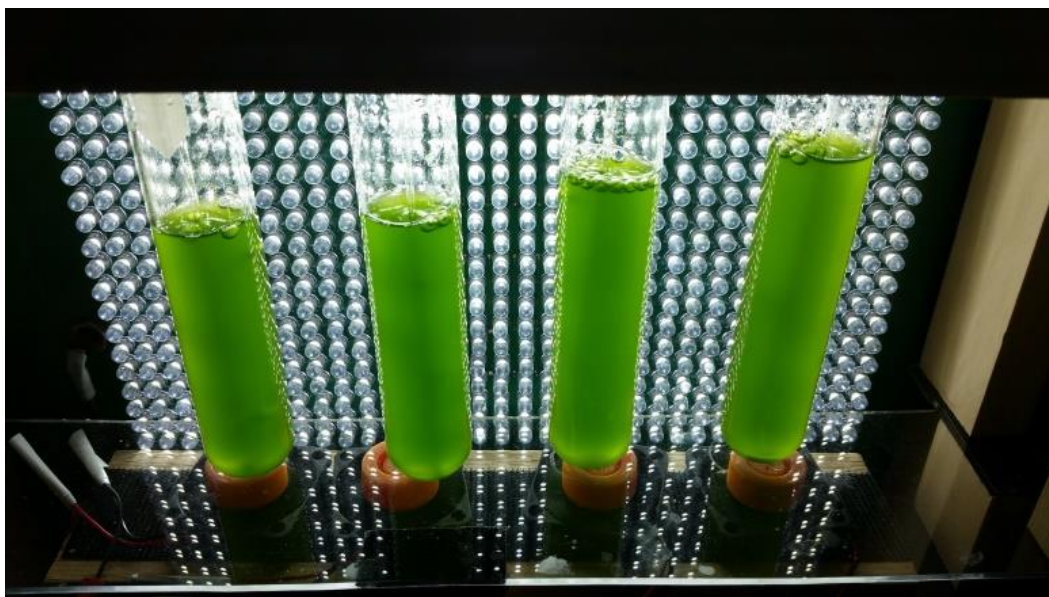
**Figure 2.3:** 50 mL test tube set-ups in operation.

Analyses for the experiments reported in Chapter 3 were performed on algae and their external medium over the course of 5 days following removal of an aliquot of algal culture from Roux bottles, centrifugation, and resuspension in fresh medium to a standard optical density of 2.5 at 750 nm ( $OD_{750}$ ). Over the 5 days of subsequent experimental exposures, algal cultures

were allowed to grow and reach higher densities. This approach was used for an initial phase of experimentation that led to the generation of the data presented in Chapter 3. For the experiments described in Chapter 4, culture density, light environment, and nutrient availability were standardized more closely by more frequent adjusting of culture density via additional centrifugation, resuspension in fresh medium, and density adjustment steps. The latter procedure allowed the cultures to be maintained at densities similar to the initial culture density over the duration of an experiment. Specifically, cultures (with an initial OD<sub>750</sub> of 2.0) were maintained in test tubes for approximately 1.5 days (Fig. 2.4) for acclimation to the experimental conditions, and subsequently diluted (by addition of culture medium and visual assessment) to a similar greenness as initial cultures prior to the 1.5 days of growth (Fig. 2.5). Thirty mL of the resulting diluted cultures were transferred to sterile 50 mL centrifuge bottles, gently centrifuged (2 mins, 706 g), and pellets resuspended in 30 mL of fresh medium and returned to test tubes and experimental conditions for the duration of a subsequent 24-h experimental period (Fig. 2.6). This additional, turbidostat-like protocol was used for the generation of data for Chapter 4 since self-shading and nutrient availability could potentially affect growth, light use efficiency, and glycerol accumulation and release.

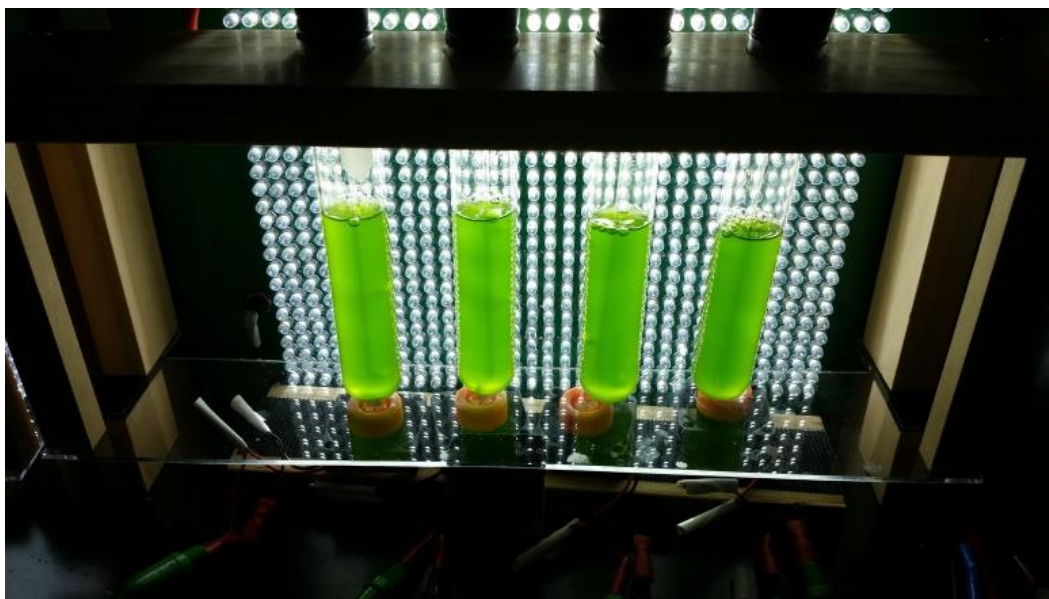


**Figure 2.4:** 30 mL culture after 1.5 days of growth for pre-acclimation to the test tube environment.



**Figure 2.5:** Diluted cultures visually set to similar greenness by introduction of fresh medium prior to centrifugation.

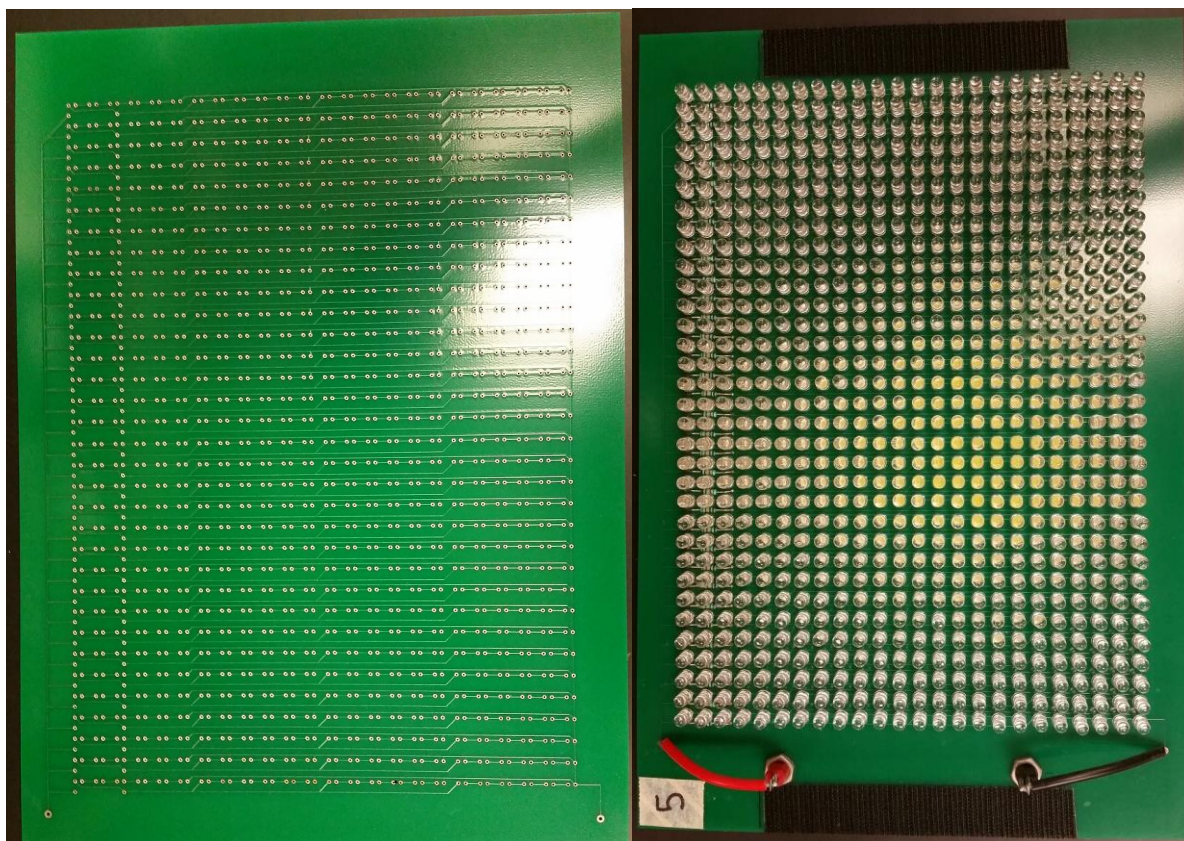




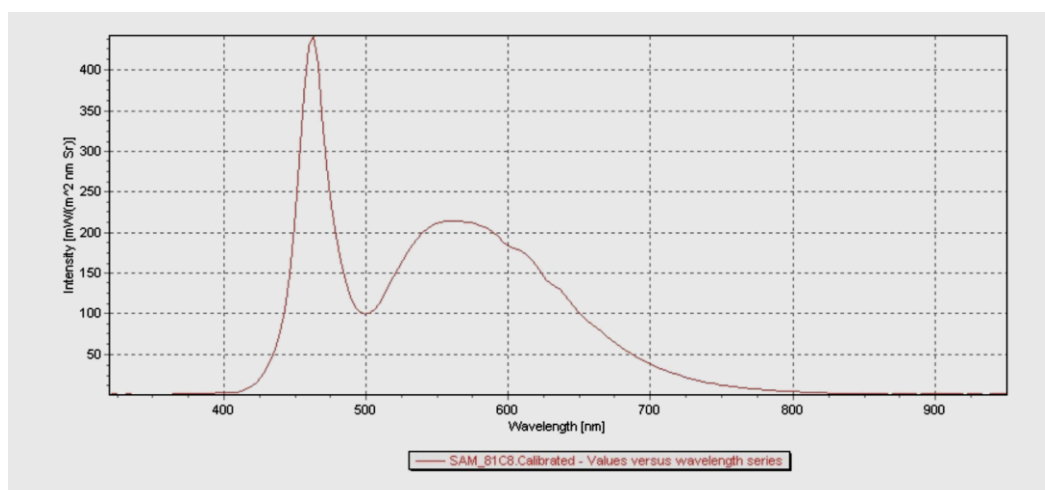
**Figure 2.6:** Standardized, resuspended cultures at the beginning of a 24-hour experiment.

## 2.2 Light source for experiments conducted in tubes: LED arrays

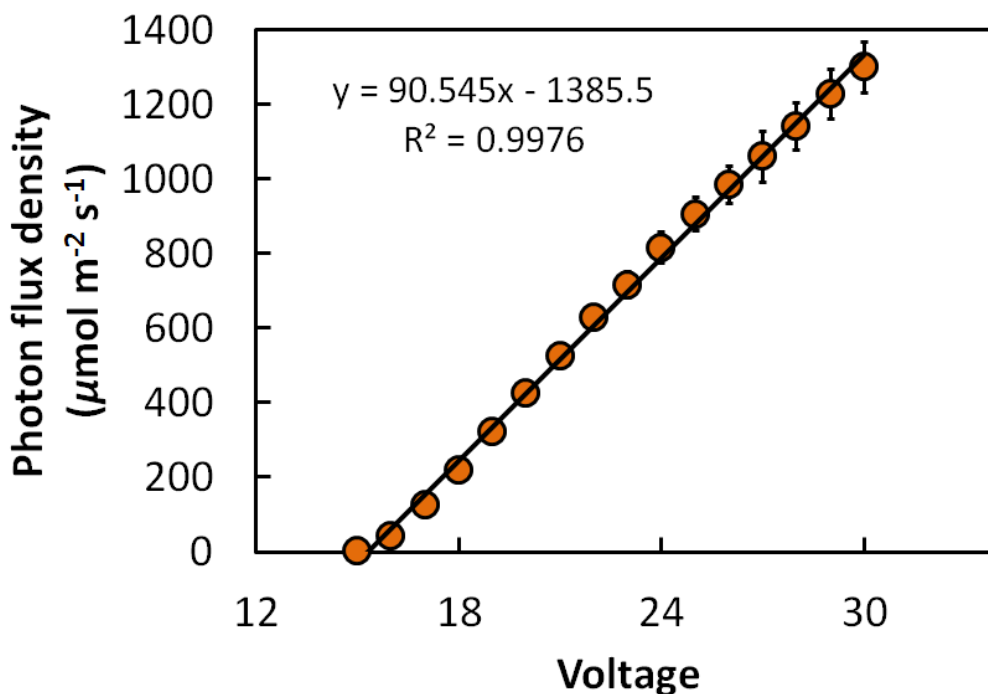
Custom-designed circuit boards (see left image in Fig. 2.7, Sunstone, Mulino, OR, USA) were fitted with 792 white light-emitting diodes (LEDs; C503C-WAN, CREE Inc., Durham, NC, USA) and 33 124-ohm resistors (Vishay Intertechnology, Malvern, PA, USA) each to produce arrays (see right image in Fig. 2.7) utilized as the light source for precise control of incident light flux. LEDs with a favorable emission spectrum (Fig. 2.8) for chlorophyll (with a peak in the blue region and strong representation in the red region of the visible spectrum) were selected. Light intensity (photon flux density, PFD) was measured in  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$  using a digital light meter (LI-250A, Li-Cor Biosciences, Lincoln, NE, USA). PFD was linearly dependent on the voltage in the networked array (Fig. 2.9) provided by 32-volt DC power supplies (HY3003D, Mastech, Pittsburgh, PA, USA).



**Figure 2.7:** Custom-designed circuit board prior to LED and resistor attachment (left). Completed LED array (right).



**Figure 2.8:** Emission spectrum of C503C-WAN LED, from CREE Inc., Durham, NC, USA



**Figure 2.9:** Photon flux density in  $\mu\text{mol m}^{-2} \text{s}^{-1}$  of photosynthetically active radiation at the surface of the 50 mL test tubes as dependent on voltage.

The LED arrays were used to provide continuous light of a constant intensity for the experiments described in the present thesis. For experiments involving a simple light/dark cycle, the power supplies were connected to a 24-hour on/off timer.

### 2.3 Buffer system

To prevent fluctuations in pH in the external medium under continuous bubbling with 5%  $\text{CO}_2$ , several buffer methods were utilized and compared by monitoring medium pH prior to any algal introduction. To avoid confounding total organic-carbon analyses, sodium bicarbonate was used instead of an organic-carbon-based buffer. All culture media used for Roux bottle and tube

cultures were buffered with 60 mM NaHCO<sub>3</sub> after it had been determined that use of 100 mM NaHCO<sub>3</sub> led to an excessively high pH of above 8, which is toxic to *Chlamydomonas*.

## 2.4 Separation of algae from culture medium

Two different strategies, centrifugation and filtration, were evaluated for their efficacy in separating algae from their culture medium, while avoiding rupture of algae and release of internal organic carbon compounds into the medium. Analysis of total-organic-carbon (see section 2.5.1) was used for initial comparison of various centrifugation speeds and different filters, and results were verified via analysis of glucose equivalents (see section 2.5.2).

For total-organic-carbon analysis, a culture volume was collected and rinsed two times with de-ionized water (1% NaCl added to provide osmotic balance with the internal contents of the algae, thereby avoiding rupture of the algae). Results from different methods are compared in Table 2.1 with the goal of finding a method that results in minimal total organic carbon (TOC) levels in the external medium combined with absence of visible signs of greenness in the medium. Multiple conditions were tested in incremental steps of centrifugation speed and duration as well as filtration by syringe or suction pump without replication of the individual steps. Data were evaluated for the apparent effectiveness of spinning down of algae (thus lowering TOC levels in the external medium), while avoiding rupturing of algae during their separation from the external medium (thus also lowering TOC level in the external medium). Centrifugation at 2,000 rpm for 10 minutes resulted in visibly detectable levels of chlorophyll or algal cells in the form of a slight green hue in the supernatant (presumably resulting from algae that failed to be spun down by this approach), and was thus eliminated from consideration.



Centrifugation at 4,000 rpm for 10 minutes was selected as the centrifugation method of choice to separate algae from the medium because this method resulted in the absence of chlorophyll in the supernatant, while simultaneously minimizing the level of organic carbon in the external medium, which indicated that rupturing of cells was negligible. This method was used for all sample extractions for sugar and glycerol analysis.

<b>Centrifugation</b>		<b>Filtration</b>	<b>TOC (mg L<sup>-1</sup>)</b>
Speed (rpm)	Time (min)	Pore size ( $\mu\text{m}$ )	
2,000	1	-	21
2,000	2	-	18
2,000	5	-	14
2,000	10	-	8
2,000	20	-	12
4,000	1	-	23
4,000	2	-	13
4,000	5	-	9
4,000	10	-	9
4,000	20	-	14
6,000	1	-	20
6,000	2	-	11
6,000	5	-	10
6,000	10	-	32
6,000	20	-	16
10,000	1	-	22
14,000	1	-	24
-	-	Syringe 0.22	43
-	-	Pump 0.22	33
-	-	Pump 0.45	60

**Table 2.1:** Total Organic Carbon (TOC) in the external medium in mg L<sup>-1</sup> as affected by centrifugation speed in rpm and time (minutes), or filtration type (syringe or pump) and pore size ( $\mu\text{m}$ ).

In addition, results from centrifugation at 4,000 rpm (about 1,500 g) for 10 minutes were compared with results from filtration of algae using Whatman filters (GF/D of 2.7  $\mu\text{m}$  pore size, Little Chalfont, UK) to separate algae from their external medium. Small (1.5 mL) samples from

cultures of the marine species *Chlamydomonas euryale* were subjected to either centrifugation or filtration. In addition, a corresponding sample from the same culture was boiled for 15 minutes and then centrifuged or filtered as a control, in which all algae were presumably ruptured. Table 2.2 shows the results of these comparisons that, once again, were not done in replicate.

	Without boiling		After boiling	
	Filtration	Centrifugation	Filtration	Centrifugation
<b>Glucose equivalents (mg L<sup>-1</sup>)</b>	39	29	76	67

**Table 2.2:** Glucose equivalents in filtered or centrifuged samples before and after boiling.

As expected, apparent greater levels of glucose equivalents were detected in the culture medium after boiling of the algae compared with filtration or centrifugation without boiling, further suggesting that centrifugation at this speed does not rupture the algae. Centrifugation was selected as the preferred method to separate intact algae from their culture media for the purpose of assessing carbohydrate release from the algae into the external medium.

## 2.5 Analyses of reduced-carbon compounds in the medium

### 2.5.1 Total organic carbon

Measurement of Total Organic Carbon (TOC) was performed using a TOC meter (Shimadzu 5000A, Kyoto, Japan). 0.7 or 0.8 mL of each sample of centrifuged supernatant or filtered medium (see table 2.1), three volumes of methanol standards of known carbon

concentration in deionized water, and three samples of laboratory deionized water were diluted with 50 mL hyper-purified deionized water and placed in the TOC meter. The results were analyzed by adjusting the readouts to compensate for trace amounts of organic carbon in the laboratory deionized water samples, and the sample dilution volumes, and compared to the standard curve of known organic carbon to yield TOC on a  $\text{mg L}^{-1}$  basis.

### 2.5.2 Analysis of glucose equivalents

Supernatants were assayed for glucose equivalents after the method of DuBois *et al.* (1956). 100  $\mu\text{L}$  of sample and 100  $\mu\text{L}$  of de-ionized water were combined in a 2 mL plastic vial, to which 200  $\mu\text{L}$  of 50  $\text{g L}^{-1}$  phenol was added, followed immediately by addition of 1 mL  $\text{H}_2\text{SO}_4$  (97%) to hydrolyze all soluble carbohydrates in the sample to monosaccharides. Pentoses form furfural, while hexoses form hydroxyl-methyl furfural, both of which have a yellow color that can be quantified by measuring optical density at 483 nm. Vials were mixed using a vortex genie for 1 or 2 seconds; the resulting solution was incubated for 10 minutes at room temperature and then placed in a water bath at 35°C for 30 minutes to stop the reaction and cool the sample. Finally, optical density was determined at 483 nm in a Beckman DU-64 spectrophotometer (Brea, CA, USA) and calibrated using a standard curve of known sugar concentrations (D-Glucose) to determine the concentration in glucose equivalents in the sample.

### 2.5.3 Glycerol analysis

Glycerol, both released into the medium and accumulated intracellularly, was determined using colorimetric free glycerol assay kits; the BioVision Free Glycerol Assay Kit (San Francisco, CA, USA) for experiments in Chapter 3 and the BioAssay Systems EnzyChrom Glycerol Assay Kit (Hayward, CA, USA) for experiments in Chapter 4. All glycerol samples were diluted either 1:10 or 1:20 with deionized water prior to analysis to keep the results within

the standard curve of the assay. The BioVision kit comes with four small vials of reagents; a glycerol standard, enzyme mixture, probe reagent, and dimethyl sulfoxide (DMSO), and a larger vial of assay buffer. The small vials were briefly centrifuged to prevent loss of products upon opening of vials. Then 220  $\mu\text{L}$  (pipette volume accuracy was verified using a scale) of anhydrous DMSO were added to the glycerol probe vial. The glycerol enzyme mix was prepared by adding 220  $\mu\text{L}$  assay buffer to the glycerol enzyme vial. Both vials were gently mixed by turning vials over and back slowly for a few minutes. A standard curve was generated by adding 0, 2, 4, 6, 8, or 10  $\mu\text{L}$  of 1 mM glycerol standard to separate wells on a 96-well flat-bottom plate. All wells were brought to 50  $\mu\text{L}$  total volume by adding the appropriate volume of assay buffer. 4  $\mu\text{L}$  of each of the final samples to be tested and 45  $\mu\text{L}$  assay buffer were added to separate wells. The total number of samples were counted, including standard curve samples, and 2 were added (example – 25 test samples + 6 standard curve samples + 2 = 33 total). To generate the reaction mix, the previous number was multiplied by 46  $\mu\text{L}$  (to determine the total amount of assay buffer) and then multiplied by 2  $\mu\text{L}$  (to determine the total amount of glycerol probe and glycerol enzyme mix to add to a suitably sized vial;  $33 \times 46 = 1518$   $\mu\text{L}$  assay buffer,  $33 \times 2 = 66$   $\mu\text{L}$  glycerol probe,  $33 \times 2 = 66$   $\mu\text{L}$  glycerol enzyme mix). Then 50  $\mu\text{L}$  reaction mix was added to standard curve wells and final sample wells. After several minutes, the standard curve wells were compared with the final sample wells to ensure that samples were within the range of the standard curve. Following completion of this procedure for all samples, the plate was incubated in the dark for 30 minutes, after which absorbance was determined at 570 nm using a Tecan Safire 2 plate reader (Männedorf, Switzerland).

The EnzyChrom kit worked in a similar fashion, but the reagents were partitioned in the small vials in a different manner. The EnzyChrom kit came with an enzyme mix, ATP, dye

reagent, and glycerol standard. On a 96-well flat-bottom plate, 4  $\mu\text{L}$  each of known glycerol concentrations (0, 0.3, 0.6, and 1 mM) were added to the plate, followed by 4  $\mu\text{L}$  of each diluted sample. For each reaction well (standards + samples + 4 extra to ensure excess reagent), 100  $\mu\text{L}$  assay buffer, 2  $\mu\text{L}$  enzyme mix, 1  $\mu\text{L}$  ATP, and 1  $\mu\text{L}$  dye reagent were mixed in a tube, and 100  $\mu\text{L}$  of the resulting solution was pipetted into each reaction well. The plate was incubated for 20 minutes in the dark, after which absorbance was determined at 570 nm using the Tecan Safire 2 plate reader.

## 2.6 Intracellular glycerol analysis

Samples for the quantification of intracellular glycerol levels were prepared using algal pellets from the centrifugation of samples for released glycerol analysis and put through a series of two rinses described below. After removing any remaining supernatant left over from the released glycerol supernatant extraction by careful pipetting, the pellets were gently resuspended in fresh sterile medium of the same salt concentration as the sample and centrifuged again. The supernatants were removed by pipette, discarded, and the pellets were resuspended in medium and centrifuged again. After discarding the supernatants, the pellets were resuspended with 1 mL deionized water and boiled for 10 minutes in a large beaker half-filled with deionized water. The boiled samples were centrifuged and the resulting supernatants were collected for glycerol analysis. This protocol is similar to that described by Chow *et al.* (2013), with an extra round of rinsing (centrifugation and resuspension in medium) and a lower centrifugation force (1,500 g instead of 10,000) and time (10 minutes instead of 20).

## 2.7 Species evaluated

### 2.7.1 Marine species

Four strains of marine *Chlamydomonas* were evaluated. The strains were obtained from the Culture Collection of Algae at The University of Texas at Austin (UTEX, <https://utex.org/>). Information provided about the strains by UTEX, including recommended growth media, the source of the strains, and other key details can be found below:

- *C. augustae* Media: Sea Water Enriched Solution (½ SWES)
- *C. euryale* UTEX # 2274  
Media: Artificial Seawater Medium  
Origin: Qingdao  
Collection: M. Li  
Isolation: R.A. Lewin  
Deposition: (1981)  
Relatives: CCMP219 aka CCMP MACC0054, CCMP WTLEWIN
- *C. hedleyi* UTEX # 2848  
Media: Modified Artificial Seawater Medium  
Origin: Key Largo Sound, Florida, USA  
Description of Location: Foraminiferan *Archaias angulatus*  
Collection: (1969)  
Isolation: J.J. Lee  
Deposition: T. Pr\_schold (2002)

Relatives: ATCC 50216

- *C. provasolii* UTEX # 2849

Media: Modified Artificial Seawater Medium

Origin: Key Largo Sound, Florida, USA

Description of Location: Foraminiferan *Cyclorbiculina compressa*

Collection: (1978)

Deposition: T. Pr\_schold (2002)

Relatives: ATCC 50217

All marine strains were transferred into these respective liquid media to produce larger amounts of algae over successively larger flask and culture volumes. The two marine species were then transferred to Modified Okamoto Medium to standardize the culture medium between species. For a complete list of media components, see Appendix A.

### 2.7.2 *Freshwater species*

In addition, two freshwater strains of *Chlamydomonas* were studied:

- *C. reinhardtii* UTEX # 2244

Media: Minimal Medium for Growth

Origin: Amherst, Massachusetts, USA

Isolation: G.M. Smith

Isolator Number: cc-125 wild type mt+ a.k.a. Levine's clone of  
137c(+)

Deposition: CGC (8/6/1980)

Relatives: SAG 34.89; CGC CC-125; UTCC 11; ?CCAP 11/32C

- *C. segnis* UTEX # 1638

Media: Minimal Medium for Growth

Origin: Dauphin Islands, Alabama, USA

Description of Location: Soil

Isolation: T.R. Deason (1965)

Deposition: T.R. Deason (Fall 1967)

Relatives: SAG 2.75

Also Known As: Formerly *Chlamydomonas gymnogama* (Starr & Zeikus 1987)

Notes: Homothallic (Deason 1967)

The freshwater species were transferred to liquid media (Minimal Medium for Growth, MMG; see Appendix A) from the agar slants provided by the supplier and slowly moved to larger culture flask and culture volumes to obtain larger amounts of algae for experimentation.

## 2.8 Sugar/polysaccharide release into the medium

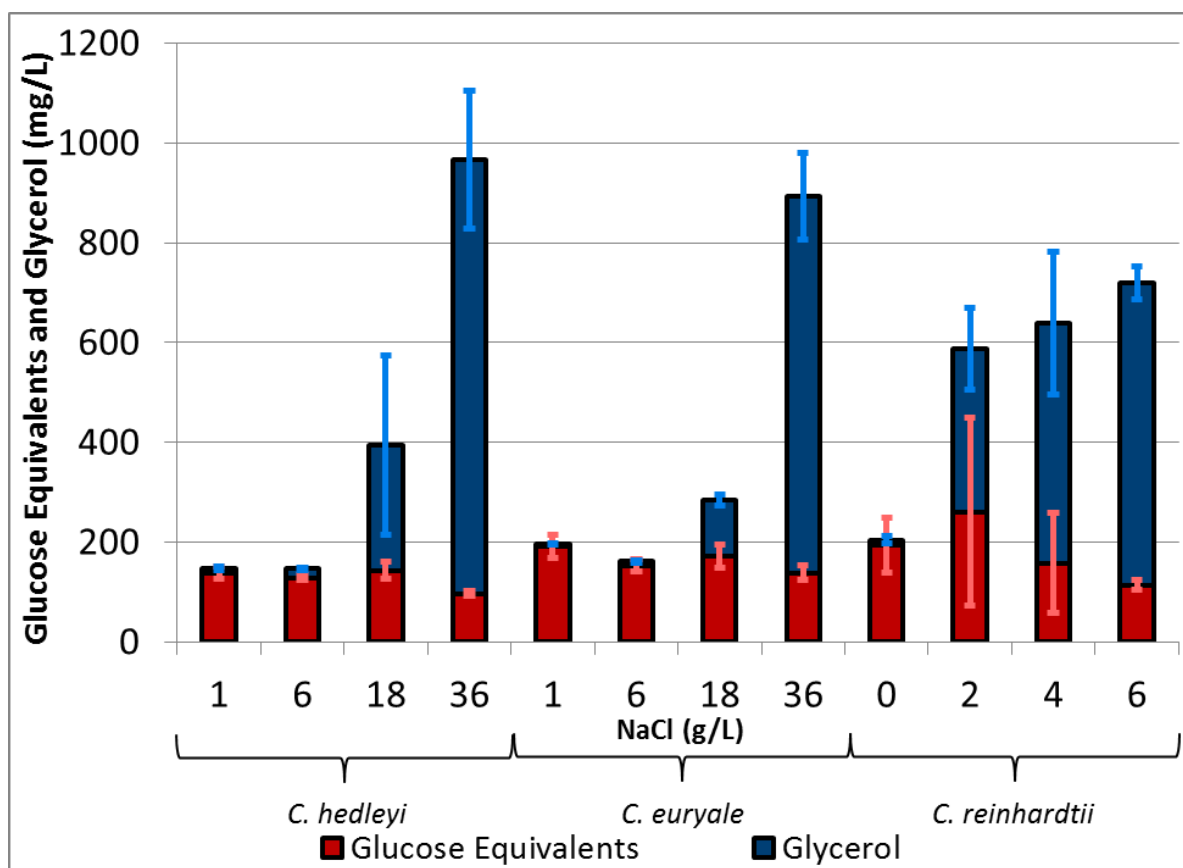
Since *Chlamydomonas* exudes polysaccharides and does not secrete simple sugars (Allard & Tazi 1993), it is likely that the glucose equivalents detected were released by the algae as polysaccharide-based mucilage. The only conditions under which putatively large glucose equivalents were detected in the medium, as was the case in the freshwater strain *C. segnis* (data not shown), were likely stressful to the homothallic alga, leading to apparent sexual reproduction (evident as zygospore state viewed under the microscope), and associated gelatinous mucilage



secretion seen in the supernatant of centrifuged samples. Zygospore formation represents the transition into the dormant state associated with extremely low levels of photosynthesis. Since it formed zygospores extremely readily, *C. segnis* was eliminated as a candidate for further experimentation.

### 2.8.1 *Comparison of glycerol release versus glucose equivalents*

The level of glycerol release into the external medium by species of *Chlamydomonas* responded to differences in the concentration of salt in the external medium, whereas the secretion of sugars and sugar polymers was unaffected by external salinity (Fig. 2.10). Since the purpose of this study was to compare algae under conditions with and without a sink for the products of photosynthesis, triggering glycerol release using medium salinity was the viable choice.



**Figure 2.10:** Levels of glycerol (blue) and other carbohydrates (as glucose equivalents; red) in the culture medium after five days in liquid culture at different NaCl concentrations for three species of *Chlamydomonas* at  $200 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ .

## CHAPTER 3

### ENVIRONMENTAL MANIPULATION OF GROWTH AND ENERGY CARRIER RELEASE FROM FRESHWATER AND MARINE *CHLAMYDOMONAS* SPECIES

#### 3.1 Abstract

While many microbial species produce glycerol as an internal osmoregulant, only some species release glycerol into the external medium. The present study compared synergistic effects of light intensity and salinity on rates of glycerol release and growth in two marine species of *Chlamydomonas* (*C. euryale* and *C. hedleyi*) versus a freshwater species (*C. reinhardtii*). High light intensity stimulated both glycerol release and algal growth in all species, presumably by stimulating photosynthesis and thereby the production of the energy carrier glycerol. The freshwater species exhibited a lower salinity threshold than the marine species for both glycerol release and growth retardation. These findings suggest (i) that there is competition between the production and release of glycerol into the medium versus the internal use of the products of photosynthesis for algal growth and (ii) that the freshwater species has a greater propensity for glycerol leakage into the external medium under saline conditions than the marine species. Furthermore, conditions that stimulated glycerol release increased the maximal rate of photosynthesis, suggesting that synthesis (from the direct products of photosynthesis) and removal of glycerol from the cell may alleviate feedback inhibition on photosynthetic capacity. The stimulation of glycerol synthesis and release by *Chlamydomonas* species via environmental manipulation offers an attractive option for continuous algal energy-carrier production as a feedstock for biofuel generation under conditions eliminating energy-carrier consumption by

algal growth, while circumventing feedback inhibition of photosynthesis as well as the need for algal harvest and regrowth employed for extraction of lipid or carbohydrate feedstocks.

### 3.2 Introduction

The ability of many non-photosynthetic and photosynthetic microbes to tolerate salinity is associated with the intra-cellular accumulation of osmotically active solutes compatible with continued metabolic function (Brown & Simpson 1972). One such compatible osmoregulant is the three-carbon sugar alcohol, glycerol (Borowitzka & Brown 1974). Internal accumulation of glycerol apparently helps confer salinity tolerance, and some microorganisms keep glycerol confined within their cells (Craigie & McLachlan 1964; Borowitzka & Brown 1974), while others continuously leak significant amounts of glycerol into the external medium (Vijalkishore & Karanth 1984; Parekh & Pandey 1985; Grizeau & Navarro 1986; Ahmed & Zidan 1987; León & Galván 1994, 1995; Miyasaka *et al.* 1998; Chow *et al.* 2013). Stimulation of the internal accumulation of glycerol in *Dunaliella salina* was used in early studies on algal energy-carrier production (Ben-Amotz & Avron 1981). In turn, the production of energy carriers like glycerol from photosynthetic microbes has received recent attention because of the potential to continuously and non-destructively collect energy carriers released into the medium that could be used as feedstock for generating biofuels or other high value products (Chow *et al.* 2013).

Current approaches to generating algal biofuels typically involve (1) growing algae to high densities under conditions favorable for growth, followed by (2) subjecting the algae to severe stress that arrests growth, which (3) induces accumulation of energy-rich lipids (oils) that can serve as a feedstock for biodiesel. The growth-arresting stress also typically inhibits algal photosynthesis (Baldisserotto *et al.* 2014; Yang *et al.* 2014), thereby inhibiting further *de novo*

production of energy-rich molecules from atmospheric CO<sub>2</sub>. Siphoning off large amounts of energy-rich products of photosynthesis released by algae into the culture medium could be a more energetically favorable approach to producing biofuels compared to the (1) energy-intensive transportation, pretreatment, and enzymatic breakdown of plant feedstocks or (2) the energy-consuming harvesting of algae, extraction of lipids from those algae, and investment into the regrowth of algal cultures. Moreover, glycerol is a versatile feedstock for the generation of not only biofuels (e.g., ethanol and hydrogen), but also high-value biochemicals (e.g., 1,3-propanediol, 1,2-propanediol, 2,3-butanediol, dihydroxyacetone, propionate, 3-hydroxypropionic acid, succinic acid, citric acid, lactic acid, polyhydroxybutyrate, polyhydroxyalkanoate, phenyl alanine, astaxanthin, rhamnolipids; for reviews, see da Silva *et al.* 2009; Choi *et al.* 2011; Ganesh *et al.* 2012; Metsoviti *et al.* 2012; Yu *et al.* 2012), by non-photosynthetic microbes that readily take up, and utilize, glycerol as their main energy source (Solomon *et al.* 1995; Barbirato & Bories 1997; Menzel *et al.* 1997). To move industrial glycerol-based biofuel production to the scale needed to contribute significantly to transportation fuel demands – without competing for arable land areas suitable for food production – sustainable, large-scale algal glycerol production independent of freshwater resources is needed. See Chapter 5 for more on potential industrial applications.

Furthermore, release of energy carriers from algae may prevent feedback inhibition of photosynthesis and thereby permit higher photosynthetic rates, and thus higher rates of potential feedstock production. Internal accumulation of the products of photosynthesis is known to exert feedback inhibition by repressing major photosynthetic genes (Krapp & Stitt 1995; Adams *et al.* 2013b; Körner 2013; Fatichi *et al.* 2014). Record-high photosynthetic capacities, amounting to a tripling of what had been thought of as the maximal photosynthetic capacity for this species,

were recently reported for the model plant *Arabidopsis* manipulated to possess exceptionally large sugar-exporting leaf veins (Adams *et al.* 2013a; Cohu *et al.* 2013a,b). It was concluded that the maximal capacity of photosynthesis is determined by active adjustments on the part of the organism to balance investment in carbon-fixing enzymes against the finite, rather limited ability to export, utilize, and/or store the products formed in photosynthesis – and that the true ceiling of photosynthesis lies far higher than currently recognized (Adams *et al.* 2013a; Cohu *et al.* 2013b; Demmig-Adams *et al.* 2014a). By stimulating the release of the photosynthetically derived osmoregulant glycerol from microalgae, this fundamental tenet may be tested in a carbon-storage and -export system that is simplified and more easily manipulated compared to that of plants.

The specific hypotheses tested in this chapter are:

- (i) If glycerol acts as a compatible solute, increasing medium salinity will be associated with increased glycerol synthesis. The corresponding null hypothesis is that if glycerol does not act as a compatible solute, medium salinity will have no effect on glycerol synthesis.
- (ii) If salinity and light act synergistically in stimulating glycerol release, there will be significant interaction between salinity and light intensity as well as a significant effect of either parameter on glycerol release. The corresponding null hypothesis is that salinity and light do not act synergistically in stimulating glycerol release.

### **3.3 Materials and methods**

#### *3.3.1 Microalgae and culture maintenance*

The green algal species *Chlamydomonas reinhardtii* (UTEX #2244; freshwater), *C. euryale* (UTEX #2274; marine), and *C. hedleyi* (UTEX # 2848; marine) were obtained from the Culture Collection of Algae (<http://web.biosci.utexas.edu/utex/Search.aspx>). All cultures were grown in axenic conditions; glassware was autoclaved, media were filter-sterilized (Millex-GP filters, Millipore, Billerica, MA, USA), and culture transfers were performed within a laminar flow cabinet (ESCO Airstream, Hatboro, PA, USA). All flask cultures were continuously agitated on orbital shaker tables (to facilitate access to CO<sub>2</sub> and to deter clumping of the algae) under aerobic conditions and maintained at 20°C with continuous illumination by cool white fluorescent lamps (photon flux density, PFD, of 20  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ ) in a growth chamber (E-15, Conviron, Winnipeg, Manitoba, Canada). The freshwater species was maintained in minimal medium for growth (MMG) and the marine species were maintained in modified Okamoto medium (MOM; Miura *et al.* 1986), both buffered with 10 mM tricine and NaOH (pH 7.9) and containing 7.48 mM NH<sub>4</sub>Cl, 1.24 mM KH<sub>2</sub>PO<sub>4</sub>, 1.94 mM K<sub>2</sub>HPO<sub>4</sub>, 0.1 mg L<sup>-1</sup> vitamin B1 and 0.001 mg L<sup>-1</sup> vitamin B12. MMG also contained 0.34 mM CaCl<sub>2</sub>, 0.41 mM MgSO<sub>4</sub>·7H<sub>2</sub>O, and 1 mL L<sup>-1</sup> Hutner's trace metals (Hutner *et al.* 1950). Prior to experiments, the marine species were maintained in one of three NaCl concentrations (86 mM, 308 mM, or 513 mM). See Appendix A for a complete list of media components.

### 3.3.2 Low-density cultures of marine species

In an initial growth experiment, the two marine species were grown at low culture density in 1 L Roux bottles (Pyrex, Czech Republic) with 0.5 L culture volume (MOM with 616 mM NaCl). Roux bottles were fitted with two-hole rubber stoppers, through which nine-inch Pasteur pipettes were fed, with gas sterilizing filters (Aervent 50, Millipore, Billerica, MA, USA)

secured to both the inlets and outlets. Media used in Roux bottles, and in subsequent Roux bottle pre-culturing and test tube experimental phases, contained 60 mM  $\text{NaHCO}_3$  as a buffer (starting pH of 7.9) instead of tricine and NaOH. Each Roux bottle was inoculated with 25 mL of the algal flask cultures with 513 mM NaCl. Cultures were continuously bubbled with 2% (v/v)  $\text{CO}_2$  in air (to overcome any possible  $\text{CO}_2$  substrate limitations to photosynthesis), stirred (with magnetic stir bars at 100 revolutions per minute), and grown under continuous illumination of  $100 \mu\text{mol photons m}^{-2} \text{s}^{-1}$  (at the bottle surface, provided by panels of white LEDs) at  $20^\circ\text{C}$ . Samples (for, e.g., cell counts and glycerol essays) were extracted through a side tube that remained sealed at all other times. Culture samples for glycerol concentration analysis were centrifuged (1,476 g for 10 minutes), after which the supernatant was decanted and frozen at  $-20^\circ\text{C}$  for subsequent glycerol analysis.

### 3.3.3 Pre-culturing in Roux bottles for experiments in 50 mL tubes

Prior to the experiments with high-density cultures described in section 3.3.4, each species was pre-cultured at a pH of 7.9 in 1 L Roux bottles (prepared as described above) to obtain sufficient quantities of algae. *Chlamydomonas reinhardtii* was pre-cultured in MMG without salt for all experiments. The two marine species were pre-cultured in MOM medium at various salt concentrations (17, 103, 308, and 616 mM NaCl) after step-wise transfer from seawater salinity to the respective lower NaCl concentrations to avoid rupturing cells. 50 mL of flask-culture algae were added to each Roux bottle, and bottles were continuously stirred, bubbled with air containing 5% (v/v)  $\text{CO}_2$ , illuminated with cool white fluorescent lamps (continuous PFD of  $150 \mu\text{mol photons m}^{-2} \text{s}^{-1}$  at the Roux bottle surface), and maintained at  $20^\circ\text{C}$ . After approximately five days of growth, culture density (as optical density at 750 nm,



OD<sub>750</sub>) was determined with a Beckman DU-64 spectrophotometer (Brea, CA, USA). To achieve a target OD<sub>750</sub> of 2.5 for the beginning of the experiments described in section 3.3.4, an appropriate culture volume from each Roux bottle was gently centrifuged (1,377 g for 2 minutes) using a Beckman J-21B centrifuge (Brea, CA, USA) to remove the medium, and then re-suspended with the appropriate volume of sterilized experimental medium (MMG with or without salt for *C. reinhardtii* and MOM with various salt concentrations for the two marine species).

#### 3.3.4 *Experimental growth of high-density cultures in 50 mL test tubes*

After re-suspension, 44 mL of culture (pH of 7.9) was added to 50 mL test tubes with two-hole rubber stoppers fitted as described above for the Roux bottles. Throughout the experiments, cultures were stirred (magnetic stir flea at 100 revolutions per minute) to keep algae from settling at the bottom, with the majority of mixing accomplished by continuous bubbling with air containing 5% CO<sub>2</sub>. The cultures were maintained at 20°C; samples were extracted from the side-tube for various assays.

#### 3.3.5 *Culture density*

Culture density was determined as cell counts per volume of medium (using a microscope and hemocytometer) and/or as OD in the spectrophotometer. For the initial Roux-bottle experiment, OD was measured at 660 nm. For all subsequent experiments in 50 mL tubes, OD was measured at 750 nm as a better estimate of actual cell number not confounded by chlorophyll content. A standard curve was established for cell count versus OD<sub>750</sub> for each species.

### 3.3.6 Chlorophyll extraction and quantification

Chlorophyll was extracted at 4°C using dimethylformamide (DMF) and quantified for each culture following the protocol of Porra *et al.* (1989).

### 3.3.7 Photosynthetic oxygen evolution

Light- and CO<sub>2</sub>-saturated rates of photosynthetic O<sub>2</sub> evolution were determined in 1.2 mL volumes of medium containing 25 µg chlorophyll per mL and 60 mM NaHCO<sub>3</sub> using a Hansatech liquid phase oxygen electrode (DW2/2; Norfolk, England, UK) maintained at 20°C. The suspension was exposed to saturating light (PFD of 1,000 µmol photons m<sup>-2</sup> s<sup>-1</sup> at the surface of the chamber) until O<sub>2</sub> evolution rate was stable for several minutes, after which the light was turned off and respiration rates determined as the rate of O<sub>2</sub> consumption.

### 3.3.8 Glycerol analysis

100 µL of thawed supernatant samples were diluted 1:10 with 900 µL of deionized water in 2 mL microcentrifuge tubes, thoroughly mixed, and 4 µL of each sample was placed in wells on a flat-bottom 96-well plate. The 96-well plate was analyzed in a Tecan Safire 2 plate reader (Männedorf, Switzerland). The assay was run and glycerol concentrations were determined according to the colorimetric protocol of the BioVision Free Glycerol Assay Kit (San Francisco, CA, USA).

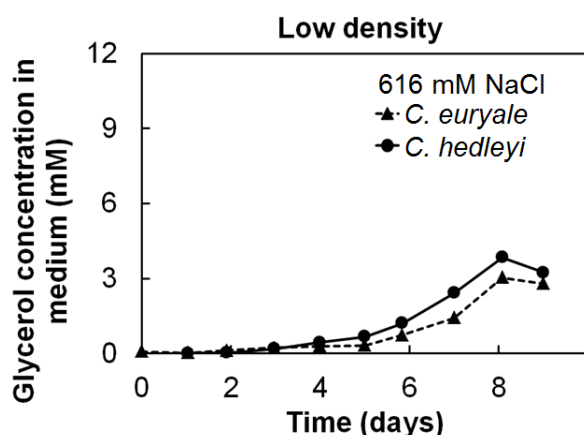
### 3.3.9 Statistical analyses

Differences among mean values were determined through ANOVA coupled with Tukey-Kramer tests using JMP statistical software (SAS Institute, Cary, North Carolina).

### 3.4 Results

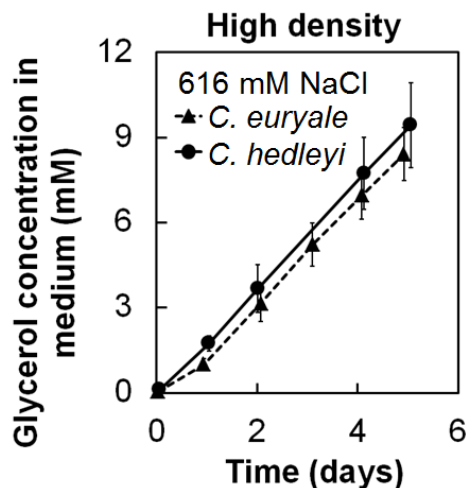
#### 3.4.1 Glycerol release into the external medium in marine species of *Chlamydomonas*

Two marine *Chlamydomonas* species, *C. euryale* and *C. hedleyi*, were found to release glycerol into the medium when maintained at a NaCl concentration (616 mM) approximately corresponding to seawater levels of salinity (Fig. 3.1, 3.2). The two species exhibited very similar responses (Fig. 3.1, 3.2); while glycerol levels in the external medium increased gradually over time in low density cultures (Fig. 3.1), dense cultures (pre-cultured in larger containers at the same salinity) exhibited pronounced, near-linear glycerol accumulation in the external medium over a period of five days (Fig. 3.2). The latter experimental protocol (assessing glycerol accumulation in the medium over five days in dense cultures) was used for all subsequent experiments.



**Figure 3.1:** Time course of glycerol release to the external medium by low density cultures of the marine *Chlamydomonas* species at a salinity level (616 mM NaCl) similar to that of seawater under  $100 \mu\text{mol photons m}^{-2} \text{s}^{-1}$  at  $20^\circ\text{C}$ . Cell density progressed from ODs of 0.07 to 4.20 and

0.06 to 2.12 for *C. euryale* (filled circles and line) and *C. hedleyi* (filled triangles and dashed line), respectively. Mean values  $\pm$  standard deviation ( $n = 3$ ) shown.

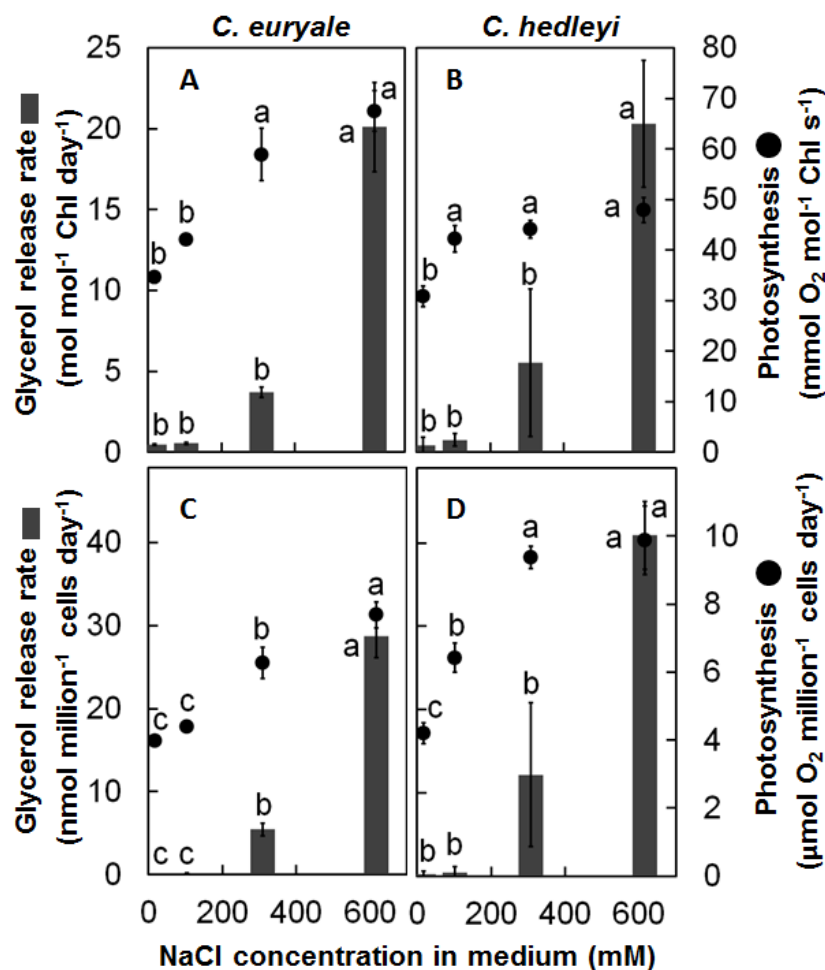


**Figure 3.2:** Time course of glycerol release to the external medium by high density cultures of the marine *Chlamydomonas* species at a salinity level (616 mM NaCl) similar to that of seawater under  $200 \mu\text{mol photons m}^{-2} \text{s}^{-1}$  at  $20^\circ\text{C}$ . Cell density progressed from ODs of 2.31 to 8.01 and 2.21 to 5.82 for *C. euryale* (filled circles and line) and *C. hedleyi* (filled triangles and dashed line), respectively. Mean values  $\pm$  standard deviation ( $n = 3$ ) shown.

The rate of glycerol release into the external medium depended on medium salinity in both marine species (Fig. 3.3 A-D), irrespective of whether glycerol release was expressed relative to the chlorophyll content of the culture (Fig. 3.3 A,B) or relative to the number of cells (Fig. 3.3 C,D). While glycerol release rates were negligible at very low salt levels (17 mM) in the medium, seawater levels of NaCl triggered glycerol release into the medium (Fig. 3.3). In addition to the rates of glycerol release into the medium, Figure 3.3 also shows corresponding photosynthetic capacities of the cultures as dependent on the salinity of the culture medium. In both marine species, maximal rates of photosynthesis increased with increasing salinity on either a chlorophyll (Fig. 3.3 A,B) or cell basis (Fig. 3.3 C,D). Maximal photosynthesis rates

approximately doubled from very low salinities (17 mM) to salinity levels (616 mM NaCl)

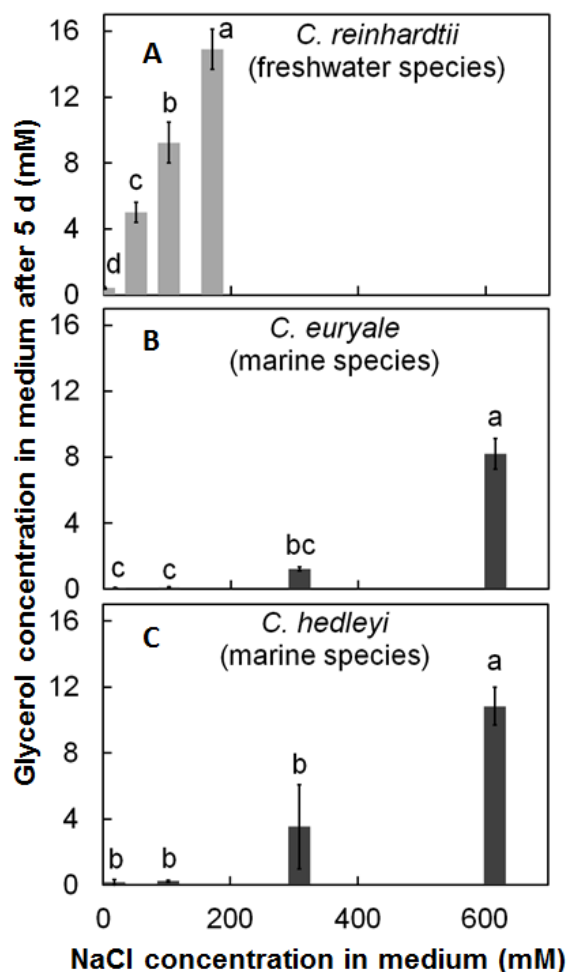
similar to those of seawater.



**Figure 3.3:** Glycerol release rate and photosynthetic capacity on either a chlorophyll basis (A,B) or a cell basis (C,D) at four different salinity levels for the marine species *C. euryale* (A,C) and *C. hedleyi* (B,D). Cumulative glycerol release into the external medium over five days was expressed as glycerol release rate per day; light- and CO<sub>2</sub>-saturated photosynthetic capacity (measured from cell-suspension aliquots corresponding to 25 μg of chlorophyll in an oxygen electrode) was ascertained 12 hours after the start of the experiment. 44 mL cultures were maintained over the five-day experiment under a PFD of 200 μmol photons m<sup>-2</sup> s<sup>-1</sup> at 20°C and continuously bubbled with 5% CO<sub>2</sub> in air. Mean values ± standard deviation (n = 3) shown, with significant differences among the means indicated by different lower case letters, i.e., those means sharing a common letter are not significantly different from each other.

### 3.4.2 Comparison of the salinity dependence of glycerol release into the external medium in freshwater versus marine species of *Chlamydomonas*

Figure 3.4 shows a comparison of glycerol release into the medium as dependent on medium salinity for the freshwater species *Chlamydomonas reinhardtii* versus the two marine species, *C. euryale* and *C. hedleyi*. Stimulation of glycerol release into the medium was seen in the freshwater species (Fig. 3.4 A) at much lower salinities than in the two marine species (Fig. 3.4 B,C). *Chlamydomonas reinhardtii* did not exhibit positive growth at salinities above 200 mM NaCl (see Fig. 3.7). Maximal levels of glycerol release into the medium were similarly high or higher in the freshwater species at about one-third seawater salinity compared to glycerol release at approximate seawater salinity in the two marine species (Fig. 3.4 A-C).



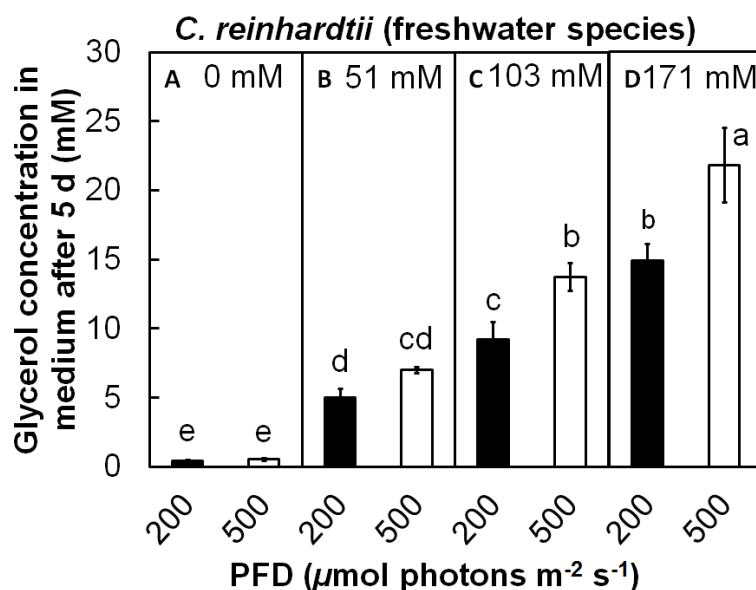
**Figure 3.4:** Final glycerol concentration in the culture medium after five days for the freshwater species *C. reinhardtii* (A) and for the marine species *C. euryale* (B) and *C. hedleyi* (C) over a range of salinities (from very low levels to levels similar to seawater salinity). Over the course of the experiment, 44 mL cultures were exposed to a PFD of  $200 \mu\text{mol photons m}^{-2} \text{s}^{-1}$  at  $20^\circ\text{C}$  and continuously bubbled with 5%  $\text{CO}_2$  in air. Mean values  $\pm$  standard deviation ( $n = 3$ ) shown, with significant differences among the means indicated by different lower case letters, i.e., those means sharing a common letter are not significantly different from each other.

### 3.4.3 Interaction of light intensity and salinity

Synergies between medium salinity and growth PFD are explored in Figures 3.5 and 3.6.

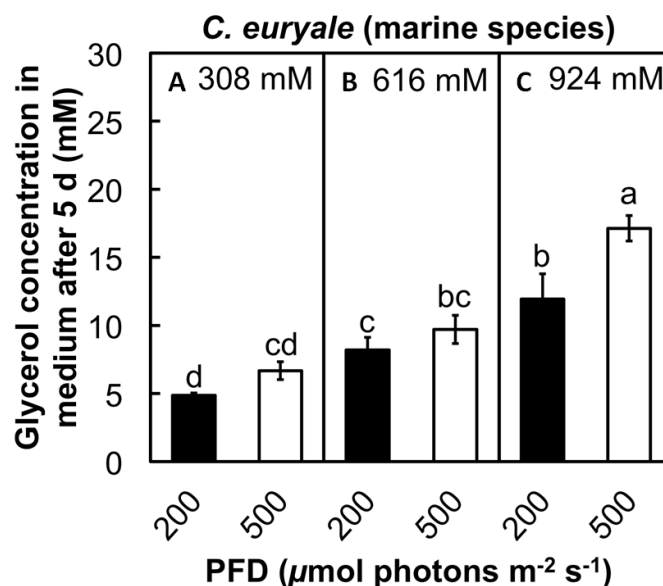
Glycerol release into the medium was stimulated not only by higher salinity but also by higher growth PFDs in the freshwater species *C. reinhardtii* (Fig. 3.5 A-D) as well as the marine species *C. euryale* (Fig. 3.6 A-C). Specifically, at a given salinity, glycerol release was stimulated by a

higher growth PFD (Figs. 3.5 and 3.6). Likewise, at a given growth PFD, glycerol release was stimulated by higher salinity (Figs. 3.5 and 3.6). Statistical analysis revealed a significant effect of salinity on glycerol release, a significant effect of light intensity on glycerol release, and a significant interaction between salinity and light (as evidence for a synergistic effect). These effects were verified using least squares multiple regression analysis, yielding p-values of less than 0.05 for salinity, light intensity, and the interaction between salinity and light for both the freshwater and marine species.



**Figure 3.5:** Glycerol concentration in the external medium after five days for the freshwater species *C. reinhardtii* cultured under two different PFDs each (200 [solid columns] and 500 [open columns]  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ ) over a range of different salinities (A 0 mM, B 51 mM, C 103 mM, and D 171 mM). 44 mL cultures were maintained at 20°C and continuously bubbled with 5% CO<sub>2</sub> in air. Mean values  $\pm$  standard deviation ( $n = 3$ ) shown, with significant differences among the means indicated by different lower case letters, i.e., those means sharing a common letter are not significantly different from each other.

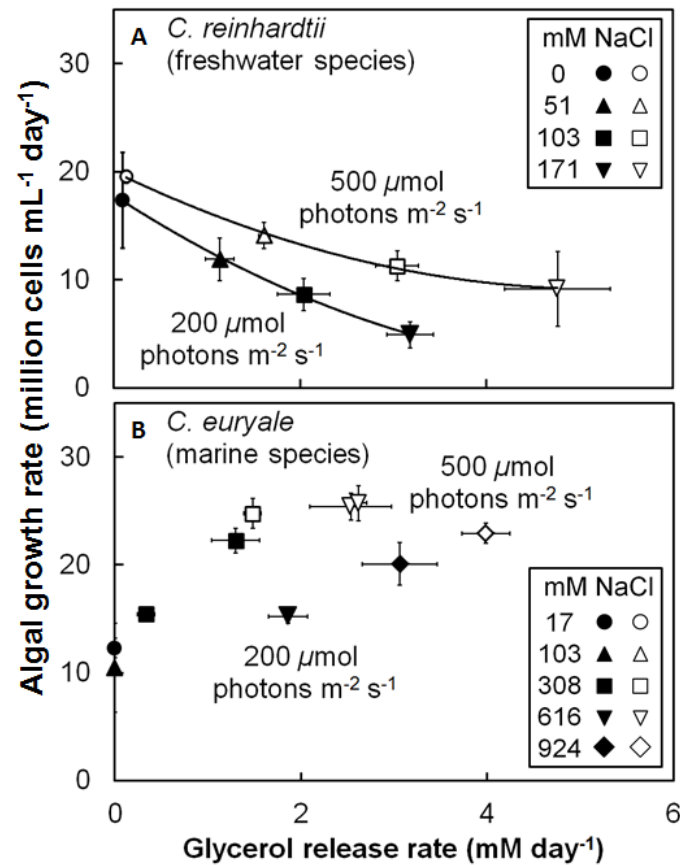




**Figure 3.6:** Glycerol concentration in the external medium after five days for the marine species *C. euryale* cultured under two different PFDs each (200 [solid columns] and 500 [open columns]  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ ) over a range of salinities (A 308 mM, B 616 mM, and C 924 mM). 44 mL cultures were maintained at 20°C and continuously bubbled with 5%  $\text{CO}_2$  in air. Mean values  $\pm$  standard deviation ( $n = 3$ ) shown, with significant differences among the means indicated by different lower case letters, i.e., those means sharing a common letter are not significantly different from each other.

Figure 3.7 depicts the relationship between algal growth rates and glycerol release rates in the freshwater species *C. reinhardtii* (Fig. 3.7 A) and the marine species *C. euryale* (Fig. 3.7 B). The relationship between algal growth rates and glycerol release rates was affected by growth PFD in that a higher growth PFD supported a higher algal growth rate at a given salinity for both the freshwater and the marine species. The relationship between algal growth rates and glycerol release rates differed between the freshwater and the marine species *C. euryale* (with similar results obtained for *C. hedleyi* versus *C. euryale*; data not shown); while the freshwater species exhibited decreasing growth rates at increasing glycerol release rates, the growth rates of the marine species were independent of salinity at the higher light intensity. At the lower light intensity, growth rates under the three highest salinities were higher than those under the two

lowest salinity levels in the marine species (Fig. 3.7 B). Pre-acclimation of *C. reinhardtii* to higher salinities (51 mM NaCl) during the pre-culturing phase in the Roux bottles had no effect on algal growth rates in response to 51, 103, or 171 mM NaCl under continuous illumination with either 200 or 500  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$  (data not shown).



**Figure 3.7:** Relationship between algal growth rate and glycerol release rate into the external medium (over the course of five days) for the freshwater species *C. reinhardtii* and the marine species *C. euryale* at two different PFDs each (200 and 500  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ ) over a range of salinities (see key to symbols in each panel for specific levels of NaCl). Solid symbols within the dashed perimeter = 200  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$  and open symbols within the solid perimeter = 500  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ . 44 mL cultures were maintained at 20°C and continuously bubbled with 5%  $\text{CO}_2$  in air. Mean values  $\pm$  standard deviation (n = 3) shown.

### 3.5 Discussion

#### 3.5.1 *Species-dependent adaptations in apparent glycerol metabolism and glycerol release into the external medium*

It is generally accepted that glycerol is produced as an internal osmoregulant to balance lower osmotic potentials under saline conditions in the medium (Craigie & McLachlan 1964; Brown & Simpson 1972; Borowitzka & Brown 1974). Continuous release of photosynthetically produced glycerol into the external medium was reported for the freshwater green alga *Chlamydomonas reinhardtii* (Léon & Galván 1994, 1995) in response to moderate salinity levels (up to salinities of about one-third seawater strength). Subsequent studies reported glycerol release into the external medium for a marine *Chlamydomonas* species (the W-80 strain not identified to the species level; Miyasaka *et al.* 1998) as well as in a single *Dunaliella* species. *Dunaliella tertiolecta* continuously released glycerol into the medium at very high salinities of 8 X full seawater (4 M NaCl; Grizeau & Navarro 1986 and references cited therein; see also Chow *et al.* 2013), while another species, *D. salina*, accumulated glycerol internally but did not release glycerol into the external medium (Ben-Amotz & Avron 1981; Borowitzka & Borowitzka 1988). Other freshwater green algae tested, i.e., *Chlorella vulgaris* and *Monoraphidium braunii*, did not release glycerol into the external medium (Léon & Galván 1995). The present study is the first to compare glycerol release into the medium for freshwater and marine species of the same genus over a range of salinities under otherwise identical conditions and to report (i) triggering of glycerol release by higher salinities as well as (ii) a greater propensity to continue growth at equivalent levels of glycerol release into the medium in marine versus freshwater species.

The results shown in Figures 3.4, 3.5, and 3.6 demonstrate a clear relationship between glycerol release (synthesis) and salinity of the external medium, supporting the hypothesis that glycerol acts as a compatible solute in both the freshwater and marine species of *Chlamydomonas*.

### 3.5.2 Why and how may microbes release glycerol into the medium?

Why would an organism leak energy carriers it has produced through the energy-driven process of photosynthesis? Is it simply an inability to prevent the loss of glycerol into the external medium? Release of glycerol from photosynthetic microbes to a non-photosynthetic host is a common mechanism for energy transfer in symbioses between a photosynthetic symbiont and a host providing shelter, nutrients, and CO<sub>2</sub> (Muscatine 1967; Venn *et al.* 2008; Lee *et al.* 2010; Suescún-Bolívar *et al.* 2012). For instance, foraminifera are often found in symbiosis with an alga, but are heterotrophic in the absence of photosynthetic symbionts. *Chlamydomonas hedleyi* used in the present study was, in fact, isolated from a symbiosis with the non-photosynthetic foraminifer host *Archaias angulatus* (Lee & Zucker 1969; Lee *et al.* 1974), and it may be the release of glycerol in full-strength seawater salinities that makes *C. hedleyi* (and possibly other marine species of *Chlamydomonas*) attractive photosynthetic symbionts to non-photosynthetic hosts. The majority of newly fixed carbon can be transferred from the alga to the host in these symbioses – as much as 90% of the energy-rich products of photosynthesis from the dinoflagellate *Symbiodinium* can be transferred to its coral host, and glycerol release from *Symbiodinium* is furthermore stimulated by increased levels of osmotica in the culture medium (Suescún-Bolívar *et al.* 2012). In addition, signals from the non-photosynthetic host can inhibit the storage of starch (used as a source of energy overnight) on the

part of the photosynthetic symbiont and this, coupled with the stimulation of glycerol release, provides a possible mechanism for inhibiting growth (by arresting cell division) of the symbiotic alga (Grant *et al.* 2006).

The symbiotic algae do not grow or divide, are long-lived, and supply a continuous stream of energy carriers to their host (Davies 1984; Lee 2006; Lee *et al.* 2010). The greater rates of glycerol release in dense compared to dilute culture observed by us may be related to the fact that, when participating in a symbiotic relationship, the algae are closely packed together as well as in physical contact with their non-photosynthetic host (akin to quorum sensing; see Teplitski *et al.* 2004; Ianora 2006; Natrah *et al.* 2011; Imai 2012). Furthermore, the propensity for *Chlamydomonas* to release glycerol appears to be a trait common to members of this genus regardless of whether or not particular species partner with a host in a symbiotic relationship. Future studies should examine these traits more broadly across additional species, as well as consider differences in the evolutionary history of symbiotic relationships for different genera and species of algae with contrasting features of glycerol release.

What is the mechanism for the movement of glycerol across the cellular membrane of a microbe? Research into the same phenomenon in yeast likely provides an answer. Osmotolerant non-photosynthetic yeast continuously released glycerol into the external medium when supplied with glucose under certain conditions (Vijalkishore & Karanth 1984; Parekh & Pandey 1985). Transport proteins, referred to as aquaporins or aquaglyceroporins in animals and major intrinsic proteins (MIPs) in plants and algae, function in water and glycerol transport across membranes (Agre *et al.* 1998; Anderberg *et al.* 2011). These MIPs are related to the glycerol transporter, FPS1, in yeast (Luyten *et al.* 1995), and yeast mutants lacking FPS1 accumulated more intracellular glycerol, while FPS1 overexpression enhanced glycerol production and release into

the external medium (Luyten *et al.* 1995). Mutants affecting portions of FPS1 involved in regulation of the channel also exhibit unregulated glycerol efflux (Hedfalk *et al.* 2004). Moreover, a membrane transporter of the MIP family present in *C. reinhardtii* was shown to function as a glycerol transporter in transgenic yeast (Anderca *et al.* 2004). Aquaglyceroporins are thus attractive candidates for the mechanism of glycerol release into the medium, and should be targeted by future studies. Our present findings suggest that the numbers, properties, and/or regulation of such aquaglyceroporins may (1) be subject to salinity acclimation and (2) vary between freshwater and marine species as well as between taxa with and without symbiosis-forming members.

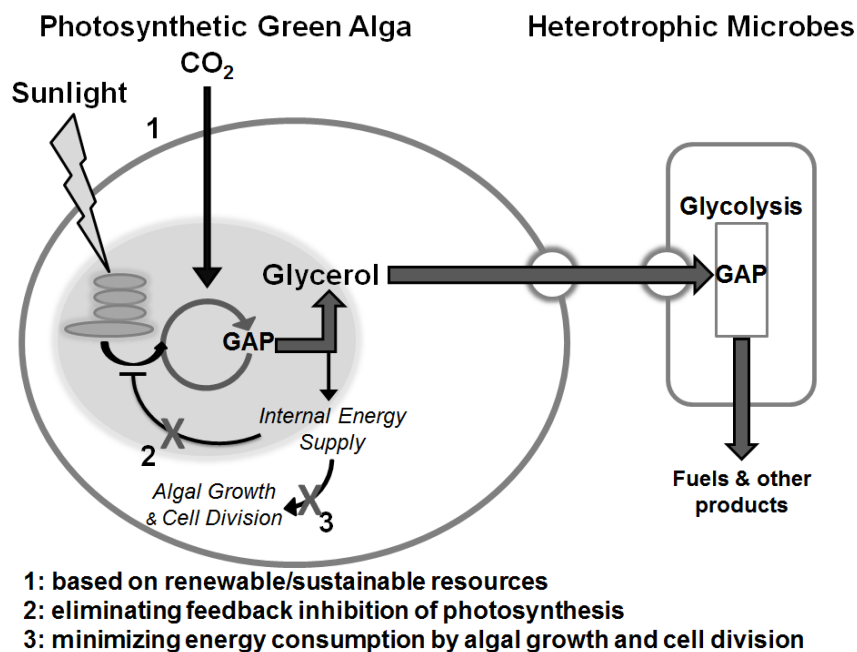
### 3.5.3 *Relationship between glycerol release, maximal photosynthetic capacity, and light availability*

We have shown that glycerol release into the medium by *Chlamydomonas* is stimulated not only by greater salinity but also by greater growth light intensity, with effects that are more than additive. These findings support the hypothesis that growth light intensity and salinity act synergistically in stimulating glycerol release. Higher growth PFD stimulated glycerol release into the medium and also led to higher growth rates at a given salinity, suggesting that higher growth PFDs stimulate internal photoproduction of glycerol in photosynthesis (increased light availability increases the availability of NADPH for CO<sub>2</sub> fixation and glycerol production, which results from the NADPH-dependent conversion of dihydroxyacetone phosphate to glycerol). This should, in turn, provide a greater pool of energy carriers for algal growth. It is therefore possible that glycerol is released or leaked into the external medium as a side effect of increased internal glycerol biosynthesis under more saline conditions.

At a constant light intensity, increasing salinity increased the maximal rate of photosynthesis. We thus propose that glycerol synthesis and release into the external medium prevents build-up of NADPH by continuously consuming NADPH in glycerol production, thereby releasing any feedback inhibition on photosynthesis that would be caused by accumulation of NADPH and energy-carriers. This draining of energy-carriers should stimulate the capacity of photosynthesis, analogous to the greater rates of photosynthesis observed in plant leaves with a greater capacity to export sugars (Adams *et al.* 2013a, 2014; Cohu *et al.* 2013b, 2014; Muller *et al.* 2014a,b). The results presented here indicate that the maximal rate of photosynthesis and algal tolerance of high light intensities are stimulated by conditions leading to glycerol release. Similarly, Suescún-Bolívar *et al.* (2012) found that sudden osmotic stress triggered not only glycerol release in symbiotic dinoflagellates from the genus *Symbiodinium* but also increased expression (increased levels of mRNA) of ribulose biphosphate carboxylase and glycerol 3-phosphate dehydrogenase. Moreover, Chow *et al.* (2013) likewise found that glycerol release into the medium could serve as an apparent sink for photosynthesis, allowing high rates of photosynthesis to be maintained even when *Dunaliella tertiolecta* was prevented from growing. The freshwater species in the present study exhibited reduced growth in the presence of salt in the medium, indicating either a direct inhibition of processes underlying growth or competition between allocation of energy carriers formed in photosynthesis to glycerol synthesis and release versus the support of algal growth. In contrast, the marine species did not show a reduction of growth under salinities up to and just above the salinity level of seawater, and are thus presumably able to retain more of the synthesized glycerol internally and/or to synthesize more glycerol. Glycerol accumulation versus release is analyzed in Chapter 4.

### 3.5.4 Implications for algal production of energy carriers

Triggering release of energy carriers into the external environment, as opposed to internally accumulating energy carriers like starch or oils, promises the advantage of allowing the algae to continuously convert high levels of solar energy into energy carriers without experiencing feedback inhibition (Fig. 3.8). Triggering export of photosynthetically produced energy carriers from single-celled algae and other photosynthetic microbes may thus be a feasible approach to enhance photosynthetic productivity and increase the viability of algae as continuous generators of biofuel feedstocks and other valuable chemical products. Such an approach would negate the need to harvest, digest the diverse biomass components, and regrow algae, and, by working with long-lived mature cultures, minimize the nutrient requirements for culture growth. The industrial application of these findings is discussed further in Chapter 5.



**Figure 3.8:** Schematic depiction of a photosynthetic alga (of the genus *Chlamydomonas*) that converts (1) solar energy and atmospheric CO<sub>2</sub> into a stream of continuously released glycerol feedstock that can support heterotrophic microbes in the production of biofuels and other products. Continuous release of newly fixed reduced-carbon-compounds (energy carriers) prevents feedback inhibition of photosynthesis (2); glycerol release diverts energy from algal



growth and cell division to glycerol production (3). GAP=glyceraldehyde-3-phosphate, an intermediate of the CO<sub>2</sub>-fixing Calvin-Benson cycle of photosynthesis as well as of glycolysis. GAP can be directly converted to glycerol and *vice versa*.

Glycerol has been shown to be a versatile feedstock, readily taken up and utilized as the main energy source (after entering glycolysis; see Fig. 3.8) by a wide variety of heterotrophic microbes requiring an external energy supply (Solomon *et al.* 1995; Barbirato & Bories 1997; Menzel *et al.* 1997). Glycerol can serve as the sole energy source for non-photosynthetic microbes producing ethanol (Choi *et al.* 2011; Metsoviti *et al.* 2012; Yu *et al.* 2012) or other energy carriers or biochemicals (da Silva *et al.* 2009; Ganesh *et al.* 2012) as well as for hydrogen-producing cyanobacteria (Luo & Mitsui 1994; Bandyopadhyay *et al.* 2010; Min & Sherman 2010). Recent interest in using glycerol as a feedstock for microbial energy has been driven by glycerol availability. However, current post-harvest production of glycerol as a minor byproduct of biodiesel production from oil-accumulating crops and algae is subject to the principal limitations of low overall photosynthetic efficiency, competition with food production for arable land and water resources, and high nutrient-input costs (Chow *et al.* 2013). Sustainable, large-scale algal glycerol production independent of freshwater resources would be needed to contribute significantly to transportation fuel demands. Although glycerol release was stimulated by the right combination of environmental triggers in all three *Chlamydomonas* species examined here, the freshwater species *Chlamydomonas reinhardtii* was stimulated to release similar levels of glycerol as the marine species at lower salinities, and putative competition by algal growth for energy carriers was eliminated more easily in the freshwater species. The use of glycerol as a sustainable bioenergy precursor is elaborated upon further in Chapter 5.

## CHAPTER 4

### ALGAL GLYCEROL ACCUMULATION AND RELEASE AS A SINK FOR PHOTOSYNTHETIC ELECTRON TRANSPORT

#### 4.1 Abstract

In response to external salinity, the freshwater species *Chlamydomonas reinhardtii* released considerably more glycerol to the external environment compared to the marine species *C. euryale*, suggesting that limitations to the ability to internally accumulate and retain glycerol as an osmoregulatory compound may be involved in the inability of the freshwater species to show positive growth at salinities exceeding 0.2 M NaCl. While the freshwater species exhibited no downregulation of photosynthesis between 0 and 0.2 M NaCl, the marine species exhibited significant upregulation of photosynthetic oxygen evolution between 0.2 and 2.0 M NaCl in the medium, indicating that internal glycerol accumulation, unlike sugar accumulation, does not interact with sugar-signaling networks that induce photosynthetic downregulation upon internal photosynthate accumulation. Continuous release of glycerol into the medium and/or internal glycerol accumulation followed by periodic glycerol release via hypo-osmotic shock are attractive options for continuous production of biomaterials and bioenergy from renewable sources in algae with high rates of solar-energy conversion to photosynthetically produced energy carriers.

## 4.2 Introduction

The overall efficiency of solar-energy conversion into energy-rich biomass by living organisms is relatively low (Blankenship *et al.* 2011; Ort *et al.* 2015), i.e., plant or algal biomass contains but a small fraction of the solar energy collected by the photosynthetic organism over its life time. The vast majority of this harvested energy is expended to fuel the myriad of metabolic reactions carried out by the organism for its own growth, cell division, and reproduction. However, the efficiency of solar-energy conversion by photosynthesizing organisms into the initial energy-rich chemical products of photosynthesis can be quite high (Demmig-Adams *et al.* 1996, 2012, 2014b). Consequently, withdrawing energy carriers from photosynthetic organisms before the energy is utilized for the organisms' own growth and development could represent a promising approach to increasing the overall efficiency and productivity of energy-carrier production by living organisms.

Examples of such an approach can be found in the symbioses between non-photosynthetic host organisms and photosynthetic microbes that live as symbionts within, or in association with, a host. Such photosynthetic symbionts expend very little energy for cell division and growth, instead releasing a large fraction of the energy-rich products of photosynthesis to the host in exchange for CO<sub>2</sub> and nutrients (Muscatine 1967; Davies 1984; Lee 2006; Venn *et al.* 2008; Lee *et al.* 2010; Suescún-Bolívar *et al.* 2012). A major energy carrier released by photosynthetic symbionts for utilization by their host is glycerol produced directly in photosynthesis; glycerol is readily used as the main energy source by a host of non-photosynthetic organisms (Solomon *et al.* 1995; Barbirato & Bories 1997; Menzel *et al.* 1997). Attractive aspects of copying this process in a nature-inspired approach include (i) the

circumvention of the need to harvest, extract, and regrow algae by virtue of simply harvesting energy carriers released by the algae and (ii) the prospect of driving up photosynthetic rates by circumventing feedback inhibition acting on photosynthesis.

Photosynthetic organisms' maximal capacity for photosynthesis is controlled by the organisms' demand of energy carriers produced by photosynthesis (Körner 2013; Fatichi *et al.* 2014). Accumulation of sugars in leaves or algae results in repression of multiple photosynthetic genes, which lowers (downregulates) photosynthetic capacity (Krapp & Stitt 1996). Conversely, efficient sugar removal from the site of photosynthesis leads to upregulation of photosynthetic genes and an increased maximal capacity for photosynthesis (for plants, see Körner 2013; Fatichi *et al.* 2014; for algae, see Chow *et al.* 2013). This phenomenon is known as source-sink regulation, with photosynthesis as the energy source and processes or sites that contribute to withdrawing, storing, and/or consuming sugars produced in photosynthesis as sinks for photosynthate (Koch 1996).

We here study the production of glycerol by green algae of the genus *Chlamydomonas* that is strongly stimulated by salinity in the external medium (León & Galván 1994, 1995; Miyasaka *et al.* 1998; Burch *et al.* 2015). Glycerol accumulation within the alga functions in osmotic adjustment, lowering the internal osmotic potential and thereby preventing excessive water loss from the alga to the saline external medium (Borowitzka & Brown 1974). We will test the hypothesis that if glycerol accumulation does not cause feedback inhibition of photosynthesis, increased intracellular glycerol will not be associated with decreased rates of photosynthesis. This postulate is based on the notion that conversion of photosynthetically produced triose dihydroxyacetone phosphate to glycerol (via glycerol-phosphate dehydrogenase) produces an osmoregulant and energy carrier (glycerol) that, unlike glucose, is not perceived as

part of the pool of photosynthetic products (sugars) detected by the signaling network that exerts feedback on photosynthesis (Sheen 2014). We predict that maximal capacity of photosynthetic electron transport is positively correlated with internal glycerol accumulation in the algae and/or glycerol release from the algae into the external medium. The corresponding null hypothesis tested is that if glycerol does cause feedback inhibition of photosynthesis, increased intracellular glycerol will lead to decreased rates of photosynthesis.

In addition to testing glycerol's role in feedback inhibition of photosynthesis, we will test the hypothesis that if glycerol acts as a sink for photosynthetic energy, glycerol production will be associated with similar or increased rates photosynthesis, regardless of growth rate. Thus, if growth rates decrease yet photosynthesis rates remain the same under conditions stimulating glycerol synthesis, it is likely that glycerol is acting as a sink for photosynthetic energy. Additionally, if growth remains unchanged yet photosynthesis rates increase under conditions stimulating glycerol synthesis, glycerol could be acting as a photosynthetic sink. The corresponding null hypothesis is that glycerol does not act as a sink for photosynthetic energy, so glycerol production will not be associated with similar or increased rates of photosynthesis, regardless of growth.

Finally, we compare and contrast the responses of a freshwater versus a marine species and test the hypothesis that the proportion of intracellular versus released glycerol determines a species' salt tolerance. If so, the marine species will have a higher proportion of intracellular versus released glycerol than the freshwater. The corresponding null hypothesis is that accumulated versus released glycerol has no effect on a species salt tolerance, so both the freshwater and marine species will exhibit similar proportions of accumulated versus released glycerol.

We place these results into the context of available approaches to continuously siphon off released glycerol versus non-destructive removal of internally accumulated glycerol for industrial applications. Chapter 5 further develops the industrial applications of the findings of this chapter.

### 4.3 Materials and methods

#### 4.3.1 *Species*

The freshwater species *Chlamydomonas reinhardtii* (UTEX #2244) and the marine species *Chlamydomonas euryale* (UTEX #2274) were obtained from the Culture Collection of Algae at the University of Texas, Austin (<https://utex.org>). Culture maintenance and pre-culturing in Roux bottles was performed as described in Burch *et al.* (2015), except that the marine species was maintained in flasks and pre-cultured in Roux bottles at 0.5 M NaCl.

#### 4.3.2 *Experimental conditions and sampling protocol*

After pre-culturing in Roux bottles, 30 mL culture volumes were standardized to an Optical Density at 750 nm ( $OD_{750}$ ) of 2.25 and transferred to 50 mL test tubes. Test tubes and growing conditions were as described in Burch *et al.* (2015), with a continuous stream of 5% CO<sub>2</sub> (in ambient air) delivered to cultures and an incident light intensity at the surface of the test tubes of either 200 or 500  $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ . Experimental media for *C. reinhardtii* contained 0, 0.1, or 0.2 M NaCl, while media for *C. euryale* contained 0.2, 0.5, or 2 M NaCl. Cultures were grown in test tubes for approximately 1.5 days for acclimation to the experimental conditions, and subsequently diluted (by visual assessment) to a similar greenness as initial

cultures prior to the 1.5 days of growth. Thirty mL of these diluted cultures were transferred to sterile 50 mL centrifuge bottles, gently centrifuged (2 mins, 706 g), and pellets resuspended in 30 mL of fresh medium and returned to test tubes and experimental conditions for the duration of the subsequent 24-h experiment. Samples for OD<sub>750</sub>, cell counts, chlorophyll, and intracellular and released glycerol were collected at 0 and 24 hours of the experimental exposure of diluted cultures in test tubes. Samples for determination of rates of CO<sub>2</sub>- and light-saturated photosynthetic oxygen evolution and respiration were collected after 24 hours. Samples for the quantification of intracellular glycerol levels were prepared from algal pellets using centrifugation, rinsing, and boiling protocols described by Chow *et al.* (2013). Supernatants were frozen for subsequent analysis of glycerol released into the medium. Samples were processed and analyzed as described in Burch *et al.* (2015) for OD<sub>750</sub>, cell count, chlorophyll, and rates of photosynthetic oxygen evolution and respiratory oxygen uptake.

#### 4.3.3 *In-situ chlorophyll fluorescence*

In-situ chlorophyll fluorescence measurements were taken by appressing the end of a fiber optic cable against the test tubes perpendicular to the incident growth light and recording fluorescence yields obtained from a portable PAM-2000 fluorometer (Walz, Effeltrich, Germany) on a chart recorder (Kipp & Zonen B.V., Delft, The Netherlands). Effective photon yield of photosystem II under the incident light intensity ( $[F_m' - F]/F_m'$ ) was calculated as described in Demmig-Adams *et al.* (1996). Measurements were taken 2.5 hours (200  $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$  only) and 24 hours (for both 200 and 500  $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ ) after the start of the experimental period with diluted cultures in fresh medium.

#### 4.3.4 *Glycerol analysis*

Glycerol concentration was determined colorimetrically using an EnzyChrom Glycerol Assay Kit (BioAssay Systems, Hayward, CA, USA). For assessment of glycerol in the external medium, 100  $\mu$ L of supernatant was diluted 1:10 with 900  $\mu$ L of deionized water in 2 mL microcentrifuge tubes, thoroughly mixed, and 4  $\mu$ L of each sample were placed in wells on a flat-bottom 96-well plate. For the determination of intracellular glycerol levels, samples were diluted 1:20 to account for the higher glycerol concentrations.

#### 4.3.5 *Glycerol addition to the culture medium*

Cultures with glycerol added to the experimental medium were prepared as above, but after the cultures were diluted and gently centrifuged, they were resuspended in fresh sterile medium to which 25 mM glycerol was added. Twenty five mM glycerol was chosen to provide slightly more glycerol in the surrounding medium than would be accumulated after several days of glycerol release by a dense culture (data not shown), but was also a low enough concentration as to not significantly change the osmotic potential of the culture medium compared to cultures without glycerol added.

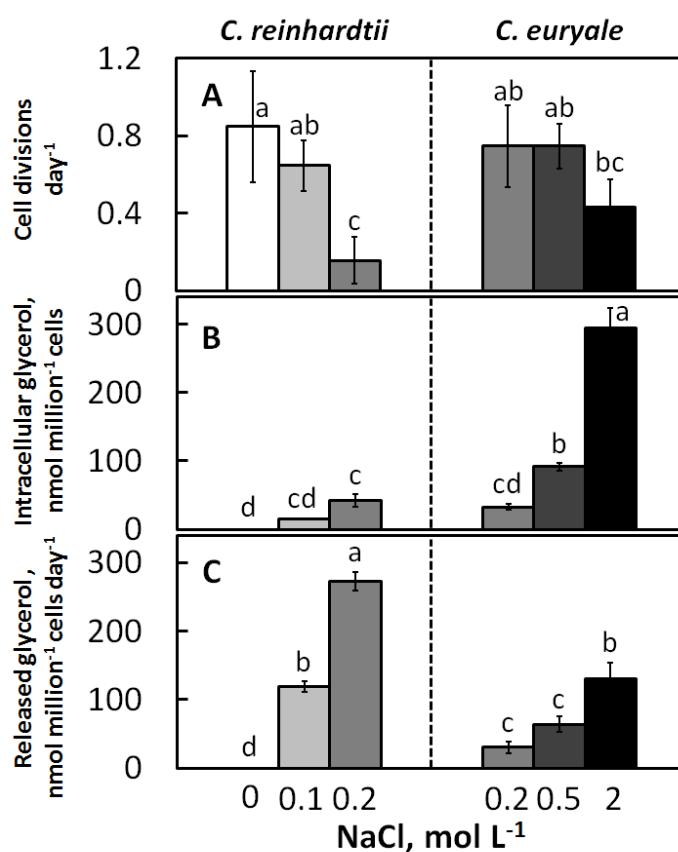
### 4.4 Results

#### 4.4.1 *Response of growth rate, glycerol accumulation, and glycerol release rate to external salinity*

We grew the freshwater species *C. reinhardtii* and marine species *C. euryale* over a range of salinities in the external medium from optimal conditions for growth to partial or near-



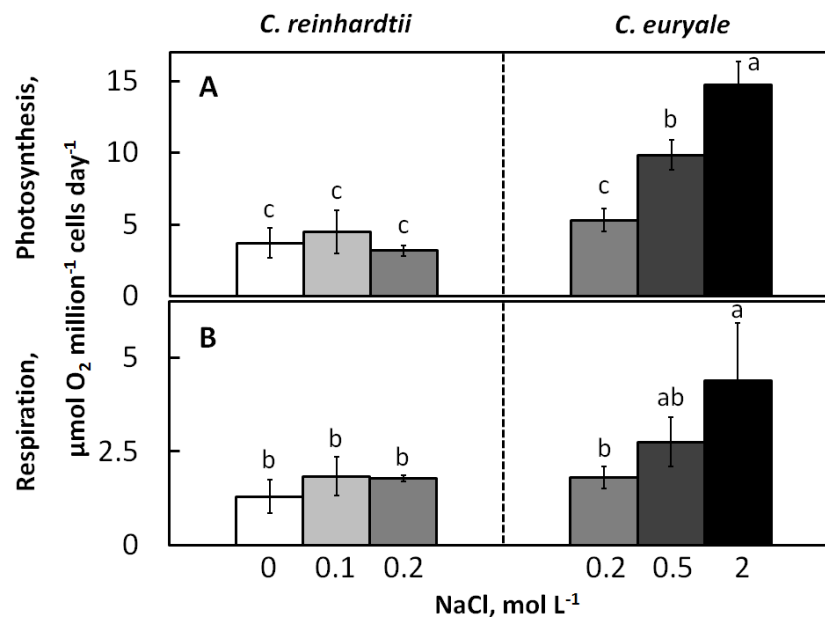
complete inhibition of algal growth. Increased salinity level led to a decrease in algal growth rate (rate of cell divisions per day) that was dramatically more pronounced in the freshwater than the marine species (Fig. 4.1 A). While the freshwater species exhibited almost complete growth inhibition at an external salinity of 0.2 M NaCl (corresponding to less than half of seawater salinity; Fig. 4.1 A), the marine species showed similar growth rates at 0.2 and 0.5 M NaCl and a growth-rate reduction of less than 50% under four times the salinity of seawater (2 M NaCl; Fig. 4.1 A). The marine species accumulated high levels of glycerol internally under 2 M NaCl (Fig. 4.1 B). Conversely, the freshwater species released considerably more glycerol into the external medium than the marine species under a salinity level of 0.2 M NaCl (Fig. 4.1 C).



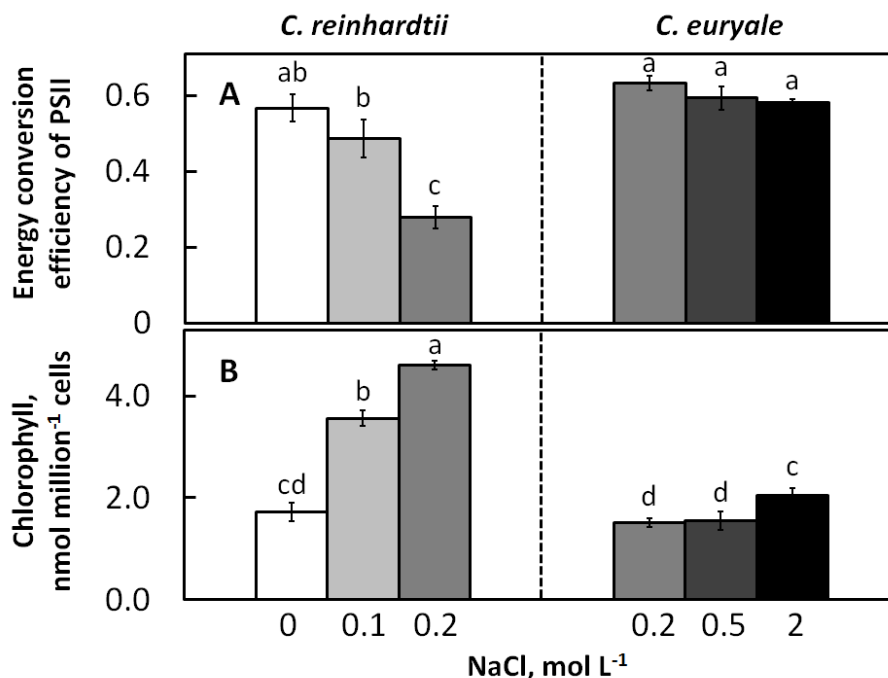
**Figure 4.1:** Effect of external salinity on (A) algal growth rate (cell division rate), (B) internal glycerol accumulation, and (C) glycerol release into the external medium in the freshwater species *C. reinhardtii* (left) and the marine species *C. euryale* (right). Mean values  $\pm$  standard deviation (n = 3) shown, with lower-case letters indicating significant differences (p < 0.05).

#### 4.4.2 *Response of photosynthesis, respiration, solar energy conversion efficiency, and chlorophyll content to external salinity*

There was no difference in maximal capacity of photosynthesis (light- and CO<sub>2</sub>-saturated rates of linear electron transport as net oxygen evolution) or cellular respiration in the freshwater species over the range of 0 to 0.2 M NaCl in the external medium (Fig. 4.2), and thus no evidence for photosynthetic downregulation. In the marine species, increasing salinity between 0.2 and 2.0 M NaCl actually resulted in substantial increases in maximal photosynthesis rates and cellular respiration rates (Fig. 4.2). The efficiency of the conversion of light absorbed in photosystem II (PS II) into PS II photochemistry, measured via chlorophyll fluorescence as  $(F_m' - F)/F_m'$ , decreased with increasing salinity over the range employed here for the freshwater species but not for the marine species (Fig. 4.3 A). Chlorophyll content per cell increased strongly with increasing salinity in the freshwater species, but not in the marine species, over the range of salinities employed here (Fig. 4.3 B).



**Figure 4.2:** (A) Light- and CO<sub>2</sub>-saturated rate of linear electron transport (photosynthetic oxygen evolution) and (B) cellular respiration (oxygen uptake) as affected by external salinity in the freshwater species *C. reinhardtii* (left) and the marine species *C. euryale* (right). Mean values  $\pm$  standard deviation (n = 3) shown, with lower-case letters indicating significant differences (p < 0.05).

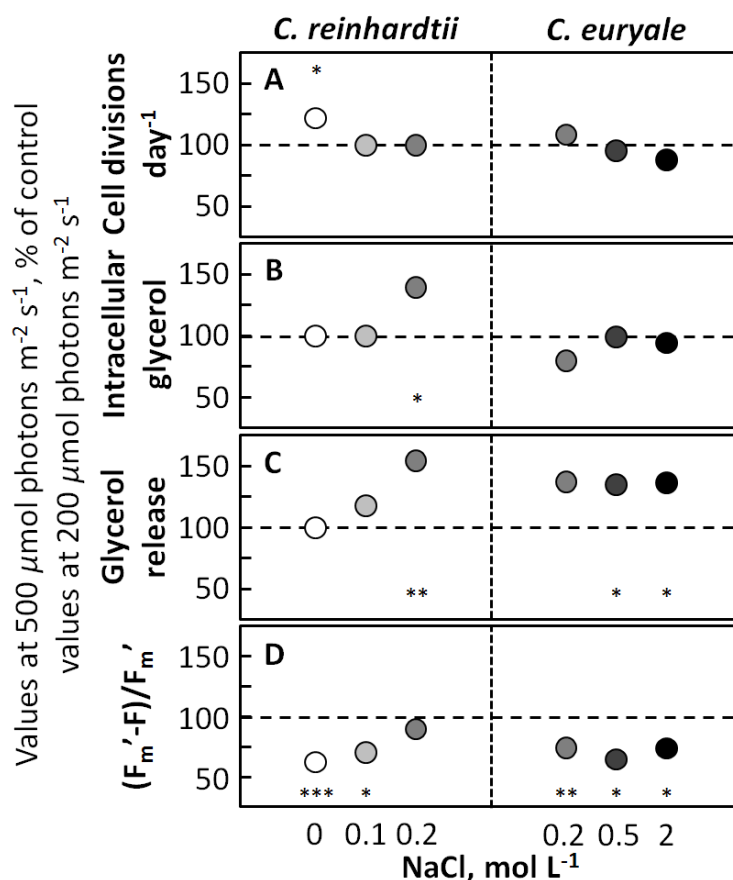


**Figure 4.3:** (A) The efficiency of the conversion of light absorbed in photosystem II (PS II) into photochemistry, measured as  $(F_m' - F)/F_m'$ , and (B) chlorophyll content per cell as affected by external salinity in the freshwater species *C. reinhardtii* (left) and the marine species *C. euryale* (right). Mean values  $\pm$  standard deviation ( $n = 3$ ) shown, with lower-case letters indicating significant differences ( $p < 0.05$ ).

#### 4.4.3 Effect of growth light intensity

Compared to a growth light intensity of  $200 \mu\text{mol photons m}^{-2} \text{s}^{-1}$  used for the experiments described in Figures 4.1-4.3, a growth light intensity of  $500 \mu\text{mol photons m}^{-2} \text{s}^{-1}$  had little effect on algal growth rate (cell division rate) in response to increasing salinity in either species (Fig. 4.4 A). However, the higher growth light intensity resulted in significantly greater intracellular glycerol accumulation in the freshwater species under 0.2 M NaCl, but had no significant effect on the marine species (Fig. 4.4 B). The higher versus the lower growth light intensity likewise resulted in a greater level of glycerol release in the freshwater species under 0.2 M NaCl and under 0.5 M and 2.0 M NaCl in the marine species (Fig. 4.4 C). Lastly, the higher growth light intensity led to somewhat lower efficiencies of the conversion of light

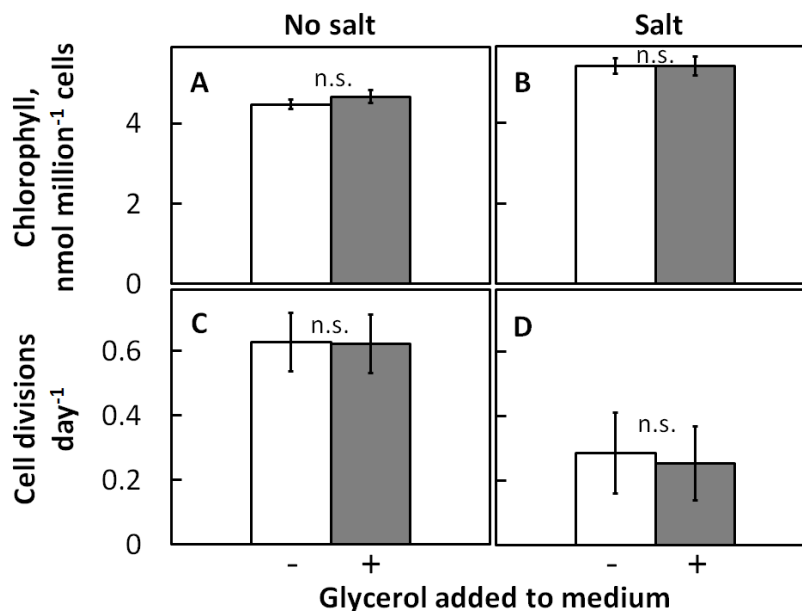
absorbed in PS II into photochemistry, measured as  $(F_m' - F)/F_m'$ , in the freshwater species under 0 and 0.1 M (but not under 0.2 M NaCl) and under all salinities tested in the marine species (Fig. 4.4 D).



**Figure 4.4:** Effect of growth light intensity (metrics determined for algae grown under 500  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$  and expressed as a percentage of the metrics determined for algae grown under 200  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ ) on (A) algal growth rate (cell division rate), (B) intracellular glycerol accumulation, (C) glycerol release rate, and (D) the efficiency of the conversion of light absorbed in PS II into photochemistry, measured as  $(F_m' - F)/F_m'$ , at various external salinity levels in the freshwater species *C. reinhardtii* (left) and the marine species *C. euryale* (right). \* =  $p < 0.05$ ; \*\* =  $p < 0.01$ ; \*\*\* =  $p < 0.001$  from non-paired, two-tailed  $t$ -test.

#### 4.4.4 Effect of experimental glycerol addition to the external medium

Addition of glycerol to the external medium did not affect chlorophyll content (Fig. 4.5 A,B) or growth rate (Fig. 4.5 C,D) in *C. reinhardtii* under either no added NaCl (Fig. 4.5 A,C) or 0.2 M NaCl in the external medium (Fig. 4.5 B,D).



**Figure 4.5:** Effect of 25 mM glycerol addition (+) to the external medium on algal (A,B) chlorophyll content and (C,D) growth rate in the absence (No salt) or presence (Salt = 0.2 M NaCl) of external salinity in *C. reinhardtii*. n.s. = not significantly different.

#### 4.4.5 Statistical analyses

Data were subjected to ANOVA analysis coupled with Tukey-Kramer tests or non-paired, two-tailed *t*-tests in order to determine differences among mean values using JMP software (SAS Institute, Cary, NC, USA).

## 4.5 Discussion

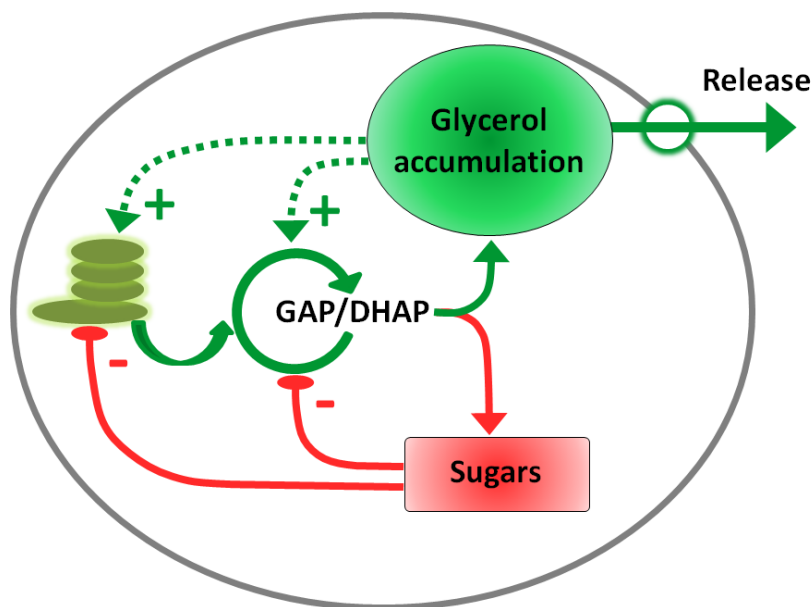
### 4.5.1 Differential proportions of glycerol accumulation versus glycerol leakage/release from algal cells in a freshwater versus a marine species

The freshwater *Chlamydomonas* species, *C. reinhardtii*, lost twice as much glycerol to the external environment compared to the marine species, *C. euryale*, when both were grown in 0.2 M NaCl. This apparent limitation to the ability to accumulate glycerol as an internal

osmoregulatory compound may be involved in the inability of the freshwater species to acclimate to, and show positive growth at, salinities exceeding 0.2 M NaCl (which is less than half the salt concentration of seawater). This supports the hypothesis that the proportion of accumulated versus released glycerol determines a species' salt tolerance. The effect of growth light intensity further expounds differences in the response of the freshwater and marine species to salinity. The finding that photochemical efficiency (and presumably photosynthesis rate) as well as glycerol release and internal glycerol accumulation under 0.2 M NaCl in the freshwater species were increased by growth at a higher versus a lower light intensity suggests that glycerol accumulation may be limited not only by the inability of the freshwater species to effectively retain synthesized glycerol, but also by a limited rate of glycerol synthesis. There is no evidence for similar limitations in the marine species under the range of experimental conditions used here.

#### *4.5.2 Differential response of photosynthetic electron transport to salinity in a freshwater versus a marine species*

The higher photosynthesis rate (rate of linear electron transport), and associated greater internal glycerol accumulation, in the marine species under higher salinity supports the hypothesis that glycerol accumulation is not causing photosynthetic repression – unlike what would be expected from sugar accumulation (Fig. 4.6). Increasing glycerol synthesis over a wide range of increasing salinities in the marine species could represent a mechanism to facilitate the continued utilization of absorbed excitation energy, thereby preventing a backup of electrons in the photosynthetic electron transport chain even when algal growth ceases or decreases. This supports the hypothesis that glycerol may act as a sink for photosynthetic energy.



**Figure 4.6:** Schematic depiction of the effect of glycerol accumulation and/or release on photosynthesis compared to internal sugar accumulation. Sugar accumulation leads to feedback inhibition of photosynthesis, while glycerol accumulation and release either does not affect linear electron transport rate (freshwater species) or results in elevated rates of photosynthesis (marine species). GAP, glyceraldehyde phosphate; DHAP, dihydroxyacetone phosphate. GAP and DHAP are the triose-phosphate products of photosynthetic carbon fixation and the immediate precursors of sugars or (DHAP) glycerol.

It is curious that the freshwater species lowers its PS II photochemical efficiency (the efficiency with which light absorbed in PS II is converted to photochemistry) in response to increasing salinity, while at the same time increasing overall chlorophyll content and thus increasing apparent overall light absorption. In contrast to the situation in the marine species, the maximal rate of linear electron transport in the freshwater species did not increase with salinity, which makes it attractive to speculate that the combination of increased chlorophyll content with decreased PS II energy conversion efficiency may be due to an increase in cyclic electron flow around photosystem I (PS I) in the freshwater species, with the increased chlorophyll level delivering excitation energy primarily to PS I. Unlike linear electron flow, cyclic flow is not detectable via the measurement of photosynthetic oxygen evolution presented here (and would



also not be detectable as CO<sub>2</sub> uptake), but does increase the ratio of ATP to NADPH formed (Fork & Herbert 1993). Increased cyclic electron flow around PS I in response to sudden salt stress was demonstrated in cyanobacteria, algae, and plants (Fork & Herbert 1993; see also Liu & Shen 2006; Yang *et al.* 2006). Increased levels of cyclic electron flow would be a way to produce ATP under conditions where linear electron transport may be limited by electron acceptor availability (as a result of limited glycerol production rates) in the freshwater species. Both cyclic electron flow, and especially linear flow supported by glycerol synthesis (as is apparently the case in the marine species) would be expected to result in greater algal tolerance of high light intensities.

Both the higher photosynthesis rate and greater internal glycerol accumulation in the marine species and the absence of downregulation of photosynthetic capacity in the freshwater species under elevated salinity support the hypothesis that glycerol is not causing photosynthetic repression. These findings are also consistent with the absence of an effect of experimental glycerol addition to the external medium on algal growth rate or chlorophyll content; either glycerol is not taken up from the medium into the algal cell, or if it is taken up, it exhibits no discernible internal effect. This gives new meaning to the term compatible solute – as a solute that does not interact with intracellular signaling networks modulating photosynthesis rate in response to the demand for photosynthate, in contrast to the downregulation of photosynthesis when sugars accumulate (Fig. 4.6).

#### 4.5.3 *Implications of the differential responses between a freshwater and a marine species in the contexts of ecology and industrial application*

The findings presented here support the view that marine species, compared to freshwater species, are better at osmoregulation via internal glycerol accumulation under high salinities. Exchange of energy carriers and nutrients between photosynthetic and non-photosynthetic organisms is common in symbioses found in marine environments (Muscatine 1967; Davies 1984; Lee 2006; Venn *et al.* 2008; Lee *et al.* 2010; Suescún-Bolívar *et al.* 2012). However, the greater glycerol release rates of the freshwater species in response to minor increases in salinity level reported here suggest that freshwater species could be adept at exchanging energy carriers and nutrients with other members of microbial communities.

The fact that both internal accumulation and release of glycerol into the external medium apparently avoids photosynthetic downregulation is relevant for the use of algae for production of energy carriers or materials in an industrial context (see Chapter 5). Being able to harvest energy carriers released from the algae circumvents energy- and resource-intensive harvesting, digesting, and regrowth of algae necessary in many other approaches to the generation of feedstocks for biofuels and biomaterials. Glycerol can serve as the feedstock for multiple products generated by non-photosynthetic microbes and cyanobacteria (Luo & Mitsui 1994; da Silva *et al.* 2009; Bandyopadhyay *et al.* 2010; Min & Sherman 2010; Choi *et al.* 2011; Ganesh *et al.* 2012; Metsoviti *et al.* 2012; Yu *et al.* 2012). Unlike starch or lipid accumulation, internal accumulation of glycerol avoids photosynthetic downregulation in glycerol-producing *Chlamydomonas* (Fig. 4.6). Glycerol can be harvested either via continuous release (the candidate of choice for this approach would be the freshwater *Chlamydomonas* species) or via periodic draining of glycerol from cells (the candidate of choice for this approach would be the marine *Chlamydomonas* species). It has been demonstrated that hypo-osmotic shock treatment

can quickly and non-destructively drain large amounts of glycerol from microbial cells (Fujii & Hellebust 1992; Kayingo *et al.* 2001).

## CHAPTER 5

### POTENTIAL APPLICATIONS IN THE CONTEXT OF BIOENERGY/BIOMATERIALS

#### 5.1 Introduction

Human society relies on many different types of resources for support. Photosynthesis by plants and photosynthetic microbes supports or supported the generation of a large portion of these resources, including food, materials, and energy sources. Likewise, most ecosystems on this planet are supported by photosynthesis performed by primary producers (photosynthesizing plants or microbes). Despite the vast quantity of resources derived from photosynthesis, the *overall* efficiency of biological solar-energy conversion into the final biomass of the primary producers is relatively low (Blankenship *et al.* 2011; Ort *et al.* 2015). This low overall conversion efficiency is due to the fact that the biomass accumulated by photosynthetic organism represents only a small fraction of the solar energy absorbed by the organism, while a large fraction of the energy is consumed by the myriad of metabolic reactions that support the photosynthetic organism's own growth, maintenance, and reproduction. Despite its overall relatively low solar-energy conversion efficiency, photosynthesis is a highly desirable production method because it is sustainable in that it utilizes the resources of virtually limitless sunlight and renewably generated CO<sub>2</sub>.

This thesis has elucidated how energy captured through photosynthesis in *Chlamydomonas* can be funneled into accumulation and/or release of the energy carrier glycerol, rather than used for growth. Additionally, photosynthesis was upregulated under conditions that

strongly promoted glycerol synthesis in a marine *Chlamydomonas* species, which would constitute a further improvement of overall solar-energy conversion efficiency into glycerol in this system. Release of glycerol by *Chlamydomonas* species and subsequent conversion to useful products by other, non-photosynthetic organisms represents a potential applied aspect of this thesis that goes beyond the fundamental findings presented in Chapters 3 and 4. Glycerol's features of providing an apparent sink for photosynthesis and representing a photosynthetic product that does not cause feedback inhibition of photosynthesis could be exploited for improved overall production efficiencies of other valuable products of photosynthesis, and could also lead to an improved understanding of primary productivity in natural ecosystems. This chapter (Chapter 5) focuses primarily on bioenergy production through glycerol release, potential practical applications, and methods that could further improve overall photosynthetic conversion efficiency of product production.

## 5.2 Biofuels

Renewable energies are needed to phase out fossil fuels and limit net increases in atmospheric CO<sub>2</sub> concentrations (Chisti 2007; Pate *et al.* 2011). While solar and wind energy are employed to decrease fossil fuel use for electricity generation, these limitless energy sources are not suitable to replace liquid transportation fuels at current electricity prices and as long as batteries lack the high energy-to-volume and energy-to-weight ratios of liquid transportation fuels (Park *et al.* 2012). The energy sources (biofuels or electricity generated from wind or photovoltaics) presently available or close-to-being available are not at the scale and/or cost needed to replace fossil-fuel-based energy (Graves *et al.* 2011; Park *et al.* 2012). Ethanol

produced from sugars (e.g., sugarcane) or starches (e.g., corn), or biodiesel derived from oil-seed crops like soybean and canola (i) do not approach the scale necessary to displace a large percentage of liquid fossil fuel use (Chisti 2007), (ii) compete with food production on arable land and lead to increased food prices (Wallington *et al.* 2012), and (iii) require high levels of fossil fuel input (Chisti 2007; Pate *et al.* 2011). Although it is more efficient, plant-based cellulosic ethanol nonetheless requires significant amounts of land, water, and fertilizer, and is energy-intensive and expensive to process (Chisti 2007; Zhang *et al.* 2010; Hulatt *et al.* 2012).

The production of glycerol by green algae described in this thesis presents a transformative approach to continuous feedstock production by photosynthetic algae. Glycerol is suitable to supply the energy for a wide variety of biofuel-producing microbes dependent on external feedstock supply. This approach is supported directly by sunlight and atmospheric CO<sub>2</sub> and maximizes algal photosynthesis (by prevention of the feedback inhibition of photosynthesis caused by internal accumulation of oil or starch in the algae) and algal high light tolerance.

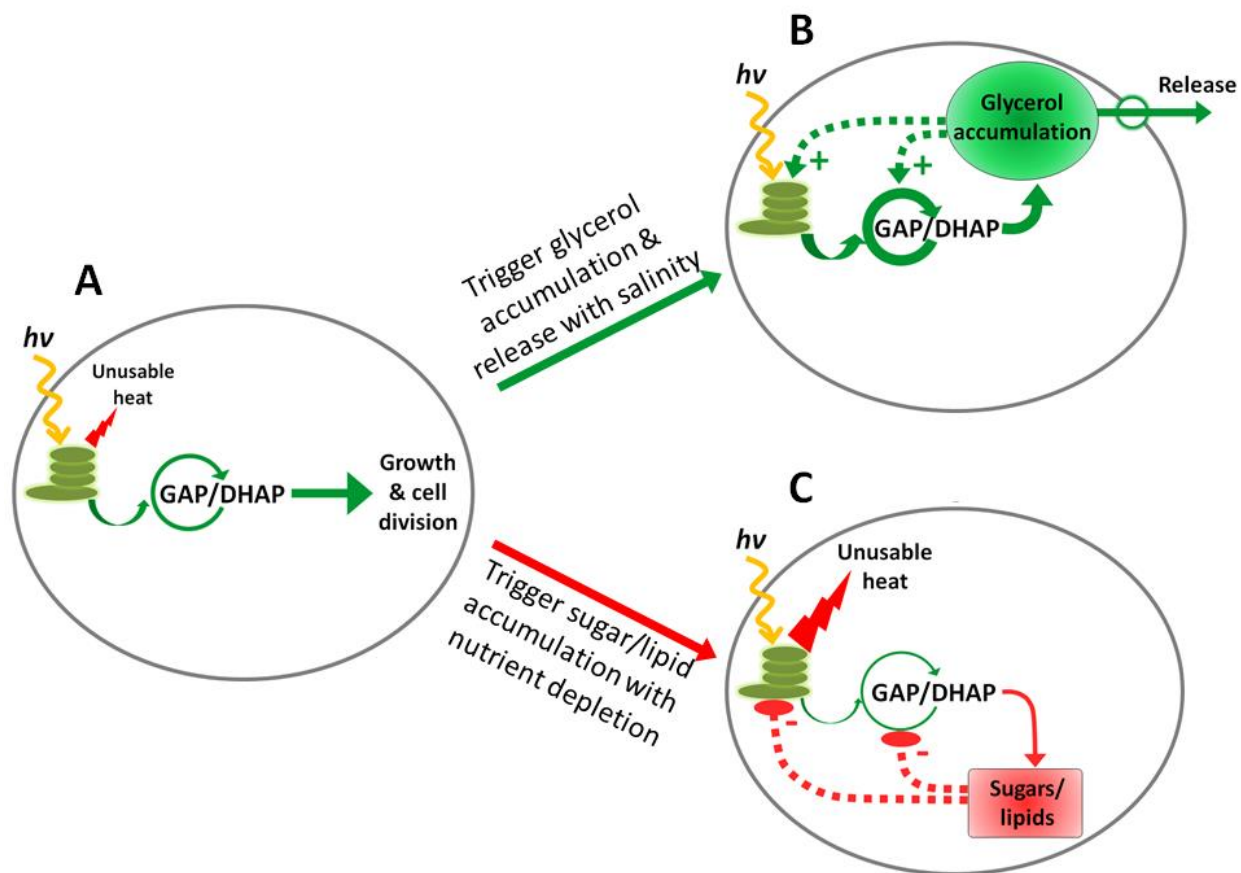
### 5.2.1 *Comparison of algal glycerol production with other sources of biofuels*

Biofuels derived from microalgae have the potential to solve many of the problems associated with plant-based biofuels (Amer *et al.* 2011). Production of oils (neutral fats = triacylglycerides = TAG) from microalgae is possible on non-arable land, and uses land more efficiently than plant-based biofuels (Amer *et al.* 2011; Sun *et al.* 2011). Algal TAG-based biofuels are currently produced by growing high-density cultures under conditions favorable for growth (see Fig. 5.1 A), then adjusting culture conditions to cause algal stress that triggers TAG accumulation and decreases growth (see Fig. 5.1 C). Unfortunately, algal stress also inhibits photosynthesis, eliminating *de-novo* production of energy-rich molecules from atmospheric CO<sub>2</sub>.

After extraction from the harvested algal biomass, the lipids are then converted to biodiesel. The harvesting, extraction, and purification processes are energy-intensive, and recycling or disposal of nutrients from the harvested biomass is costly (Sun *et al.* 2011). Life-cycle-analyses for plant- and algal TAG-based biofuel production processes (Pate *et al.* 2011) indicate that both require large fossil fuel inputs (Hulatt *et al.* 2012) to supply the nitrogen necessary for biomass growth, with the environmental effects of nitrogen runoff in waterways and the ocean being cause for concern (Ansari *et al.* 2011). Dependence on nitrogen fertilizer is a major obstacle to self-sufficiency and scalability for algae-based production of transportation fuels and other high value products: The nitrogen required to support algae-based biodiesel production at a scale to meet current US needs for transportation fuels would exceed current US production of urea-nitrogen by nearly 100-fold (Batan *et al.* 2010).

As shown in Chapter 4, production of energy-rich glycerol, whether accumulated internally or released to the surrounding medium, does not cause photosynthetic repression in *Chlamydomonas* (see Fig. 5.1 B). Indeed, glycerol accumulation and release in the marine species led to **increased rates** of photosynthesis. This increase is likely due to not only increased demand for the products of photosynthesis (greater sink pressure) but also because glycerol may not contribute to signaling networks that inhibit photosynthesis (as discussed in Chapter 4). Additionally, collection of glycerol released into the culture medium (via already available low-cost membrane separation processes; Gin *et al.* 2008; Carter *et al.* 2012; Wiesenauer & Gin 2012) eliminates energy-intensive harvest, breakdown, and re-growth of biomass associated with other biofuels. Elimination of the need to re-grow microbial cultures preserves the energy and nutrients contained in the algal biomass. The amount of nitrogen required to support continuous

secretion of glycerol by mature algal cultures is thus much lower than for approaches involving harvest and conversion of algal biomass.



**Figure 5.1:** Schematic depiction of (A) initial algal growth, (B) bioenergy production through glycerol release triggered by culture medium salinity, and (C) biofuel production through sugar/lipid accumulation with nutrient depletion, and the corresponding effect on photosynthesis of glycerol accumulation/release versus sugar/lipid accumulation.

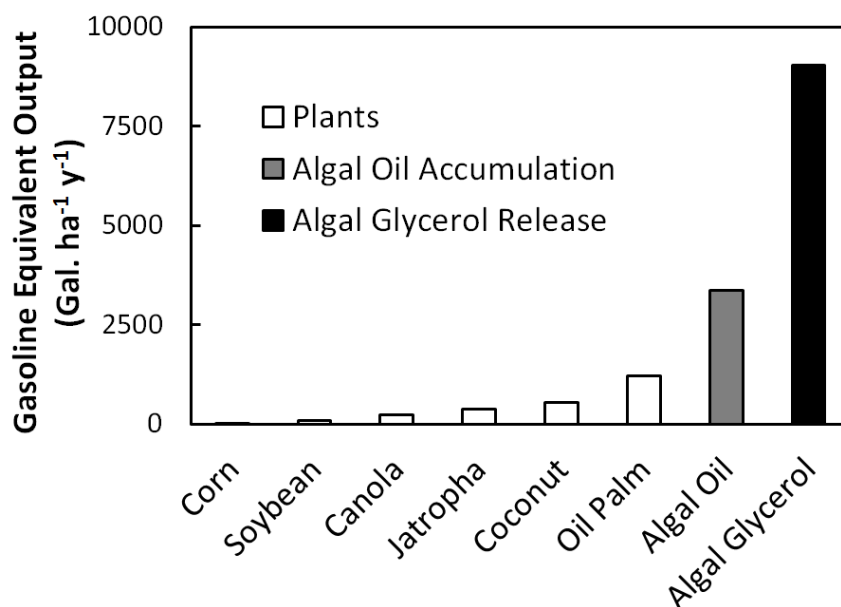
The system described in this thesis (green algae that internally accumulate and/or release glycerol) serves as a novel system for energy carrier production. Figure 5.2 depicts estimated gross energy output of fuel per cultivated area for corn, oil seeds, algal TAG, and the novel algal glycerol release. Data for plants were taken from Chisti (2007), algal oil accumulation data were taken from Sun *et al.* (2011), and data for algal glycerol were extrapolated from experiments performed for this thesis. To extrapolate our experimental results, glycerol production per area



under cultivation was estimated by first calculating glycerol release rate per surface area of the experimental vessel. Production rate was further adjusted to account for differences in growth light intensity outdoors versus our continuously illuminated cultures. Energy content of the produced glycerol in gasoline equivalent energy was extrapolated using data from Arechederra *et al.* (2007), and decreased by 50% to account for estimated energy conversion losses inherent to biological conversion of glycerol to other bioproducts by non-photosynthetic microbes. For a complete overview of input values, conversions, and sources, see Table B.1 in Appendix B. For comparison, Ben-Amotz *et al.* (1982) calculated maximum theoretical glycerol production rates of  $16 \text{ g m}^{-2} \text{ day}^{-1}$  in *Dunaliella* using accumulation, while our calculated rates for glycerol release were  $18.1 \text{ g m}^{-2} \text{ day}^{-1}$ . The glycerol release results in this thesis were comparable to those of Miyasaka *et al.* (1998) and León and Galván (1994;1995). Energy output per area is higher for algal glycerol release compared with all other approaches. In addition, none of the energy outputs shown in Figure 5.2 include the required inputs of fossil-fuel-based energy (for fertilizer) to support algal growth (the production of nitrogen-containing algal biomass). As shown in Chapter 4, both freshwater and marine species eventually exhibited decreased growth rates at high enough culture salinities. This suggests that mature glycerol-producing cultures may require low inputs of nitrogen fertilizer, which could further dramatically widen the difference in *NET* energy output from algal glycerol release compared to other approaches to plant- or algae-based biofuels.

The biological parameters governing glycerol accumulation and release (e.g., algal species, salinity level, growth light intensity) described in this thesis can be utilized to optimize glycerol production from *Chlamydomonas* for biofuel production by (i) minimizing algal cell division and growth and (ii) maximizing photosynthesis rates and glycerol production with

respect to salinity and light. The rapid recent progress in using glycerol as a versatile feedstock for microbial production of ethanol (Choi *et al.* 2011; Metsoviti *et al.* 2012; Yu *et al.* 2012), hydrogen (see Ganesh *et al.* 2012 for *E. coli*; see Luo & Mitsui 1994, Min *et al.* 2010, and Bandyopadhyay *et al.* 2010 for cyanobacteria), and various valuable biochemicals (da Silva *et al.* 2009; Ganesh *et al.* 2012) has been inspired by glycerol availability as a byproduct of biodiesel. However, it is important to note that current biodiesel production, and thus production of glycerol (as a minor byproduct of biodiesel production from oil-accumulating crops), is subject to the principal limitations of crop plants detailed at the beginning of this section and in section 3.5.4. Our algal-based production of glycerol thus ties into the rapidly expanding development of glycerol-based fuels and materials motivated by glycerol being a byproduct of biodiesel production. In this context, it is important to keep in mind that current glycerol production from biodiesel and biodiesel production itself are far from the scale needed to meet transportation fuel demands.

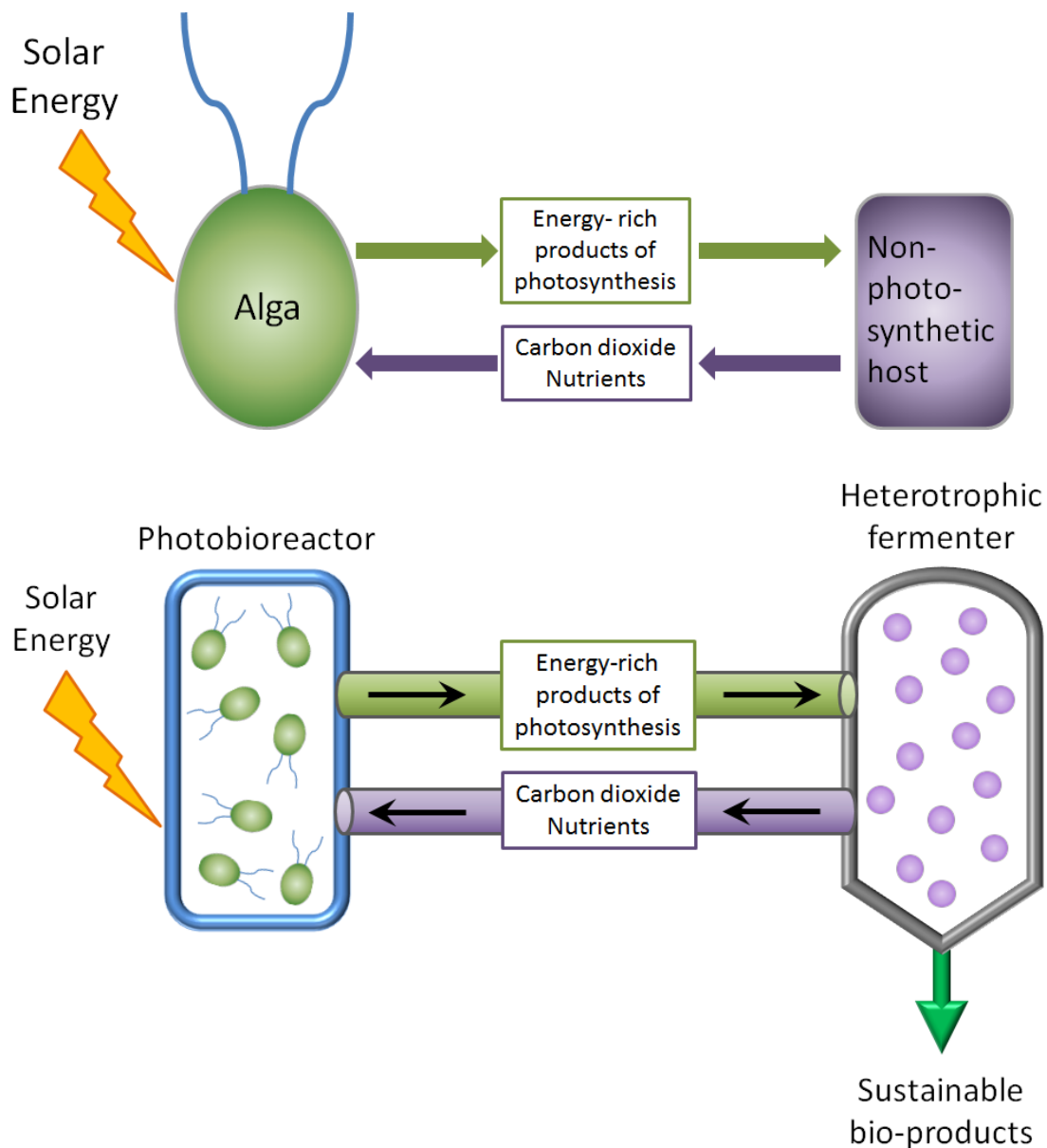


**Figure 5.2:** Gross energy output (in gasoline equivalents) for biofuel crops and algae. Due to required energy input in the form of nitrogen fertilizer, *net* energy outputs for plant and algal oil

production will be much smaller than shown, while input of nitrogen fertilizer for algal glycerol would presumably be much lower due to minimal algal growth in the mature cultures. Data for plants (Chisti 2007), algal oil (Sun *et al.* 2011), and algal glycerol (this thesis) were extrapolated to represent outdoor cultivation based on Chisti (2007), de Souza *et al.* (2010), Thomas (2000), Elsayed *et al.* (2003), Arechederra *et al.* (2007), and Amer *et al.* (2011). See Table B.1 in the appendix for further detail on the calculations of this figure.

### 5.2.2 Possible practical applications in a nature-inspired design

As discussed throughout this thesis, photosynthetic algal symbionts provide energy-rich products of photosynthesis to a non-photosynthetic host in exchange for CO<sub>2</sub> and nutrients. One potential industrial application of this natural system would be to replace the non-photosynthetic host in this system with an industrial heterotrophic fermentation unit (see Fig. 5.3). In this dual-reactor design, the renewable photosynthetic energy captured by algae and released into the medium would be removed from the photobioreactor unit and transferred to a fermenter for microbial processing into a variety of potential products (see previous section and Chapters 3 and 4). CO<sub>2</sub> and other nutrients produced in the fermenter would then be captured and fed back into the photobioreactor. Algal glycerol secretion serves as a proof-of-concept for how a continuous stream of reduced-carbon can be produced at an enhanced photosynthesis rate and effective quantum yield of electron transport by providing a photosynthetic sink. In addition, glycerol accumulation within the cell apparently does not interact with the sugar-sensing signaling networks that can cause photosynthetic repression (Chapter 4), further improving glycerol production as a model system. This approach will thus eliminate the two principal causes of the inefficiency of natural photosynthesis, i.e., the “wasting” of the vast majority of photosynthetic energy on growth and cell division and the capping of maximal photosynthesis rates by feedback limitations.



**Figure 5.3:** Schematic depiction of a photosynthetic algal symbiont and non-photosynthetic host as a model for a dual-reactor industrial production design with a photobioreactor producing renewable energy carriers for a heterotrophic fermenter.

### 5.2.3 Applications and advantages of algal immobilization

To facilitate the separation of algae from their external medium for the harvesting and quantification of glycerol released into the external medium, embedding of algae in beads and

sheets of alginate was explored. Immobilization is the process of embedding algae in a larger (macroscopic) structure to facilitate handling of the microscopic algae.

Alginate is obtained from brown algae, with the characteristics of the alginate varying slightly depending on brown-algal species (Moreno-Garrido 2008). Based on the stability results of Moreira *et al.* (2006), alginate from *Laminaria hyperborea* (FMC Biopolymer, Protanal LF 10/60, Drammen, Norway) was utilized in all immobilization trials presented in this thesis. Immobilization of algae was achieved by mixing a concentrated solution of algae into an aqueous sodium alginate solution and then hardened in a solution containing strontium chloride (4% weight to volume) as a polymerizing cation. To produce beads and sheets that were stable in salt water, strontium instead of calcium was chosen as the polymerizing cation since this substitution had been shown to increase bead stability, while, however, slightly decreasing algal growth rate and maximum cell density in the matrix (León & Galván 1995; Moreira *et al.* 2006).

Immobilization has several technical advantages over free-swimming algae. Since the immobilized algae are completely embedded in alginate, it is a simple matter to separate the medium, into which the glycerol is released, from the algae. In fact, once established, it should be possible to continuously pass fresh medium through a photobioreactor containing bead- or sheet-embedded algae, and to pass glycerol-laden medium on to microbes in a heterotrophic fermenter or for further purification to concentrated glycerol (membrane separation techniques for the separation of glycerol from water and salt are available; Gin *et al.* 2008; Carter *et al.* 2012; Wiesenauer & Gin 2012). In addition, embedding in alginate may help keep unwanted photosynthetic and non-photosynthetic organisms from contaminating the glycerol-secreting *Chlamydomonas*, and the facile continuous removal and replacement of the medium surrounding alginate-embedded *Chlamydomonas* should also discourage establishment of any unwanted

contaminating microorganisms. This approach of embedding in beads or sheets to facilitate the harvesting of glycerol on an industrial scale may be a viable future approach.

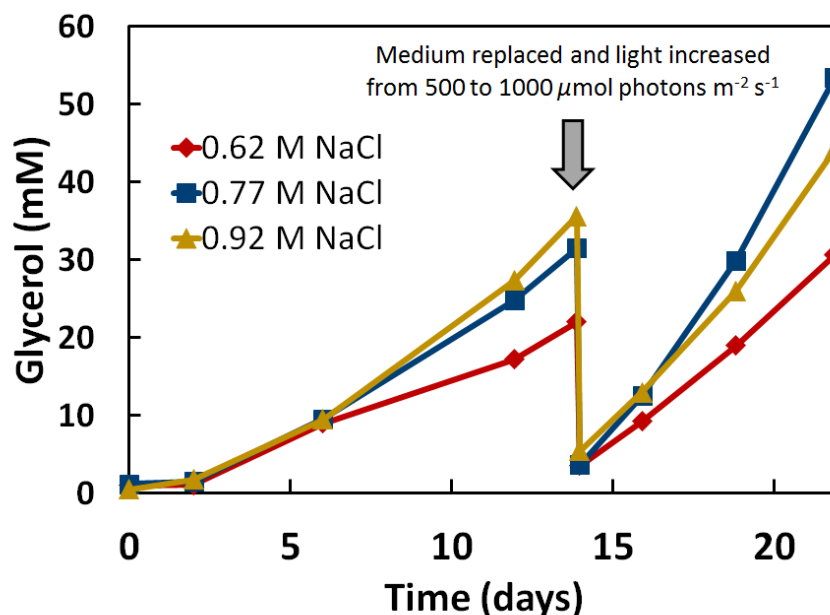
#### 5.2.4 *Bead immobilization*

To produce beads of immobilized algae, a concentrated algal solution produced through gentle centrifugation of Roux bottle pre-cultured algae was added to a solution of autoclave-sterilized alginate and mixed thoroughly. The algae-laden alginate solution was slowly dripped from a sterile syringe into a chilled (4°C) solution of 4% strontium chloride using the method of Moreira *et al.* (2006) to produce beads of strontium-alginate-immobilized algae. After immobilization, beads were placed into 140 mL bubble columns, and stirred by bubbling with 5% CO<sub>2</sub> (mixed with air through a flat dispersion stone; Pyrex, Czech Republic).

##### 5.2.4.1 Glycerol release in bead-immobilized *Chlamydomonas hedleyi*

The symbiont *C. hedleyi* was bead-immobilized (5.8% alginate) and tested over a period of 22 days (Fig. 5.4), during which the algal beads remained stable despite the hypersaline conditions of 0.62, 0.77, and 0.92 M NaCl (salt is a known de-polymerizer of alginate beads). Algae were not observed outside the beads in the liquid portion of the medium, indicating that the matrix successfully held the algae over the course of the experiment. The medium was replaced with fresh medium on day 14 to replenish nutrients, and the light intensity was increased from 500 to 1,000  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ . The beads were circulated by bubbling with 5% CO<sub>2</sub> added to air, and the temperature was 20°C. The initial volume of beads per total

culture volume was  $0.17 \text{ mL bead mL}^{-1}$ , but that ratio increased as samples were taken throughout the experiment.



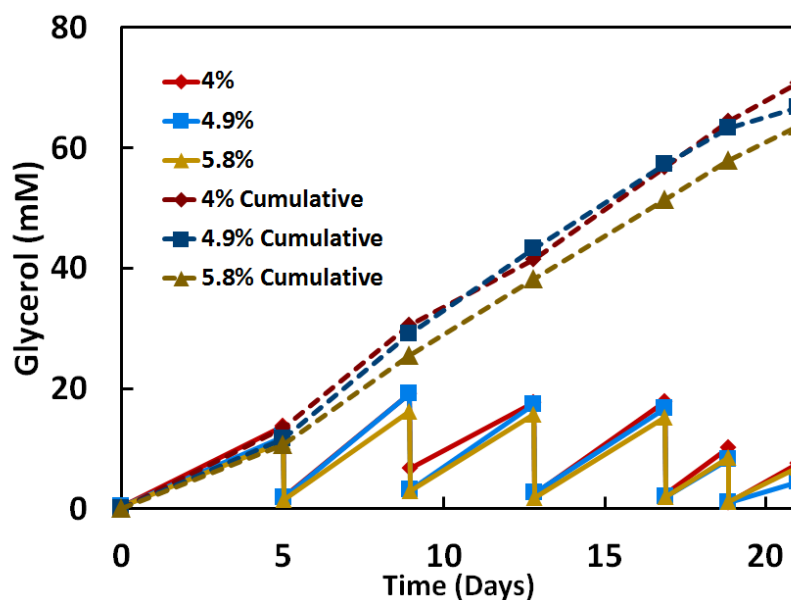
**Figure 5.4:** Time course of changes in estimated glycerol concentration in the medium at three salinities (0.62, 0.77, and 0.92 M NaCl) in the marine species *C. hedleyi* embedded in strontium-alginate beads. On day 14 (black arrow), the medium was replaced with fresh medium and the photon flux density was simultaneously increased from 500 to 1000  $\mu\text{mol m}^{-2} \text{s}^{-1}$ . The apparent increases in glycerol release rate at a given light intensity are likely due to decreases in culture volume, and thus increases in bead-to-total volume-ratio, as medium samples were extracted (days 2, 6, 12, 16, and 19). While samples were removed without fully replacing the medium here, all other immobilization experiments were done while replacing medium to keep medium volume constant.

#### 5.2.4.2 Bead-immobilized *Chlamydomonas reinhardtii* at three alginate concentrations

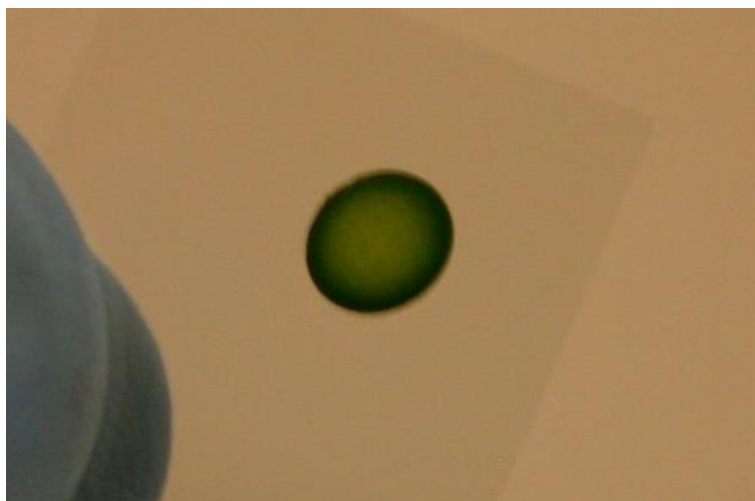
Bead-immobilized cultures of the freshwater species *C. reinhardtii* were tested over a period of 21 days at a constant light intensity of 500  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ . Continuously high rates of glycerol secretion in a medium with 0.17 M NaCl were observed over the course of the experiment (Fig. 5.5). Cultures were continuously bubbled with 5%  $\text{CO}_2$  added to air, and kept

at 20°C. The volume of alginate beads per total culture volume was 0.17 mL bead mL<sup>-1</sup>. During the experiment, the culture medium was completely removed and replaced five times. This semi-continuous culturing method was employed to keep the algae provided with ample nutrients, and to approximate a production process where the glycerol-containing medium is regularly removed and replaced with fresh or recycled medium. The times at which the medium was replaced (with glycerol-free medium) are easily recognized by the immediate drop in glycerol concentration (Fig. 5.5). Different concentrations of alginate (all remained structurally intact) were tested and similar results were obtained for 4% to 5.8% alginate (Fig. 5.5). Figure 5.5 also shows cumulative glycerol release over the entire period, illustrating the potential for continuous, steady glycerol production. Figure 5.6 below shows a cross section of a single alginate bead at the end of this experiment. Green algae were concentrated near the surface of the beads during the experiment; light intensity may have been limiting to photosynthesis past the first millimeter of the beads (from the surface inwards), leading to their migration to the surface region, or it is possible that algae died in the center of the beads due to insufficient light or limitations in gas and/or nutrient diffusion into/out of the beads.





**Figure 5.5:** Time course of glycerol release and cumulative glycerol production (dashed lines) for *C. reinhardtii* embedded in beads prepared with 4, 4.9, or 5.8% alginate, respectively. Culture conditions were  $500 \mu\text{mol photons m}^{-2} \text{s}^{-1}$  of continuous light in 140 mL bubble columns with 120 mL total culture volumes (beads + medium) over 21 days at 0.17 M NaCl in the medium. Medium was removed and fresh medium added on days 5, 9, 13, 17, and 19.



**Figure 5.6:** Cross section of a *C. reinhardtii* strontium-alginate bead at the end of the 21-day experiment shown in Figure 5.5. The bead diameter was approximately 4.8 mm.

To compensate for the dead-zone in the center of the beads, it is possible to produce beads of different diameters (Fig. 5.7) by dripping the alginate solution from syringes with different sized tips. Beads of immobilized *C. reinhardtii* with average bead diameters of 4.49, 3.78, and 2.76 mm were produced and tested over a period of 16 days in 0.17 M NaCl medium. The smaller beads led to higher total chlorophyll in the culture (about 84 nmol Chl mL<sup>-1</sup> in the large beads seen in Fig. 5.6, about 178 nmol Chl mL<sup>-1</sup> in the small beads) because the volume of algae/alginate was the same but the number of resulting beads was much higher. This suggests that the smaller bead diameters may have decreased the central dead-zones seen in the larger beads. The overall glycerol secretion rate was slightly higher in the smaller beads, but further study would be needed to determine the exact effect of bead size on productivity. The smallest beads remained stable over the course of the 16-day experiment, despite their higher surface area to volume ratio.



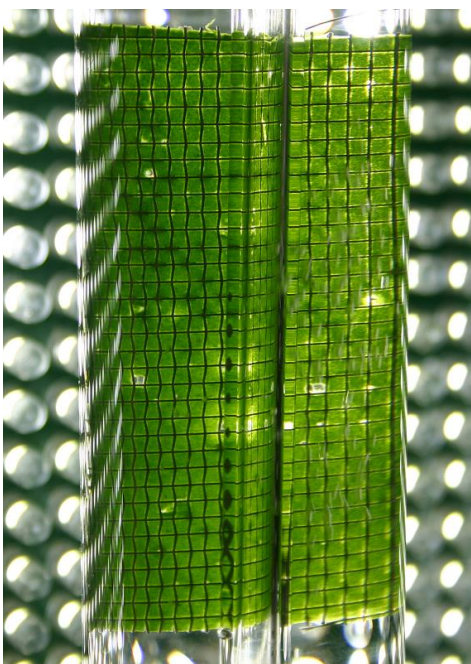
**Figure 5.7:** From left to right; large, medium, and small beads of immobilized *C. reinhardtii* in bubble columns.

#### 5.2.5 Sheet immobilization

To produce sheets of immobilized algae, a concentrated algae/alginate solution was spread across an aluminum mesh screen using a sterile 25 mL glass pipette and submerged in a chilled solution of 4% strontium chloride using similar methods to those of Kosourov and Seibert (2009). The resulting sheet was then trimmed to size and placed in 140 mL bubble columns. Figure 5.8 shows an initial thin sheet within a bubble column immediately after preparation, and Figure 5.9 shows a mature sheet after 12 days of additional algal growth.



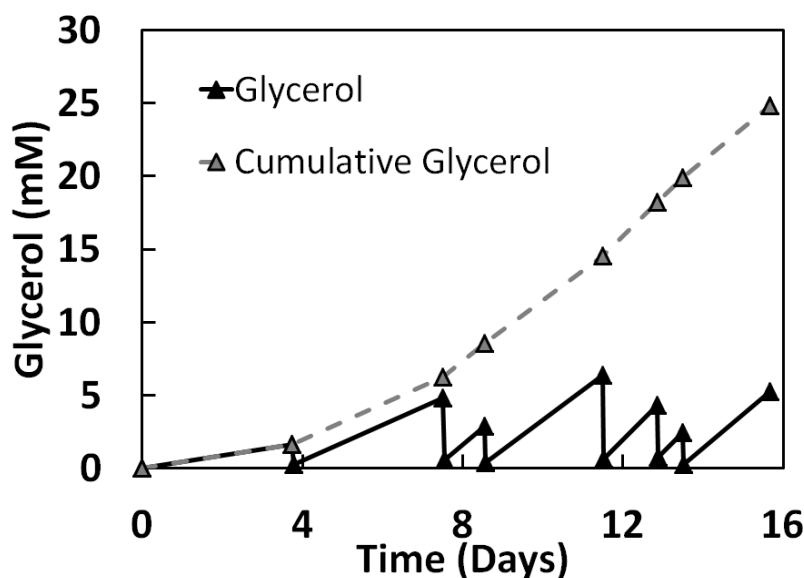
**Figure 5.8:** *Chlamydomonas reinhardtii* immobilized in a strontium alginate sheet immediately after formation.



**Figure 5.9:** The same sheet depicted in Figure 5.8, but 12 days after generation.

#### 5.2.5.1 Glycerol release by *Chlamydomonas reinhardtii* immobilized in a thin sheet

A similar experiment as shown in Figure 5.5 was undertaken with thin sheets (cf. Fig. 5.10). Preliminary data from the latter experiment indicate that rates of glycerol release on a chlorophyll basis for thin sheets ( $70 \text{ mol glycerol mol}^{-1} \text{ Chl day}^{-1}$ ) may even be higher than for beads ( $57 \text{ mol glycerol mol}^{-1} \text{ Chl day}^{-1}$  from data shown in Fig. 5.5). Conversely, glycerol release rates per liter of medium were lower for the thin sheets containing low algal densities ( $2.28 \text{ mM glycerol day}^{-1}$ ) versus beads with high algal densities ( $4.44 \text{ mM glycerol day}^{-1}$  from data shown in Fig. 5.5). The volume of alginate per total culture volume was very low for the sheet, at only  $0.012 \text{ mL sheet mL}^{-1} \text{ culture}$ , compared to  $0.17 \text{ mL bead mL}^{-1} \text{ culture}$ . However, in this preliminary comparison, light intensity was not the same ( $200$  and  $500 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$  on the surface of the bioreactors for sheets and beads, respectively) and a different percent alginate was used ( $5.8\%$  for sheets versus  $4.9\%$  for beads). For a direct comparison of glycerol production rates of algae embedded in sheets versus beads, light-saturated rates of glycerol production would need to be determined and compared for the two systems. Because the sheet-immobilized algae remain stationary in relation to the growth light, instead of tumbling or circulating around like the beads or non-immobilized free-algal cells, they do not experience periods of self-shading. This, combined with the thickness of the immobilized sheets studied, meant that the sheets would likely be light saturated at lower light intensities than both the bead and non-immobilized cultures studied.



**Figure 5.10:** Time course of glycerol release and cumulative glycerol production (dashed lines) for *C. reinhardtii* embedded in a sheet of 5.8% alginate. Culture conditions were  $200 \mu\text{mol photons m}^{-2} \text{s}^{-1}$  of continuous light in 140 mL bubble columns with 101 mL total culture volume (sheet + medium) over 16 days at 0.17 M NaCl in the medium. Medium was removed and fresh medium added on days 4, 7, 9, 11, 13, and 14.

### 5.2.6 Pros and cons of bead versus sheet immobilization

Bead-embedded algae may be the preferred form of immobilization for certain applications depending on bioreactor configuration, whereas other applications may benefit from sheet-embedding. Since beads would remain mobile within the bioreactor, removal of the beads from the system for testing, replacement, or adjustment would likely be easier than in fixed sheets. This mobility could also be a hindrance since beads would need to be actively moved continuously within the system to ensure uniform light absorption on the surface of the beads. If utilizing multiple species each immobilized in different beads (see polyculture section below) adjusting the ratio of one species to another within the system would be a simple matter of adding or removing beads. Immobilized sheets with stratified layers of species could not be adjusted after the sheet is produced, unless the sheets are produced independently. While species

ratios can be actively adjusted in beads in culture, they would lack the strategic stratification of species possible in sheets (see section 5.3.2 below). Sheets would presumably have higher light absorption per illuminated area because there would be no temporary open areas for light to travel through as there are with beads moving in a circulating culture. Additionally, fixed sheets could be precisely positioned relative to the sun to maximize light absorption, but sheets run a higher risk of damage from too much light. Total medium depth (volume) in a sheet-based bioreactor could likely be lower than in a bead-based reactor because beads may require more medium to maintain proper circulation.

Studying immobilized beads and sheets in the laboratory also comes with pros and cons for each type of immobilization. While uniform beads are simple to produce and manipulate in the laboratory, they lack some of the beneficial characteristics of sheets. In beads, it is difficult to calculate productivity per area based on incident light absorption because light escapes through the spaces between beads. Calculating productivity per area for sheets is simple. We also found that sheets could be placed into the apparatus for measuring oxygen evolution in leaves so that maximal rates of photosynthesis can be measured, whereas measuring maximal photosynthesis rates in beads is problematic because it is difficult to achieve full light saturation.

### **5.3 Polyculture**

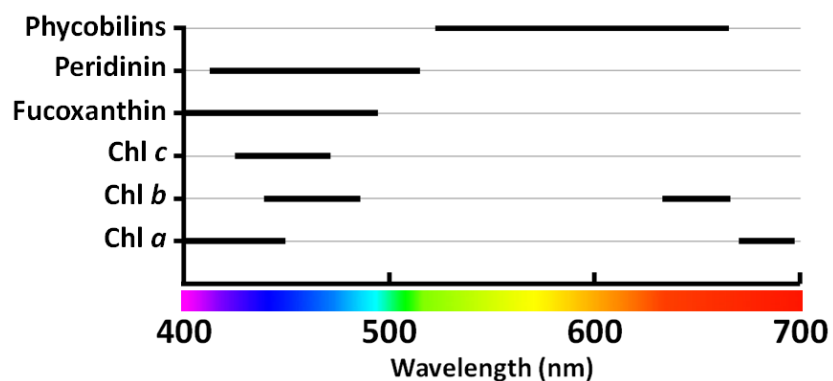
Data from both native ecosystems and experimental systems show that more diverse communities are more productive than monocultures (*e.g.*, Tilman *et al.* 1997, 2001). It is also well established for natural communities of photosynthetic microbes that increasing species

diversity not only drives higher productivity, but also stabilizes community composition by bolstering resistance to invasion by other organisms (Ptacnik *et al.* 2008). Random species mixes are unlikely to produce useful productivity gains so successful polycultures will instead need to be carefully assembled using ecological principles (Shurin *et al.* 2013).

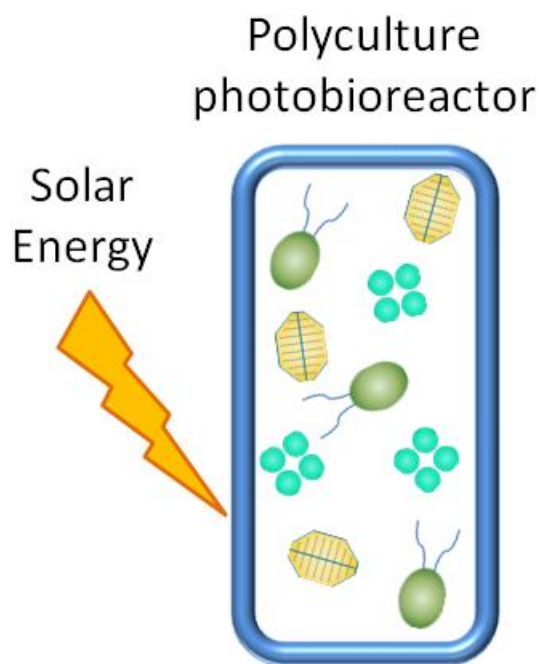
### 5.3.1 *Solar spectrum utilization*

To maximize light absorption across the visible light spectrum, species from groups with complementary light-harvesting pigments (green algae, diatoms, cyanobacteria, and dinoflagellates/prymnesiophytes; Fig. 5.11) could be combined, while selecting candidates that all release energy carriers and/or other desirable products. For instance, including a N<sub>2</sub>-fixing cyanobacterium that releases reduced-nitrogen compounds in exchange for energy carriers (see Agawin *et al.* 2007) could drastically decrease the nitrogen requirements of both the photobioreactor and the fermenter. Indeed, reduced-nitrogen compounds could potentially be a co-product of an industrial application. Figure 5.12 depicts a photobioreactor, like that of Figure 5.3, growing a mix of different algal species in liquid culture to maximize the utilization of the entire solar spectrum by the reactor.





**Figure 5.11:** Full utilization of the photosynthetically active (400-700 nm) spectrum in polycultures of several photosynthetic microbe groups: Absorption ranges (as 50% of maximal absorbance) for light-harvesting pigments in green algae (Chl *a* & *b*), diatoms (Chl *a* & *c*, and fucoxanthin), dinoflagellates (Chl *a* & *c*, and peridinin), and cyanobacteria (Chl *a* & three phycobilins). Chl = chlorophyll.



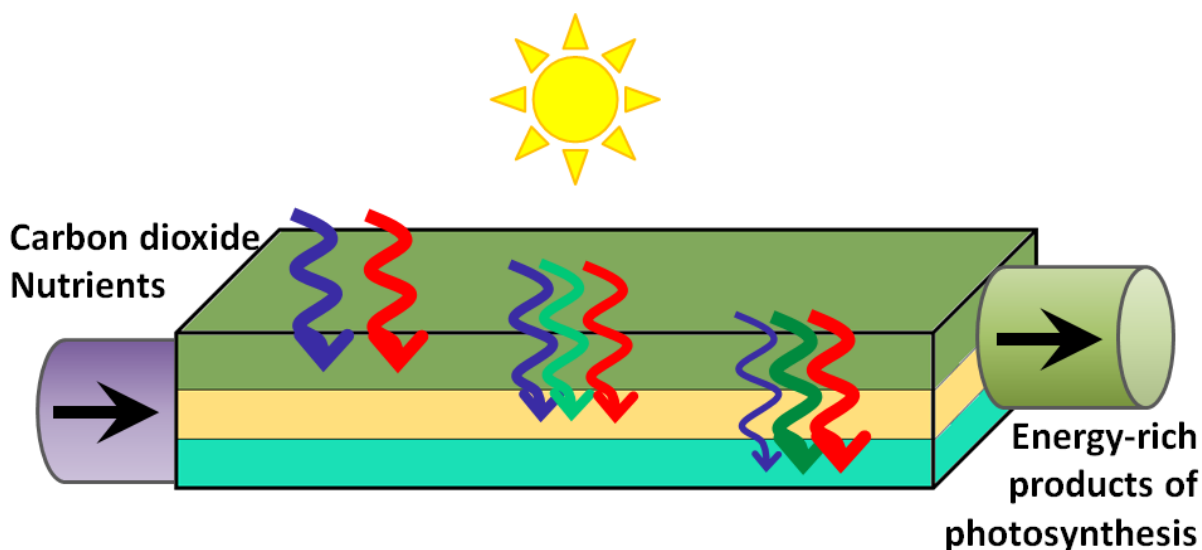
**Figure 5.12:** Schematic depiction of a photobioreactor containing a polyculture of different photosynthetic microbes (green algae depicted by green ovals, cyanobacteria depicted by blue-green structures, and diatoms/dinoflagellates depicted by golden structures).

### 5.3.2 *Stabilizing polycultures and improving productivity through immobilization*

In practice, it is difficult to maintain stable liquid polycultures in the laboratory because one species tends to take over the culture. This could be due to one species outcompeting the others for resources such as light or nutrients. Since laboratory conditions tend to be relatively static, the selected growth temperature, light intensity, and other culture conditions like medium composition could naturally favor one species over another. While selecting polyculture community members that have complementary pigments may lead to much more stability within a liquid polyculture, immobilization may offer an easy solution. By growing each species separately, then combining them at the desired ratio in the soluble alginate, it may be possible to dramatically improve polyculture stability. As mentioned in section 5.2.6, by producing immobilized beads of individual species and then combining them in the desired ratio in the culture, it could be simple to set and adjust the community make-up of a polyculture.

While simple immobilization of strategically selected species in the same or different alginate beads could yield substantial productivity gains over mono-cultures, and improved community stability over liquid polycultures, strategically layering species in a sheet could lead to even higher gains. Within the water column and in microbial mats, photosynthetic microbes tend to organize themselves based on their photosynthetic needs and abilities to exploit different intensities and colors of sunlight. Using this organization as a template, one could immobilize different algal genera in a stratified sheet (see Fig. 5.13) that optimizes their individual light intensity and light quality requirements. For instance, green algal species that can tolerate high light would be immobilized in the sun-facing layer, whereas cyanobacteria that are able to absorb wavelengths of light not utilized by green algae would be placed in the layer furthest from the incident sunlight. In this way, each stratified layer of algal genera would receive not only

optimal wavelengths of light but also optimal light intensity. Stratified sheets could be produced by following the sheet preparation process above for the first layer, then lightly depolymerizing the surface to free up the edges of the polymer matrix and adding another layer of alginate with the next species and dipping in the polymerizing cation solution again.



**Figure 5.13:** Schematic depiction of different species stratified in an immobilized sheet, where photosynthetic microbes are layered by their ability to utilize different portions of the solar spectrum. Composition of the layers and their positioning can be optimized for full use of the photosynthetically active wavelengths of sunlight by the different layers in combination.

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## APPENDIX A

### MEDIA COMPONENTS

<b>Minimal Medium for Growth (MMG)</b>		
	<b>Component</b>	<b>mM</b>
<b>Beijerinck</b>	NH <sub>4</sub> Cl	7.48
	CaCl <sub>2</sub>	0.34
	MgSO <sub>4</sub> ·7H <sub>2</sub> O	0.41
<b>Phosphate buffer</b>	KH <sub>2</sub> PO <sub>4</sub>	1.24
	K <sub>2</sub> HPO <sub>4</sub>	1.84
<b>Hutner's Trace Metals</b>	Na <sub>2</sub> EDTA·2H <sub>2</sub> O	0.134
	ZnSO <sub>4</sub> ·7H <sub>2</sub> O	0.077
	H <sub>3</sub> BO <sub>3</sub>	0.184
	MnCl <sub>2</sub> ·4H <sub>2</sub> O	0.026
	FeSO <sub>4</sub> ·7H <sub>2</sub> O	0.018
	CoCl <sub>2</sub> ·6H <sub>2</sub> O	0.007
	CuSO <sub>4</sub> ·5H <sub>2</sub> O	0.005
	(NH <sub>4</sub> ) <sub>6</sub> Mo <sub>7</sub> O <sub>24</sub> ·4H <sub>2</sub> O	0.0008
<b>Vitamins</b>	Vitamin B1	0.1 mg L <sup>-1</sup>
	Vitamin B12	0.001 mg L <sup>-1</sup>

**Table A.1:** Components of Minimal Medium for Growth in mM, except for vitamins B1 and B12, which are in mg L<sup>-1</sup>

<b>Modified Okomoto Medium (MOM)</b>		
	<b>Component</b>	<b>mM</b>
<b>Beijerink</b>	NH <sub>4</sub> Cl	7.48
	CaCl <sub>2</sub>	0.34
	MgSO <sub>4</sub> ·7H <sub>2</sub> O	0.41
<b>Phosphate buffer</b>	KH <sub>2</sub> PO <sub>4</sub>	1.24
	K <sub>2</sub> HPO <sub>4</sub>	1.84
<b>A<sub>5</sub> Trace Metals</b>	Na <sub>2</sub> EDTA·2H <sub>2</sub> O	0.171
	ZnSO <sub>4</sub> ·7H <sub>2</sub> O	0.00087
	H <sub>3</sub> BO <sub>3</sub>	0.042
	MnCl <sub>2</sub> ·4H <sub>2</sub> O	0.009
	FeSO <sub>4</sub> ·7H <sub>2</sub> O	0.07
	CoCl <sub>2</sub> ·6H <sub>2</sub> O	0.000042
	CuSO <sub>4</sub> ·5H <sub>2</sub> O	0.00032
	Na <sub>2</sub> MoO <sub>4</sub>	0.000097
<b>Vitamins</b>	Vitamin B1	0.1 mg L <sup>-1</sup>
	Vitamin B12	0.001 mg L <sup>-1</sup>
<b>Other</b>	NaCl	500

**Table A.2:** Components of Modified Okomoto Medium in mM, except for vitamins B1 and B12, which are in mg L<sup>-1</sup>

<b>Modified Artificial Seawater (MASW)</b>		
	<b>Component</b>	<b>mM</b>
<b>Beijerink</b>	NH <sub>4</sub> Cl	0.5
	CaCl <sub>2</sub>	2.04
	MgSO <sub>4</sub> ·7H <sub>2</sub> O	9.9
	NaNO <sub>3</sub>	11.8
<b>Phosphate buffer</b>	KH <sub>2</sub> PO <sub>4</sub>	0.37
<b>P-IV Trace Metals</b>	Na <sub>2</sub> EDTA·2H <sub>2</sub> O	2
	ZnCl <sub>2</sub>	0.037
	MnCl <sub>2</sub> ·4H <sub>2</sub> O	0.21
	FeCl <sub>3</sub> ·6H <sub>2</sub> O	0.36
	CoCl <sub>2</sub> ·6H <sub>2</sub> O	0.0084
	Na <sub>2</sub> MoO <sub>4</sub> ·2H <sub>2</sub> O	0.017
<b>Vitamins</b>	Vitamin B1	0.335 mg L <sup>-1</sup>
	Vitamin B12	0.135 mg L <sup>-1</sup>
	Biotin	0.025 mg L <sup>-1</sup>
<b>Other</b>	NaCl	510
	Tricine	5.58
	KCl	8

**Table A.3:** Components of Modified Okomoto Medium in mM, except for vitamins B1 and B12, which are in mg L<sup>-1</sup>

**APPENDIX B****CALCULATIONS FOR FIGURE 5.2**



Energy content (MJ kg <sup>-1</sup> )	Biodiesel 37.13	Source <a href="#">de Souza et al 2010</a>							
Conversion factor (l biodiesel kg <sup>-1</sup> )	0.912 Biodiesel								
Energy content (MJ kg <sup>-1</sup> )	33.86256	Calculated from de Souza							
Energy content (MJ kg <sup>-1</sup> )	TAG 38	Source <a href="#">Elisayed et al. 2003</a>							
Energy content (MJ kg <sup>-1</sup> )	Gasoline 132	Source <a href="#">Thomas 2000</a>							
Energy content (MJ kg <sup>-1</sup> )	Glycerol 18	Source <a href="#">Avechederra et al. 2007</a>							
Output	L oil ha <sup>-1</sup> y <sup>-1</sup>	Source	Biodiesel output (l ha <sup>-1</sup> y <sup>-1</sup> )	Energy output (MJ ha <sup>-1</sup> y <sup>-1</sup> )	Energy output gal gasoline equivalent (gal. gasoline ha <sup>-1</sup> y <sup>-1</sup> )				
Corn	172	Chisti 2007	137.6	4659.5	35.3				
Soybean	446	Chisti 2007	356.8	12082.2	91.5				
Canola	1190	Chisti 2007	952	32237.2	244.2				
Jatropha	1892	Chisti 2007	1513.6	51254.4	388.3				
Cocunut	2689	Chisti 2007	2151.2	72845.1	551.9				
Oil palm	5950	Chisti 2007	4760	161185.8	1221.1				
	kg TAG ha <sup>-1</sup> yr <sup>-1</sup>	Calculated using Sun et al., 2011							
Algal oil current	11680			443840	3362.4				
		25% TAG content, 80% harvest efficiency, 80% lipid recovery							
		Source							
Algal glycerol release	g l <sup>-1</sup> day <sup>-1</sup> 0.615	Our experiments							
	Light ( $\mu\text{mol photons m}^{-2}\text{s}^{-1}$ ) 500	Surface area (m <sup>2</sup> ) 0.00425	Light J m <sup>-2</sup> s <sup>-1</sup> 112.65	Glycerol g day <sup>-1</sup> 0.076875	Glycerol g m <sup>-2</sup> day <sup>-1</sup> 18.1	Light multiplier 2.01	Expected glycerol g m <sup>-2</sup> day <sup>-1</sup> 36.3	Expected glycerol (MJ ha <sup>-1</sup> y <sup>-1</sup> ) 2384179	Algal glycerol gas, equiv. energy [gal. gasoline ha <sup>-1</sup> y <sup>-1</sup> ] 18062
	Average light intensity (J m <sup>-2</sup> s <sup>-1</sup> ) 226	Source <a href="#">Amer et al., 2011</a>							Algal glycerol decreased by 50% for conversion losses 9031

**Table B.1:** Input values, sources (green), conversion factors, and final results (orange) that were used to calculate values in Figure 5.2